FINAL PROJECT REPORT

Project Title: Optimization of release strategies for sterile codling moth

PI: Dr. Larry Gut (Posthumous)
Organization: Michigan State University
Telephone:
Email:
Address:
City/State/Zip: East Lansing, MI 48824

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

Key Person(1): Nathan Moses-Gonzales
Organization: M3 Consulting Group
Telephone: (480) 239-8942
Email: nmosesgo@m3cg.us
Address: 10009 Mallet Dr.
City/State/Zip: Dayton, OH 45458

Cooperators: Dustin Krompetz (M3), Julianna Wilson (MSU)

Total Project Request: Year 1: $125,000 Year 2: $125,000 Year 3:

Organization Name: Michigan State University **Contract Administrator:** Diane Cox
Telephone: 517-884-4243 **Email address:** contractteam2@osp.msu.edu

WTFRC Budget: ***none***

Item	2019	2020	2021
Salary: Project Manager[1]	$25,168	$25,671	
Benefits: Project Manager[1]	$3,084	$3,270	
Supplies: Project Manager[1]	$4,248	$3,559	
Wages: Time Slip Staff[2]	$19,040	$19,040	
Benefits: Time Slip Staff[2]	$4,809	$4,809	
Project Vehicle[3]	$4,900	$4,900	
Fuel[3]	$7,000	$7,000	
Misc. Field Supplies[4]	$4,751	$4,751	
Travel[5]	$7,000	$7,000	
SIR CM, delivery and release[6]	$45,000	$45,000	
Total	**$125,000**	$125,000	0

Footnotes: [1]Project Manager: Rob Curtiss: 50% Salary ($25,168 +2% increase in year 2) + Fringe ($3,084 yr 1; $3,270 yr 2) + supplies ($4,248 yr 1; $3,559 yr 2) to be matched 1:1 by FFAR Fellowship. [2]Time Slip Staff: Two technicians at $14/hr for 40 hr/wk 17 weeks ($19,040) + 0.0765 fringe rate ($1,456.56) +Health @419/mo. [3]Project vehicle: One rental vehicle @$1,225/month for 4 months (Enterprise Car Rental) + Fuel: 240-300 miles/day*3 days/week* $0.54/ mile (MSU 2019 Mileage rate). [4]Misc. Field Supplies: Traps, Liners, lures, etc. [5]Travel: PI and Key person 2 travel to WA field sites 2x/year @$1,750.00/ trip/ person. [6]Sterile CM Delivery and Release: M3 Consulting Group Sterile Codling Moths + Delivery, and UAS release missions throughout field season and LIDAR missions for 3 orchards.

Original Project Goal and Objectives

The overall project goal was to provide information to form a best management practices recommendation for the use of sterile codling moths on a farm-scale in Washington State.

1 – Determine if orchard factors impact dispersion of SIR CM – **Completed 2019-2020**
   a) Determine impact of orchard architecture on distribution of released CM,
   b) Determine impact of topography on distribution, and
   c) Correlate topography and architecture impacts on distribution of CM.

2 – Determine if release factors impact dispersion of SIR CM – **Completed 2020-2021**
   a) Determine the optimal target release altitude,
   b) Determine if distributed or point releases are optimal for dispersion, and
   c) Determine the optimal time of release.

Significant Findings and Benefits to the Industry

- Significant Finding: Orchard architecture is an important factor in the dispersion and recapture of sterile codling moths
  - Benefits: Simplified release tactics may be employed in orchards with trellised trees
- Significant Finding: Orchard topography is not a significant factor in the dispersion and recapture of sterile codling moths
  - Benefits: Releases of sterile moths do not need to be modified for orchards with steep slopes
- Significant Finding: Release altitude, among those tested, is not a significant factor in recapture or dispersion of sterile codling moths
  - Benefits: Release by Unmanned Aerial Vehicle is effective at any altitude up to 100ft
- Significant Finding: Moths released by hand at a single central location in a 10-acre orchard distribute to the edges of the plot, and fewer leave the area than when they are released in a spread-out pattern
  - Benefits: For blocks up to 10-acres, simplified releases should be employed to ensure that moths that were purchased are not lost
- Significant Finding: The time-of-day moths are released impacts recapture
  - Benefits: Releasing moths before noon appears to give them the best chance at survival and dispersion; avoid releases late in the day to prevent loss of moths

Results

For all experiments, sterile moths, obtained from the OKSIR facility in BC, Canada, were released into commercial apple orchards in Washington State to measure their recapture and dispersion under several test conditions. Releases were replicated up to 18 times per treatment and the average percent recapture was compared among treatments. In addition, the aggregation of the released population was calculated by Morisita's Index of Dispersion (Iδ) to determine the degree to which the population dispersed throughout the orchard. ** Iδ is interpreted as follows: 0-1= random dispersion, 1=even dispersion, and >1= aggregated dispersion (higher numbers indicate more aggregation).** When appropriate we also analyzed per trap average recapture distance to determine how far moths dispersed.

Objective 1: Determine if orchard factors impact dispersion of SIR CM

Obj. 1a: Orchard Architecture: Trellised vs. free standing orchards under mating disruption

Recapture: Significant differences in recapture were found between the three tree training systems ($F=17.624$, $df=2$, $P<<.001$). Tukey's HSD test showed that significantly more moths were

recaptured in both types of trellised orchards than standard planted orchards, but recapture was not different among trellised orchards (Table 1). The mean recapture of moths in standard planted blocks was 1.6% ± 0.3, in V-trellised blocks was 9.2% ± 1.5, and in Vertical-trellised blocks was 9.1% ±1.5.

Aggregation: Significant differences in aggregation were found among the three tree training systems (F=6.871, df=2, P=.0025) (Table 1). Fisher's LSD test showed that released moths aggregated significantly more around the release point in standard planted orchards than in either trellised training system. The mean Iδ for moths released in standard planted blocks was 2.57 ± .44, in V-trellised blocks was 1.59 ± .09, and in Vertical trellised blocks was 1.39 ± .05. There was no significant difference in degree of aggregation between the trellised blocks.

	Training system	***Replicates***	***ANOVA***	***Mean ± SEM***
Percent Recapture	STD-32	19	F=17.624	1.6 ± 0.3 a
	V-Trellis	18	df=2,52	9.2 ± 1.5 bc
	Vertical Trellis	18	P<<0.001	9.1 ± 1.5 bc
Iδ - Absolute	STD-32	14	F=6.871	2.57 ± 0.44 a
	V-Trellis	17	df=2,45	1.59 ± 0.09 bc
	Vertical Trellis	17	P=0.0025	1.39 ± 0.05 bc

Table 1. Average % recapture and Iδ for sterile codling moths released in three tree training systems.

Distance: Moths released in trellised orchards were captured significantly more than in standard planted single, stand-alone tree orchards. Distance from point of release analysis determined that for each distance, 10m (F= 18.115, df=2, P<<0.001), 20m (F= 17.176, df=2, P<<0.001), 30m (F=14.434, df=2, P<<0.001), and 40m (F=12.370, df=2, P<<0.001) from the point of release, there were significant differences among the three tree training systems. Significantly more moths were captured in both trellised systems than in standard planted orchards, and moth recapture in the two trellised orchard types were not different at any of the distances. The estimated maximum population recapture distance of between 55-65 meters from the point of release was estimated as the point at which the three trend lines x-intercept on Figure 1.

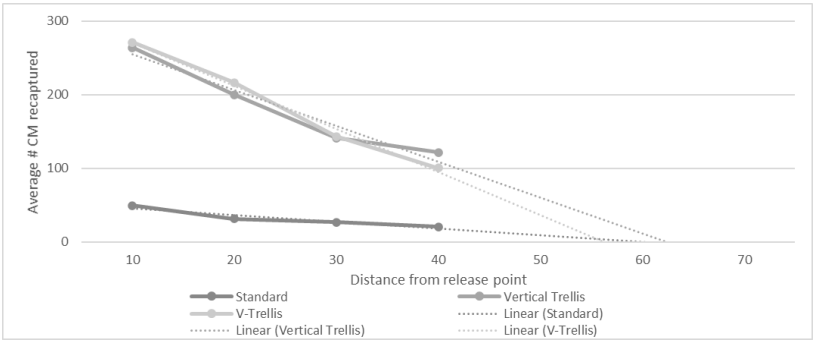

Figure 1. The average number of codling moths captured in apple orchards with three tree architectures at four distances from release.

Directionality: There were significant differences found in the direction of sterile moth dispersal in the standard planted orchards (F=2.570, df=7, P=0.0159), but not in the Vertical trellis training system (F=0.404, df=7, P=0.8986), nor the V-trellised system (F= 1.387, df=7, P=0.2157). Moths released in trellised orchards exhibited a slight, but not statistically significant directional preference for movement up and down rows, while those released in standard planted blocks had a minor but significant Westerly preference as revealed by Tukey's HSD. However, the overall impact on dispersion is minimal as moths dispersed to the edges of the blocks in all directions in the three tested tree training systems (Fig. 2).

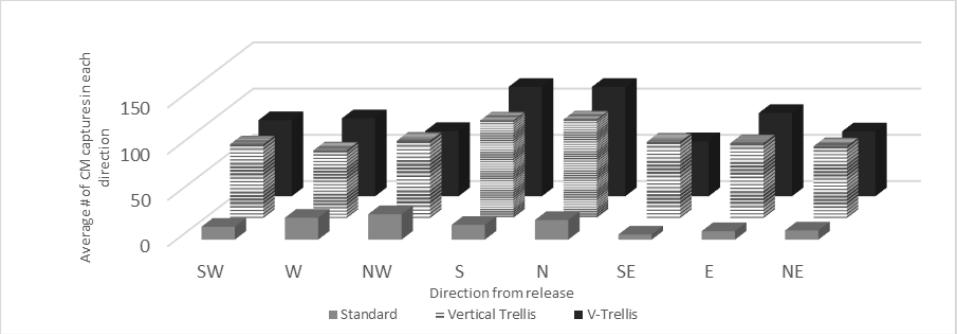

Figure 2. The average number of sterile *C. pomonella* captured at each direction in apple orchards with three different tree training systems. Traps to the North and South are up and down rows while those to the West and East are across rows.

Obj. 1b: Orchard Topography: Steep slope vs flat planar orchards

Recapture: Significantly more sterile moths (F=30.991, df=1, P<0.001) were recaptured in plots on a slope (4.8%±0.9) than without a slope (0.9%±0.3) (Table 2). However, the flat plots were not trellised, and as demonstrated in Obj. 1a, plots of this type typically have lower rates of recapture.

Aggregation: There was no significant difference in aggregation in the two treatments based on absolute recapture (F=3.128, df=1, P=0.0878). The Iδ of absolute number of sterile moths recaptured on orchards with a hill was 1.53 ± 0.10, and for orchards with less than a 1° slope was 2.90 ± 0.90 (Table 2). There were significant differences in the average trap percent of total capture between the two treatments (F=5.366, df=1, P=0.0281). The aggregation index for traps on the hill was 1.45 ± 0.13 was less than that for traps in a flat orchard was 3.29 ± 0.91, indicating that moths dispersed in a less aggregated manner.

	Orchard Slope	***# Reps***	***ANOVA***	***Mean ± SEM***
Percent Recapture	14° steep hill	18	F=31.763 df=1, 34 P<<0.001	4.8±0.9 a
	1° Flat planar	18		0.8±0.3 b
Iδ - Absolute	14° steep hill	18	F=3.128 df=1, 28 P=0.088	1.53±0.10
	1° Flat planar	12		2.95±0.97
Iδ - % of total	14° steep hill	18	F=5.366 df=1, 28 P=0.028	1.45±0.13 a
	1° Flat planar	12		3.35±0.99 b

Table 2. Average % recapture and Iδ for sterile codling moths released in orchards with two slopes.

Direction uphill/downhill: In the two apple orchard blocks with a 14° slope, the traps at the bottom of the slope recaptured a slightly lower but not significantly different number of moths than the traps at the top of the hill (F=3.305, df=1, P=0.078). In the flat blocks, the traps corresponding to the hilltop traps and the hill bottom traps were also not significantly different from each other (F=0.652, df=1, P=0.425). These data indicate that sterile codling moths do not exhibit a significantly elevated uphill movement (Fig. 3).

The traps down slope from the central release point did not capture significantly different numbers of moths than the traps up the slope in the apple orchards on the 14° slope (F=1.261, df=1, P=0.269). The orchards on the flat area also did not capture significantly different numbers of moths in the traps corresponding to uphill and the traps corresponding to downhill (F=0.152, df=1, P=0.700). Further confirmation that *C. pomonella* do not prefer uphill dispersal (Fig. 3).

Direction across slope: In the two orchard blocks with a 14° slope, the traps at each extreme side of the block did not recapture different numbers of sterile codling moths (F=0.208, df=1,

P=0.651). Likewise, the traps on each side of the release point across the slope also did not recapture different numbers of moths (F=0.015, df=1, P=0.905) (Fig. 3).

In the orchard blocks with a flat planar slope, the traps that correspond to those on the extreme sides of hill blocks did not recapture different numbers of moths (F=0.250, df=1, P=0.620), nor did all traps on each side of the release point (F=0.100, df=1, P=0.754) (Fig. 3).

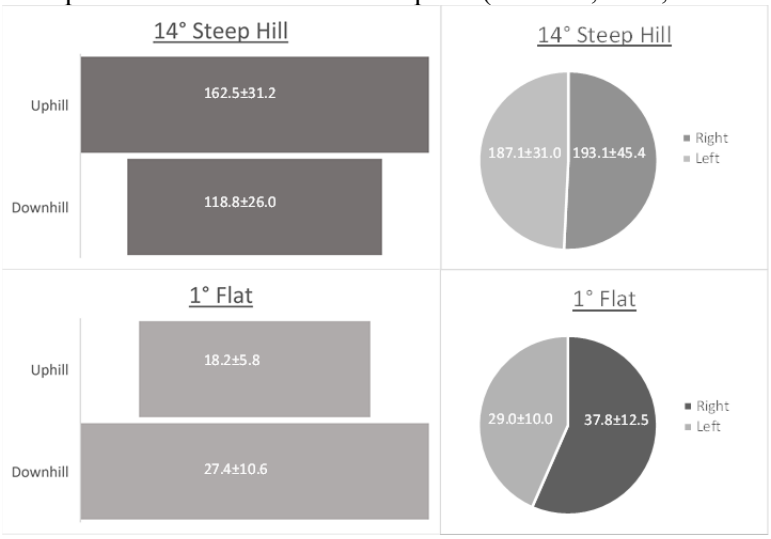

Figure 3. Sterile moth dispersion on steep slopes and flat ground.

Obj. 1c: Orchard Architecture vs. Topography

Because Orchard Topography was not found to be a significant factor in the dispersion of sterile codling moths, and orchard architecture was significant, no correlation between the two factors was found.

Objective 2: Determine if release factors impact dispersion of SIR CM

Obj. 2a: Optimal target release altitude

Recapture: There were no significant differences in recapture of moths released by UAS at 30-35m altitude, 20-25m altitude, 10-15m altitude, or by hand at 0-5m altitude (F=1.0562, df=3, P=0.3833). The mean proportion of moths recaptured did not exceed 2.5% in any treatment (Table 3).

Dispersion: There were no significant differences found among the four treatments based on absolute aggregation (F=0.5726, df=3, P=0.6377) or aggregation by percent of recapture (F=0.815, df=3, P=0.4964) Overall, all four strategies for releasing sterile *C. pomonella* centrally resulted in 1.1-2.4% recapture and moderate aggregation around the point of release (Table 3).

	Release altitude	*# Reps*	*ANOVA*	*Mean (± SEM)*
Percent Recapture	0-5 m (hand)	8	F=1.0562 df=3, 28 P=0.3833	2.4 ± 0.62
	10-15 m (UAS)	8		2.1 ± 0.70
	20-25 m (UAS)	8		1.1 ± 0.39
	30-35 m (UAS)	8		1.5 ± 0.60
Iδ - Absolute	0-5 m (hand)	8	F=0.5726 df=3, 28 P=0.6377	2.27 ± 0.34
	10-15 m (UAS)	8		2.14 ± 0.24
	20-25 m (UAS)	8		2.90 ± 0.48
	30-35 m (UAS)	8		2.25 ± 0.65
Iδ - % of Total Captured	0-5 m (hand)	8	F=0.815 df=3, 28 P=0.4964	2.32 ± 0.36
	10-15 m (UAS)	8		2.18 ± 0.28
	20-25 m (UAS)	8		3.20 ± 0.58
	30-35 m (UAS)	8		2.66 ± 0.67

Table 3. Average % recapture and Iδ of sterile codling moths after release at four altitudes.

Distance: Three of the four release strategies resulted in significant differences in recapture by distance (Fig. 4). There were significant differences in recapture by distance when sterile *C.*

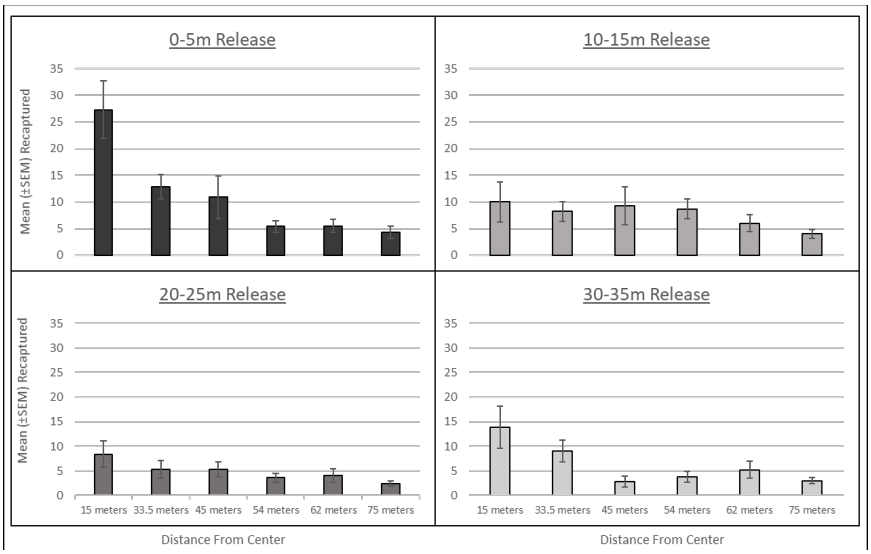

Figure 4. Mean (+/- SEM) per trap sterile moth recapture at 5 distances from release at four altitudes

pomonella were centrally released by hand at 0-5m altitude (F=9.9738, df=5, P<<0.0001), by UAS at 10-15m altitude (F=4.5111, df=5, P=0.0007), and by UAS at 30-35m altitude (F=4.5364, df=5, P=0.0007). Hand release resulted in traps at 33.5m recapturing significantly more moths than those at 75m. Following release by UAS at 10-15m altitude, traps closest to the release point (15m) recaptured significantly more sterile *C. pomonella* than traps at 33.5m, 54m, 62m, and 75m. Following release by UAS at 30-35m altitude, more sterile codling moths were recaptured in traps 15m from the center than in traps 45m, 54m, 62m, and 75m away. Although the one-way analysis of variance did not indicate significant differences in recapture by distance following UAS release at 20-25m altitude (F=1.8878, df=5, P=0.0995), there were more moths recaptured in traps closest to the center of the block than in those furthest from the center.

Obj. 2b: Distributed or point releases

Recapture: There were significant differences in moth recapture between by the four treatments (F=4.8407, df=3, P=0.0047) (Table 4). Significantly lower recapture, 0.8%±0.3, was recorded in hand-nine even points releases than the highest recapture, 3.6%±0.7, in the UAS evenly spread treatment. There were no significant differences in recapture between the hand-center release (1.6%±0.7) and hand-nine even points release, the hand-center release and UAS Center release (2.5%±0.6), or the hand-center release and the UAS even release.

Dispersion: There were significant differences in Iδ indices calculated using the absolute number recaptured in traps for the four treatments (F=2.9316, df=3, P=0.0431) (Table 4). Fisher's LSD test revealed differences in aggregation between hand-center (mean Iδ=2.64±0.44) and hand-nine even points (mean Iδ= 1.68±0.22), and UAS-even (mean Iδ=1.43±0.14) releases, indicating that sterile moths released at the center of the orchard were significantly more aggregated around the central point of release than those released in a spread-out pattern. Moths released by hand-at nine evenly spaced points and UAS-center (mean Iδ= 1.87±0.27) were not significantly more or less aggregated from each other or the hand center release or UAS 9 release point treatments.

There were significant differences in dispersion based on Iδ indices calculated using the proportion of total recaptured moths (F=4.021, df=3, P=0.012) (Table 4). Moths released by hand at the center of blocks were highly aggregated (average Iδ=3.25±0.56), those released by hand at nine even points were more dispersed (average Iδ=2.36±0.44), followed by releases by UAS at the center (average Iδ=1.94±0.28), and the greatest dispersion was for moths released by UAS spread throughout the orchard approximating the hand release at nine evenly spaced points (average Iδ=1.40±0.17). However, Fisher's LSD test revealed that only moths released by hand at the center

were significantly more aggregated on average than those released by both UAS methods. There were no significant differences in dispersion using proportion recapture found between the two hand releases, nor between the two UAS releases.

	Release method	*ANOVA*	*Mean (± SEM)*
Percent Recapture	**Hand release - Center**	F=4.8407 df=3, 52 P=0.0047	1.6 ± 0.7 a,b,c
	Hand release - 9 Points		0.8 ± 0.3 b
	UAS release - Center		2.5 ± 0.6 a,b,c
	UAS release - ~9 Points		3.6 ± 0.7 c
Iδ - Absolute	**Hand release - Center**	F=2.9316 df=3, 47 P=0.0431	2.64 ± 0.44 a
	Hand release - 9 Points		1.74 ± 0.28 b
	UAS release - Center		1.87 ± 0.27 a,b
	UAS release - ~9 Points		1.43 ± 0.14 b
Iδ - % of Total Captured	**Hand release - Center**	F=4.0213 df=3, 48 P=0.0124	3.25 ± 0.56 a
	Hand release - 9 Points		2.36 ± 0.44 a,b
	UAS release - Center		1.94 ± 0.28 b
	UAS release - ~9 Points		1.40 ± 0.17 b

Table 4. Average % recapture and Iδ of sterile codling moths after release by hand or UAV at orchard center or manually distributed.

Distance: There were significant differences in recapture by distance from the center of the orchard (F=4.6925, df=5, P=0.0004) for moths released by hand at a single central location (Fig. 5). More moths released at the center of test orchards were recaptured at the closest traps (15m) than at traps 62m, and 75m away (Fig. 5). In addition, moths were significantly more likely to be captured in traps 33.5m from the release than those 75m from the center.

The effect of distance from the center on recapture *within* treatments was variable. Although average recapture was low at all trap distances from the center, there were no significant differences in capture by distance (F=1.5887, df=5, P=0.1634) when moths were released at nine evenly spaced locations. Sterile *C. pomonella* released by UAS at the center of the orchards were significantly more likely (F=3.8202, df=5, P=0.0023) to be captured in traps 15m from the center than those 54m and 75m away (Fig. 5). In addition, they were less likely to be recaptured in traps 75m from the center than in traps 33.5m away from the point of release. There were no significant recapture differences by distance from the center of the block when moths were released by UAS approximating nine evenly spaced points in the orchards (F=0.7507, df=5, P=0.5862); recapture was highest at all distances for this release method among the treatments (Fig. 5).

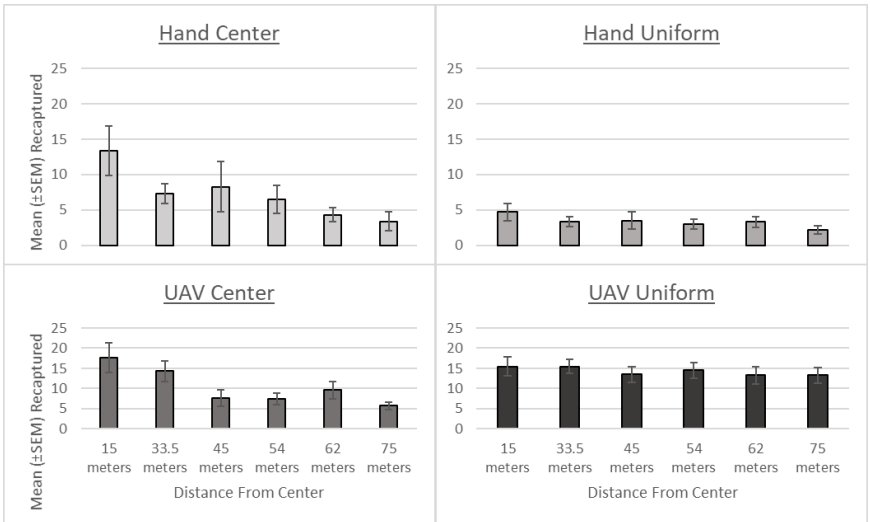

Figure 5. Mean (+/- SEM) per trap sterile moth recapture after release by hand or UAV at center or manually distributed.

The effect of distance from the center on recapture *between* treatments also was variable. There were significantly different treatment impacts on moths recaptured 15m from the center of the orchard (F=5.2226, df=3, P=0.0021). Fewer moths were captured at this distance when they were released by hand at nine locations than by UAS at the center, and UAS approximating the nine locations. There were significant treatment effects on recapture of moths at 33.5m from the center of the orchard (F=15.9328, df=3, P<<0.0001). Fewer sterile *C. pomonella* were recaptured when they were released by hand at nine evenly spaced points than by UAS at the center, and by UAS approximating nine evenly spaced points. There were also significantly fewer recaptured when they were released by hand at the center than UAS at the center, or UAS at nine evenly spaced locations. There were no significant differences in recapture between the two hand released methods at this distance from the center. At a distance of 45m from the center of the orchard, there were significant differences in recapture of moths by treatment (F=6.0036, df=3, P=0.0008); fewer moths were recaptured when released by hand at a single central location and released by hand at nine evenly spaced points than when they were released by UAS approximating nine evenly spaced points. There were significant differences (F=14.5591, df=3, P<<0.0001) in recapture of released moths among the four treatments at a distance of 54m from the center of the orchard. Traps at this distance recaptured significantly more sterile codling moths when they were released by UAV in a pattern approximating nine evenly spaced points than by hand at the center, by UAS at the center, and by hand at nine points. Also, significantly fewer moths released by hand at nine evenly spaced locations were recaptured than those released by UAS at the center of the orchard, and by UAS approximating nine evenly spaced points. Additionally, significantly fewer of the moths released by UAS at the center were recaptured than those by UAS approximating nine evenly spaced points. There were significant differences (F=11.5004, df=3, P<<0.0001) in recapture of moths among release strategies at a distance of 62m from the center of the orchard. Fewer moths released by hand at the center of the orchard were recaptured than moths released by either UAS at the center or UAS approximating the nine evenly spaced points. Likewise, Tukey's HSD test showed that significantly fewer moths released by hand at nine evenly spaced points were recaptured than when moths were released both by UAS at the center and UAS approximating the nine evenly spaced points. At a distance of 75m from the center of the orchard, there were significant differences (F=19.7768, df=3, P<<0.0001) in recapture of moths among the treatments. As indicated by Tukey's HSD test of recapture of sterile *C. pomonella* at this distance from the center of the orchard, more moths released by the UAS approximating nine evenly spaced points were recaptured than those released by hand at the center, hand at nine evenly spaced points, and UAS at the center of the orchard. Also, significantly fewer moths released by hand at the center and by hand at nine evenly spaced locations were recaptured than those released by UAS at the center of the block.

Obj. 2c: Release Time

Recapture: There were significant treatment differences found in the recapture of moths released at different times of the day (F=4.1328, df=5, P=0.0039). Releases conducted at 12:00 resulted in numerically higher recapture than all other treatments and Tukey's HSD test indicated significantly more sterile *C. pomonella* were recaptured from releases conducted at 12:00 than releases at 11:00, 15:00, and 19:00 (Table 5).

Dispersion: Analysis of absolute recaptures revealed significant differences in Iδ indices among the treatments (F=4.1124, df=5, P=0.0061). The highest value and thus extent of aggregation was found for releases conducted at 19:00 - dispersion indices at 21:00, 18:00, 11:00, 12:00, and 15:00 were all significantly lower than at 19:00, but were not different from each other (Table 5).

Time of release had a significant effect on dispersion (F=5.7990, df=5, P=0.0005) when Iδ indices were calculated based on the proportion of a replicate's total capture (Table 5). The aggregation indices for moths released at 11:00 (Iδ=2.60), 12:00 (Iδ=1.81), 15:00 (Iδ=2.65), 18:00 (Iδ=3.17), and 21:00 (Iδ=3.24) were not significantly different from each other, but all were significantly less aggregated than releases conducted at 19:00 (Iδ=8.33).

	Treatment	*# Reps*	*ANOVA*	*Mean (± SEM)*
Percent Recapture	**1100 release**	7	F=4.1328 df=5, 41 P=0.0039	1.4 ± 0.9 a
	1200 release	10		3.7 ± 0.8 b
	1500 release	10		1.2 ± 0.6 a
	1800 release	4		1.2 ± 0.7 a,b
	1900 release	6		0.4 ± 0.2 a
	2100 release	10		1.7 ± 0.3 a,b
Iδ - Absolute	**1100 release**	4	F=4.1124 df=5, 29 P=0.0061	1.96 ± 0.38 a
	1200 release	10		1.90 ± 0.19 a
	1500 release	5		1.80 ± 0.21 a
	1800 release	4		2.91 ± 0.40 a
	1900 release	3		8.01 ± 3.56 b
	2100 release	9		3.13 ± 0.88 a
Iδ - % of Total Captured	**1100 release**	5	F=5.7990 df=5, 36 P=0.0005	2.60 ± 0.61 a
	1200 release	10		1.81 ± 0.19 a
	1500 release	9		2.65 ± 0.41 a
	1800 release	4		3.17 ± 0.47 a
	1900 release	4		8.33 ± 2.55 b
	2100 release	10		3.42 ± 0.80 a

Table 5. Mean (± SEM) % recapture and Iδ for sterile *C. pomonella* released at six different times of the day. Means with the same letters are not significantly different (Tukey's α = 0.05).

Distance: Five of the six release times resulted in significant differences in recapture by distance. When sterile codling moths were released at 12:00 they were more likely to be captured in traps close to the center of the orchard than traps farther from the center (F=16.8678, df=5, P<<0.0001). Tukey's HSD indicated that traps 15m from the center recaptured significantly more sterile *C. pomonella* than those 33.5m, 45m, 54m, 62m, and 75m away. Traps 33.5m from the center of the orchard recaptured significantly more sterile *C. pomonella* than those 75m away from the center, and traps 45m from the orchard center recaptured more than those 62m, and 75m away when moths were released at 12:00. Recapture at all distances when moths were released 1500 day was low, but there were significant differences in the distance from the center where moths were recaptured (F=2.4175, df=5, P=0.0374). Traps 15m from the center of the orchard were significantly more likely to recapture sterile moths than traps 75m away from the point of release. Captures were low when sterile moths were released at 19:00, but there were significant effects globally on recapture based on distance of the trap from the center of the orchard (F=2.6104, df=5, P=0.0283), and Tukey's HSD revealed that moths were more likely to be recaptured in traps 15m from the release point than moths 54m, 62m, and 75m away. Significantly more *C. pomonella* released at 21:00 were recaptured in traps close to the center of the block (F=12.7680, df=5, P<<0.0001) than in traps farther away, and Tukey's HSD test showed that traps within 15m of the center of the orchard recaptured significantly more sterile moths than those 33.5m, 45m, 54m, 62m, and 75m, and traps 33.5m from the center captured more than those 54m and 75m. Although ANOVA indicated that for the 18:00 time of release, the distance from the central release point moths are recaptured was significant globally (F=2.5206, df=5, P=0.0366), overall captures were low, and the Tukey's test did not reveal significant differences among the trap distances. There was not a significant effect on recapture at the six trapping distances when sterile moths were released at 11:00 (F=0.9806, df=5, P=0.4339), but numerically there were more captured in traps close to the center.

All six trap distances resulted in significant differences in recapture by time of release and generated the following results: 15m from the center (F=9.4846, df=5, P<<0.0001), 33.5m from the center of the orchard (F=10.4666, df=5, P<<0.0001), 45m from the center of the orchard (F=7.1408,

df=5, P<<0.0001), 54m from the center of the orchard (F=11.6886, df=5, P<<0.0001), 62m from the center of the orchard (F=8.1937, df=5, P<<0.0001) and 75m from the center of the orchard (F=5.5968, df=5, P<<0.0001). Traps placed 15m from the center recaptured more moths when they were released at 12:00 than at 11:00, 15:00, 18:00, 19:00, and 21:00. Also, significantly more moths were recaptured when they were released at 21:00 than 19:00. Traps placed 35m from the center recaptured more moths when they were released at 12:00 than at 15:00, 18:00, 19:00, and 21:00. In addition, moths released at 11:00 and 21:00 were recaptured significantly more than those released at 19:00. Traps placed 45m from the center recaptured more moths when they were released at 12:00 than at 11:00, 15:00, 19:00, and 21:00. Traps 54m from the central release point recaptured significantly different numbers of moths depending on the time of the day they were released, and more of the moths released at 12:00 were recaptured than moths that were released at 11:00, 15:00, 18:00, 19:00, and 21:00. Also, the 19:00 release had significantly lower recapture than the 1100 release and the 21:00 release. Traps placed 65m from the center recaptured more moths when they were released at 12:00 than at 15:00, 18:00, 19:00, and 21:00. In addition, significantly fewer moths released at 19:00 were recaptured than those released at 21:00. Traps placed 75m from the center recaptured more moths when they were released at 12:00 than at 15:00, 18:00, 19:00, and 21:00.

Discussion

Orchard Factors

Our results from these experiments indicate that tree training system and topography impact the dispersal of sterile codling moths, and there are implications for understanding the behaviors of wild moths in these orchard systems. While many studies have focused on the maximum distance that a few CM will travel after release, few have explored the dispersion of a population of moths within the orchard close to the point of release and none have compared dispersion in different orchard training systems or on slopes. Moths released at the center of blocks with trellis disperse more readily than those released into blocks with large, widely spaced single-planted trees. We demonstrated that moths do not prefer dispersing up and down rows versus across them in all tree training systems tested, indicating that the direction of dispersion is not impacted by rows. Hills do not impact dispersion by causing more moths to move upslope than down.

Early studies exploring intra-orchard self-dispersion of released sterile codling moths (i.e., Howell and Clift, 1974, Mani and Wildbolz, 1977) did not explore the impact of planting system on dispersal, nor did they ascertain more accurate information on within-orchard movement, the impacts of topography, or other orchard conditions on the whole population of released moths. We have assessed the impact of tree training systems on released sterile codling moth recapture, aggregation, and dispersion and found that greater dispersion and recapture of moths is found in orchards with trellis compared to standard planted orchards. This is the first study to test the idea that rows may act as barriers to self-dispersal of released sterile codling moths – they do not.

Previous studies have shown that landscape-level elevational differences are an important factor in the capture of wild *C. pomonella* (Vernon et al. 2006). However, our results refine the notion that elevation may impact the capture of moths by scaling down the test area to single plots and comparing on-farm recapture at different elevations on a hill. We found that released moths are *not* more likely to disperse uphill than downhill or in either direction across the slope, and in flat plots there is also no preference for movement in a directional manner.

It is clear from these experiments that moths released into orchards with trellised trees are recaptured at higher rates than those released into orchards with large old stand-alone trees. This may be explained by the complexity of the systems and canopy density: trellised canopies are less dense and less three dimensionally complex. In trellised systems, not only is there more space for moths to move, but there is also less canopy to interact with as they move from one point to the next. In addition, the odor plume emitted from lures in traps may be more apparent to moths and more defined in orchards with trellis due to the reduced complexity and three-dimensional structure of the canopy.

Our results show that 1) sterile codling moths disperse from the point of release and some moths reached the furthest traps in all directions from the central release point, and 2) that aggregation around the point of release is typically moderate. These data suggest that although some moths may travel far and leave the block, the majority remain in the target area and are available to provide farm-scale control of existing wild populations when they are released at the center of the orchard. Self-dispersal of released sterile codling moths, regardless of orchard conditions, is vital to successfully integrating them into existing management systems. The current study has demonstrated that over short distances the layout and architecture of the orchard impacts how CM disperse throughout the landscape when orchards are treated with pheromone mating disruption. We estimated shorter maximum dispersive distances, less than 245 ft (75 meters), for the population of released moths under mating disruption than studies conducted in the absence of mating disruption, well over 650 ft (200 meters) (i.e., Tremmaterra, 2004; Basoalto et al., 2010).

From a practical standpoint, the movement of sterile codling moths into and out of orchards is of great importance to growers as they strive to make a SIT program economically viable. Farmers do not want to learn that the insects they released have all flown away, nor do they want to suffer the disappointment of knowing that the insects did not fly to the areas of the farm where they were needed most. Our results support employing different release tactics depending on orchard training system. Although moths dispersed to the edges of plot in all directions, it is evident that moths do not disperse as readily in orchards with old stand-alone tree plantings. In these types of plantings, it may be prudent to not release moths at the center of the block for every release: either employ several distributed release points for each weekly release or rotate release points throughout the season based on a schedule or damage sampling. It is equally clear that more moths disperse to the edges of trellised blocks than in stand-alone tree blocks, and release at the center is optimal to retain the maximum number of moths within the target block. It may be possible to utilize fewer sterile moths in orchards with trellis to affect the same level of control as in blocks with large stand-alone trees. Orchards with trees partly on steep hills, or wholly situated on steep slopes should have sterile moths released at the center of the slope because there is not a significant preference for uphill dispersion.

In summary, *C. pomonella* move up and down rows just as readily as they move across them in several planting systems. Rows do not act as a barrier for *C. pomonella*. Rather, released sterile codling moths disperse much more readily in trellised orchards, suggesting that this planting system is highly conducive to the sterile insect technique. Farmers with trellised orchards will find that a single point release at the center of a 10-acre bock is sufficient to treat the whole block with sterile codling moths. Orchardists with apple blocks on hills need to understand the ways that sterile codling moths disperse in an orchard with uphill sections and modify releases accordingly.

Release Factors

There are practical and economic considerations when comparing the use of UAS versus manual releases of sterile *C. pomonella*. A practical advantage of the UAS method over hand release is that pesticide reentry intervals do not impact the ability to release moths in a timely manner. On the other hand, weather conditions and proximity to “no-fly” areas may impede the use of UAS. Wind or rain may delay the application of sterile moths because the aircraft used to release the moths are expensive and precautions to avoid crashes are necessary and may impact the cost of the program.

Regardless of the various methods of release employed to deliver sterile insects, typically the goal is to distribute them quickly and uniformly throughout the target orchards without compromising quality and survival. Knowing that sterile *C. pomonella* are capable of flights of up to 250m, at the outset of this study we hypothesized that on a farm-scale, uniform distribution of moths may not be necessary.

To determine the best means of farm-scale sterile *C. pomonella* release we compared recapture and dispersion of moths following their deployment by hand or by UAS and from a central location vs multiple uniformly spaced sites. Our results show that *C. pomonella* adults released by hand at evenly spaced locations in orchard blocks were recaptured significantly less than when

released by UAS at 35m altitude on a flight path approximating the same locations. Although percent recapture was found to be different in our releases at/above nine evenly spaced points, there was no difference in the degree of aggregation/dispersion found by either release method (Hand or UAS). However, when we compared hand releases at the center of the orchard to hand releases at nine evenly spaced points and also UAS release approximating the same points, we found there was a higher degree of SIT moth aggregation about the center of the block when moths were released by hand at the center than when they were released at nine evenly spaced points by hand or UAS, indicating that more sterile *C. pomonella* were retained in the targeted orchard block when they were released at the center. In addition, release altitude was not significant to *C. pomonella* dispersion or recapture. These findings indicate that *C. pomonella* dispersal capabilities and size may be beneficial for UAS release with less impact from winds and forces associated with aerial release.

Moths released at different times of the day behaved generally like those in other trials – when released at the center of the orchard they dispersed throughout the 4.05ha area. Moths released at noon were recaptured at a higher rate than those released at other times of the day while also not having major differences in dispersion. Causes of this may be: 1) moths released late in the evening do not have sufficient time to acclimatize to field conditions before cessation of normal evening activities, thus are subject to 24 hours of potential mortality causes before conditions are appropriate for dispersing, 2) moths released in the middle afternoon are susceptible to shock caused by the rapid transition from chill-coma to >95°F causing either direct mortality or non-lethal damage resulting in low response rates, and 3) moths released at noon do not experience the previous two conditions at their release time and have sufficient time to acclimatize to temperatures and day lengths in the field. Typically, the warmest part of the day at our field sites was from 13:00-17:00. Successful mating usually occurs between 18:00 and 20:00 and longevity decreases with increasing temperatures. Moths released at or after typical evening mating periods do not experience the cues to elicit a mate-finding response. These data show that late afternoon and evening releases should be avoided, and moths should be released by noon the day they are received.

Application costs are a major consideration when implementing any SIT program and are acutely important for commercial applications of the technique by individual farms. For the past few years, several research programs have explored UAS as a means of delivering sterile *C. pomonella* to target areas. A UAS flying at about 30 meters above the orchard can distribute 30,000 sterile moths over a 6ha orchard in less than 10 minutes. However, the cost remains high. Season-long release of moths is currently accomplished in Washington State by a commercial applicator for over $1100/ha. Wider adoption of this method of release will require new approaches that focus on improved efficiencies. Releasing only during peak generation flight may provide a significant cost savings and still provide substantial *C. pomonella* population suppression. Similarly, releasing fewer than the currently accepted full release density of 2000 mixed sex sterile moths per hectare may be more cost-effective. SIT is density dependent and thus operates much like pheromone-based mating disruption. With mating disruption, the fraction of added control achieved by applying 1000 rather than only 750 dispensers per acre may not be worth the added cost. The same may be the case with SIT, adding more moths to achieve the theoretical overflooding ratio of 40:1 sterile to wild males for eradication may not be worth the additional cost when used in conjunction with other management techniques. Also, deployment of SIT females in combination with synthetic pheromone mating disruption may have an additive impact on confusing wild males and increasing suppression and control.

Our findings reveal that hand-applying sterile *C. pomonella* is a viable option. Hand and aerial release of moths provided similar recapture and dispersal of released moths. Labor costs for hand application versus the costs of paying for a UAS or service to release moths should be compared when deciding which approach is best for a given situation. In the approximately 10-ac experimental blocks, an individual could walk from the truck to the pre-marked center of the orchard and back for a hand release in less than five minutes and walk to the nine evenly spaced locations in 10-15 minutes. In contrast, UAS releases applied moths to 10-ac plots in five minutes from the time the flight began to when it returned for all methods tested.

Conclusions

The sterile insect technique for codling moth in Washington State is a valuable new management tool that should be carefully employed. Our studies have shown that released moths are vulnerable to a variety of conditions that may impact their effectiveness as a control tactic. The two main factors that we studied, those inherent to the orchard, and those controllable during the release were both found to be significant in the recapture and dispersion of released sterile codling moths. Users of SIT for on-farm *C. pomonella* control should carefully plan where and when to release moths to maximize their effectiveness. Based on the findings reported herein, a single release at the center of a 10-ac orchard either manually or by UAS at any altitude is sufficient to allow moths to disperse independently to the edges of the block while retaining the maximum number of moths within the targeted treatment area. Requiring only a single central release in each 10-ac orchard plot should reduce the cost of application. Additionally, moths should be released prior to noon. If moths are unavailable for release in the morning, applicators should consider holding them in cold storage until the next day. Our findings have shown that trellised orchards are more conducive to the spread of sterile moths than large single plantings, moths do not only move up slope in orchards with hills, hand release is not better or worse than UAS release at any altitude, and moths should be released by noon to ensure they have the best chance at being effective on the farm.

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Executive Summary

Project title: Optimization of release strategies for sterile codling moth

Key words: Codling Moth, Dispersion, Sterile Insect Technique, Integrated Pest Management

Abstract: Sterile codling moth release as a control tactic recently became commercially available to Washington apple farmers. A better understanding of how released sterile codling moths behave on-farm in the presence of existing control tactics was necessary before this technology is recommended for adoption on an industry-wide scale. We studied how both factors inherent to modern orchards and factors that may be controlled at release impact dispersion and recapture of released sterile moths. We found that dispersion and recapture are impacted by orchard training systems, release locations, and release time of day, but not impacted by release altitude and orchard slopes. We demonstrate that sterile codling moth release is highly conducive to use in modern trellised orchards. Moths are flexible in the release and orchard conditions they will tolerate; they quickly distribute throughout the orchard when released at a single central location despite facing a variety of conditions. Based on the assumption that high moth recapture and slightly aggregated dispersion equates with effective on-farm control, we recommend use of a single central release location before noon in 10-ac trellised apple orchards. Unmanned aerial systems or hand releases are both effective tactics for deploying moths within orchards.

Final report prepared and submitted to WTFRC by Dr. RT Curtiss on behalf of Dr. Larry Gut who passed away before the conclusion of the project.

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

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

Cooperators: Jacqueline Serrano, USDA-ARS, Wapato, WA (Scientific Collaborator); Dr. Jason Pitts, Baylor University, Waco, TX (Scientific Collaborator)

Report Type: Continuing Project Report

Project Duration: 3 Year

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

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

WTFRC Collaborative Costs: None

Budget 1
Primary PI: William B. Walker III
Organization Name: USDA-ARS
Contract Administrator: Chuck Myers
Telephone: (510) 559-5769
Contract administrator email address: Chuck.Myers@ars.usda.gov
Station Manager/Supervisor: Rodney Cooper
Station manager/supervisor email address: rodney.cooper@usda.gov

Item	2018	2021	2022
Salaries	$37,306	$39,282	$41,322
Benefits	$13,804	$14,535	$15,288
Wages			
Benefits			
Equipment			
Supplies	$5,000	$5,000	$5,000
Travel			

Miscellaneous			
Plot Fees			
Total	$56,110	$58,817	$61,610

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

AMENDMENT

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

OBJECTIVES

1) Identify and clone Odorant Receptors expressed in the abdomen tip of codling moth females.
Over thirty odorant receptors (OR) were identified in a transcriptome generated from abdomen tips of codling moth females. Most of these have been cloned during this past year. Gene expression assays have also been conducted to compare OR expression in mated versus non-mated codling moth females. In year three, several of the cloned ORs will be used in expression assays (not funded by this proposal) to determine activating plant compounds.

2) Knock-out OR genes using CRISPR/Cas9 genome editing.
CRISPR/Cas9 genome editing is fully developed for codling moth, and is being used to knock-out genes expressed in the female abdomen tip. Laboratory populations of codling moth with inactive OR genes are being generated for use in oviposition assays.

3) Determine which genes are essential for oviposition site selection.
The hypothesis tested here will be that inactive OR genes for specific plant volatiles will alter female codling moth oviposition site selection. An oviposition assay for codling moth is under development and will be used to assess the effects of OR gene knock-out populations. In year one, initiation of testing and adapting the oviposition assay was done, however, this provided unsatisfactory results. The ARS Pacific Area Office provided $35,000 for a flight tunnel system that will monitor behaviors using cameras and specialized behavioral analysis software. This system was set up for use in January 2019.

SIGNIFICANT FINDINGS

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

METHODS

The CRISPR (clustered regularly interspaced palindromic repeats)/Cas9 (CRISPR associated protein 9) genome editing system has been used extensively in the past few years to study protein function in many organisms. Interest in the CRISPR/Cas 9 system to control insects or to overcome insecticide resistance is now coming of age with substantial investments in this technology by Bayer Crop Sciences. A CRISPR/Cas 9 genome editing system for use in codling moth is fully developed and has been successfully used to knock-out an OR gene thought to be involved in codlemone detection (Garczynski et al., 2017). These methods are being used to knock-out OR genes expressed in codling moth females to evaluate protein function and determine which genes are critical for oviposition site selection by codling moth females.

1) Identification and cloning ORs expressed in the abdomen tips of codling moth females.
Gene transcripts encoding ORs expressed in antennae of codling moth males and females have been identified in transcriptomes (Walker et al., 2016). Recently, it was discovered that a candidate pheromone receptor is expressed in the abdomen tip of codling moth females (Garczynski et al., 2017). To determine the extent of OR expression, a female abdominal tip transcriptome was prepared late in 2017. Initial analysis of the transcriptome revealed that 38 potential OR transcripts are present, 28 of which are also found in the antennae (Walker et al., 2016, Garczynski and Walker, Unpublished data).

Many of the ORs expressed in the codling moth abdomen tip are related to those activated by plant volatiles in other moths (de Fouchier et al., 2017).

To confirm the initial identifications, PCR has been performed with oligonucleotide primers designed to amplify the full-length transcript of each individual OR found in the transcriptome. Once expression is confirmed by PCR, the full-length transcript sequence will be determined. DNA sequence information gained from this step will be used in subsequent steps to design guide RNAs (see below). Additionally, all odorant receptors that show sex-biased expression in codling moth antennae were also assayed with PCR for expression in the female abdomen tip, even if they were not identified as expressed in the transcriptome. The rationale for this is that the candidate pheromone receptor initially detected by PCR in the female abdomen tip was identified in the transcriptome Garczynski et al., 2017); furthermore, genes that show sex-biased expression are hypothesized to have substantial impact on sex-specific behaviors.

In order to further assess the importance of individual odorant receptors in female abdomen tip, a quantitative real time PCR (qRT-PCR) study has been conducted to evaluate relative expression levels of odorant receptor genes in abdomen tip of unmated versus mated females. It is hypothesized that odorant receptor genes that show higher expression levels in mated versus unmated females are likely to play important role in mediating egg-laying behaviors since egg-laying necessarily happens after mating. One potential problem with this approach is that not all OR genes may be expressed at sufficiently high levels to be detected by qRT-PCR assay. None-the-less, preliminary results indicated that sufficiently many ORs expressed in female abdomen tip could be detected by qRT-PCR to warrant further examination by this approach.

2) Design and production of single guide RNAs.

Single guide RNAs (sgRNA) contain a target-specific nucleotide sequence of the gene of interest along with nucleotide sequence necessary for Cas9 (a protein that cleaves DNA) binding. Using the DNA sequence information above, the genome region containing target sites of the codling moth OR genes of interest will be identified in the published codling moth genome (Wan et al., 2019). With the gene sequence information, a minimum of five regions containing the features needed for CRISPR/Cas9 genome editing will be targeted for sgRNA design and production. Knowing OR gene structure from the codling moth sequenced genome makes designing sgRNAs a relatively easy task. This process has been streamlined through development of a web-based software, CRISPOR, that analyzes target gene input genomic sequence, and provides output of the best candidate sgRNA sequences from the target gene sequence (Concordet and Haeussler, 2018).

To generate sgRNAs specific to the selected codling moth OR genes, overlapping oligonucleotide primers containing the appropriate nucleotide sequence features will be designed and synthesized. These oligonucleotide primers will then be amplified in PCR reactions to generate a DNA template that will be used to produce sgRNAs in a test tube reaction. The sgRNAs are generated from the DNA template with an *in vitro* transcription kit which makes RNA from a DNA template. Once the sgRNAs are produced and purified, they are ready for use in genome editing experiments. Routine procedures for generating sgRNAs have already been developed in the laboratory (Garczynski et al., 2017).

3) CRISPR/Cas9 genome editing of codling moth OR genes.

CRISPR/Cas9 genome editing takes place when an appropriate sgRNA and Cas9 protein are present in the same cell. For this to occur, sgRNA and Cas9 mRNA are co-injected into early-stage eggs (freshly laid, within first 30 minutes after egg-laying). After injection, the Cas9 mRNA is translated to Cas9 protein by normal cellular processes, and the sgRNAs are transported to the cell nucleus by the Cas9 protein. Once in the cell nucleus, the Cas9 protein/sgRNA complex binds to the target gene and the double-stranded nuclease of Cas9 cleaves the gene creating a mutation that can ultimately result in the loss of protein function. Procedures for performing CRISPR/Cas9 genome editing in codling moth have already been developed (Garczynski et al., 2017). To knock-out our selected OR genes, target specific sgRNAs along with Cas9 mRNA will be co-injected into freshly laid eggs. The genome editing

takes place shortly after injection. At this stage, DNA will be extracted from neonate larvae to analyze the effectiveness of the genome editing (see below). It is important to note, that this initial analysis step is necessary because the efficiencies of individual sgRNAs are not equal, and is why at least five sgRNAs are designed for each target gene. The results of this initial analysis will identify the best sgRNAs for large scale knock-out experiments.

4) Analyzing the effectiveness of CRISPR/Cas9 genome editing using molecular techniques.
Two molecular techniques, DNA sequencing and high-resolution melt (HRM) analysis, are used to determine the effectiveness and extent of CRISPR/Cas9 genome editing, and to verify that the mutations made will knock out protein production (Garczynski et al., 2017). For each molecular technique, oligonucleotide primers surrounding the region of the gene being targeted are designed for PCR amplification. These oligonucleotide primers are first used in PCR reactions to amplify genomic DNA of treated and untreated insects, then the PCR products are cloned and sequenced to verify CRISPR/Cas9 generated mutations. Direct analysis through next-generation sequencing serves as a fast high-throughput alternative to the cloning and sequencing approach. A bioinformatic approach, using a software called CRISPResso (Clement et al., 2019) has been developed to facilitate streamlined analysis of the massive amount of data generated in this manner. Our laboratory has recently acquired a next-generation sequencing machine, which has been utilized in initial CRISPR experiments on codling moth ORs.

Once success of the CRISPR/Cas9 genome editing technique is verified by DNA sequencing, DNA from larger numbers of injected insects are analyzed with HRM analysis. To perform this assay, PCR amplifications of the targeted gene are done using a real-time PCR machine and the resultant PCR products are subjected to HRM analysis. HRM analysis is a high-throughput technique that is used to detect minor changes in the mutated gene when compared to its unaltered counterpart. Once CRISPR/Cas9 genome editing is verified molecularly, analyses of the effects of these mutations will be assessed using bioassays.

5) Assays to determine the effects of CRISPR/Cas9 genome editing on egg laying.
Oviposition in moths is a two-step process; females must first find their host plant, and after host recognition, oviposition sites need to be identified (Honda 1995). Ovipositing females use plant volatiles to locate the host plants and then use contact evaluation of plants to detect less or non-volatile chemical compounds to determine suitability for egg laying (Honda 1995). For codling moth, oviposition is stimulated by apple odor (Wearing 2016), and several volatiles in apple odors including α-farnesene, β-farnesene and nonanal have been specifically identified as stimulants (Sutherland et al. 1977, Witzgall et al. 2005). ORs expressed in the female antennae are thought to play important roles in host plant finding, bringing the moth in proximity of a suitable oviposition site. The hypothesis to be tested in this project evaluates whether specific ORs expressed in the abdomen tip are important for close-range evaluation in determining plant suitability for egg laying.

To determine if an OR gene knocked out by CRISPR/Cas9 genome editing has an effect on codling moth oviposition, a bioassay will be used. The assay uses jars or cups in which 10 mated females are placed in the presence or absence of varying concentrations of a volatile compound (see below for compounds to be tested). After one hour, females are removed from their container and eggs are counted. It is expected that oviposition stimulants will result in a greater amount of eggs laid vs controls. To determine which ORs play key roles in detecting oviposition stimulants, CRISPR/Cas9 edited females will be placed in the cups in the presence or absence of varying concentrations of volatile compounds and a positive result would be fewer eggs laid compared to unedited females. To determine statistical significance, full factorial analysis with concentration, treatment, and their interaction as the dependent variables. All statistical analyses will be done using SAS 9.4 with the GLIMIX procedure.

There are at least 64 volatile compounds found in headspace collections from apple at different phenological stages (Bengtsson et al 2001). Using the assay above, apple volatile compounds that produce antennal activity in females will be tested (Bengtsson et al 2001). These compounds are (Z)3-

hexenol, butyl butanoate, propyl hexanoate, hexyl propanoate, butyl hexanoate, hexyl butanoate, hexyl 2-methyl-butanoate, hexyl hexanoate, methyl salicylate, benzyl alcohol, 4,8-dimethyl-1,3,(E)7-nonatriene, β-linalool, β-caryophyllene, (E)-β-farnesene, germacrene D, (Z,E)-α-farnesene and (E,E)-α-farnesene, all of which are commercially available. In addition, compounds found in apple leaves will be also be tested, including a mixture of Theaspirane, (2R, 5R) and (2S, 5R), and geraniol, which acts as a codling moth repellant (Wei et al 2004). Initially, volatiles previously identified as oviposition stimulants, α-farnesene, β-farnesene and nonanal (Witzgall et al., 2005) will be tested and candidate ORs for these compounds will be edited.

6) Expected Outcomes.

Completion of this project will identify ORs expressed in the abdomen tips of female codling moth that play key roles in oviposition site selection. This information, along with identification of ORs expressed in female antennae that have key roles in attracting females to oviposition sites, will provide targets that may be manipulated for codling moth control. Examples can include: 1) development of new compounds that are more potent attractants which can be used to trap females; 2) development of compounds that block receptor activity to prevent females from finding oviposition sites; and 3) development of a system that attracts females (using a more potent attractant from example 1) to a platform that contains oviposition stimulants enticing the females to lay eggs away from apple. In the long term and when socially acceptable, these targets can be knocked out in wild populations using CRISPR/Cas9 technology are currently being commercially developed (Bayer Crop Sciences and DuPont are working on this technology).

RESULTS AND DISCUSSION

Identification and cloning odorant receptors expressed in female codling moth

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

We were not able to detect or confirm expression in adult female abdomen tips for 14 of the 38 ORs (36%) identified in the abdomen tip transcriptome. It is possible that some of these ORs are not expressed in adult female abdomen tip, but rather only in the abdomen tip of pupal stage, since pupal abdomen tips were also used to generate the source transcriptome. Given the overall objectives of this project, we did not conduct any PCR screening of ORs in the pupal stage. Alternatively, failure to detect OR expression by PCR in the female adult abdomen tips may be due to technical reasons, for example faulty oligonucleotide primers, or expression below detection thresholds. To rule these out, for ORs that we were unable to detect during initial PCR assays, we designed and tested additional oligonucleotide primers, and moreover, each primer set was subjected to serial rounds of PCR amplification in order to detect expression of transcripts that were otherwise below threshold detection levels after the first round of detection.

It was previously reported that a candidate pheromone receptor, OR1, was detected in female codling moth abdomen tips (Garczynski et al., 2017). Interestingly, we did not observe OR1 transcripts in our female abdomen tip transcriptome. It may be the case that OR1 displays restricted expression in codling moth abdomen tip neurons below thresholds levels for detection in our transcriptome. Because of this, we decided to conduct additional PCR screening of all candidate pheromone receptors, regardless of whether they were identified in our female abdomen tip transcriptome. In our previously published codling moth antennal transcriptome (Walker et al., 2016), a majority of these candidate

pheromone receptors displayed either male-specific or female-specific antennal expression and are thus hypothesized to influence sex-specific behaviors such as egg-laying in case of female specific ORs. Furthermore, one candidate pheromone receptor, OR3 was previously reported to be activated by host-plant volatiles, not pheromones (Bengtsson et al., 2014), opening up the possibility that other candidate pheromone receptors may also respond to host-plant odorants, and mediate behaviors such as egg-laying.

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

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

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

Oligonucleotide primers were designed for qRT-PCR assay to assess relative expression levels of 19 ORs in abdomen tip of unmated versus mated codling moth females. Initial testing using multiple sets of primers for each OR revealed that for 10 of these 19 ORs (53%), expression was not consistently detectable with the qRT-PCR assay. We thus pursued fully replicated qRT-PCR assays of the remaining 9 ORs (47%) from this subset to determine if mating affects their expression. It was determined that for one of the ORs, expression was significantly increased in abdomen tip of mated versus non-mated female codling moth, and for one other OR, expression was significantly decreased in abdomen tip of mated versus non-mated female codling moth. These two ORs will be subjected to further research on their role in codling moth egg-laying behavior through assays on OR protein function as well as CRISPR knock-out experiments. None of the other seven ORs that we assayed showed differences in expression in abdomen tip of mated versus unmated codling moth females.

Functional characterization of receptors expressed in female abdomen tip

Prior to initiation of this project, research was being conducted on functional characterization of ORs that displayed female-specific expression in antennae of codling moth. One of these female-specific antennal ORs, OR22, was determined to be activated by aldehyde odorants present in apple volatile collections, including nonanal (Walker et al., unpublished data), which has been shown to stimulate egg-laying behavior in codling moth (Witzgall et al., 2005). Further research on the functional capacity of OR22, in collaboration with the laboratory of Dr. Jason Pitts at Baylor University, has revealed that OR22 is also strongly activated by specific lactone compounds, in addition to activation by aldehydes present in apple volatile collections. Lactones are known to provide distinct odor characteristics present in other fruit that codling moth can infest, such as peaches and plums, however detection of these types of compounds by codling moth has not been previously reported.

Furthermore, our research on abdomen tip expression of ORs in female codling moth revealed that OR22 is also expressed in female abdomen tip, in addition to the antennal female-specific expression profile previously reported (Walker et al., 2016). Therefore, it is hypothesized that OR22 may have a dual role in detection of odorants that mediate both host-seeking behavior at as distance as well as close-range oviposition behavior. Current research efforts are focused on CRISPR editing of OR22 to disrupt its functionality and evaluate specific roles it plays in mediating olfactory behaviors in codling moth females.

Preliminary CRISPR experiments on female-specific odorant receptors

Five different guide RNAs (sgRNAs) were designed to target editing of different genomic regions of the codling moth OR22. Approximately 200 freshly laid codling moth embryos were injected with each sgRNA combined with the CAS9 mRNA. 40-60% of all injected embryos survived injections through at least neonate larval hatching. Larvae were provided with unrestricted access to standard artificial diet. In order to assess relative efficacy of CRISPR editing for each sgRNA, two different biological samples containing approximately 10 pooled third-instar larvae were taken from each sgRNA cohort. Genomic DNA was extracted from each sample, the appropriate genomic region was PCR amplified and subjected to high-throughput DNA sequencing in order to evaluate degree of editing induced by each sgRNA.

CRISPResso analysis revealed that substantial CRISPR editing was induced by three of the five sgRNAs in one or both of the biological samples selected for each sgRNA. These three sgRNAs have been selected for further experimentations. Current efforts are focused on using these sgRNAs to edit OR22 and generate stable colonies that are lacking in functional expression of the OR22 gene.

Conclusions

Analysis of OR expression in the female codling moth transcriptome has been completed. A repertoire of over thirty ORs expressed in the abdomen tip has been confirmed through molecular cloning and sequencing analysis. Rigorous quantitative expression analysis has resulted in identification of two ORs that display expression patterns in the abdomen tip that are modulated by mating. A single OR that is expressed in both female antennae and abdomen tip is observed to respond to fruit-derived odorants, including some that have not been previously reported to be detected by codling moth. Research is planned during the year 3 field season to evaluate how these odorants affect codling moth behavior in apple orchards. Current efforts in the laboratory are focused on CRISPR editing of the OR22 gene and subsequent consequences on olfactory behaviors when this gene is disrupted. Further research is planned for later in year 3 aimed at CRISPR-editing disruption of other OR genes that display mating-affected expression patterns in the abdomen tip. Collaborative research efforts will be aimed at identification of odorant ligands that activate these receptors, as well as testing the effects of CRISPR-based disruption of them on codling moth oviposition behaviors.

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

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

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

Cooperators: Dr. Johannes Jehle, Julius Kühn-Institut, Darmstadt, Germany (Scientific Advisor, CpGV expert); River Bioscience, Port Elizabeth, South Africa (CrpeNPV supplier)

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Budget 1
Primary PI: William Walker
Organization Name: USDA-ARS
Contract Administrator: Chuck Meyers
Telephone: 510-559-5769
Contract administrator email address: Chuck.myers@usda.gov
Station Manager/Supervisor: Rodney Cooper
Station manager/supervisor email address: rodney.cooper@usda.gov

Item	2021	2022	2023
Salaries	$40,089	$41,425	$42,762
Benefits	$14,031	$14,499	$14,967
Wages			
Benefits			
Equipment			

Supplies	$4,076	$4,076	$4,075
Travel			
Miscellaneous			
Plot Fees			
Total	$58,196	$60,000	$61,804

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.

Admixtures of different strains of CpGV will be used to co-infect codling moth cell culture lines. This will yield viruses that are genetic hybrids, with properties of the different viruses combined in novel ways. Isolates of these hybrids will be cultivated, exposed to codling moth larvae and screened for efficacy. Those displaying enhanced effectiveness will be genetically characterized and selected for formulation and further assay, with eventual applicability in both conventional and organic orchards. For authorized use in organic orchards intended products would be submitted to appropriate Material Review Organizations for official registration.

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

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

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

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

SIGNIFICANT FINDINGS

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

METHODS

1) CpGV Hybridization Studies

Procedures: Five CpGV strains, CpGV-M (A), CpGV-E2 (B), CpGV- I07 (C), CpGV-I12 (D), and CpGV-S (E), representative of the five different known CpGV genomic subtypes, A through E (Eberle et al., 2009, Gebhardt et al., 2014), will be obtained and used for genetic hybridization experiments. To establish baseline mortality metrics to compare with genetic hybrids, infection assays will be done with each of the five strains independently, using newly hatched, neonate larvae, as it is this stage in which larvae externally feed on leaves before entering the apple; non-infected neonate larvae will also be assayed as a further control. To standardize viral titers, each strain will be cultivated through infection of a codling moth-derived cell culture line, Cp14 (Winstanley and Crook, 1993). Infected cell culture can then be used to directly inoculate codling moth larval diet (artificial diet or apple leaves). Dose response and time-course studies will be conducted, in which larvae will be permitted to feed on virus-inoculated food, for varied lengths of time (10 minutes, 30 minutes, 1 hour, 2 hours, 5 hours), and viral dosage. Larval death rates after three hours post infection (hpi), average post-exposure time to morality and percentage of larvae dead due to granulovirus infection will be measured: precise measurements

may be taken with use of video tracking equipment present in our laboratories. These will provide mortality metrics that may inform applicability of treatment in orchard settings (Hinsberger et al., 2020) wherein successive generations of codling moth larvae experience shorter periods of external leaf-feeding before entering the apple (Burgerjon, 1986).

For the genetic hybridization experiments, aliquots of all five strains will be mixed in equal ratios and used to inoculate and infect a codling moth-derived cell culture line, Cp14 (Winstanley and Crook, 1993). After a sufficient viral inoculation period (5-8 days), infected cell culture material will be used as substrate for viral plaque assays (Harrison and Lynn 2008) that have been developed to allow individual viral hybrid genotypes to be purified. Pure isolate strains may then be re-cultured and subsequently used for codling moth infection assays, which will be conducted as described in the preceding paragraph for the parental viral strains. For hybrid strains that are more effective against codling moth larvae than parental strains, genotyping experiments will be performed to determine how different parental strains have hybridized, and which genes have been affected. This would include analysis of restriction fragment profiles of hybrids as compared to parental strains (Winstanley and Crook, 1993) as well as DNA sequencing.

Expected Results: During the first year of the project, initial experiments will be conducted, aimed at generating hybrid strains of CpGV in Cp14 cell culture. While hybridization of different strains of CpGV have, to our knowledge, never been studied, it is expected that hybrid strains will be recovered for further utility in larval infection assays. These assays will initially be conducted with larvae from an in-house codling moth colony that has no known resistance to CpGV. During the second and third years of the project, and in consultation with local growers, codling moth specimens from local orchards with suspected/demonstrated CpGV resistance or diminished CpGV efficacy will be collected, reared for one generation, and emergent neonate larvae will be tested in the same way as our laboratory colony. Furthermore, specimens from lab colonies known to be resistant to CpGV (Asser-Kaiser et al., 2007, Sauer et al., 2017a, 2017b) will be obtained and tested in our quarantine laboratory to examine how hybrid viral strains may facilitate resistance-breaking. These experiments would be critical to assess if hybrids that contain genetic material from multiple parental strains are able to successfully infect resistant codling moth populations that their corresponding parental viral strains cannot. Considering that five different CpGV genome group subtypes have been identified and different resistance-breaking patterns have been observed across these different groups, it is expected that hybrid stains cultivated across the subgroups may yield improved efficacy against codling moth larvae.

Potential Problems and Contingencies: While insect cell cultures are generally immune from contamination by human viruses, bacterial contamination may occur, as may other issues with culturing the cells in suitable growth media. Substantial issues with cell culture propagation, while not anticipated, may thus hinder the research. With that in mind, it is widely known within the field of research on insect baculoviruses that the insect larvae themselves may be used to cultivate baculovirus stock. In this case, multiple parental strains may be used to co-infect individual codling moth larvae, after which potential hybrids may then be purified from infected larvae and used for further research. It has previously been demonstrated for other GV types that genetically hybrid virus isolates may be purified from infected larvae (Smith and Crook, 1993) without the use of cell culture cultivation and plaque assay purification. In this case larger fifth-instar larvae would be used to culture the virus. This methodology may be wholly suitable for laboratory research within the scope of this project if persistent problems are encountered with the cell-culture.

In order for genetic hybridization of different viral strains to occur during cell culture experiments, it is required that individual cells be infected with two or more viral particles of different strains at the same time. To our knowledge, this has not previously been directly studied with different strains of CpGV, so it is uncertain to what degree it may happen. For NPV baculoviruses it has been reported that hybridization can occur at very high frequencies (Hajós et al., 2000). Hybridization has also been reported for GVs, with indirect evidence for hybridization of CpGV with another GV viral type (Jehle et al., 2003). It is thus likely that our hybridization experiments will be successful after optimization.

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

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

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

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

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

Potential Problems and Contingencies: Based upon previous experience of Walker and Neven with viral genetic engineering and insect culture-based viral production, it is anticipated that the goals

of this objective will be achieved. However, potential methodological problems may arise. The Bac-to-Bac baculovirus engineering system is standard to the point of being commercialized, so no problems are anticipated in the genetic engineering phase of the project. However, if problems do arise in the methodology, other methods of viral genetic engineering, such as homologous recombination (Hilton et al., 2008) may be utilized to insert the *Hvt* gene into the CpGV bacmid.

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

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

3) CpGV and CrpeNPV co-infection studies.

Procedures: It is hypothesized that co-infection of CpGV and CrpeNPV in codling moth larvae will result in enhanced infectivity and mortality, above and beyond that observed with CpGV alone. This principle has been demonstrated in other viral/host infection systems, such as with co-infection of fall armyworm, *Spodoptera frugiperda*, with a GV and NPV (Cuartas-Otálora et al., 2019). For these experiments, the most effective parental CpGV strain identified from objective one will be utilized, and CrpeNPV will be obtained from the source laboratory that initially reported it (Marsberg et al., 2018). To establish baseline mortality metrics to compare with the co-infection studies, infection assays will be done with CpGV and CrpeNPV individually, using neonate larvae. For both CpGV and CrpeNPV, virus titers will be established through infection of Cp14 cell culture, as it has been demonstrated that CrpeNPV is fully capable of being cultivated in Cp14 (Wennmann et al., 2019). Codling moth larval co-infection studies of CpGV and CrpeNPV will be conducted with an empirically assessed range of different ratios of the two virus types, combined.

Infected cell culture will be used to directly inoculate codling moth larval diet (artificial diet or apple leaves). Varied-ratio and time-course studies will be conducted, in which larvae will be permitted to feed on viral-inoculated food for varied lengths of time (10 minutes, 30 minutes, 1 hour, 2 hours, 5 hours), and ratio mixtures. If an optimal ratio of CpGV and CrpeNPV is identified, further optimization trials will be conducted via dose-response assays at the optimal ratio. Larval death rates after three hours post infection (hpi), average post-exposure time to morality and percentage of larvae dead due to granulovirus infection will be measured; precise measurements may be taken with use of video tracking equipment present in our laboratories.

Expected Results: During the first year of the project, optimization of CpGV and CrpeNPV co-infection parameters will be assessed with experiments conducted in an in-house codling moth colony that has no known resistance to CpGV nor CrpeNPV. Given that co-infection of moth larvae with GV and NPV viruses has been reported to increase viral efficacy (Cuartas-Otálora et al., 2019), it is expected that we will observe this in codling moth as well. During the second and third years of the project, and in consultation with local growers, codling moth specimens from local orchards with suspected/demonstrated CpGV resistance or diminished CpGV efficacy will be collected, reared for

one generation, and emergent neonate larvae will be tested in the same way as our laboratory colony, using optimally determined ratios and dosages as determined during the first year's experiments. As a positive control, specimens from lab colonies known to be resistant to CpGV (Asser-Kaiser et al., 2007, Sauer et al., 2017a, 2017b) will be obtained and tested in our quarantine laboratory to examine how mixtures of CpGV and CrpeNPV may facilitate resistance-breaking by the engineered viral strain.

Potential Problems and Contingencies: One issue with culturing baculoviruses in different cell culture types concerns the long-term genomic stability of different viruses cultivated in cell cultures derived from non-host insects. While it has been demonstrated that CrpeNPV can be cultivated in the codling moth derived Cp14 cell culture, this has not been examined in long term studies (Wennmann et al., 2019). Genomic instability may lead to diminishing returns with respect to virulence against target insects. This can be assessed during the course of this project with continuous cultivation of CrpeNPV in Cp14 and periodic efficacy trial experiments conducted on codling moth larvae each year, to determine if there is a decrease in efficacy against codling moth larvae. Genomic instability may also be directly assessed through examination of restriction fragment patterns for CrpeNPV DNA in conjunction with the efficacy trials.

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

RESULTS AND DISCUSSION

Administrative delays on multiple fronts have effectively prevented the initiation of this project until current time. Primarily, there has been a substantial delay in hiring technical support staff funded by this project that is essential to perform research for all of the objectives. The process to initiate hiring on our side was launched months ago directly after the funding became available, however it has still not been completed due to administrative delays in processing by the HR department. This is currently being resolved, as documents required to formally announce the position were received by us to confirm from HR in the closing days of December. It is therefore anticipated that the hiring process will be completed within the next month. Additionally, specific CpGV genetic materials required for genetic engineering of spider toxin (objective 2) are only available from a single laboratory in Germany. Thus, a Material Transfer Agreement (MTA) was required to obtain these materials, and there were delays associated with formalizing the agreement. In December, the agreement was finalized, and necessary materials will soon be received. This will allow the proposed genetic engineering of CpGV to commence in our genetics laboratory even prior to onboarding of new technical support staff, such that when the technical support is in place, experimentation with the genetically engineered CpGV may begin immediately. Finally, in the process of attempting to obtain the novel CrpeNPV (objective 3) from the researchers who reported its discovery, it came to our knowledge that a company in South Africa, River Bioscience, was in the process of commercializing this virus for usage against false codling moth and other crop pest insects. Thus, it has been required to negotiate a Material Transfer Research Agreement in order to obtain the CrpeNPV for our research purposes. This agreement has been more difficult to resolve than the aforementioned MTA, and thus the process of finalizing the agreement is still ongoing. It is anticipated that this agreement shall be completed in the coming next months. Thus, at present time there are no results to present and discuss.

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

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

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

Cooperators: Steve Arthurs (BioBee), Chuck Weaver (Parabug), Mike Brown (Gebbers Farms), Nick Willett (Gilbert Fruit) [note: pear grower cooperators will be specified in pear report]

Report Type: Continuing Project Report

Project Duration: 2-Year

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

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

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

Other funding sources:	**Awarded**
Amount:	$29,968
Agency Name:	Western IPM Center, project initiation grant
Notes:	This project expands the efforts in this grant by providing support to conduct grower input sessions and a needs assessment survey. The WIPMC grant will also be used to start a grant team and stakeholder advisory group that will submit a federal grant application to expand this work (likely to USDA OREI). The data collected in this grant

will be used as preliminary data in the OREI submission. The results in this report are due to this grant award.

Other funding sources: **Awarded**
Amount: $348,733
Agency Name: Western SARE
Notes: This is a complementary (non-overlapping) project, specifically focusing on earwig releases in apple and pear, on the ground and by drone.

WTFRC Collaborative Costs: none

Budget 1*
Organization Name: USDA-ARS **Contract Administrator:** Chuck Myers
Telephone: 510-559-5769 **Email address:** Chuck.Myers@usda.gov
Station Manager/Supervisor: Rodney Cooper **Email Address:** rodney.cooper@usda.gov

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

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

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

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

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

OBJECTIVES

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

2. Determine cost-effectiveness and efficacy of natural enemy release by drone. One method for reducing natural enemy release labor costs is to conduct releases by drone. However, the ability of natural enemies to survive release by drone into orchards and whether this method significantly decreases natural enemy abundance relative to hand-releases is unknown. We will compare released predator abundance, pest control levels, and labor costs for releases by hand and by drone of lacewings and mealybug destroyers in apples.

SIGNIFICANT FINDINGS

- We were unable to hire a qualified postdoc in time to start this work in 2021 due to multiple reasons: timing of receipt of Fresh and Processed Pear funding, delays in WSU grant processing, USDA COVID restrictions (we are currently unable to have non-citizens cleared to work in the building, so all non-citizen applicants were unable to qualify), and a limited number of qualified applicants, which seems to be a general trend for hiring postdocs at this time.

- To address this issue, we expanded our search to include an associate in research position to manage this project, which would only require a M.S., expanding our candidate pool. We have identified a suitable candidate and are in the process of making an offer to her.

- Because of funding through Western IPM Center (see "Other funding sources") we were able to still collect some data which will inform how we conduct this project starting in 2022. All results in this report are the result of the related WIPMC project.

- We were able to conduct a second year of work in 2021 on mealybug destroyer releases in large, one-acre apple plots, comparing drone versus ground releases of 1,000 mealybug destroyers per acre to a no-release control. Although some mealybugs were detected in the fruit at harvest, during the growing season, there were <0.04 mealybugs/trap. We also found very few mealybug destroyers immediately after release (1 day) and no mealybug destroyers 8 days after release; they likely dispersed due to lack of prey. This indicates that mealybug numbers must be fairly substantial for releases to work.

- This contrasted with our 2020 results, where releases of mealybug destroyers decreased mealybug populations. However, in that study, there were up to 6 mealybugs/trap during the growing season.

- In 2021, we also tested releases in apple of two species of lacewings as eggs or larvae: *Chrysoperla rufilabris* and *Chrysoperla carnea*. Through examining the materials, we found that the *C. carnea* larvae (which came from a different insectary than the eggs) were actually *C.*

externa. While lacewings in the *C. carnea* species group are suited to our arid climate, *C. externa* is not. This quality control issue was reported to the insectary.

- A release of *C. carnea* as eggs (100,000/acre) was the most successful treatment at suppressing woolly apple aphid and green apple aphid in this study. A release of *C. rufilabris* larvae were also effective (20,000/acre). Seasonal counts of aphid colonies were reduced by 57% and 43%, respectively.

- As part of the WIPMC project, we also collected survey data on apple and pear grower perspectives of releasing natural enemies in tree fruit. To date, the survey has collected 127 responses. Four stakeholder input sessions have been conducted (Omak, Wenatchee, Yakima, and Hood River) and a final session will be conducted in Medford on January 7th. Feedback from the survey will be used to determine future research directions and to obtain federal funding to expand the work in this project.

METHODS

This work will now initiate in 2022 due to hiring difficulties in 2021. All work will be conducted by the associate in research, supervised by Schmidt-Jeffris. The associate will also conduct the pear portion of the work, under the guidance of Nottingham. The summer technician (WSU) will assist with plot set up and data collection in all Wenatchee-area trials. The USDA technician will assist with data collection, plot set up, and processing of PCR/lab samples. The pear work will mirror the work conducted in apple, with pear psylla as the target pest, with some changes to the natural enemies released. Details will be provided in the pear grant, the work described here is for the apple portion of the study. BioBee (Steve Arthurs) will supply the natural enemies for releases and Chuck Weaver (Parabug) will pilot the drone in Obj. 2.

1. Improve retention of released natural enemies.

This two-year (2022-2023) study will be conducted in a commercial organic apple orchard in Wapato, WA (Gilbert Fruit) which was selected due to annually high woolly apple aphid (WAA) pressure. The grower annually conducts natural enemy releases for WAA control. The release day will target when aphid populations begin to rise and the typical timing the grower releases lacewings, approximately mid-May. There will be a total of six treatments made of combinations of lure use and food supplements: use of a methyl salicylate lure (Predalure) – yes/no × commercially available food supplements – Nutrimite (pollen), Artemac (brine shrimp cysts), or none. Each combination will be replicated in the orchard 5 times for a total of 30 plots. Each plot will consist of 0.5 acres, which is the maximum plot size for this orchard. There will be a minimum of 3 rows or 500 feet between plots. One week prior to release, we will conduct precounts of aphids by counting the number of WAA, green apple aphid, and rosy apple aphid colonies per 4 shoots on 10 trees in the center of the plot. Treatments will be assigned to plots using pre-release levels of aphids to ensure initial aphid abundance does not differ between treatments for a fair comparison. At this point, one methyl salicylate lure will be added to four trees in the center of each plot to allow the volatiles sufficient time to dissipate prior to releasing the natural enemies. One week after this, we will apply Nutrimite and Artemac to the 10 center trees of each plot at the insectary recommended rate. Artemac will be applied by tying tape with attached cysts to trees and Nutrimite will be applied by using the insectary-recommended handheld blower. Then, we will release two natural enemy species across the entire trial at insectary recommended release rates: 100,000 *Chrysoperla carnea* eggs per acre (green lacewing, BioBee) and 5,000 *Orius insidiosus* per acre (minute pirate bug, BioBee), using typical ground-releases by ATV. Post-release sampling will occur at 3, 7, and 14 days after release, with additional sampling on alternating weeks if treatment differences continue to be observed. Aphids will be sampled as previously described. Beat tray samples will be collected from the 10 center trees of each plot. All natural enemies from the tap counts will be collected and stored in ethanol.

Lacewings and *Orius* collected will be identified to species in the laboratory to determine if they are from the insectary. These specimens and all other natural enemies collected will be used for gut content analysis to determine: 1) if released beneficials are consuming pests at high rates and 2) if either released beneficials or resident natural enemies are consuming the nutritional supplements. We will also place three sticky cards on trees within the center of each plot to count key natural enemies to species (released *O. insidiosus* and *C. carnea*, but also resident lacewings, other anthocorids, and syrphid adults).

2. Determine cost-effectiveness and efficacy of natural enemy release by drone.
This two-year (2022-2023) study will be conducted in a commercial organic apple orchard in Mattawa, WA (Gilbert Fruit). This orchard has annual problems with mealybugs and WAA and was the site of a 2020 pilot study releasing mealybug destroyers. We propose to continue this work and get a better sense of efficacy by increasing plot sizes to one acre and sampling from the center of plots. We also would like to test efficacy of lacewing releases for WAA control, although they should also provide some control of mealybugs. The treatments will be 1) mealybug destroyer drone release, 2) mealybug destroyer ground release, 3) lacewing (*C. carnea*) drone release, 4) lacewing ground release, and 5) no-release control. There will be five one-acre replicates per treatment (25 plots total). One week prior to release, mealybug and aphid counts will occur and treatments will be randomized based on pest levels. We will use the release rate of 2,000 mealybug destroyers per acre and release at the "early" timing used in the previous study. This is also roughly when WAA populations begin to increase, so the mealybug destroyers and lacewings will likely be released on the same day. Lacewings will be released at the 20,000 larvae per acre rate. Ground releases will be conducted by ATV and the amount of time spent conducting the release in each replicate will be recorded. Mealybugs and mealybug ovisacs will be counted by two methods: shoot samples and burlap samples; these were the most effective methods in the 2020 trial. Shoot samples will consist of 30 shoots collected from 10 trees in the center of each plot. Burlap samples will consist of a 15 cm wide piece of burlap tied to the main limb of the tree, on 30 trees per plot (Grasswitz and Burts 1995). They will be evaluated one week after placement. Aphids will be counted using the 5-minute search previously described. All stages of lacewings and mealybug destroyers will be sampled with beat trays; beat samples will be conducted on 10 trees in the center of each plot. Lacewings and mealybug destroyers collected will be stored in alcohol and examined in the lab to confirm that they are the released species. All sample types will then be collected once weekly for four weeks following releases. Fruit damage and infestation (i.e., insects in the stem or calyx end) will be assessed prior to harvest by examining 5 fruit from the 10 center trees of each plot. We will compare cost of release by drone versus by ground for each species and use pest control levels to determine which release method has the best combination of cost effectiveness and efficacy.

Methods used in the 2021 field studies

These methods have some overlap with the Obj. 2 methods described above and will allow us to make modifications to improve these trials. Because of the preliminary work we conducted in 2021 while unable to hire a postdoc for this project, we have identified some key areas for improvement. First, we will examine differences in releases of *C. carnea* versus *C. rufilabris* for aphid management. We identified better locations with higher pest pressure to conduct these trials in 2022. We determined that sampling for adult lacewings will need to be more intensive (more traps, use of lures) to ascertain if released juveniles establish. Finally, instead of timed aphid counts, we will use number of colonies per shoot on sampled trees, as this method was more effective during our 2021 trial.

Mealybug destroyer releases

In 2021, we conducted releases of mealybug destroyers in an organic, commercial apple orchard in Pateros, WA. Plots were 1 acre, separated by at least 208 feet, and each treatment was replicated 5

times. The treatments were 1) ground release of 1,000 mealybug destroyers per acre, 2) drone release at dusk of 1,000 mealybug destroyers per acre, and 3) a no-release control. Releases were scheduled to be conducted on 12 May 2021, but were delayed due to shipping issues and were instead conducted on 25 May 2021. Mealybugs were sampled by tying one burlap strip trap to 20 center trees in each plot and by collecting one shoot sample from each of these trees. Mealybug destroyers were sampled by tap sampling 10 trees from the center of each plot (3 taps per tree). Samples were conducted once weekly, with the final sample collected on 7 July 2021.

Lacewing releases

In 2021, we conducted releases of lacewings in an organic, commercial apple orchard in Pateros, WA. Plots were 0.25 acres, separated by at least 104 feet, and each treatment was replicated 5 times. The treatments were single releases of 1) 100,000 *Chrysoperla rufilabris* eggs/acre, 2) 20,000 *C. rufilabris* larvae/acre, 3) 100,000 *C. carnea* eggs/acre, 4) 20,000 *C. carnea* larvae/acre, and 5) no-release control. Releases were conducted on 5 May 2021. Aphids were sampled by counting the number of colonies (WAA) or infested leaves (green apple aphid) on 3 1-foot shoots per tree, for 12 trees located in the center of each plot. Rosy apple aphid was not present. Lacewings were sampled by performing tap counts on one limb from each of the 12 trees (3 taps per limb). Collected adults and larvae were placed in vials with ethanol for later identification. Adult lacewings were also sampled by hanging two sticky cards in each plot. Counts were conducted once weekly, with the final sample collected on 30 June 2021.

RESULTS AND DISCUSSION

These results are from the WIPMC grant conducted in 2021, which will be used to inform the work done on the project objectives initiated in 2022. An excellent benefit of conducting these initial trials is that we have identified more suitable locations to conduct this research, which have very large populations of both aphids and mealybugs, especially compared to our previous research sites.

Mealybug destroyer trial. Neither release treatment lowered mealybug counts compared to the control. This is likely because mealybug populations were very low (<0.04 mealybugs per trap, compared to up to 6 mealybugs per trap in the 2020 trial). Low mealybug populations likely caused the low establishment of mealybug destroyers. Although mealybug destroyers were found in low numbers through most of the growing season in the preliminary 2020 trial, in the 2021 trial we were unable to find any mealybug destroyers the week after release or any weeks following. Our results indicate that mealybug destroyers are only effective predators when mealybug populations are higher and therefore may only be useful in orchards where there is a serious, reoccurring issue with this pest.

Lacewing trial. The *C. rufilabris* were the species that were advertised. The *C. carnea* eggs came from an insectary in Mexico, whereas the larvae came from an insectary in Canada. The *C. carnea* eggs were indeed a species in the *carnea* species group (molecular work will be needed to determine exactly which species). However, the *C. carnea* larvae were *C. externa*, a species not in the *carnea* species group (Fig. 1). This is a known issue with insectaries, as lacewings within the genus

Chrysoperla, especially in the *C. carnea* species group, are very difficult to identify without the expertise of a specialized taxonomist.

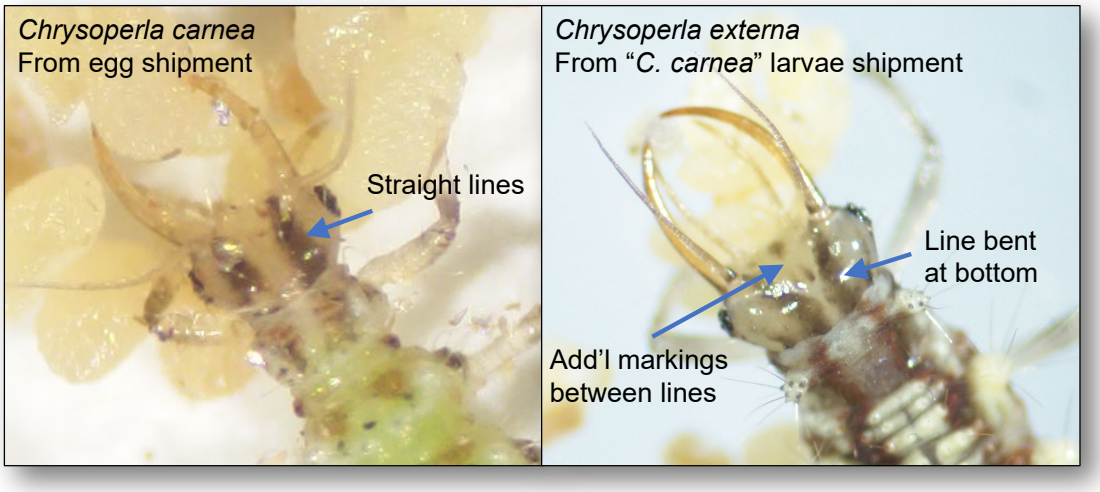

Fig. 1. Distinguishing between lacewing species received in shipments from insectaries. Head capsule markings are the most common way to distinguish between species in *Chrysoperla*.

The *C. carnea* eggs and *C. rufilabris* larvae resulted in lower aphid populations compared to the control, whereas the other two treatments did not ("*C. carnea*" larvae and *C. rufilabris* eggs) (Fig. 2-4). More trial work will be needed to determine if this is a consistent pattern; there is always the possibility that a particular order of insects is going to be better or worse than another, due to either changes in health of a colony or differences in how the package is stored during shipping. Because the larvae were refrigerated overnight (to prevent cannibalism), while eggs were stored at room temperature (to encourage egg hatch) differences in treatments may also be due to variation in storage requirements.

Lacewing larvae from the releases were found up to a month after the release occurred. We recovered *C. rufilabris* from the "*C. rufilabris* larvae" treatment the most often, but never found any *C. rufilabris* larvae in the treatment where eggs were released. We found larvae of the correct species in all other plots. We also found several species of native, non-released *Chrysopa* lacewings, which appear to have a healthy population in the orchard. *Chrysopa* larvae were not found until three weeks after our releases and then in lower numbers than our released lacewings. This indicates that our treatments gave this orchard a headstart in aphid management compared to the no-release control. All adult lacewings that were found during the course of the trial were *Chrysopa*, therefore we do not yet have evidence that the juvenile lacewings released ever fully developed. However, recovery of lacewings in general was low, so they many have been present and not found. We will use these results to inform our sampling efforts for 2022-2023. In particular, adult sampling will be more intensive (we kept this minimal in 2021 to avoid removing too many released lacewings from plots) and we will bait at least one trap per plot with lacewing lures.

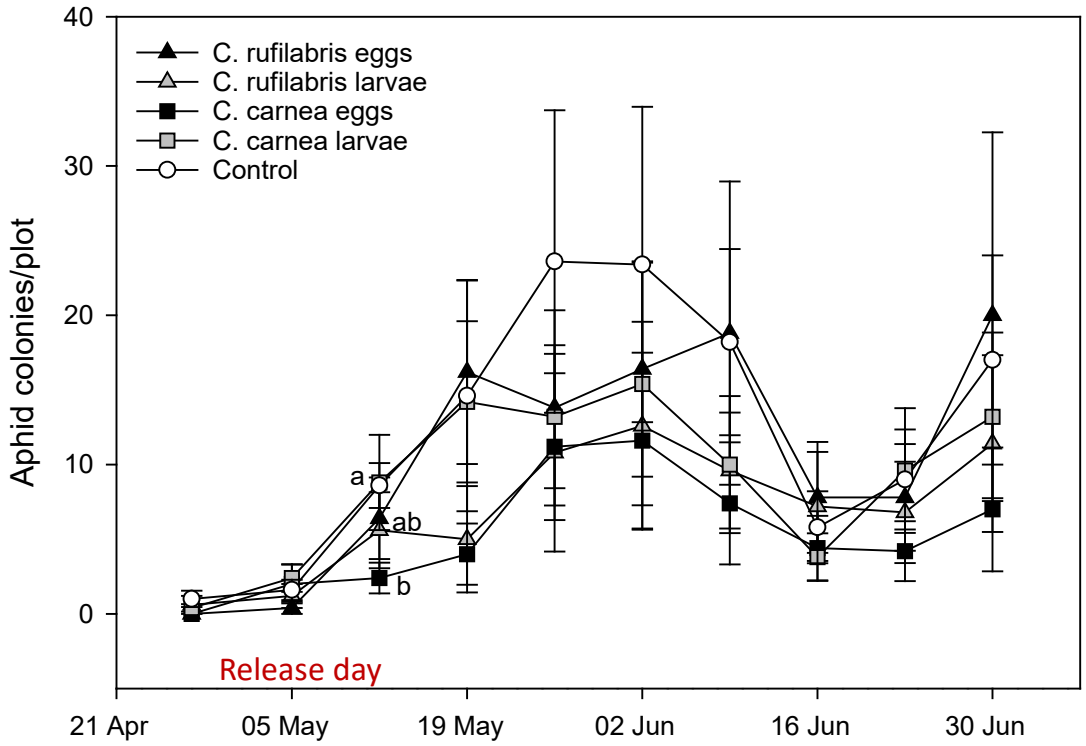

Fig. 2. Aphid colonies (WAA and green) per plot in lacewing release trial.

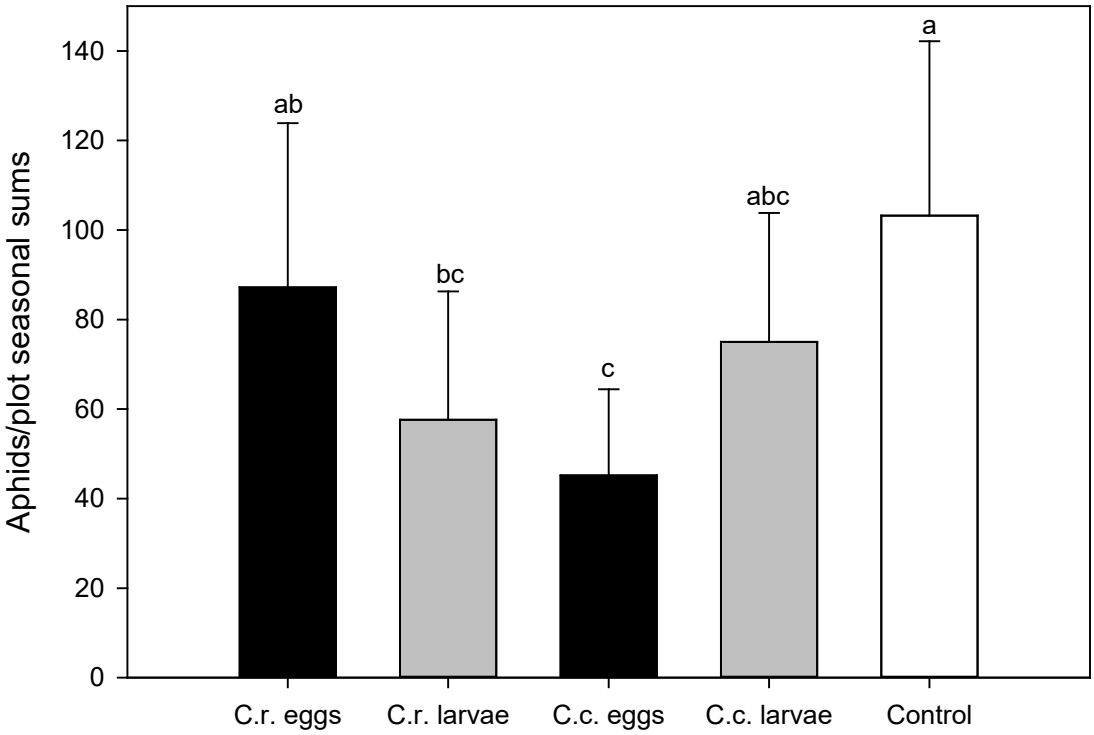

Fig. 3. Aphid colonies (WAA and green) per plot summed across the season in lacewing release trial.

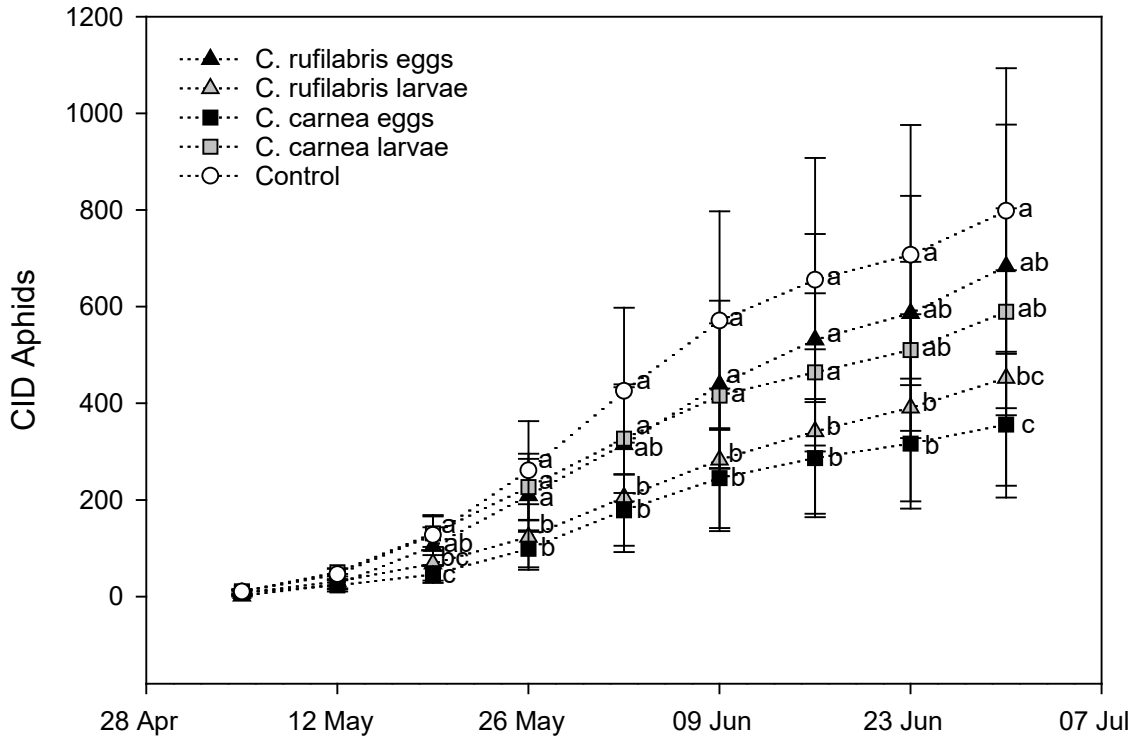

Fig. 4. Cumulative insect days (CID) for aphids in the lacewing release trial.

Project/Proposal Title: Integrated control of brown marmorated stink bug

PI: Elizabeth H. Beers
Organization: WSU-TFREC
Telephone: 509-679-1010:
Email: ebeers@wsu.edu
Address: 1100 N. Western Ave.
Address 2:
City/State/Zip: Wenatchee, WA 09901

Cooperators: None

Report Type: Continuing Project Report

Project Duration: 3 Years

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

Other related/associated funding sources: Awarded
Funding Duration: 2017 - 2021
Amount: $9,164,908; years 3-5 (current): $5,477,389; WSU/Beers 2019-2021 total direct budget: $156,047
Agency Name: USDA-NIFA-SCRI

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

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Elizabeth Beers
Organization Name: Washington State University
Contract Administrator: Stacy Mondy
Contract administrator email address: arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

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

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

Objectives

1. *Investigate the efficacy and non-target effects of insecticide infused netting as a means of monitoring and control of BMSB.* Captures of BMSB in interior traps in blocks protected by attract and kill (A&K) traps were consistently lower than in blocks not protected by traps. Fruit damage in the protected blocks was 50% lower than the unprotected blocks.
2. *Redistribute Trissolcus japonicus (the samurai wasp) where established BMSB populations are identified, and monitor its establishment and non-target effects.* A total of 4,130 *T. japonicus* were released in Washington in 2021, with release sites including Puyallup, Spokane, and the mid-Columbia. The total number of wasps released (2017-2021) is 13,469 in both urban and agricultural areas. Yellow sticky cards were deployed in 2021 (data collection still in progress) to determine establishment from previous releases.
3. *Determine development of BMSB on shrub-steppe plants.* A second year's study of development on shrub-steppe plants indicated clearly there is a dietary penalty for BMSB development on eastern Washington's native plants. This studied was followed by an additional study on the adult reproductive stage to verify if the lower adult weight correlated with poorer fecundity. We will continue these studies in 2021 using native stink bugs.
4. *Track the invasion of BMSB in Washington State*. The online reporting system for BMSB finds in the state recorded 74 reports in 2021 (variable numbers of individuals/report). We also did targeted sampling of BMSB in areas where it was known to co-occur with native species; the 219 specimens collected in this sampling effort will be subjected to gut content analysis to determine niche overlap of BMSB and native stink bugs.

Significant Findings

- We released 4,130 *T. japonicus* in two urban areas (Puyallup, Spokane) and two agricultural areas (both in Klickitat counties) in 2021.
- Using PCR and morphological methods, we found that *T. japonicus* had higher total impact (reproductive and non-reproductive) on the native spined soldier bug (a predator) than on BMSB.
- Deployment of insecticide-infused netting attract-and-kill (A&K) traps resulted in a 50% reduction in BMSB fruit damage in protected blocks.
- The addition of lights to pheromone traps did not increase trap capture.
- BMSB fed on native shrub-steppe plants from the egg to adult stage were smaller, slower to develop, and had poorer survivorship when compared to BMSB fed on plants typical of the mid-Atlantic. In addition, the resulting western-diet adults were shorter-lived and laid fewer eggs.
- A total of 74 reports of BMSB were made from citizens around the state in 2021, compared to 61 in 2020, and 146 in 2019; Additional target sampling yielded ca. 100 specimens. A total of 29 Washington counties have now reported BMSB (no change from 2019).

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

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

Thus, development of tactics that are compatible with biological control are the highest priority for BMSB.

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

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

Plate 2. LLIN net in a pear

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

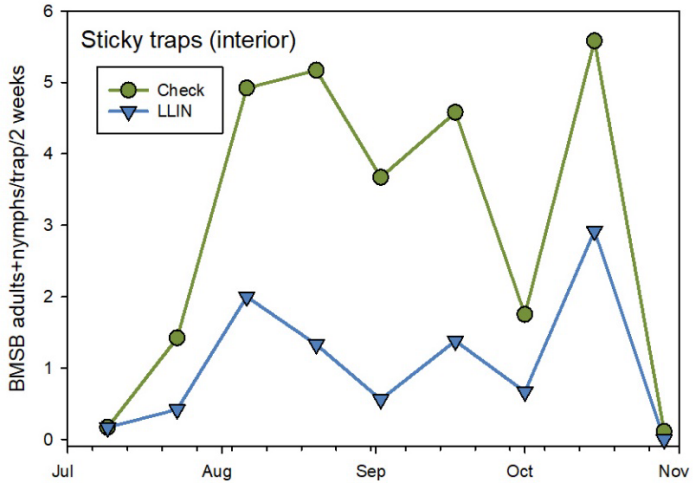

Fig. 1. BMSB captured in the interior of plots protected by A&K traps (LLIN) versus the unprotected (check) plots.

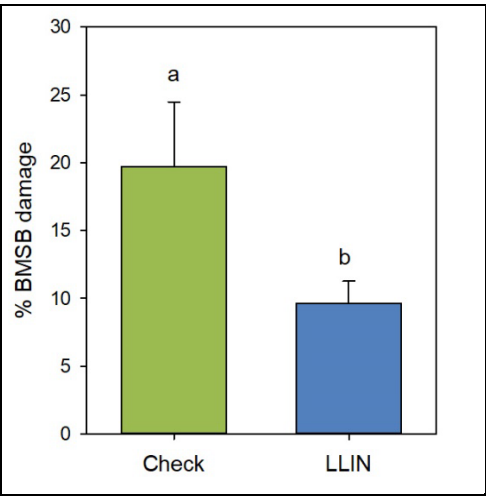

Fig. 2. Fruit damage caused by BMSB in LLIN versus check plots.

1b. Physical exclusion, net barriers. This objective was completed in 2019.

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

2a. Redistribute the samurai wasp in Washington State. *Methods:* Because the majority of reports of BMSB come from urban areas, we focused our release efforts there. In 2021, we released *T. japonicus* (mostly as adults, occasionally as parasitized eggs close to hatch) in Puyallup and Spokane. Our goal for release in those sites is that when BMSB spread from urban to agricultural areas (the pattern observed in other parts of the nation), their parasitoid will move with them. This may effectively constitute a *pro-active* release for agriculture. In addition, we released *T. japonicus* in two agricultural areas where is has been detected for one or more years, both in Klickitat County. We deployed 18 yellow sticky traps to determine establishment of *T. japonicus* in several previous release sites (data collection still in progress).

Plate 2. Release of the samurai wasp in Washington

Results: We released a total of **4,130** adult *T. japonicus* in June-August of 2021, in Puyallup (700), Spokane (1,600), and Klickitat counties (1,830) (Plate 2, Fig. 3). The numbers released were dependent on the availability of egg masses from the BMSB colony. All of the 2021 releases were from the 2020 collection from one of the original sites in Vancouver.

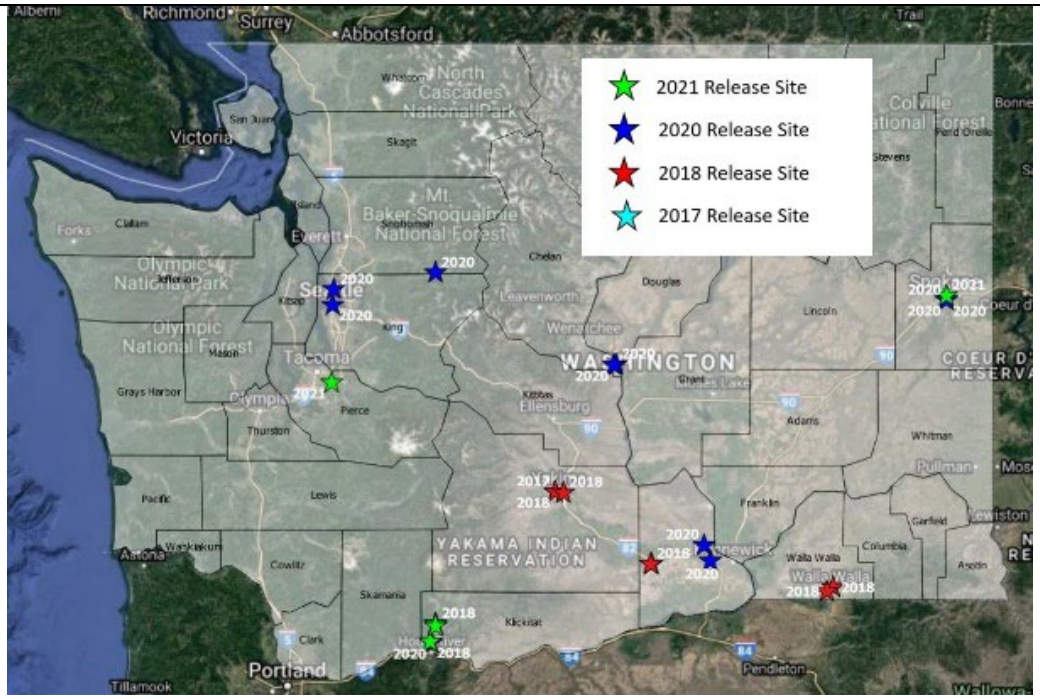

Fig. 3. Release sites of the samurai wasp in Washington, 2017-2021.

Although data collection is still in progress, we have an (unconfirmed) ID of 11 T. japonicus from the sticky traps. All were from a group of traps in the White Salmon area where releases were made in 2018 and 2020 (Fig. 4).

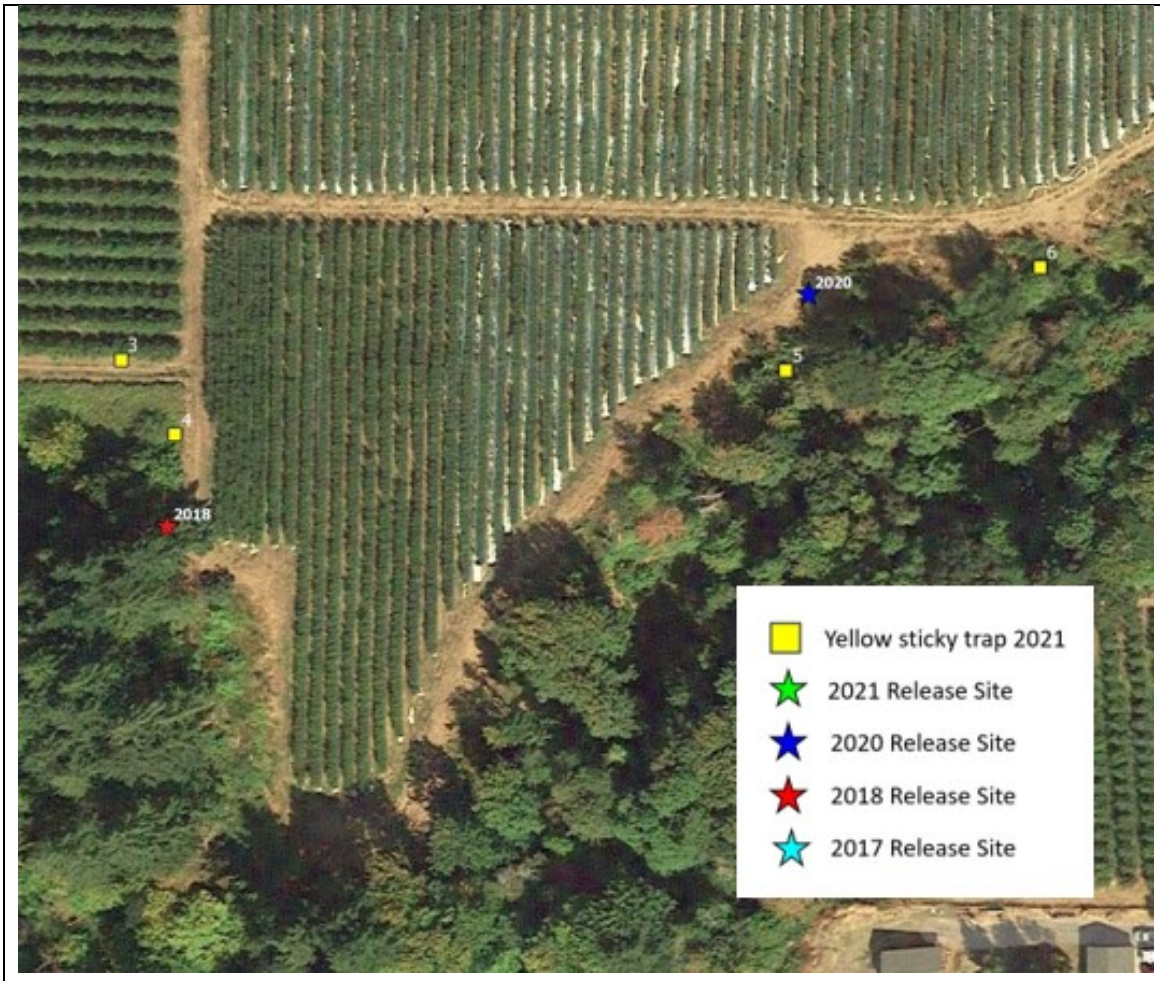

Fig. 4. Follow-up sampling positive sites today in 2021 (sticky traps nos 4,5 and 6).

Plans for 2022: We plan to release *T. japonicus* in the four counties with the highest numbers of reports in 2021 (King, Pierce, Snohomish, Thurston) and in an apparently well-established population in Leavenworth, WA.

2b. Determine permeability of net enclosures to *T. japonicus*. This objective was deferred due to COVID-19 staffing/travel restrictions.

2c. Determine the effects of host plant and canopy height by *T. japonicus*. This objective was deferred due to COVID-19 staffing/travel restrictions.

2d. Determine the non-target effects of the samurai wasp on native stink bugs.
Methods: In the summer of 2019 we deployed sentinel stink bug egg masses of three native stink bugs (*Euschistus conspersus Chinavia hilaris*, and *Podisus maculiventris*) and compared them to BMSB to determine attack rate of *T. japonicus*. After allowing completion of egg hatch or development of a parasitoids, we characterized the eggs individually using a combination of morphological and PCR methods. The morphological methods used a classification scheme based on appearance where a normally hatched egg and one producing an adult parasitoid were assessed; unhatched eggs were

classed based on appearance and subjected to PCR with the new *T. japonicus* primer developed by my lab (Dr. Kacie Athey). The combination of these methods allowed us to evaluate both reproductive (emerged adult parasitoid) and non-reproductive (egg is killed by the parasitoid, but no adult parasitoid is produced) impacts. The latter can be a hidden, but potentially very important non-target effect of a parasitoid, and is an emerging criterium in evaluating natural enemies for classical biological control programs.

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

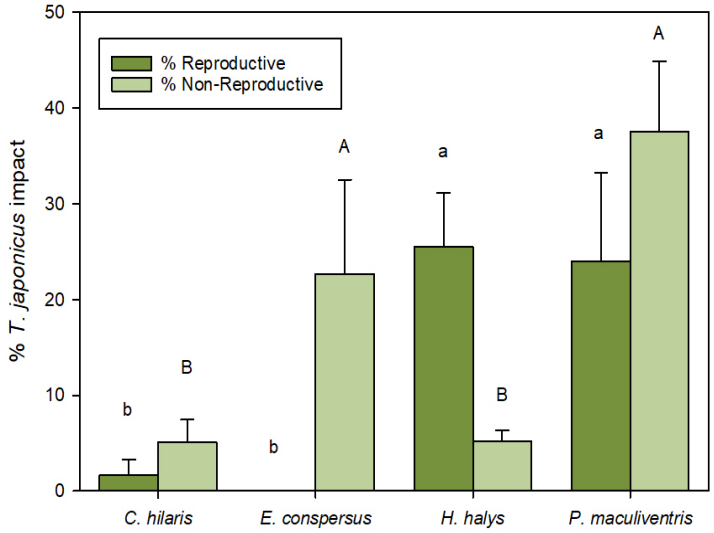

Fig. 5. Total parasitoid impact of *T. japonicus* on BMSB and 3 native stink bugs.

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

Methods: In 2019, we compared BMSB raised from the egg to the adult stage on either a typical colony diet (carrots, sunflower seeds, peanuts, bean plants) or plants native to Eastern Washington's sagebrush steppe habitat. Understanding the dietary limitations for development in different regions of the country should help us predict the relative risk of population buildup. The 2019 results indicated clearly that nymphs were slower to develop to the adult stage when fed on native plants, and that survivorship was significantly reduced. However, BMSB is unlikely to encounter a typical 'colony' diet in the wild (they do not have access to carrots and peanuts), so we followed up in 2020 with similar study using plants typical of the mid-Atlantic region compared to our native plants. The mid-Atlantic (or 'Eastern' plants) were cuttings taken from residential areas of Wenatchee, while the native or 'Western' plants were cuttings taken from unmanaged habitats (No. 2 Canyon, Horse Lake Preserve). The assemblage of Eastern plants was fairly consistent throughout the study (maple, tree of heaven, catalpa), while the Western plant assemblage changed as the various species bore fruit. The Western diet was more varied, and included serviceberry, chokecherry (Plate 3), bitterbrush, currant, Oregon grape, elderberry, snowberry, and wild rose at various points during the season. In all cases, both foliage and fruit structures were included in the cuttings; the latter is believed to be essential for the development of BMSB. We followed the nymphal development from 1st instars through adults,

Plate 3. BMSB nymph on chokecherry.

noting developmental time, adult weight, and survivorship. Adult weight is believed to associated with reproductive success of the adult; to test this, we took 10 male/female pairs from each of the two diet regimes, and allowed them to mate and lay eggs until the death of the female. This gave us the important measures of fecundity (eggs/female) and longevity.

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

Following up on previous work done using molecular gut content analysis on BMSB, we collected sympatric specimens of BMSB and native stink bug in two areas of Washington where our past research has indicated that they co-occur. Gut content analysis will provide insight into the subject of niche competition between our native stink but species and the invasive BMSB. Collections will continue in the summer of 2022, and PCR analysis in the winter of 2022.

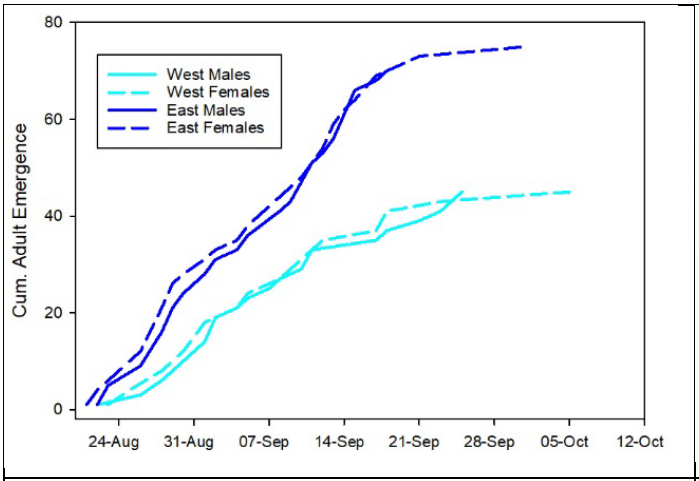

Fig. 6. Adult emergence and survivorship of nymphs fed on host plants from the eastern US vs the arid western US.

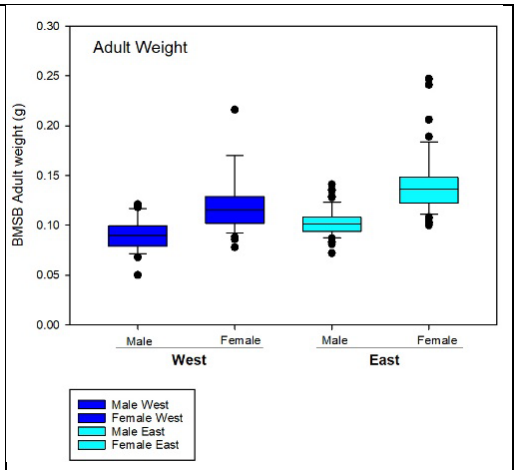

Fig. 7. Weight of surviving adults fed Eastern and Western plant diets.

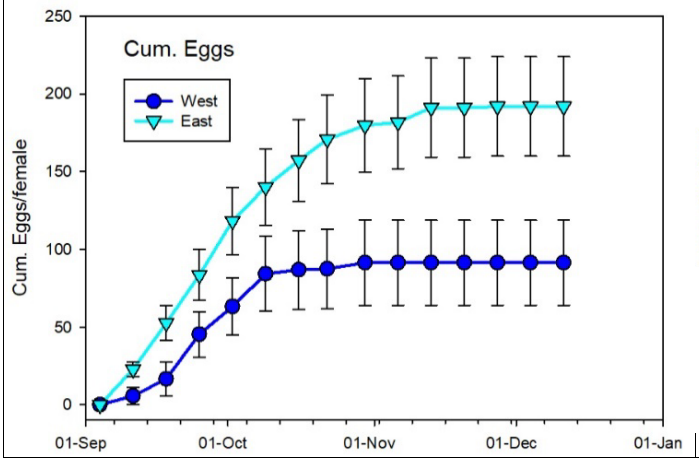

Fig. 8. Fecundity (cumulative eggs/female) from BMSB adults fed on either Eastern or Western plant diet.

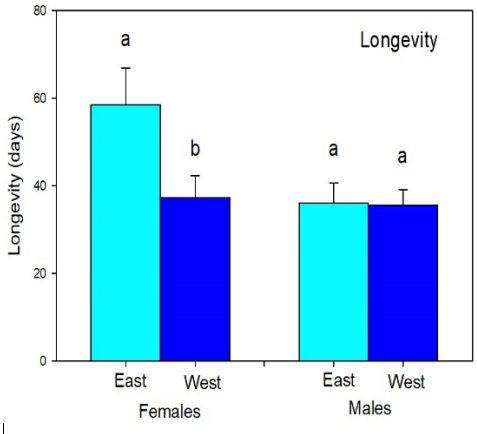

Fig. 9. Adult longevity of BMSB fed on either Eastern or Western plant diet.

Objective 4. Track the invasion of BMSB in Washington State

Methods: Trapping the same site in 2019 and 2020 allowed us to make comparisons of annual population differences. This site had both pheromone+LLIN traps and the pheromone+sticky card traps in both years. Captures in the LLIN traps were 93 to 99% lower in 2020 (1 to 3 BMSB/traps/season) when compared to 2019 (44 to 104 BMSB/trap/season) (Fig. 10). Sticky trap catches in 2020 were 39% lower than in 2019. These data indicate that BMSB populations (and possibly damage potential) will likely vary from year to year in Washington. This is consistent with observations in the mid-Atlantic area, which has also noted yearly fluctuation in insect pressure from this pest.

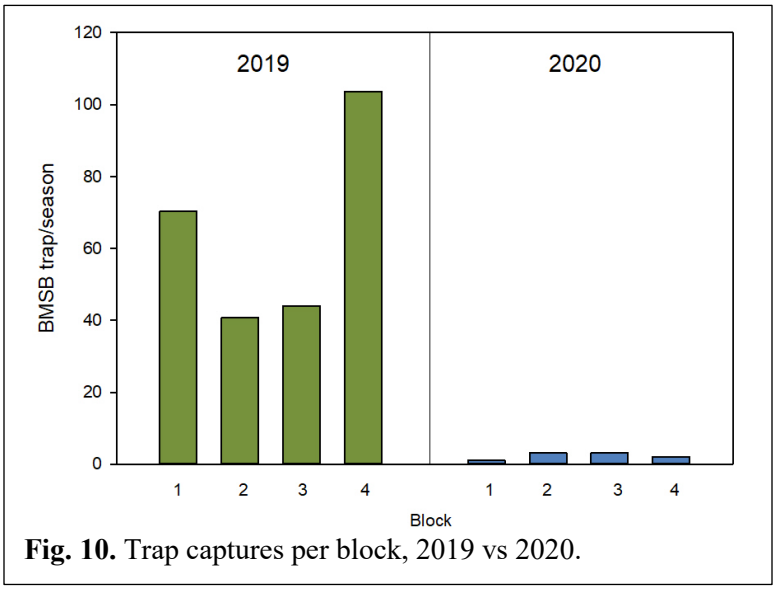

Fig. 10. Trap captures per block, 2019 vs 2020.

Results: BMSB has been found in 29 (out of 39) counties in Washington state; there were no new counties reported in 2021 (Figure 11).

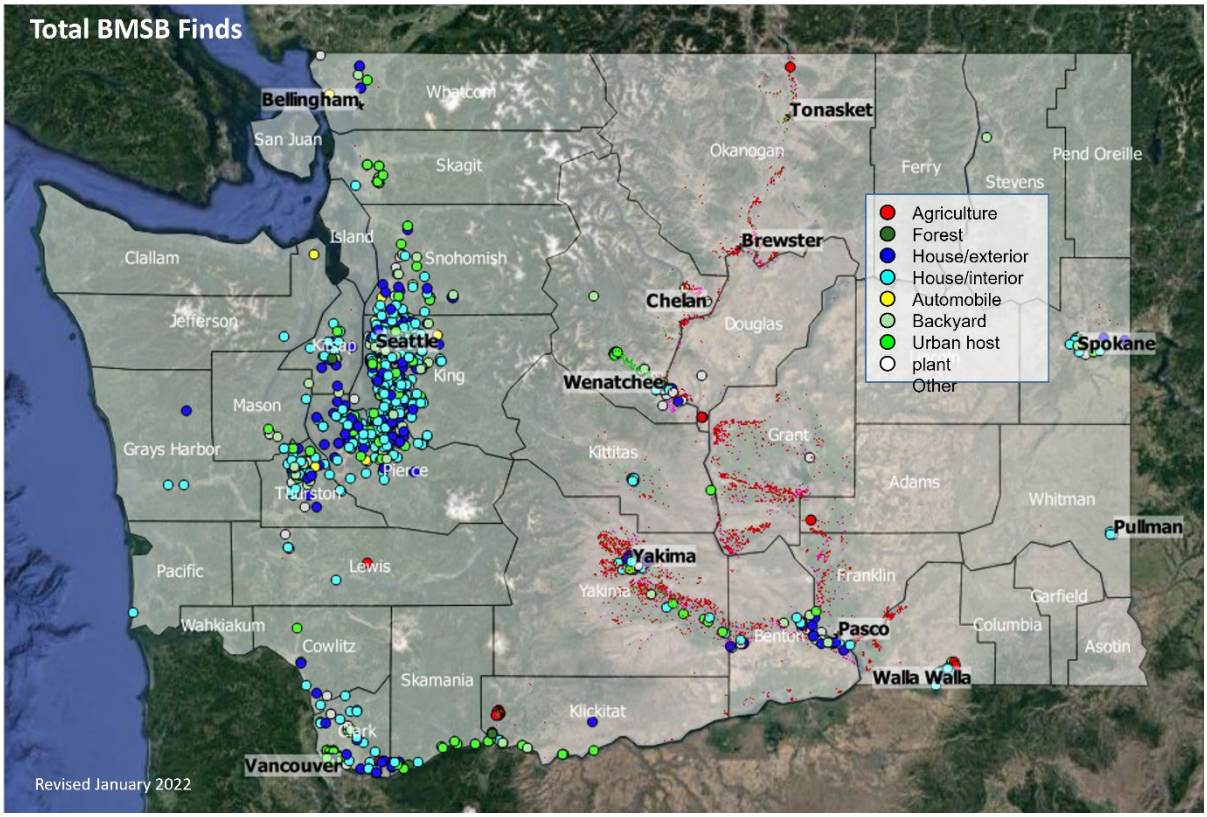

Fig 11. BMSB detection by GPS location. Red, green and magenta pixels represent apple, pear, and sweet cherry acreage, respectively.

The BMSB reporting system logged 74 reports in 2021 (as compared to 61 in 2020 and 146 in 2019. Most of the 2021 reports (64, 88%) came from counties west of the Cascades (Clark, King, Kitsap, Lewis, Mason, Pierce, Snohomish, Thurston), with the remaining 9 reports from Chelan, Benton, Kittitas, Yakima, Spokane and Whitman counties (Fig. 12).

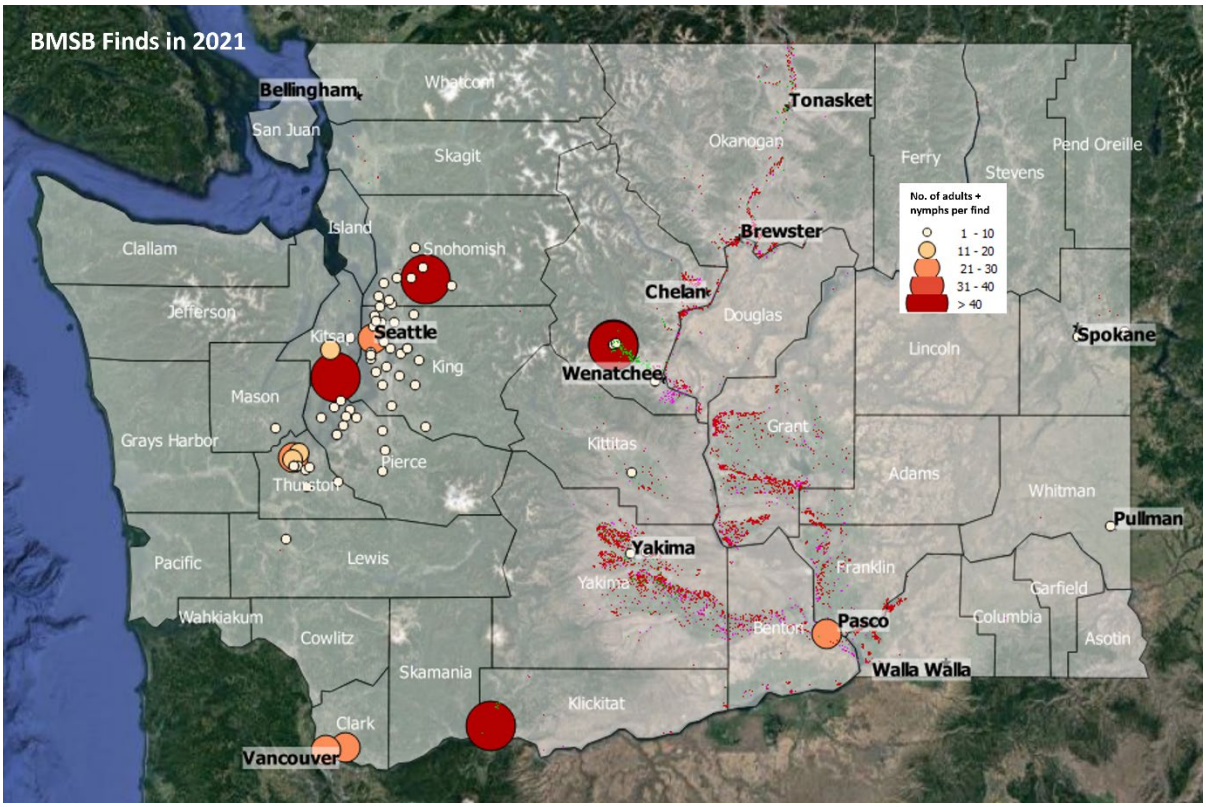

Fig. 12. BMSB reports in 2021; 74 individual reports, ranging from 1 to 50 BMSB (adults+nymphs).

In addition to the reports from homeowners, we collected an additional 219 BMSB in our targeted sampling for gut content analysis: 103 from several sites in Leavenworth, and 116 from the Vancouver/White Salmon are.

Based on discussions with Justin Bush of the Washington Invasive Species Council (WISC), the BMSB reporting function will be transferred to this organization starting January 1, 2022. This organization has an ongoing mission to track a wide range of invasive species, with greater mapping capability (viz., EDDMapS). Because the WTFRC and SCRI projects are nearing completion, the WISC can provide a long-term archive for this information.

WTFRC INTERNAL PROJECT – BUDGET SHARED FOR INFORMATIONAL PURPOSES ONLY

CROP YEAR: 2021

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

Project Title: Pesticide Residues on WA Apples

PI: Tory Schmidt
Organization: WTFRC
Telephone: (509) 665-8271 x4
Email: tory@treefruitresearch.com
Address: 1719 Springwater Ave.
City/State/Zip: Wenatchee, WA 98801

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

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

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

2021 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 eleven insecticide/acaricides and eight fungicides and one plant growth regulator according to either an "aggressive" protocol intended to generate the highest possible residues while observing label guidelines (maximum label rates at minimum retreatment and pre-harvest intervals) or a "standard" protocol following more typical industry use patterns for rates and timings. Fruit samples were collected at commercial maturity on August 26 and delivered the next day to Pacific Agricultural Labs (Sherwood, OR) for chemical residue analysis.

TRIAL DETAILS

- 14th 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 0.21 inches of rain fell on the trial block after the application of Ethephon, but there was no measurable precipitation in July or August, when all other materials were sprayed

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 2021.

Chemical name	Trade name	Application rate	Application timing(s)	Measured residue	US MRL[1]	India MRL[1]	Lowest export MRL[1]
		oz per acre	*dbh*	*ppm*	*ppm*	*ppm*	*ppm*
ethephon	Ethephon 2SL	32	121 (Apr 27)	<0.1	5	0.01*	0.8 (many)
flutianil	Gatten	8	35	<0.01	0.15	0.01*	0.01 (many)
isofetamid	Kenja 400SC	12.5	35	0.083	0.6	0.01*	0.2 (Kor)
abamectin	AgriMek SC	4.25	35	<0.01	0.02	0.01*	0.01 (many)
benzovindiflupyr	Aprovia	7	35	0.013	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	**0.013**	0.2	0.01*	0.01 (many)
diazinon	Diazinon 50W	16	35	<0.01	0.5	0.01*	0.1 (Can)
spinetoram	Delegate WG	7	35 & 21	0.011	0.2	0.01*	0.05 (many)
spinosad	Entrust	3	35 & 21	<0.01	0.2	0.01*	0.1 (many)
tolfenpyrad	Bexar	27	35 & 21	**0.20**	1	0.01*	0.01 (many)
myclobutanil	Rally 40WSP	10	35 & 21	0.27	0.5	0.01	0.5 (many)
fenpropathrin	Danitol	18	35 & 21	**0.26**	5	0.01*	0.01 (many)
difenoconazole	Inspire Super	12	28	0.030	5	0.01	0.5 (China)
cyprodinil	Inspire Super	12	28	**0.057**	1.7	0.01*	0.05 (Indo)
cyflufenamid	Torino	6.8	28	**0.012**	0.06	0.01*	0.01 (Thai)
buprofezin	Centaur WDG	34.5	28	0.68	3	0.01*	1 (Tai)
afidopyropen	Versys	3.5	28 & 14	<0.05	0.02	0.01*	0.01 (many)
bifenazate	Acramite 50WS	16	14	0.18	0.7	0.01*	0.2 (China)
phosmet	Imidan 70-W**	92	14	**2.8**	10	0.01*	2 (Tai)
mefentrifluconazole	Cevya	5	14	**0.089**	1.5	0.01*	0.01 (many)
cyclaniliprole	Verdepryn	11	14	**0.061**	0.3	0.01*	0.01 (many)

[1] Top markets for WA apples with established MRLs; 29 Sept 2021. 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 2021.

Chemical name	Trade name	Application rate	Application timing(s)	Measured residue	US MRL[1]	India MRL[1]	Lowest export MRL[1]
		oz per acre	*dbh*	*ppm*	*ppm*	*ppm*	*ppm*
ethephon	Ethephon 2SL	32	79 (June 8)	0.14	5	0.01*	0.8 (many)
benzovindiflupyr	Aprovia	7	35	0.015	0.2	0.01*	0.2 (many)
pydiflumetofen	Miravis	3.4	35	**0.036**	0.2	0.01*	0.01 (many)
isofetamid	Kenja 400SC	12.5	35 & 21	0.012	0.6	0.01*	0.2 (Kor)
diazinon	Diazinon 50W	16	35 & 21	0.043	0.5	0.01*	0.1 (Can)
abamectin	AgriMek SC	4.25	28	<0.01	0.02	0.01*	0.01 (many)
tolfenpyrad	Bexar	27	28 & 14	**0.39**	1	0.01*	0.01 (many)
fenpropathrin	Danitol	18	28 & 14	**0.46**	5	0.01*	0.01 (many)
difenoconazole	Inspire Super	12	21 & 14	0.088	5	0.01	0.5 (China)
cyprodinil	Inspire Super	12	21 & 14	**0.19**	1.7	0.01*	0.05 (Indo)
flutianil	Gatten	8	21 & 14	**0.026**	0.15	0.01*	0.01 (many)
myclobutanil	Rally 40WSP	10	21 & 14	0.43	0.5	0.01	0.5 (many)
spinosad	Entrust	3	21 & 14	<0.01	0.2	0.01*	0.1 (many)
cyclaniliprole	Verdepryn	11	21 & 7	**0.053**	0.3	0.01*	0.01 (many)
phosmet	Imidan 70-W**	92	21 & 7	**6.1**	10	0.01*	2 (Tai)
cyflufenamid	Torino	6.8	14	**0.023**	0.06	0.01*	0.01 (Thai)
buprofezin	Centaur WDG	34.5	14	**1.1**	3	0.01*	1 (Tai)
spinetoram	Delegate WG	7	14 & 7	<0.01	0.2	0.01*	0.05 (many)
afidopyropen	Versys	3.5	14 & 7	<0.05	0.02	0.01*	0.01 (many)
bifenazate	Acramite 50WS	16	7	0.12	0.7	0.01*	0.2 (China)
mefentrifluconazole	Cevya	5	7 & 1	**0.37**	1.5	0.01*	0.01 (many)

[1] Top markets for WA apples with established MRLs; 29 Sept 2021. https://nwhort.org/export-manual/, https://bcglobal.bryantchristie.com/

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

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

CONCLUSIONS

As we have observed in every study since 2011, no spray program produced a residue that exceeded the tolerance level set by the US Environmental Protection Agency; these findings are further evidence that apple growers following directions on product labels should expect their fruit to be in full compliance for domestic sales regarding pesticide residues. Several products we tested, however, did produce **residues which exceed Maximum Residue Levels** (MRLs) set in important export markets for Washington apples including: **Miravis, Bexar, Danitol, Inspire Super, Gatten, Verdepryn, Imidan 70-W, Torino, Centaur WDG, and Cevya**. India has yet to post tolerances for most pesticides used by WA apple growers; in the absence of a posted MRL, the default tolerance in India is 0.01 ppm, essentially meaning that any product which produced a detectable residue in our study would potentially violate India's standards. Trade representatives from the USDA and Northwest Horticultural Council continue to work with Indian authorities to encourage them to post more MRLs, which should make compliance more feasible.

Our 2021 study reports findings for the first time for mefentrifluconazole, pydiflumetofen, and cyclaniliprole, as well as ethephon applied at standard timings for chemical thinning (late April) or promotion of return bloom (early June). While ethephon is an old product, Canada is expected to reduce its current MRL for ethephon on apples from 3.0 to 0.1 ppm in late 2022; our first year of results at these timings indicate no detectable residues for apples treated with ethephon at chemical thinning timing and a relatively modest residue for fruit sprayed in early June, well below most current export MRLs from other countries.

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

WASHINGTON TREE FRUIT RESEARCH COMMISSION

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

Project Title: Pre-bloom resistance induction for fire blight suppression

PI: Kenneth B. Johnson
Organization: Oregon State University,
Telephone: (541) 737-5249
Email: johnsonk@science.oregonstate.edu
Address: Dept. Botany & Plant Pathology

Address 2: Cordley Hall 2082
City/State/Zip: Corvallis, OR 97331-2902

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Budget 1
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Organization Name: OSU Ag. Res. Foundation
Contract Administrator: Dan Arp
Telephone: (541) 737-3228
Contract administrator email address: Charlene.Wilkinson@oregonstate.edu

Item	2020	2021
Salaries Faculty Res. Assist. 3.5 mo	17,860	18,396
Benefits OPE 61%	10,895	11,221
Wages undergraduate $12.50/hr	1,111	1,143
Benefits OPE 8%	89	93
Equipment		
Supplies	1,000	1,030
Travel	1,500	1,545
Miscellaneous		
Plot Fees	2,000	2,060
Total	$34,455	$35,488

Footnotes: 3% inflation in year 2

OBJECTIVES

Obj. 1a: Determine if prohexadione-Ca or acibenzolar-S-methyl applied prior to bloom achieves blossom blight control, and 1b. determine if prebloom Ph-Ca enhances effectiveness of Actigard treatments during bloom.

Obj. 2. Determine if Regalia applied prebloom influences blossom blight suppression.

Obj. 3. In greenhouse, investigate synergy between Ph-Ca and Actigard for fire blight suppression, and resistance induction by Regalia for fire blight control.

SIGNIFICANT FINDINGS:

- Kudos/Apogee (prohexadione-Ca), Actigard, or Regalia applied at prebloom timings did not provide significant suppression of fire blight in flowers compared to a non-treated control.

- Kudos applied as a solitary treatment at timings of first pink, full pink and 10% bloom, did not reveal a pattern related to time of treatment and level of suppression.

- In 2020, Actigard sprayed three times during bloom reduced the incidence of infection compared the water-treated control (45 and 65 % reduction in pear and apple, respectively). In2020, a prebloom Kudos treatment appeared to enhance the level of suppression obtained by the Actigard treatment program. But in 2021, the degree of suppression obtained with a 3-spray Actigard program (75%) was not improved with a prebloom treatment of Apogee.

- In greenhouse, an Actigard spray reduced fire blight canker expansion in potted ELMA.26 by 28%, and the effect of Actigard was not improved with the addition of an Apogee spray. A single Actigard trunk paint treatment reduced canker expansion by 55% and was not improved with the addition of an Apogee spray.

- In greenhouse, among spray and trunk paint treatments of Apogee, Actigard, Regalia, and LifeGard, only Actigard trunk paint treatments showed sustained expression of apple defense genes, PR-1, and PR-2.

- In greenhouse, treatments with the organic materials, Regalia and LifeGard, had no effect on fire blight canker expansion in potted ELMA.26.

RESULTS

Experimental design for orchard trials. In 2020 and 2021, objectives 1 and 2 were conducted in a 6/7-yr-old and a 2/3-yr-old blocks of Gala apple located at the Oregon State University, Botany and Plant Pathology Field Laboratory near Corvallis. In 2020, additional treatments were evaluated in older (20-yr-old) blocks of Gala apple and Bartlett pear.

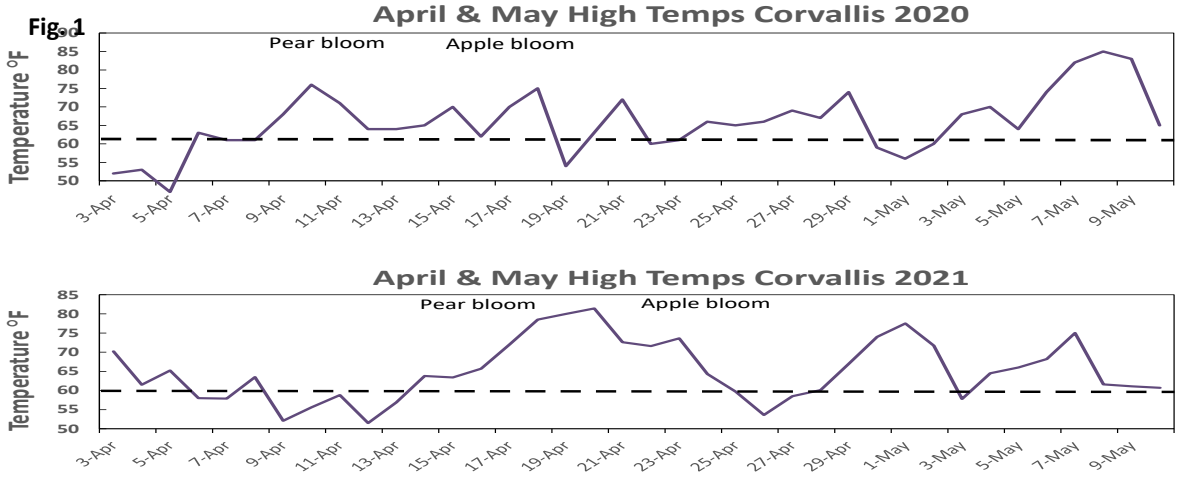
Experimental design for orchard trials. Experiments were arranged in randomized complete block designs with 9 to 14 replicates of single-tree plots in young tree blocks, and with 4 replicates of single-tree plots in older tree blocks. Prior to bloom, flower cluster density on individual trees was counted. These counts along with tree location in orchard were considered in the assignment of trees to blocks in the plot design. On the date of treatment, suspensions of each product in water were prepared at the specified rate and applied to near run-off with backpack sprayers in early morning under calm wind conditions. The amount of material suspension sprayed was 0.5 to 1.0 liter/tree in young tree trials, and 3 liters/tree in the older tree trials. On an evening near full bloom, re-suspended freeze-dried inoculum of pathogenic *E. amylovora* 153N (streptomycin and oxytetracycline sensitive

pathogen strain) prepared at a concentration of 1 x 10^6 CFU/ml was fogged onto the trees. In the 2020 young tree trials, because of asynchronous bloom, the inoculation was repeated to ensure adequate disease pressure.

Disease assessment and data analysis. During bloom, treated trees were evaluated for phytotoxic responses from sprayed treatments. Beginning 2 to 3 weeks after petal fall, fire blight incidence was assessed by counting and removing blighted flower clusters from each tree. The number of blighted flower clusters was divided by the total number of clusters on each tree. Incidence of fire blight was subjected to analysis of variance (Analyze-It, v. 3.0, Leeds, UK).

Weather in spring 2020. Temperatures during primary bloom of both pear and apple were moderately favorable for fire blight development (Fig.1). Six days between 9 and 21 April had a maximum daily temperature > 70°F. On inoculated control trees, epiphytic pathogen populations were high with measured sizes exceeding 1 x 10^6 colony forming units (viable cells per flower) on flowers sampled near petal fall. These high populations on non-treated or water-treated control flowers resulted in infection incidences that averaged 31% of total clusters (the range was 14 to 41% among the four trials mentioned in this report). *Weather in spring 2021.* Temperatures during primary bloom of apple were moderately favorable for fire blight development. Four days between 21 and 30 April had a maximum daily temperature > 70°F; rain occurred on 24 and 25 April as apple trees entered full bloom. For the water-treated control, pathogen populations on flowers resulted in moderate incidences of infection averaged 9% of total clusters (the range was 5% in the 7-yr-old Gala block and 12% in the 2-yr-old Gala block).

Fig. 1



Objective 1a: Determine if Ph-Ca or Actigard applied prior to bloom achieves blossom blight control, and **Objective 2:** Investigate if Regalia applied prebloom influences blossom blight suppression.

Rationale. Wallis and Cox (2020) achieved very good suppression of fire blight from a single application of prohexadione-Ca (Ph-Ca, 6 oz. /100 gal.) prior to bloom. Questions that arose from their study include: 1) can this repeated under western conditions?, 2) what is the optimal timing of Ph-Ca treatment?, 3) could prebloom treatment with Ph-Ca would improve the efficacy of Actigard treatments during bloom (without antibiotics), and 4) could organic resistance-inducing materials applied prebloom, e.g., Regalia, achieve this suppression response.

Approach. In young tree trials in 2020, the timing of Kudos (prohexadione calcium, Fine Americas, Walnut Creek, CA) was varied from first pink to 10% bloom. In addition, Actigard 50WG (acibenzolar-S-methyl, Syngenta Crop Protection, Greensboro, NC), and Regalia (extract of

Reynoutria sachalinensis, Marrone Bio, Davis, CA) were each evaluated at the timing of full pink. In large tree trials in 2020, Kudos was applied at full pink and Actigard was applied at 70% bloom, full bloom, and petal fall. An additional treatment added Stimplex (0.01% cytokinin, 128 fl. oz. /100 gallons, Acadian Seaplants Ltd., Dartmouth, Nova Scotia) at timings of prebloom (with Kudos) and petal fall (with Actigard). Regalia was evaluated as a solitary treatment at full pink and petal fall.

In 2021, the only materials applied prebloom (full pink only) were Apogee (prohexadione calcium, BASF Ag, Research Triangle Park, NC) and Regalia. In addition, preboom treatments of Apogee were supplemented with bloom period sprays of Actigard, and prebloom Regalia treatments were supplemented with bloom period treatments of LifeGard (*Bacillus mycoides* isolate J, Certis Biologicals, Columbia, MD) gallons and an additional Regalia treatment at petal fall.

Observations in 2020. Trees used in the study averaged 82 flower clusters per tree in the 6-yr-old block and 57 flower clusters per tree in the 2-yr-old block. None of the materials (Kudos, Actigard, and Regalia) applied at prebloom timings produced symptoms of phytoxicity. Symptoms of fire blight were first observed on 5 May. For a pathogen-inoculated trial, incidence of infection was moderately high with non-treated control trees in the 6-yr-old block averaging 31 fire blight strikes per tree (41% of total clusters); in the 2-yr-old block, non-treated control trees averaged 23 strikes per tree (39% of total clusters) (Table 1). In both orchard blocks, while all treatments had fewer infections per tree than the non-treated controls, the overall suppression of infection from sprayed materials was only fair (17 to 39% in the 6-yr-old block) to poor (6 to 17% in the 2-yr-old block) and not statistically significant ($P > 0.05$) different from the non-treated control. Kudos, which was applied as a solitary treatment at timings of first pink, full pink and 10% bloom, did not reveal a pattern related to time of treatment and level of suppression.

		Date treatment applied*			**6-yr-old trees**	**2-yr-old trees**
Treatment	Rate **per 100 gal**	8 April First pink	11 April Full pink	14 April 10% bloom	% blighted floral clusters**	% blighted floral clusters**
Non-treated	-	--- [§]	---	---	**41** [#] (31 strikes)	**39** [#] (23 strikes)
Kudos [y]	6 oz.	X	---	---	25	34
Kudos [y]	6 oz.	---	X	---	34	36
Kudos [y]	6 oz.	---	---	X	28	26
Kudos [y] Actigard	2 oz. 3.2 oz.	---	X	---	31	32
Regalia [z]	256 fl. oz.	---	X	---	33	37

***On the evenings of 15 and 19 April, a motorized tank sprayer equipped with a hand wand was used to lightly fog a suspension of freeze-dried cells of *Erwinia amylovora* strain, which was prepared at 1 x 10^6 CFU per ml (0.1 to 0.2 liters per tree). ** Transformed arcsine($\sqrt{x}$) prior to analysis of variance; non-transformed means are shown. 'X' indicates material was sprayed on that specific date; '---' indicates material was not applied on that specific date. [#] Means within a column did not differ significantly ($P > 0.05$) based on analysis of variance ($F > 0.05$). [y] Amended 1:1 with ammonium sulfate and Regulaid (16 fl. oz. per 100 gallons). [z] Amended with BioLink Spreader-Sticker: 4 fl. oz. per 100 gallons.**

In the 2020 large tree trials, the 20-yr-old Bartlett pear trees averaged 455 flower clusters per tree, and the 20-yr-old Gala apple trees averaged 430 flower clusters per tree. In pear, infection incidence was moderate with fire blight infections on water-treated trees averaging 67 strikes per tree (15% of total clusters); in apple, infection incidence was moderately high with water-treated trees averaging 126 strikes per tree (31% of total clusters) (Table 2). Based on percent blighted flower clusters and compared to the water-treated control, the solitary treatments of Kudos, Actigard or Regalia treatments did not reduce infection incidence significantly ($P < 0.05$). In contrast, in both trials, the combination program of Kudos (prebloom) and Actigard (during bloom) reduced significantly the incidence of infection compared the water-treated control (55 and 65% reductions in pear and apple, respectively). Regalia (1 gallon per 100 gallons of water) at the timings of pink and petal fall reduced incidence of infection (% blighted flower clusters) by 50% in the apple trial ($P< 0.05$).

Table 2. Evaluluation of prebloom PH-Ca and within-bloom Actigard treatments for fire blight control in 20-year-old Bartlett pear and Gala apple, Corvallis, 2020.

20-year-old Bartlett pear

Treatment	Rate per 100 gallons water	Date treatment applied* 3 Apr Full white bud	7 Apr Mid-bloom	10 Apr Full bloom	14 Apr Petal Fall	Number of blighted clusters per tree**	Percent blighted floral clusters***
Water	---	---	---	**X§**	**X**	**67 a #**	**14.5 a**
Actigard	2 oz.	---	X	X	X	52 a	11.8 ab
Kudos	6 oz.	X	---	---	---	74 a	19.1 a
Kudos	6 oz.	X	---	---	---	33 b	7.9 b
Actigard	2 oz.	---	X	X	X		
Kudos	6 oz.	X	---	---	---	32 b	8.7 b
Actigard	2 oz.	---	X	X	X		
Stimplex	128 fl. oz.	X	---	---	X		
Regalia	128 fl. oz	X	---	---	X	57 a	12.9 ab

20-year-old Gala apple

Treatment	Rate per 100 gallons water	*Date treatment applied** 10 Apr Full-pink	14 Apr Mid-bloom	17 Apr Full bloom	21 Apr Petal Fall	Number of blighted clusters per tree**	Percent blighted flora clusters***
Water	---	---	---	**X§**	**X**	**126 a #**	**30.7 a**
Kudos	6 oz.	X	---	---	---	137 a	35.5 a
Kudos	6 oz.	X	---	---	---	46 b	10.8 b
Actigard	2 oz.	---	X	X	X		
Kudos	6 oz.	X	---	---	---	57 ab	14.7 b
Actigard	2 oz.	---	X	X	X		
Stimplex	128 fl. oz.	X	---	---	X		
Regalia	128 fl. oz	X	---	---	X	66 ab	14.7 b

*** Trees inoculated on 8 (pear) or 15 (apple) April with 1 x 10^6 CFU/ml *Erwinia amylovora* strain Ea153N (streptomycin- and oxytetracycline-sensitive fire blight pathogen strain). ** Transformed log(x + 1) prior to analysis of variance; non-transformed means are shown. *** Transformed arcsine($\sqrt{x}$) prior to analysis of variance; non-transformed means are shown. § X indicates material was sprayed on that specific date; --- indicates material was not applied on that specific date. # Means within a column followed by same letter do not differ significantly (P = 0.05) based on Fischer's protected least significance difference.**

Observation in 2021. Trees used in the study averaged 138 flower clusters per tree in the 7-yr-old block and 149 flower clusters per tree in the 3-yr-old block. None of the materials (Apogee, Actigard, Lifeguard and Regalia) produced symptoms of phytoxicity. Symptoms of fire blight were first observed on 10 May. For a pathogen-inoculated trial, disease intensity was light to moderate with fire blight infections on water-treated control trees averaging 9 strikes per tree (5.4% of total clusters) in the 7-yr-old block and 17 strikes per tree (12.9% of total clusters) in the 3-yr-old block (Table 3). Averaged over both orchard blocks and compared to the water control, treatment with Actigard (or this material in a program with Apogee) provided a high level of infection suppression (75%). In contrast, the incidence of infection on trees treated with Apogee only was similar to those that received water. As solitary materials, Regalia (2 applications) and LifeGard (3 applications) provided intermediate levels of suppression (38 and 48%, respectively). The combination programs of Regalia and LifeGard increased the level of infection suppression to a mean of 59%. One application of FireWall (streptomycin) provided 92% infection suppression relative to the water-treated control.

		Date treatment applied				**7-yr-old**		**3-yr-old**		**Combir**
Treatment	Rate **per 100 gal**	19 Apr Full pink	22 Apr 70% bloom	26 Apr Full bloom	29 Apr Petal fall	% blighted floral clusters		% blighted floral clusters		% blighte floral cluste
Water-treated	-	--- [§]	X	X	X	**5.4[#]** 9 strikes	**b**	**12.9** 17 strikes	**a**	**9.1**
FireWall 50	2.3 oz.	---	-[w]-	X	-[w]-	0.8	e	0.7	f	0.7
Apogee [y]	12 oz.	X	-[w]-	-[w]-	-[w]-	9.7	a	8.0	b	8.7
Apogee [y]	6 oz.	X	---	---	---	3.2	bc	2.6	de	2.9
Actigard	2 oz.	---	X	X	X					
Apogee [y]	12 oz.	X	---	---	---	1.1	de	1.9	ef	1.6
Actigard	2 oz.	---	X	X	X					
Actigard	2 oz.	---	X	X	X	0.8	e	2.9	de	2.0
Regalia [z]	128 fl. oz.	X	-[w]-	-[w]-	---	4.0	bc	6.9	bc	5.7
Regalia [z]	64 fl. oz.	---			X					
Regalia [z]	128 fl. oz.	X	---	---	---	5.4	b	4.3	cd	4.8
Regalia [z]	64 fl. oz.	---	---	---	X					
LifeGard	13.5 oz.	---	X	X	X					
Regalia [z]	256 fl. oz.	X	---	---	---	1.0	de	4.2	cd	2.9
Regalia [z]	64 fl. oz.	---	---	---	X					
LifeGard	4.5 oz.	---	X	X	X					
LifeGard [z]	13.5 oz.	---	X	X	X	2.6	cd	6.3	bc	4.7

**** Transformed arcsine($\sqrt{x}$) prior to analysis of variance; non-transformed means are shown. 'X' indicates material was sprayed on that date; '---' indicates material was not applied on that specific date; and '-[w]-' indicates water was sprayed on that date. [#] Means within a column did not differ significantly ($P > 0.05$) based on analysis of variance ($F > 0.05$). [y]Amended 1:1 with ammonium sulfate and Regulaid (16 fl. oz. per 100 gallons). [z] Amended with BioLink Spreader-Sticker: 4 fl. oz. per 100 gallons.**

Box plots of the 2021 data are an alternative method to visualize infection incidence (Fig. 2). Relative to the water-treated control and to prebloom Apogee only, the response of individual trees to the Actigard treatments (with or without a prebloom Apogee treatment) was consistent and nearly as effective as streptomycin (Firewall). Moreover, with 14 replications, the responses observed from Regalia, LifeGard, and combination programs of these materials also showed a consistent response, although to a lesser degree of suppression than observed with Actigard.

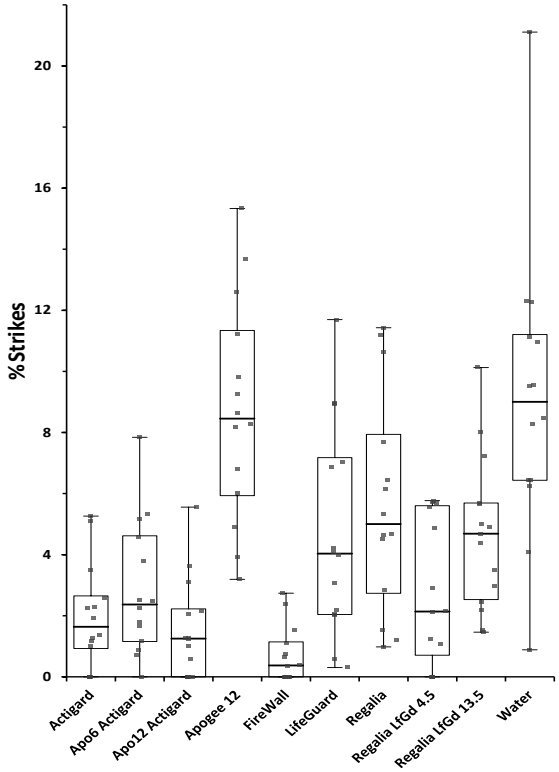
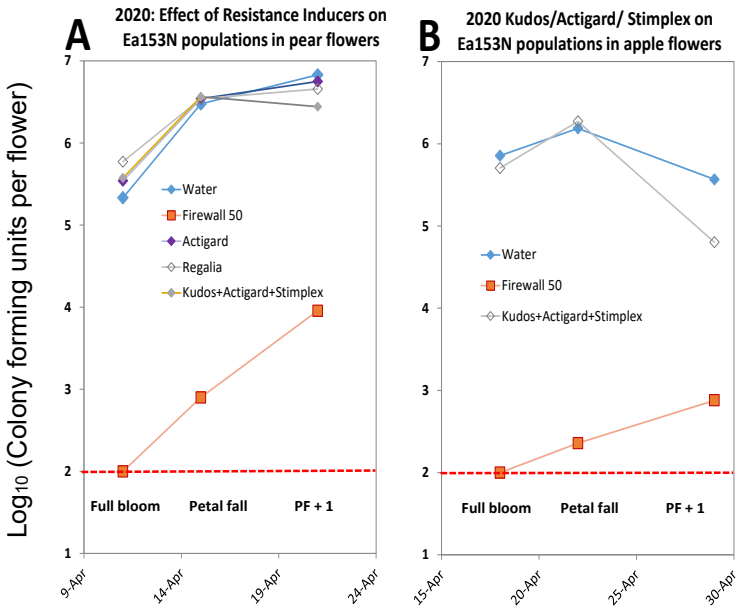


Fig 2. Box plots of percent infected flower clusters in Gala apple trees treated prebloom with potential resistance-inducing materials. The 7-yr-old (6 replication) and 3-yr-old (8 reps) orchards were located near Corvallis, OR with each treatment applied to a total of 14 replicate trees during April 2021. Within each box, horizontal line is the treatment median.

Discussion. We viewed the quality of young-tree trials reported under this objective as excellent based on the number of replications (9 per trial in 2020 and 14 in 2021), bloom density on individual trees (very good), weather for pathogen growth in flowers and level of infection (not too much, not too little). Consequently, our inability to repeat results from solitary prebloom treatment of Ph-Ca in New York was disappointing (and follows prior trials (2018 and 2019) with similar poor results). In contrast to prebloom treatment only, in both years, treatments that sequenced resistance inducing sprays through the bloom period resulted in significant disease suppression. In 2020, in pear, fire blight suppression from Actigard sprays during bloom were apparently improved when preceded by a prebloom treatment of prohexadione-Ca, but this effect of the prebloom prohexadione-CA treatment on the Actigard program was not observed in Gala apple in 2021. It should be noted that resistance inducing materials do not have any effect on epiphytic populations of the fire blight pathogen in flowers (Fig. 3). Consequently, in conventional orchards, the approaches being evaluated here would need to be complemented by antibiotic treatments. In this context, the induced-resistance from an Actigard treatment program could be expected to lessen variability in antibiotic suppression of fire blight, which can result from less than perfect coverage, pathogen resistance to the material, and very short effective residuals of antibiotic materials. We plan to continue to trial Actigard bloom programs in 2022 to better understand the significance of application frequency.

With regard to the organic material, Regalia, applying it twice in 2020 to the older apple trees resulted in a better performance than its application as solitary prebloom treatment in the younger trees. In 2021, we evaluated Regalia in an organic induced-resistance program that included the resistance-inducing material, LifeGard. And (albeit based on one year's data), the three application/multiple material program achieved a somewhat higher level of suppression than either Regalia or LifeGard individually in two-spray programs. Combination programs of Regalia and LifeGard will be investigated in 2022 trials.

Fig. 3. Effect of resistance inducers applied to A) Bartlett pear and B) Gala apple trees to suppress fire blight on the population size of *E. amylovora* strain 153N on flowers in 2020. Pathogen populations were measured by sampling five flower clusters (~25 flowers, bulked) from each replicate tree. Each sample was washed in 25-ml of sterile, de-ionized water followed by dilution plating onto nutrient agar plus nalidixic acid. Data for Firewall 50 (streptomycin) is shown for comparison. Log_{10} = 2.0 was the detection limit of the assay. Data depict mean of each treatment program on each sampling date.



Objective 3: In greenhouse, investigate further the potential synergy between Ph-Ca and Actigard for fire blight control.

Rationale. Although Kudos/Apogee (prohexadione-Ca), Actigard, Regalia and LifeGard are each marketed as materials that enhance host resistance to fire blight, to our knowledge only Actigard has been shown to increase expression of pathogenesis-related defense genes in apple. Consequently, under this objective, we attempted to slow fire blight canker expansion in potted, greenhouse-grown trees by treatment (sprays or trunk paints) of resistance inducing materials and measure if these treatments cause a change in defense gene expression.

Experimental design. In 2020, greenhouse experiments proposed did not occur owing to closure of the OSU greenhouse facility during early months of the COVID-19 pandemic. In 2021, plant material consisted of 400bareroot, 2-year-old ELMA.26 liners obtained in spring 2020 from TRECO (Woodburn, OR). The trees were greenhouse-grown in 2-gallon containers containing growth medium (Rexius Forest Products, Eugene, OR placed outside for winter 2020-21. During the summers, trees were fertilized monthly with Peters 20-10-20 (0.5 g).

The experiment was initiated when new shoots on potted trees were 12 to 14inches in length (early June 2021). Experimental design was a randomized block with 18 inoculated trees (replicates) per treatment (Table 4). Shoot tips were inoculated with a mixture of several *Erwinia amylovora* isolates suspended in water at concentration of 10^9 CFU per ml. The youngest and second youngest leaves on the actively growing shoot tip were split along mid-rib with a scissors that had been dipped in the pathogen suspension. Shoot tips were covered in a plastic re-sealable bag containing 1 ml of water for a period of 5 days. Incidence of infection, length (%) of vegetative shoot becoming necrotic, and whether or not the fire blight canker expanded into older trunk tissue were measured monthly. Relative proportion of a shoot diseased was calculated the ratio of canker length in current season growth divided by total length of the current season growth.

Leaves on non-inoculated but treated plants were sampled at 2, 4, and 7 weeks after the date of inoculation to measure expression of PR-gene indicators of resistance induction. Specifically, the relative expression of pathogenesis-related PR-genes, PR-1, PR-2, and PR-8, were quantified. Leaf samples were

frozen in liquid nitrogen and stored at −80°C until processed. Leaves were processed by grinding in liquid nitrogen and followed by RNA extraction (RNeasy mini kit for plant tissue, Qiagen, Valencia, CA), and cDNA synthesis (Superscript II RNase H–Reverse Transcriptase, Gibco BRL, Grand Island, NY). PCR primers developed by Maxson-Stein et al. (2002) were used to quantify expression of PR proteins.

Table 4. 2021 treatments of resistance inducers applied to 2-year-old ELMA 26 trees.

Treatment	Prohexidione-Ca	Timing	ASM	Timing	inoculated	no inoc.
1	12 oz./100 gal.	foliar spray (-1)			18	
2	12 oz./100 gal.	foliar spray (-8)			18	3
3			3.2 oz./100 gal.	foliar spray (-1)	18	3
4	12 oz./100 gal.	foliar spray (-1)	3.2 oz./100 gal.	foliar spray (-1)	18	
5	12 oz./100 gal.	foliar spray (-8)	3.2 oz./100 gal.	foliar spray (-1)	18	3
6	12 oz./100 gal.	foliar spray (-1)	30 g/L	**trunk paint (-1)**	18	3
7			30 g/L	**trunk paint (-1)**	18	3
	Regalia	**Timing**	**LifeGard**	**Timing**		
8	1 gallon/100 gal.	foliar spray (-1)			18	
9	1 gallon/100 gal.	foliar spray (-8)			18	3
10			13.5 oz./100 gal.	foliar spray (-1)	18	3
11	1 gallon/100 gal.	foliar spray (-1)	13.5 oz./100 gal.	foliar spray (-1)	18	
12	1 gallon/100 gal.	foliar spray (-8)	13.5 oz./100 gal.	foliar spray (-1)	18	3
13	1 gallon/100 gal.	foliar spray (-1)	60 g/L	**trunk paint (-1)**	18	3
14			60 g/L	**trunk paint (-1)**	18	3
15	1:1 concentrate	**trunk paint (-1)**			18	3
16-17	**Inoc control**	-	-	-	36	
18	**No Inoc control**	-	-	-		12

In parentheses, the treatment timings '-1' and '-8' refers days before inoculation.

Observations on fire blight progression. In spring 2021, trees acquired for the study exhibited generally uniform leaf emergence and vegetative shoot production. At inoculation (9 June), lengths of the new shoots on the trees averaged 33 cm (13"). Inoculation of the terminal shoot tips with *E. amylovora* incited fire blight in 254 of 255 trees (99.7%). In the first month after inoculation, for control trees, the expanding canker consumed ~2/3 of the vegetative shoot tissue below the inoculation site (Fig. 1). From July to August, cankers in control trees continued to expand, extending into woody stem tissue on 64% of trees. Canker expansion slowed in the woody tissue. On 5 August, an average of 80% of shoot tissue on inoculated control trees showed symptoms of fire blight. Non-inoculated control trees did not show fire blight symptoms.

Treatments that suppressed canker expansion included Apogee sprays (15% suppression), Actigard sprays (with or without co-treatment with Apogee, 28% suppression), and Actigard trunk paints (with or without co-treatment with Apogee, 55% suppression) (Fig. 4). Organic inducers, whether applied as sprays or trunk paints in solitary or co-treatment, showed no significant reduction of canker expansion in new shoot tissue.

Observations on PR-gene expression. On the first leaf sampling date (22 June), most treatments (both organic and conventional) showed elevated expression of PR-1 and PR-2, although with the exception of Actigard trunk paint treatments, expression of defense genes relative to the untreated control was relatively small (< 10-fold) (Fig.5). For PR-2, Actigard trunk paints enhanced expression by 20 to 40-fold relative to the untreated control. On the second leaf sampling date (29 June) (Fig.5) and third leaf sample date (5 Aug) (data not shown), only the Actigard trunk paint treatments (with or without co-treatment with Apogee) showed elevated defense gene expression.

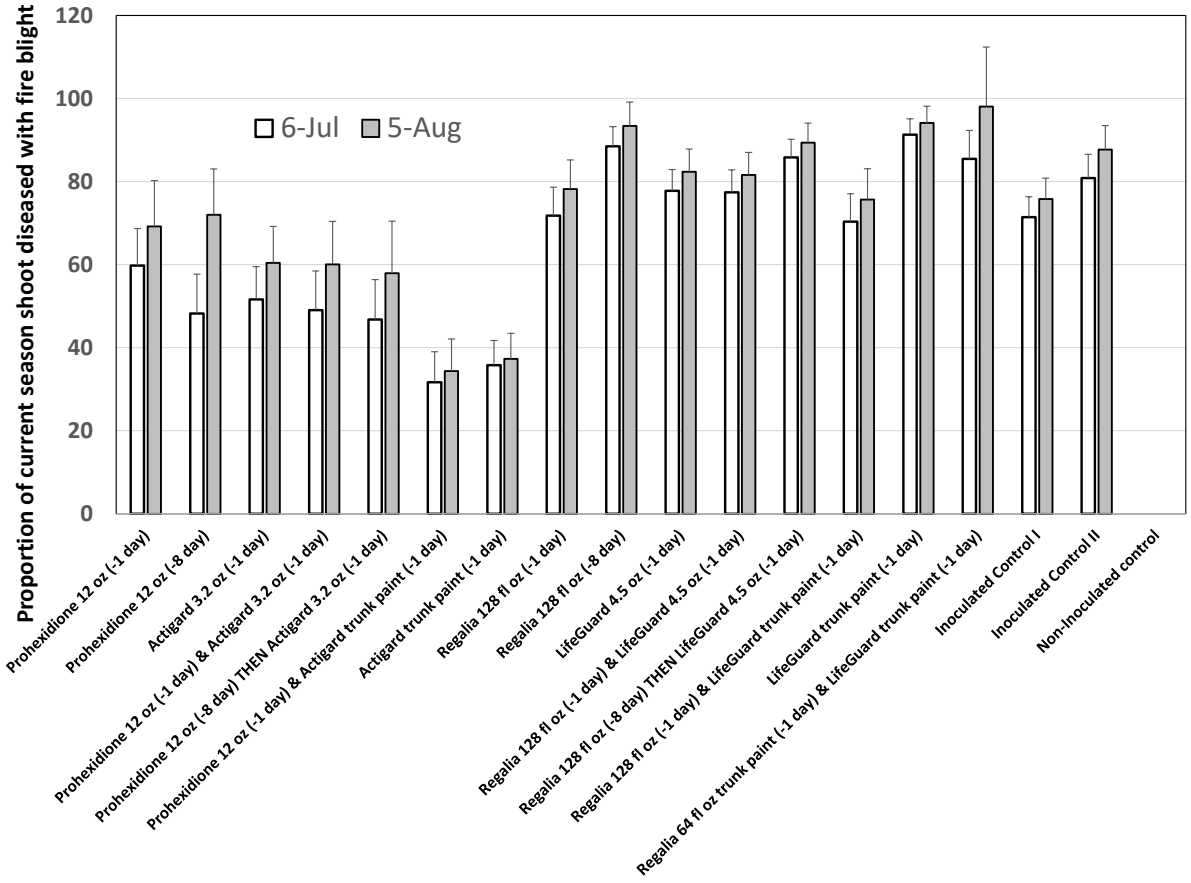


Fig. 4. Effect of applied resistance inducers on the proportion of ELMA.26 shoots diseased with fire blight. A shoot tip on the potted 2-year-old, greenhouse trees were inoculated with *Erwinia amylovora* in early June. Treatments were either a SPRAY applied to runoff at 8 or 1 day before inoculation, or a TRUNK PAINT applied to runoff at one day before inoculation. The date of inoculation was 9 June; data for 6 July (white bar) and 5 August (gray bar) are shown. Data are expressed as length of the shoot canker divided by current season shoot length. Smaller bars are the standard error of the mean (n = 18 trees). See Table 3 for additional information related to experimental design.

Discussion. From past experience and literature reports, we know that both Actigard and Ph-Ca (Kudos, Apogee) when applied to trees as solitary treatments slow/prevent the development of shoot blight during late spring and early summer. Also known are the mechanisms of suppression of Ph-Ca (inhibition of gibberellin synthesis) and Actigard (induction of systemic acquired resistance). Perhaps unsurprisingly, our experiment confirmed that both materials suppress canker expansion to a partial degree, and that Actigard induced defense gene expression and Ph-CA did not. The higher degree of defense gene induction by Actigard trunk paints relative to sprays is a result we have demonstrated previously. Consequently, the 'new information' revealed by this experiment was limited, but it did confirm our previous understanding of Ph-CA and Actigard.

For both orchard and greenhouse data, none of the experiments demonstrated additivity or synergy from combinations of Actigard and Ph-Ca. Of the two materials, Actigard results were more intriguing because the degree ofsuppression was stronger, and the understanding of its full potential in fire blight management is still incomplete. It would

behoove the industry to continue to investigate the degree to which Actigard can impact the fire blight development in susceptible cultivars. This includes a better understanding how rate and frequency of sprays contributes to suppression, as well as further investigation of concentrated trunk applications for blight prevention. Currently, trunk applications of Actigard are limited by the material cost and the EPA label. These constraints could potentially be modified if the biological data can further demonstrate the material's value.

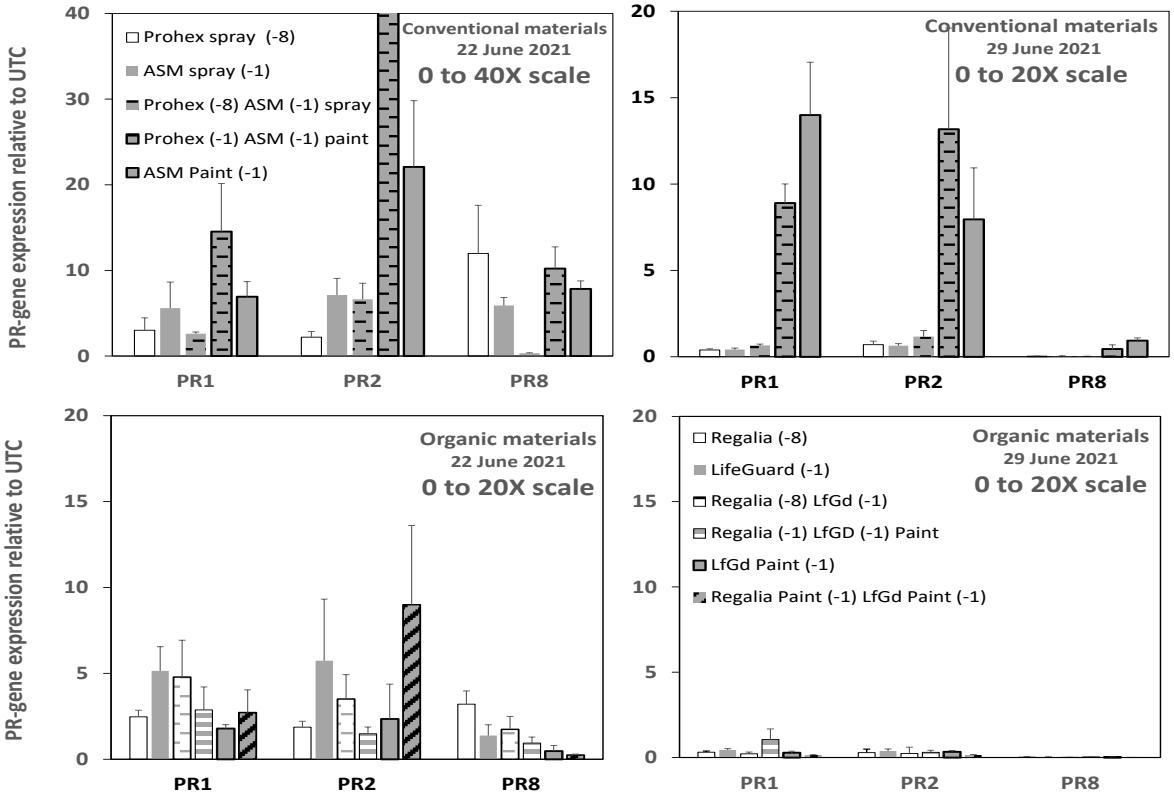


Fig. 5. Effect of applied resistance inducers on expression of pathogenesis-related (PR) genes PR-1, PR-2, and PR-8 in ELMA.26 trees. Treatments were either a SPRAY applied to runoff at 8 or 1 day before inoculation, or a TRUNK PAINT applied to runoff at one day before inoculation. Relative levels of PR protein gene expression were quantified by quantitative reverse-transcription polymerase chain reaction performed on c-DNA prepared from total RNA extracts from apple leaves sampled approximately 3, 4 and 8 weeks after the pathogen inoculation using expression of actin as the reference gene. The date of inoculation was 9 June, and data for 22 June (left panels) and 29 June (right panels) are shown. Data are expressed as length of the trunk canker divided by the height of the tree. Smaller bars are the standard error of the mean (n = 3 trees). See Table 3 for additional information related to experimental design.

EXECUTIVE SUMMARY

Project title: Refinement of practical fire blight control: Non-antibiotic and SAR

Key words: fire blight, non-antibiotic control

Abstract: Suppression of fire blight (caused by *Erwinia amylovora*) with resistance-inducing materials was investigated. Solitary, prebloom treatment of Kudos/Apogee (prohexadione-Ca), Actigard, or Regalia were of little value. Three-spray, bloom period programs of Actigard only provided up to 75% infection suppression. Organic, bloom period spray programs that combined the materials, Regalia and LifeGard, provided 59% infection suppression.

SIGNIFICANT FINDINGS:

- Kudos/Apogee (prohexadione-Ca), Actigard, or Regalia applied at prebloom timings did not provide significant suppression of fire blight in flowers compared to a non-treated control.
- Kudos applied as a solitary treatment at timings of first pink, full pink and 10% bloom, did not reveal a pattern related to time of treatment and level of suppression.
- In 2020, Actigard sprayed three times during bloom reduced the incidence of infection compared the water-treated control (45 and 65 % reduction in pear and apple, respectively). In2020, a prebloom Kudos treatment appeared to enhance the level of suppression obtained by the Actigard treatment program. But in 2021, the degree of suppression obtained with a 3-spray Actigard program (75%) was not improved with a prebloom treatment of Apogee.
- In greenhouse, an Actigard spray reduced fire blight canker expansion in potted ELMA.26 by 28%, and the effect of Actigard was not improved with the addition of an Apogee spray. A single Actigard trunk paint treatment reduced canker expansion by 55% and was not improved with the addition of an Apogee spray.
- In greenhouse, among spray and trunk paint treatments of Apogee, Actigard, Regalia, and LifeGard, only Actigard trunk paint treatments showed sustained expression of apple defense genes, PR-1, and PR-2.
- In greenhouse, treatments with the organic materials, Regalia and LifeGard, had no effect on fire blight canker expansion in potted ELMA.26.

FUTURE DIRECTIONS

- Within current labeling restrictions, Actigard requires further investigation including how rate and frequency of sprays contributes to preventative blossom blight suppression.
- Similarly, combination bloom period spray programs of Regalia and LifeGard require further investigation.
- Without regard to practicality, industry-sponsored research should learn as much as possible about the degree to which Actigard could impact the development of fire blight in susceptible apple cultivars (i.e., out-of-the-box thinking with little consideration to current material cost, pesticide labeling or application rate or technology).

FINAL PROJECT REPORT

Project Title: Integrated Fire Blight Management

PI: Tianna DuPont
Organization: WSU Extension
Telephone: (509) 293-8758
Email: tianna.dupont@wsu.edu
Address: Tree Fruit Research and Extension
Address 2: 1100 N Western Ave
City/State/Zip: Wenatchee WA 98801

Co-PI (2): Ken Johnson
Organization: Oregon State University
Telephone: (541) 737-5249 x5248
Email: kenneth.johnson@oregonstate.edu
Address: Dept. Botany & Plant Pathology
Address 2: 2082 Cordley Hall
City/State/Zip: Corvallis, OR 97321-2902

Co-PI(3): Kerik Cox
Organization: Cornell University
Telephone: (315) 787-2401
Email: kdc33@cornell.edu
Address: Cornell AgriTech
Address 2: 630 West North Street
City/State/Zip: Geneva, NY, 14456

Co-PI (4): Kari Peter
Organization: Penn State University
Telephone: 717-677-6116 Ext. 223
Email: kdc33@cornell.edu
Address: Fruit Research & Extension Ctr
Address 2: PO Box 330, 290 University
City/State/Zip: Biglerville, PA 17307

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This award was leveraged by the generous support from the WTFRC.

INTEGRATED FIRE BLIGHT MANAGEMENT

OBJECTIVES

1. Test materials to prevent bloom infections including biologicals, tank mixes, and mixes with bioregulators.
2. Demonstrate management strategies for young trees including coppers, plant defense elicitors, and Prohexodine Calcium (PhCa).
3. Test strategies to manage blocks once they are infected. Treatments will address how far back to cut, the utility of stub cuts, timeliness of cutting and the use of plant defense elicitors.
4. Provide outreach on fire blight prevention and management.

SIGNIFICANT FINDINGS

- Alum performed well in 7 of 8 blossom blight prevention trials in WA, NY, PA and OR.
- Thyme and cinnamon oil products provided intermediate control.
- Thyme oil products performed well as part of an organic program with Blossom Protect and soluble copper when applied at petal fall.
- Prohexadione calcium (Apogee/Kudos) performed best when applied 2 weeks before inoculation. 6 oz or higher rates may be important in WA/OR compared to success at the 3 oz rate in NY.
- The 40 oz rate of Serenade Opti performed no better than the 20 oz standard for blossom blight control.
- For protection of young non-bearing trees flower removal was best followed by 3 weekly applications of soluble copper (Previsto/Cueva) at 3-4 qt/ A or basic copper 1.5 lb/100 gal.
- In a replacement tree trial in OR only 42% of trees treated 3 days before infection with Actigard (vs 88% untreated, 79% preplant) developed trunk cankers.
- Timely summer cutting of fire blight infections significantly reduced the number of trees which developed rootstock blight and died from fire blight infections.
- The standard best management fire blight cutting practice where cuts are made 12 inches from the edge of the noticeably infected tissue into 2-year or older wood with sanitized loppers significantly reduced the number of new systemically caused infections compared to no-treatment controls in 8 of 9 case study trials.
- While Breaking provides a fast fire blight removal method it can leave many cankers in the orchard which provide a source for infection in subsequent years.
- In 2 of 4 case studies cutting which left a 5-inch Long Stub from structural wood significantly reduced the number of cankers on structural wood compared to flush cut or 1-inch stub.

RESULTS AND DISCUSSION

Objective 1. Test materials to prevent bloom infections including biologicals, tank mixes, and mixes with bioregulators. A number of biopesticides were tested in order to determine the efficacy of new products and try to improve the use of existing products for the control of fire blight of apple. It was hypothesized that increasing the rate for Serenade Opti might improve control. Serenade Opti provided variable control: intermediate control at low pressure Oregon 2019, 42% relative control (relative to water treated check); good New York 2019 72% relative control, low-moderate Washington 2019 24% control, 29% control Pennsylvania 2019 (Table 1). Considering timings Serenade Opti did better in trials where it was applied closer to the timing of inoculation (day of vs day before inoculation). Overall best timing for Serenade Opti was early or late bloom when the risk due to number of blooms open is less. Doubling the rate provided no additional control (Table 1). The test material Alum (aluminum potassium sulfate) has been tested for multiple years in Oregon and Washington showing significant potential but had not been tested in the eastern United States.

Relative control of fire blight by Alum with 2 to 3 applications was good in seven of eight trials (2019: WA 80%, NY 77%, PA 57%; 2020: WA 29%, OR 86%, NY 65%; 2021: WA 50%, NY 87%) (Tables 1-3). Several new essential oils have recently been marketed for fire blight control. It was critical to test these new products in order to avoid potential control failures and identify appropriate timings and rates. A 23% thyme oil product (Thymegard) with multiple post petal fall applications had provided good control but resulted in marking in WA in 2019. Application timing was adjusted to three applications with the latest application at petal fall and products were applied under fast drying conditions in 2020-21 and applied in multiple states, and it provided intermediate to moderate relative control (WA 2020 45%, WA 2021 45%, NY 2021 81%) (Table 2-3). *Note:* Frost and a freeze occurred during bloom in the 2020 Pennsylvania trial and as such due to very few surviving blooms PA 2020 data is not included in the analysis. Thyme oil was also applied as part of an organic program where Blossom Protect + Buffer Protect was applied 2x pre bloom followed by a soluble copper at full bloom just prior to Erwinia inoculation and thyme oil was applied at petal fall. This alternative organic program provided good control, comparable to the organic standard program in three of three trials (relative control organic alternative:organic standard 59:64% Washington 2021, 100:100% New York 2021, 91:93% Oregon 2021) (Tables 2-3). A new 60% cinnamon oil product (Cinnerate) was tested in four trials with 3-4 applications and provided control relative to the water treated check of 38% WA 2020, 70% NY 2020, 46% WA 2021, and 85% NY 2021 (Tables 2-3).

Objective 2. Demonstrate management strategies for young trees including coppers, plant defense elicitors, and prohexodine calcium (PhCa). Young non-bearing trees are particularly susceptible to fire blight infections and in high-risk areas growers apply preventative programs regardless of seasonal fire blight risk. Trials specific to management of non-bearing trees are important to improve efficacy and potentially reduce costs. In 2019-2020 seven trials were conducted to evaluate the use of plant defense elicitors, prohexodine calcium, coppers and flower removal for the suppression of fire blight bloom and shoot infections.

In 2019 prohexodine calcium (Kudos or Apogee) applied once at pink (10% bloom) at 6 oz/100 gal reduced infections per 100 clusters compared to the water treated check from 27 to 10 in OR, from 88 to 15 in NY, and from 94 to 65 in PA but was no different than the water treated check in WA (Table 4). In 2020 prohexodine calcium at 6 oz per 100 gal applied once at pink provided no significant reduction in infections compared to the water treated check in Oregon and two applications at 12 oz per 100 gal at tight cluster and petal fall reduced infections per 100 clusters from 77 to 11 in NY and 71 to 22 in PA (Table 5). Low rates of prohexodine calcium appear to be most useful for fire blight suppression in young or vigorously growing trees. The time between treatment and infection may also be important. For example, in 2019 the PA application at pink was 9 days before full bloom inoculation, and in Oregon application at pink was 3 days before full bloom and 9 days before when infection likely occurred at petal fall versus in New York pink was 14 days before full bloom inoculation. Further study in OR and WA conditions are necessary. A young tree planting was planted in 2021 to be used for further testing in WA in 2022.

For protection of young non-bearing trees flower removal was best followed by weekly applications of soluble copper 3-4 qt/A or basic copper at 1.5 lb/A. Flower removal at pink for young non-bearing trees reduced infections to the lowest level with 0 infections per 100 clusters in PA and 0 per 100 in NY in the 2020 trial (Table 5). Three applications of soluble copper (Previsto 3 qt, Cueva 4 qt) reduced infections per 100 clusters from 77 to 5.5 NY, and 71 to 17 PA and three applications of basic copper (1.5 lb) reduced infections per 100 clusters from 77 to 27 NY and 71 to 8.3 PA.

In Oregon in 2019 application timing of concentrated Actigard 50WG (acibenzolar-S-methyl (ASM), Syngenta Crop Protection, Greensboro, NC) treatments was evaluated on 1st-leaf Fuji apple trees as either a pre-plant or post-plant trunk spray for protection from fire blight infection. Overall, 99 of 100

(99%) of inoculated trees developed fire blight symptoms on at least one shoot. Number of infected shoots per tree was highest for untreated and pre-plant Actigard (4.1 of 5) and lowest for post-plant Actigard (3.1 of 5). By 18 September, trunk cankers developed and advanced on 88% of untreated trees and on 79% of trees treated with Actigard pre-plant. In contrast, trunk cankers developed on only 42% of trees treated with Actigard near post-plant (near inoculation). For those trees with trunk infection, by September, the average canker on a post-plant Actigard-treated trees was 78% smaller than the average canker on an untreated tree.

Objective 3. Test strategies to manage blocks once they are infected. Nine case studies were conducted to evaluate the success of fire blight cutting strategies in orchards with different scion, rootstocks, age, vigor and training system combinations.

The most important goal of timely aggressive cutting of fire blight infected material during the summer soon after infection occurs is to save the trees. If infections are not cut out and removed the fire blight bacteria can move through the tree killing large limbs, the entire scion or create rootstock blight killing the tree. Seven cutting treatments were compared to identify which would reduce the percentage of trees killed by fire blight infections. In 4 of 7 case studies where rootstock blight or tree death occurred all cutting treatments reduced the number of trees which died or acquired rootstock blight from fire blight compared to the no-treatment control (Table 10). In case studies 'New York 2019 Ever Crisp' and 'New York 2019 Idared' in 100% of no-treatment control (NTC) trees the scion died down to the resistant rootstock compared to 0 trees in cutting treatments. In case study 'Washington 2021 Pink Lady' 38% of NTC trees developed rootstock blight (which will lead to tree death) compared to 0-16% in cutting treatments. This data demonstrates that timely summer cutting of fire blight infections is critical in young or vigorous trees to avoid losing trees and orchards.

The fire blight pathogen *Erwinia amylovora* moves systemically through the tree from the point of infection. Bacterial cells in the plant's vascular system move down the branch and through the tree much more quickly than canker symptoms become visible on limbs and trunks. The recommended best management practice is to cut 12-18 inches below the visible symptoms in the tree to remove the majority of bacterial cells so that insufficient cells remain to move through the tree to new limbs where they can create new infections in young tender shoot tips. The treatment best management practice (BMP) where cuts were made 12-inches from the edge of the noticeably infected tissue into 2-year or older wood with sanitized loppers significantly reduced the number of new infections/ new cuts compared to the no-treatment control (NTC) in eight of nine case studies (Table 7). The only exception was site 'Washington 2020 Pink Lady' where trees were 14-years old, very low vigor and initial cutting was performed more than 2 weeks after symptoms first became noticeable. We hypothesized that aggressive cutting may reduce the number of new infections by removing more bacterial cells from the tree and consequently reducing the chances that sufficient cells are left to cause new infections. In treatment Aggressive branches were cut back approximately 76 cm (30 inches) from the edge of the noticeably infected tissue with sanitized loppers. Treatment Aggressive had fewer new infections than BMP at 'Washington 2019 Yarlington Mill' and 'Washington 2020 Pink Lady' but the number of new infections was no different than BMP at other sites. In site 'Oregon 2020 Gala' no new infections occurred in either BMP or Aggressive. Similarly in site "New York 2021 Gala' the number of new infections was low in both BMP and Aggressive cutting treatments. Site 'New York 2019 Evercrisp' were highly vigorous young trees where both BMP and Aggressive had three times fewer new infections than the no treatment control (NTC) but still resulted in 4-5 new infections per tree. Importantly, in site 'Washington 2021 Pink Lady' aggressive cutting resulted in excessive new growth and provided abundant susceptible tissues for bacterial infection.

When cankers caused by fire blight infections reach central leaders and main structural branches (structural wood) growers face the decision to either cut out the canker removing large parts of the tree resulting in a lost productive capacity for several years or leave cankers which are the source of new fire blight infections the following spring. It was hypothesized that by leaving a stub of 4-5" from the central leader or structural branch when cutting blight any new infections that re-ignite would be on the stub which could then be removed during winter pruning and reduce the number of cankers on structural wood. A Long Stub treatment where noticeable infections were cut back leaving a 5-inch stub of branch from the central leader or main structural branch using sanitized loppers was compared to a Short Stub where branches were cut flush or a 1-2-inch stub left. In two of four case studies where these treatments were compared 'Washington 2020 Pink Lady' and 'Washington 2021 Pink Lady' a Long Stub significantly reduced the number of cankers on structural wood. In the remaining two case studies 'Pennsylvania 2019 Gala' no cankers progressed to structural wood and in 'Washington 2019 Yarlington Mill' trees were grafts where the main structural wood was old Red Delicious interstems which are not very susceptible to symptomatic infection.

In some orchards managers are employing breaking rather than cutting to remove fire blight infected wood. This practice is designed to be quick and avoid the use of loppers which require sanitization. This practice is primarily used in V-trellis training systems where limbs are trained to a wire where they are difficult to remove. In case study trials we implemented the treatment Breaking where limbs with infections were broken back by hand, snapping the wood at the joint between the first-year growth and the second-year growth. In case study 'Washington 2021 Pink Lady' where 4th leaf trees were trained to the wire, treatment Breaking resulted in significantly more new infections than other cutting treatments, similar to the NTC (Table 7). In 3 of 9 case studies Breaking resulted in more canker tissue left in the tree at the end of the season (Table 8) compared to BMP. The larger number of remaining cankers provide a greater source of infection in the following year. In 2 of 9 case studies Breaking also resulted in significantly higher numbers of cankers on structural wood than BMP and cankers on structural wood were numerically higher in 2 additional case studies (Table 9). While Breaking provides a fast fire blight removal method it can leave many cankers in the orchard which provide a source for infection in subsequent years.

Objective 4. Provide outreach. Twenty fire blight management presentations were given in Washington between 2019 and 2021 to a total of 2291 participants. This included a talk given by the four-researcher grant team to 353 participants.

In a 2021 survey 79% of respondents managing 89,000 acres said they used WSU Extension information to inform their fire blight management decisions (N=230). 28% believed WSU information improved their control programs. 52% said they avoided a product with low efficacy. Avoiding non-efficacious fire blight programs is critical to preventing outbreaks which can kill trees and result in orchard removal. For example, one large grower removed 56,000 trees in 2018 due to fire blight. At $8 average per tree plus labor costs and 3 years of lost production he estimates one fire blight event cost their orchard over $1,000,000 in one season (approx. $18/tree). With 24 million apple trees less than four years old in Washington (WA Tree Survey 2017), a susceptible age for death from fire blight, and 20% of apple acres affected in a bad year, 52% of growers avoiding a non-efficacious product may have saved the industry $215 million in a year with high disease pressure.

For a copy of this report which contains materials and methods email tianna.dupont@wsu.edu or visit http://treefruit.wsu.edu/tianna-dupont/

Table 1. Effect of Fire Blight Materials for Prevention of Blossom Blight in 2019β**

Treatment	Rate per 100 gal	Timing	strikes per 100 clusters																
			Washington*‡ 'Red Delicious'				Oregon§ Bartlett Pear				New York¤ 'Gala'				Pennsylvania† 'Cameo'			
Streptomycin standard[y] (Firewall 17)[x] (Firewall 50)[v]	24 oz 8 oz	50% bloom, 100% bloom, petal fall	4.8	±	2.8	c	1.7	±	0.5	c	5.5	±	2.1	de	1.4	±	3.8	e
Oxytetracycline standard[y] (Fireline 17)	24 oz	50% bloom, 100% bloom, petal fall	5.7	±	3.1	c									10	±	12.5	e
Blossom Protect Buffer Protect	21.4 oz 150 oz	70% bloom, 100% bloom					2.7	±	0.7	bc	8.0	±	4.9	de				
Blossom Protect Buffer Protect Previsto or Alum	1.24 lb 8.75 lb 3 qt 8 lb	20% bloom, 80% bloom, 100% bloom, petal fall	6	±	1.1	c	2.3	±	1.4	bc								
Serenade[v]	20 oz	100% bloom -1 day, 100% bloom + 1 day, petal fall	16	±	3.2	abc	5.1	±	1.3	b	24.6	±	5.6	bc	67	±	11.9	c
Serenade	40 oz	100% bloom -1 day, 100% bloom + 1 day, petal fall	20.3	±	8.2	abc									71	±	31.7	bc
Cueva	4 qt	100% bloom -1 day, 100% bloom + 1 day, petal fall	11.5	±	4.1	abc												
Previsto	4 qt	100% bloom -1 day, 100% bloom + 1 day, petal fall	8	±	3.7	bc												
Alum	8 lb	80-100% bloom, petal fall	4.3	±	2.7						20.3		5.5	bcd	40	±	20.9	d
Water-treated check	NA	100% bloom, petal fall[x]	21		11	abc	9.0		1.3	a	88.1	±	3.3	a	94	±	5.9	a

[y] Amended with Regulaid: 30 fl. oz. per 100 gallons. Pennsylvania had an additional 80% bloom timing.

* Transformed log(x + 1) prior to analysis of variance; non-transformed means are shown.

**Values within columns followed by the same letter are not significantly different ($P < 0.05$) according to the LSMEANS procedure in SAS 9.4.

‡Inoculation was conducted on the evening of April 27, 2019 at full bloom (of king blooms), and May 1 petal fall using a suspension of freeze-dried cells of Erwinia amylovora strain 153N (streptomycin and oxytetracycline sensitive pathogen strain). 2019 application dates were: April 21 (pink), April 23 (20% bloom), April 24 and 25 (50% bloom), April 26 (full bloom minus 1 day), April 27 (full bloom), April 28 (full bloom plus 1 day), May 1, 2019 (petal fall), May 2, May 4 and May 6, and May 10, 2019.

§Oregon bartlett pear, trees inoculated on 24 April with 1 x 106 CFU/ml Erwinia amylovora strain Ea153N (streptomycin- and oxytetracycline-sensitive fire blight pathogen strain). Application dates included 10% bloom (April 23), full bloom (April 26), petal fall (May 1) of 2019.

[ɤ]New York 2019 application dates were pink (8 May), 40% bloom (13 May), 80% bloom (16 May), 100% bloom (23 May) petal fall (May 30), terminal shoot growth (5 Jun).
†Pennsylvania application dates were: Pink (17 Apr); 50% bloom (24 Apr); 80% bloom (26 Apr); 100% bloom (29 Apr); petal fall (2 May).
[x]Full bloom only in Washington. [x]Oregon had full bloom only timings. Pennsylvania had an additional 80% bloom timing. [v]Oregon.
[u]Additional application of Serenade Opti at 80% bloom and June terminal shoot growth in New York. Amended with Regulaid at 3 qt in New York.
[β]No noticeable fruit marking occurred with any treatments.

Table 2. Effect of Fire Blight Materials for Prevention of Blossom Blight in 2020[**]

Treatment	Rate per 100 gal	Timing	infections per 100 clusters Washington*[‡]	Oregon[§]	Oregon[¥]	New York[ɤ]	Pennsylvania[†]
Streptomycin standard[y] (Firewall 17)[x] (Firewall 50)[v]	28.8 oz[x] 2.7 oz[v]	100% bloom	2.8 ± 1.2 a	3.8 ± 1.5 a	1.5 ± 0.4 a	12.0 ± 2.2 bc	4.6 ± 7.5 c
Oxytetracycline standard[y] (Fireline 17)	28.8 oz[x] 16 oz[v]	50% bloom, 100% bloom, petal fall	8.2 ± 2 b	±	4.1 ± 0.6 b	27.5 ± 9.4 b	10.1 ± 9.4 a-c
Organic Standard Blossom Protect/Buffer + Soluble Copper (Previsto)	1.24 lb 8.75 lb 3 qt	50% bloom, 80% bloom, 100% bloom, petal fall	9.5 ± 1.3 bc	1.8 ± 0.4 a	---	7.0 ± 2.3 c	6.8 ± 6.2 a-c
Organic Alternative Blossom Protect/Buffer + Soluble Copper (Previsto) Thymegard	1.24 lb 8.8 lb 3 qt 2 qt	80% bloom, 100% bloom, petal fall	---	2.1 ± 0.8 a	---	---	---
Thyme Gard (0.5%)	2 qrt	80% bloom, 100% bloom +1 day, petal fall	17 ± 2.3 cd	---	---	---	4.9 ± 5.5 a-c
Alum[y]	8 lb	100% bloom, petal fall	22 ± 4.2 d		4.2 ± 1.6	28.0 ± 16.3 b	11.5 ± 6.2 ab
Cinnerate	1 qt	50% bloom, morning after inoc, petal fall	19 ± 3.5 d	---	---	24.0 ± 8.7 b	15.4 ± 26.6 a
Cinnerate	1 qt	100% bloom, petal fall	---	---	28 ± 1.7 c	---	---
Water-treated check	NA	100% bloom[x,v], +1 day[x], petal fall[x,v]	31 ± 7.1 d	24 ± 5 b	31 ± 1.7 c	80.1 ± 6.5 a	7.2 ± 3.4 a-c

[y] Amended with Regulaid: 30 fl. oz. per 100 gallons.
[x]Washington. Washington had additional 50% and petal fall applications. [v]Oregon.
* Transformed log(x + 1) prior to analysis of variance; non-transformed means are shown.
**Values within columns followed by the same letter are not significantly different ($P \leq 0.05$) according to the LSMEANS procedure in SAS 9.4.
[‡]Washington application dates were: April 14 (20% bloom), April 16 (50% bloom), April 17 (80% bloom) and April 18 (full bloom), April 19 (full bloom plus 1 day), April 22 (petal fall). Inoculation was conducted on the evening of April 18, 2020 at full bloom (of king blooms) using a suspension of 50% freeze-dried cells of *Erwinia amylovora* strain 153N (streptomycin and oxytetracycline sensitive pathogen strain) and 50% live cells, which was prepared at 24 x 10^6 CFU per ml.
[§] Oregon Golden delicious apple, application dates were 17 April and 21 April, 2020 (petal fall). On the evening of 19 April, a motorized 25-gallon tank sprayer equipped with a hand wand was used to fog a suspension (~2 liters per tree) of freeze-dried cells of *Erwinia amylovora* strain 153N (1 x 10^6 CFU per ml).
[¥] Oregon Gala apple, application dates were 17 April and 21 April, 2020 (petal fall). Inoculation was done on the evening of 15 April.
[ɤ]New York 2020 application dates were 29 April (tight cluster), 7 May (pink), 16 May (40% bloom), 20 May-(80% bloom), 22 May(100% bloom/petal fall), 29 May (petal fall/early terminal shoot growth).

[†]Pennsylvania application dates were: 4 April (tight cluster); 13 April (pink); 20 (20% bloom); 22 April (50% bloom; first inoculation); 23 April (+12 h post inoculation); 27 April (100% bloom, second inoculation); 28 April (+12 h post inoculation); 4 May (Petal fall). A frost occurred on 17 April, damaging a significant number of blossoms, thereby affecting results. In addition, the average temperature during the trial period was 49°F and no fire blight infection periods occurred.

Table 3. Effect of Fire Blight Materials for Prevention of Blossom Blight in 2021

			Infections per 100 clusters [w]					
Treatment	Rate per 100 gal	Timing	Washington* [z, y]			New York [u]		
Streptomycin standard (Firewall 17) [x]	8 oz	100% bloom	16.1 ± 2.3	a [w]				
Oxytetracycline standard (Fireline 17) [x]	16 oz	100% bloom, petal fall	17.0 ± 5.7	a				
Organic standard apple Blossom Protect + Buffer Protect Previsto	1.24 lb + 8.75 lb 3 qt	70% bloom, 100% bloom, 100% bloom + 1 day, petal fall	17.8 ± 4.5	a		0.0 ± 0.0		cd
Organic standard pear Blossom Protect + Buffer Protect Serenade Opti	1.24 lb + 8.75 lb 20 oz	70% bloom, 100% bloom, 100% bloom + 1 day, petal fall	13.9 ± 2.6	a		---		
Blossom Protect + Buffer Protect Previsto Thyme Gard [v]	1.24 lb + 8.75 lb 3 qt 2 qt	50% bloom, 100% bloom, 100% bloom + 1 day, petal fall	16.0 ± 1.9	a		0.0 ± 0.0		cd
Thyme Gard [v]	2 qt	100% bloom, 100% bloom + 1 day, petal fall	21.4 ± 3.9	ab		13.9 ± 10.8		bcd
Cinnerate + Probald Verde	32 oz + 40 oz	100% bloom, 100% bloom + 1 day, petal fall, petal fall + 3 days	17.6 ± 3.2	ab		12.8	12.8	bcd
Cinnerate	32 oz	100% bloom, 100% bloom + 1 day, petal fall, petal fall + 3 days	20.8 ± 3.7	ab		11.0	1.0	bcd
Alum [v]	*8 lb*	100% bloom, 100 bloom + 1 day, petal fall	19.3 ± 2.4	ab		10.0 ± 10.0		bcd
Jet Ag	128 oz	100% bloom + 1 day, petal fall, petal fall + 3 days	12.8 ± 1.6	a				
Oxidate 5.0 (1%)	128 oz	100% bloom + 1 day, petal fall, petal fall + 3 days	14.2 ± 1.2	a				
Water-treated check	NA	100% bloom, petal fall, petal fall + 3 days	38.6 ± 5.1	c		75.1 ± 11.1		a

[z] Application dates were: 18 Apr (70% bloom), 19 Apr (full bloom), 20 Apr (full bloom + 1 day), 23 Apr (petal fall), 26 April (petal fall + 3 days). Inoculation was conducted on the evening of 19 Apr 2021 at full bloom (of king blooms) using a suspension of 50% freeze-dried cells and 50% live cells of *Erwinia amylovora* strain Ea153 (streptomycin and oxytetracycline sensitive strain) prepared at 1 x10^6 CFU ml^{-1} (verified at 40-94 x10^6 CFU ml^{-1}).

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

[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).

[u] Application dates 24 Apr "tight cluster", 27 Apr "pink"; 2 May-20% bloom; 4 May-50% bloom; 6 May- 80% bloom; May 7 FB -1, 8 May-100% bloom/petal fall; May 9 FB + 1, 11 May-petal fall/early terminal shoot growth; 17 May- terminal shoot growth PF + .3. Trees were inoculated at 80 to 90% bloom (7 May) with *Erwinia amylovora* strain Ea 273 at 1x10^6 CFU ml^{-1} using a hand-pumped Solo backpack sprayer.

Table 4. Plant defense elicitors and prohexodine calcium for fire blight suppression in 2019.

Treatment	Rate per 100 gal	Timing	strikes per 100 clusters															
			Washington‡ Red Delicious				Oregon§ 'Gala'				New York[x] 'Gala'				Pennsylvania† 'Cameo'			
Water check	---	10% bloom, full bloom, petal fall	21	±	11	a**	26.7	±	4.25	a#	88.1	±	3.3	a**	94.1	±	5.9	a#
Untreated check	---	-----					13.8	±	1.58	bc								
Kudos[x, y] or Apogee	3oz	10% bloom	21.8	±	12.5	a	17	±	1.21	ab	17.8	±	8.1	bcd				
Kudos[x, y] or Apogee	6 oz	10% bloom	24	±	6.9	a	10.2	±	3.42	bc	15	±	4.9	bcde	65.3	±	25.8	c
Actigard[x, y]	6 oz	10% bloom					12.2	±	4.38	bc								
Kudos[x, y], Actigard[y]	2 oz, 3.2oz	10% bloom					11.2	±	3.53	bc								
Actigard	2 oz	10% bloom, full bloom, petal fall					5.33	±	2.04	c								
Regalia	**	pink, 50% bloom, petal fall													88.3	±	8.1	a
Lifegard	13.5 oz										16.3	±	3.1	bcde				

[x] Amended 1:1 with ammonium sulfate. [y] Amended with Regulaid: 16 fl. oz. per 100 gallons. [z] Amended with BioLink Spreader-Sticker: 4 fl. oz. per 100 gallons.
[#]Means within a column followed by same letter do not differ significantly ($P = 0.05$) based on Fischer's protected least significance difference.
‡ Washington inoculation was conducted on the evening of April 27, 2019 at full bloom (of king blooms), and May 1 petal fall using a suspension of freeze-dried cells of Erwinia amylovora strain 153N (streptomycin and oxytetracycline sensitive pathogen strain). 2019 application dates were: April 21 (pink/ 10% bloom), April 27 (full bloom), May 1(petal fall).
§ Gala trees inoculated on 18 April with 1 x 10^6 CFU/ml *Erwinia amylovora* strain Ea153N (streptomycin- and oxytetracycline-sensitive fire blight pathogen strain). 70% bloom (April 18), full bloom (April 20), petal fall (April 24).
†Twelve year-old 'Cameo' trees on B.9 rootstocks were used and two- tree treatments were arranged in a randomized complete block with four replications. All blossoms were inoculated on the tree, with the exception of the top 1-2 feet of the tree (could not be reached, unless with a ladder). Blossoms were inoculated late afternoon at 26 Apr with a bacterial suspension of 10^7 *Erwinia amylovora* cells/ml using a spray bottle. Blossom clusters were rated during the third week of May. Blossom clusters were rated infected if at least one blossom was dead. Due to the trees being overwhelming infection of blossoms for the majority of the treatments, shoot blight incidence was not counted.
[x]Treatment timings were: 8 May "pink" (application 1) 13 May-40% bloom (application 2); 16 May- 80% bloom (application 3); 23 May-100% bloom (application 4); 30 May- petal fall/early terminal shoot growth (application 5); 5 Jun- terminal shoot growth (application 6).
** Values within columns followed by the same letter are not significantly different ($P \leq 0.05$) according to the LSMEANS procedure in SAS 9.4 with an adjustment for Tukey's HSD to control for family-wise error.

Table 5. Effect of Products Applied for Prevention of Blossom and Shoot Blight in Young Trees on Blossom Blight in 2020.

Treatment	Rate per 100 gallons	*Timing*	Strikes per 100 clusters					
			Washington[‡] 2nd leaf WA38	Oregon[§] 6-yr-old Gala	Oregon[§] 2-yr-old Gala	New York[#] 2nd leaf Gala	Pennsylvania[†] 3rd leaf Gala	
Inoculated Check	water	100% bloom, +1 day, petal fall	0 ± 0	41 ± 6 a	39 ± 7 a	77.2 ± 4.4 a	71 ± 20.1 a	
Flower removal	NA	Pink	0 ± 0	---	---	0 ± 0 d	0 ± 0 d	
Basic Copper	1.5 lb	3 applications	5 ± 0	---	---	27.3 ± 3.3 b	8.3 ± 12.1 c	
Previsto Or Cueva	3 qt 4 qt	3 applications	0 ± 0	---	---	5.5 ± 2.1 c	17.3 ± 17.2 c	
PhCa[y z]	6 oz	tight cluster, petal fall	0 ± 0	---	---	6.5 ± 1.7 c	42.4 ± 24.0 b	
PhCa[y z]	6 oz	full pink	---	34 ± 3 a	36 ± 4 a	29.5 ± 9.7 b	---	
PhCa[y z]	12 oz	tight cluster, petal fall	0 ± 0	---	---	10.5 ± 1.0 c	21.8 ± 23.5 c	
Actigard	2 oz	10% bloom, 80% bloom, petal fall	0 ± 0	---	---	17.8 ± 2.3 bc	14.4 ± 16.1 c	
PhCa[z y] Actigard	6 oz 2 oz	full pink	---	31 ± 5 a	32 ± 5 a	20.8 ± 3.9 bc	---	
Regalia	64 oz	10% bloom (pink), 80% bloom, petal fall	0 ± 0	33 ± 7 a	37 ± 5 a	26.5 ± 1.7 b	---	
Employ	2 oz	10% bloom, full bloom, petal fall	0 ± 0	---	---	23.5 ± 2.9 b	---	
Fireline 17 (standard oxytet)	28 oz	50% bloom, 100% bloom, PF	---	---	---	10.0 ± 1.3 c	---	

[y] Amended with surfactant (Regulaid) at 16 fl oz per 100 (Oregon) 32 oz per 100 gal (Washington).

[z] Kudos amended with 1 lb of ammonium sulfate per 100 gal (Washington), 6 oz. ammonium sulfate (Oregon).

‡ Washington application dates were: April 15, pink, April 19 (20% bloom), April 21 (50% bloom), April 23 (full bloom), April 24 (full bloom plus 1 day), April 28 (petal fall). Inoculation was conducted on the evening of April 23, 2020 at full bloom (of king blooms) using a suspension of freeze-dried cells of *Erwinia amylovora* strain 153N (streptomycin and oxytetracycline sensitive pathogen strain), which was prepared at 1.3 x10^6 CFU per ml. **Only 3 cluster infections occurred in the block.**

§Oregon application dates were: 11 April full pink). Inoculation was conducted on the evening of April 23. On the evenings of 15 and 19 April, a motorized 25-gallon tank sprayer equipped with a hand wand was used to lightly fog a suspension of freeze-dried cells of *Erwinia amylovora* strain 153N (streptomycin and oxytetracycline sensitive pathogen strain), which was prepared at 1 x 10^6 CFU per ml (0.1 to 0.2 liters per tree).

#New York application dates were New York application dates were 29 Apr "tight cluster", 7 May "pink", 16 May-40% bloom, 20 May- 80% bloom, 22 May-100% bloom/petal fall, 29 May- petal fall/early terminal shoot growth.

†Pennsylvania application dates were: 6 Apr (tight cluster); 20 Apr (pink); 27 Apr (20% bloom); 1 May (50-80% bloom); 8 May (Petal fall). Frost occurred on 17 Apr and a freeze occurred on 9 May. There were no days indicating an infection period for fire blight during our trial. The average temperature was ~50°F during the test period.

**Values within columns followed by the same letter are not significantly different ($P \leq 0.05$) according to analysis of variance (F>0.05).

Table 6. Response of Fuji apples trees to inoculation with *E. amylovora* after trunk treatment of Actigard 50WG prior to or after planting.

Disease response	Untreated		Pre-plant Actigard		Post-plant Actigard	
	July 24	Sept 18	July 24	Sept 18	July 24	Sept 18
No. infected shoots post inoculation*	4.1± 1.1	-	3.9 ± 1.1	-	3.1 ± 1.2	-
Incidence of trunk canker**	85%	88%	65%	79%	39%	42%
Canker length infected trunks***	29 ± 17	49 ± 33	25 ± 20	46 ± 36	10 ± 5	11 ± 5

* Five shoots per tree were inoculated on 7 June with 1 x 10^9 CFU/ml *Erwinia amylovora* isolate mixture and were assessed for fire blight on 24 July and 18 September (± standard deviation).
** Percent of inoculated trees that developed a trunk canker (of a total of 33 trees per treatment).
*** Mean canker length (cm ± standard deviation) on trunks with symptoms; zero values not included.

Table 7. Effect of treatment on the number of new infections after initial cutting and removal of fire blight infections.

Cutting method	Washington 2019 'Yarlington Mill' 4-yr-old on Red Delicious[z]		New York 2019 'Ever Crisp' 4-yr-old on G.41[y]		New York 2019 'Idared' 7-yr-old on B.9[y]		Pennsylvania 2019 'Gala' 4-yr-old on M.7[x]		Washington 2020 'Pink Lady' 14-yr-old on M9.337[w]		Oregon 2020 'Gala' 3-yr-old on M9.337		Washington 2021 'Pink Lady' 4-yr-old on M9.337[u]		New York 2021 'RubyFrost' 3-yr-old on G.41[y]		New York 2021 'Gala' 18-yr-old on B.9 [t]	
BMP	2.6 ± 0.7	b	5.5 ± 2.3	b	2.9 ± 1.1	b	---		0.7 ± 0.3	ab	0.0 ± 0.0	a	1.5 ± 0.3	a	4.5 ± 1.5	a	0.7 ± 0.3	ab
Aggressive	0.5 ± 0.3	a	4.3 ± 1.3	b	---		---		0 ± 0.0	a	0.0 ± 0.0	a	1.6 ± 0.6	a	3.2 ± 1.1	a	0.7 ± 0.3	ab
BMP NO-sanitize	2.7 ± 0.8	b	3.9 ± 0.5	b	3.6 ± 0.8	b	---		0.3 ± 0.2	ab	0.8 ± 0.3	a	1.2 ± 0.4	a	4.8 ± 1.1	a	0.6 ± 0.3	ab
Short Stub	2.4 ± 0.6	b	---		---		0 ± 0		0.4 ± 0.2	ab	---		0.8 ± 0.4	a	8.2 ± 1.9	a	0.0 ± 0.0	b
Long Stub	1.7 ± 0.5	ab	1.9 ± 1.1	b	3.5 ± 1.2	b	0 ± 0		0.3 ± 0.2	ab	---		1.7 ± 0.3	a	---			
Breaking	2.8 ± 0.5	b	5.2 ± 0.9	b	1.4 ± 0.3	b	0 ± 0		0.9 ± 0.7	b	1.7 ± 0.5	a	5.0 ± 0.8	b	3.4 ± 0.7	a	1.1 ± 0.2	ab
NTC	7.5 ± 1.4	c	14.8 ± 1.2	a	24.5 ± 2.4	a	0 ± 0		0.5 ± 0.3	ab	6.2 ± 2.3	b	4.8 ± 1.8	b	7.8 ± 1.5	a	2.7 ± 0.4	a
BMP + ASM	---		---		---		---		---		0.0 ± 0.0	a	---		---			
Aggr NO-san	---		6.0 ± 1.3	b	1.3 ± 2.2	b	---		---		---		---		---			
Short Stub NO-san	---		---		---		0 ± 0		---		---		---		---			
Long Stub NO-san	---		---		---		0 ± 0		---		---		---		---			

[z]4-leader grafts, [y]high-density tall spindle, high vigor [x]tall spindle, low vigor, [w]tall spindle, low vigor, [u]Auvil V trained to the wire, moderate vigor, [t]Vertical axe, high vigor.

Table 8. Effect of treatment on the average length (cm) of cankers left in trees at the end of the season.

Cutting method	Washington 2019 'Yarlington Mill' 4-yr-old on Red Delicious[z]	New York 2019 'Ever Crisp' 4-yr-old on G.41[y]	New York 2019 'Idared' 7-yr-old on B.9[y]	Pennsylvania 2019 'Gala' 4-yr-old on M.7[x]	Washington 2020 'Pink Lady' 14-yr-old on M9.337[w]	Oregon 2020 'Gala' 3-yr-old on M9.337	Washington 2021 'Pink Lady' 4-yr-old on M9.337[u]	New York 2021 'RubyFrost' 3-yr-old on G.41[y]	New York 2021 'Gala' 18-yr-old on B.9[t]
BMP	0.4 ± 0.2 a	2.3 ± 0.7 ab	0.9 ± 0.3 b	---	1.1 ± 0.1 a	0.0 ± 0.0 a	1.2 ± 0.4 a	10.1 ± 6.6 ab	0.5 ± 0.3 c
Aggressive	0.0 ± 0.0 a	0.6 ± 0.4 c	---	---	0.0 ± 0.0 a	0.0 ± 0.0 a	19.7 ± 17.6 b	4.2 ± 4.2 b	0.0 ± 0.0 c
BMP NO-sanitize	0.4 ± 0.1 a	2.7 ± 0.8 ab	1.4 ± 0.6 b	---	1.1 ± 0.1 a	3.0 ± 3.0 ab	1.8 ± 0.5 a	7.9 ± 4.3 ab	0.4 ± 0.6 c
Short Stub	0.1 ± 0.1 a	---	---	16.7 ± 37.8 b	0.8 ± 0.0 a	---	0.4 ± 0.2 a	1.5 ± 1.5 b	0.0 ± 0.0 c
Long Stub	0.5 ± 0.1 a	2.5 ± 0.4 ab	1.5 ± 0.4 b	25.0 ± 43.9 b	1.0 ± 0.2 a	---	4.5 ± 3.1 ab		
Breaking	5.9 ± 3.0 b	7.5 ± 0.4 ab	1.4 ± 0.5 b	33.3 ± 47.8 b	3.2 ± 0.2 a	5 ± 2.4 b	1.9 ± 0.4 a	5.5 ± 3.0 b	12.4 ± 2.2 b
NTC	34 ± 3.7 c	12.2 ± 1.6 a	19.6 ± 1.5 a	91.7 ± 28.0 a	29.1 ± 4.3 b	13.5 ± 1.6 c	8.4 ± 2.2 ab	26.1 ± 3.8 a	29.1 ± 1.7 a
BMP + ASM	---	---	---	---	---	0.0 ± 0.0 a	---		
Aggress NO-san	---	1.1 ± 0.6 bc	0.5 ± 0.2 b	---	---	---	---		
Short Stub NO-san	---	---	---	25.0 ± 43.9 b	---	---	---		
Long Stub NO-san	---	---	---	22.2 ± 42.2 b	---	---	---		

[z]4-leader grafts, [y]high-density tall spindle, high vigor [x]tall spindle, low vigor, [w]tall spindle, low vigor, [u]Auvil V trained to the wire, moderate vigor, [t]Vertical axe, high vigor.

Table 9. Effect of treatment on the percent of strikes progressing to structural wood.

Cutting method	Washington 2019 'Yarlington Mill' 4-yr-old on Red Delicious	New York 2019 'Ever Crisp' 4-yr-old on G.41[y]	New York 2019 'Idared' 7-yr-old on B.9[y]	Pennsylvania 2019 'Gala'* 4-yr-old on M.7[x]	Washington 2020 'Pink Lady' 14-yr-old on M9.337[w]	Oregon 2020 'Gala' 3-yr-old on M9.337	Washington 2021 'Pink Lady' 4-yr-old on M9.337[u]	New York 2021 'RubyFrost' 3-yr-old on G.41[y]	New York 2021 'Gala' 18-yr-old on B.9[t]
BMP	2.2 ± 2.2 abc	2.4 ± 1.0 b	2.7 ± 0.9 b	---	11.9 ± 6.6 ab	0.0 ± 0.0 a	2.0 ± 2.0 a	2.3 ± 0.8 b	0.2 ± 0.1 b
Aggressive	0.0 ± 0.0 a	1.8 ± 0.2 b	---	---	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	1.7 ± 0.7 b	0.2 ± 0.1 b
BMP NO-sanitize	0.0 ± 0.0 a	1.6 ± 0.2 b	3.1 ± 0.4 b	---	12.4 ± 7.0 b	18.4 ± 9.0 b	3.0 ± 3.0 ab	2.4 ± 0.5 b	0.4 ±0.3 b
Short Stub	1.0 ± 1.0 ab	---	---	0 ± 0	14.4 ± 7.3 b	---	8.0 ± 7.0 ab	3.9 ± 1.1 b	0.2 ± 0.1 b
Long Stub	1.0 ± 1.0 ab	1.3 ± 0.8 b	3.9 ± 0.8 b	0 ± 0	0.0 ± 0.0 a	---	2.0 ± 2.0 a	---	---

Breaking	5.7 ± 3.1	bc	2.4 ± 0.5	b	2.3 ± 0.5	b	0 ± 0	18.7 ± 5.5	b	11.5 ± 3.0	ab	18.0 ± 7.0	b	2.0 ± 0.6	b	0.6 ± 0.5	b
NTC	7.2 ± 3.4	c	8.4 ± 0.2	a	7.4 ± 0.3	a	0 ± 0	10.5 ± 3.8	ab	63.7 ± 6.0	c	13.0 ± 6.0	ab	3.1 ± 0.5	b	1.2 ±0.1	b
BMP + ASM	---		---		---		---	---		0.0 ± 0.0	a	---		---		---	
Aggress NO-san	---		2.8 ± 0.5	b	1.8 ± 0.4	b	---	---		---		---		---		---	
Short Stub NO-san	---		---		---		0 ± 0	---		---		---		---		---	
Long Stub NO-san	---		---		---		0 ± 0	---		---		---		---		---	

*% Cuts progressing through previous season's growth were 2.8 (+/-16.7) short stub, 13.9 (+/-35.1) long stub, 27.8 (+/-45.4) breaking, 66.7 (+/-47.9) no-treatment.

Table 10. **Effect of treatment on the percentage of infected trees which develop rootstock blight in the fall or tree death in the spring.**

Cutting method	Washington 2019 'Yarlington Mill' 4-yr-old on Red Delicious[z]	New York 2019 'Ever Crisp' 4-yr-old on G.41 [y]	New York 2019 'Idared' 7-yr-old on B.9	Pennsylvania 2019 'Gala' 4-yr-old on M.7 [x]	Washington 2020 'Pink Lady' 14-yr-old on M9.337[w]	Oregon 2020 'Gala' 3-yr-old on M9.337	Washington 2021 'Pink Lady' 4-yr-old on M9.337 [u]		New York 2021 'RubyFrost' 3-yr-old on G.41[y]	New York 2021 'Gala' 18-yr-old on B.9[t]
BMP	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	---	0.0 ± 0.0		0.0 ± 0.0	a	TBD	TBD
Aggressive	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	---	0.0 ± 0.0		0.0 ± 0.0	a	TBD	TBD
BMP NO-sanitize	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	---	0.0 ± 0.0		16.7 ± 16.7	ab	TBD	TBD
Short Stub	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	---	0.0 ± 0.0		0.0 ± 0.0	a	TBD	TBD
Long Stub	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	---	0.0 ± 0.0		0.0 ± 0.0	a		
Breaking	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	---	0.0 ± 0.0		0.0 ± 0.0	a	TBD	TBD
NTC	0.0 ± 0.0	100% death	100% death	---	0.0 ± 0.0		37.5 ± 18.3	b	TBD	TBD
BMP + ASM	---	---	---	---	---		---			
Aggress NO-san	---	0.0 ± 0.0	0.0 ± 0.0	---	---	---	---			
Short Stub NO-san	---	---	---	---	---	---	---			
Long Stub NO-san	---	---	---	---	---	---	---			

TBD – To be determined in 2022 spring evaluation.

[z]4-leader grafts, [y]high-density tall spindle, high vigor [x]tall spindle, low vigor, [w]tall spindle, low vigor, [u]Auvil V trained to the wire, moderate vigor, [t]Vertical axe, high vigor.

Executive Summary

Integrated Fire Blight Management

keywords: fire blight, *Erwinia amylovora*, apple, cutting, biopesticides

Abstract: Fire blight is serious disease affecting apple and pear caused by a bacterial pathogen which infects blooms and shoots resulting and can result in tree death. In 2019 and 2020 a multi-state collaboration was initiated between Washington, Oregon, New York and Pennsylvania. Trials focused in three areas: 1) test materials to prevent bloom infections including biologicals, tank mixes, and mixes with bioregulators, 2) demonstrate management strategies for young trees including coppers, plant defense elicitors, and prohexodine calcium (PhCa) and 3) test cutting strategies to manage blocks once they are infected.

In trials testing biopesticide ability to prevent bloom infections alum performed well in 7 of 8 blossom blight prevention trials in WA, NY, PA and OR. Thyme and cinnamon oil products provided intermediate control. Thyme oil products performed well as part of an organic program with Blossom Protect and soluble copper when applied at petal fall. The 40 oz rate of Serenade Opti performed no better than the 20 oz standard for blossom blight control.

For protection of young non-bearing trees flower removal was best followed by 3 weekly applications of soluble copper (Previsto/Cueva) at 3-4 qt/ A or basic copper 1.5 lb/100 gal. Prohexadione calcium (Apogee/Kudos) performed best when applied 2 weeks before inoculation. 6 oz or higher rates may be important in WA/OR compared to success at the 3 oz rate in NY. In a replacement tree trial in OR only 42% of trees treated 3 days before infection with Actigard (vs 88% untreated, 79% preplant) developed trunk cankers.

In trials comparing cutting treatments to remove fire blight infected tissue timely summer cutting of fire blight infections significantly reduced the number of trees which developed rootstock blight and died from fire blight infections. The standard best management fire blight cutting practice where cuts are made 12 inches from the edge of the noticeably infected tissue into 2-year or older wood with sanitized loppers significantly reduced the number of new systemically caused infections compared to no-treatment controls in 8 of 9 case study trials. While Breaking treatments provided a fast fire blight removal method it left many cankers in the orchard which provide a source for infection in subsequent years. In 2 of 4 case studies cutting which left a 5-inch Long Stub from structural wood significantly reduced the number of cankers on structural wood compared to flush cut or 1-inch stub.

FINAL PROJECT REPORT **YEAR:** 2020-2021

Project Title: Development of New Biocontrol Strains from Washington Native Trees

PI: Professor Sharon Doty
Organization: University of Washington
Telephone: (206) 616-6255
Email: sldoty@uw.edu
Address: Winkenwerder Hall, Box 352100
City/State/Zip: Seattle, WA 98195-2100

Cooperators:
Dr. Achour Amiri (WSU-Wenatchee), a.amiri@wsu.edu
Dr. Tianna DuPont (WSU-Wenatchee), tianna.dupont@wsu.edu
Dr. Patricia Okubara (WSU-Pullman), patricia.okubara@usda.gov

Total Project Request: **Year 1: $46,229**

WTFRC Budget: None

Budget 1
Organization Name: University of Washington
Contract Administrator: Carol Rhodes, Director, Office of Sponsored Programs
Telephone: (206) 543-4043
Email address: osp@uw.edu

Item	2020	2021
Salaries	$28,632	
Benefits	$8,775	
Wages		
Benefits		
Equipment		
Supplies	$5,000	
Travel	$822	
Miscellaneous	$3,000	
Plot Fees		
Total	$46,229 (Total Year 1)	0

OBJECTIVES

1) Testing for biocontrol of *Erwinia amylovora* (causal agent of fire blight) using native plant microbiota from Washington State
    a. Assay development *(Completed)*
    b. Testing of our fully characterized biocontrol strains in the assays optimized in Objective 1a *(Completed)*
    c. Screening for new microbial strains with activity against *E. amylovora*. *(Completed)*
    d. Genomic sequencing of selected strains *(Completed)*
2) Testing for biocontrol of pre- and post-harvest apple fruit pathogens using native plant microbiota from Washington State
    a. Testing our fully characterized biocontrol strains *(Completed)*
    b. Screening for new microbial strains with activity against the pre- and post-harvest apple pathogens *(Completed)*
    c. Genomic characterization of selected strains *(Moved to Phase 2 grant)*

SIGNIFICANT FINDINGS

- Through this project, we isolated over a hundred new endophyte strains from the Wenatchee, Entiat, Yakima, and Methow areas
- 15 strains showed inhibition of *Penicillium expansum*. Since the strains grew in the presence of this fungus known to produce the antimicrobial compound, patulin, they may have the capacity to degrade it
- 27 strains inhibited *Botrytis cinerea*
- 21 strains inhibited *Neofabraea perennans*
- 38 strains inhibited *Phacidiopycnis washingtonensis*
- 40 strains inhibited *Erwinia amylovora*
- Several of the strains appeared to inhibit the pathogenic fungi through production of volatile compounds. This mechanism may lead to post-harvest control measures
- 11 strains were fully sequenced. Genomic analysis is required for commercialization as it provides the means to screen for potential pathogenicity and uniqueness. Genomic analysis was performed in our Phase 2 grant

METHODS

Isolation of new endophyte strains from natural areas near to the fruit tree growing areas. Doty obtained the required plant sampling permits and sampled a variety of native plants in natural sites in the Wenatchee, Entiat, Yakima, and Methow areas throughout summer and early autumn of 2020. Microbial endophyte strains (bacteria and yeast from within plant tissues) were isolated through maceration in bacterial media and multiple rounds of streak purification. Pure isolates were cryogenically-stored in glycerol at -80C.

In vitro assay for inhibition of the post-harvest decay pathogens, *Penicillium expansum*, *Botrytis cinerea*, *Neofabraea perennans*, and *Phacidiopycnis washingtonensis*. The fungal samples were obtained from the Amiri Lab at the WSU Tree Fruit Research and Extension Center in Wenatchee. Using a modification of the dual plate assay we had used previously (Kandel et al. 2017), we pipetted 10 µl fungal spore/suspended hyphae preparations to the center of agar plates containing medium appropriate for fungal growth, PDA (potato dextrose agar). The fungi were allowed to grow at room temperature until robust fungal growth was evident in the center of the plate. Endophyte isolates, which had been grown on rich media (MGL), were then spotted around the edge of the plate with up to eight isolates per plate (**Figure 1**). Due to the slower growth of *Neofabraea perennans* only up to

four isolates were spotted per plate and half the distance from the hyphae edge (**Figure 2A**). Growth of the fungus was monitored and inhibition was scored when the fungal growth reached the perimeters of the plates, except *Neofabraea perennans* which was scored based on hyphae growth disruption (**Figure 2B**).

***Erwinia amylovora in vitro* inhibition assay**. Three *Erwinia amylovora.* isolates were provided by Dr. Tianna DuPont, however after initial testing indicated the three strains displayed identical inhibition patterns, assays were carried out on a mixture of the three strains provided. 100 µl of *Erwinia amylovora* with an optical density of 0.01 at 600nm were spread onto rich medium appropriate for *Erwinia* (NYDA). Endophyte isolates were grown on MGL and then spotted onto these plates of *Erwinia amylovora*. Clear zones on the *Erwinia* lawns were scored as inhibitory activity (**Figure 3**).

RESULTS AND DISCUSSION

There were several delays to the start of our 2020 project, largely due to the restrictions of the COVID-19 pandemic. Our COVID-19 Safety Plan was approved and submitted to the Washington Department of Fish and Wildlife. We were then issued the plant sampling permits and were able to complete the sampling in early autumn 2020. Microbial isolations, strain purification, testing, and preliminary species identifications were completed in December. A no-cost extension allowed for the DNA purification and full genomic sequencing to proceed into 2021.

A total of 38 strains inhibited the growth of *Phacidiopycnis washingtonensis*, 21 strains inhibited *Neofabraea perennans*, 27 strains inhibited *Botrytis cinerea,* and 15 strains inhibited *Penicillium expansum*. Many of the strains strongly inhibited the growth of *Erwinia amylovora,* with a total of 40 inhibitory strains. (**Table 1**).

Figure 1. Inhibition of *Botrytis cinerea* by some of the endophyte strains. A) Example plate showing no inhibition of the fungus. B) Strong inhibition of the fungus by sample # 88. C) Apparent inhibition by volatiles produced by some of the strains, as indicated by the overall reduced fungal growth and the bubbling appearance of some of the samples.

A B C

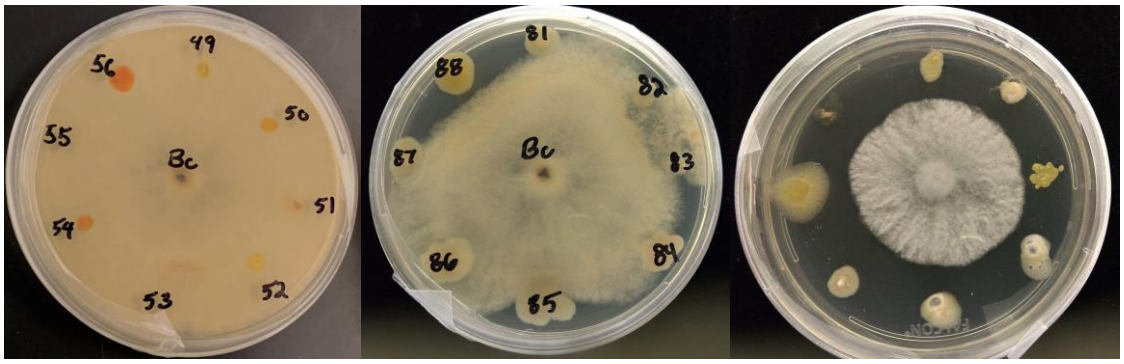


Figure 2. Inhibition of *Neofabraea perennans*. A) Example of four spotted endophyte isolates. B) Close up of disrupted hyphae growth near sample #4, with the leading edge of fungal growth becoming filiform as opposed to the smooth edge seen near sample #1.

A B

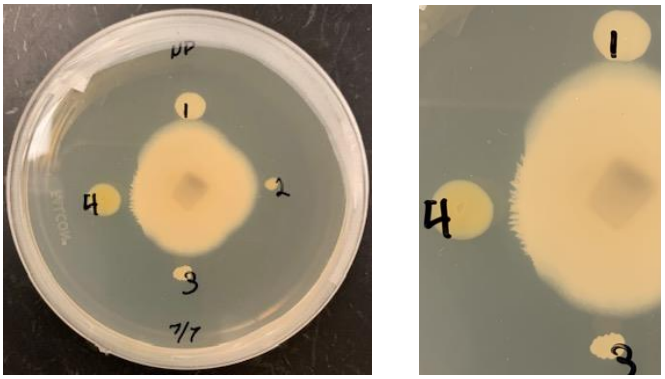


Figure 3. Inhibition of *Erwinia amylovora*. The pathogen was inhibited by several of the endophyte strains as indicated by clearing zones on the lawn of *Erwinia* growth. A. Overall screening results. B. Close up of one of the assay plates showing the strong inhibition of *Erwinia* growth.

A B

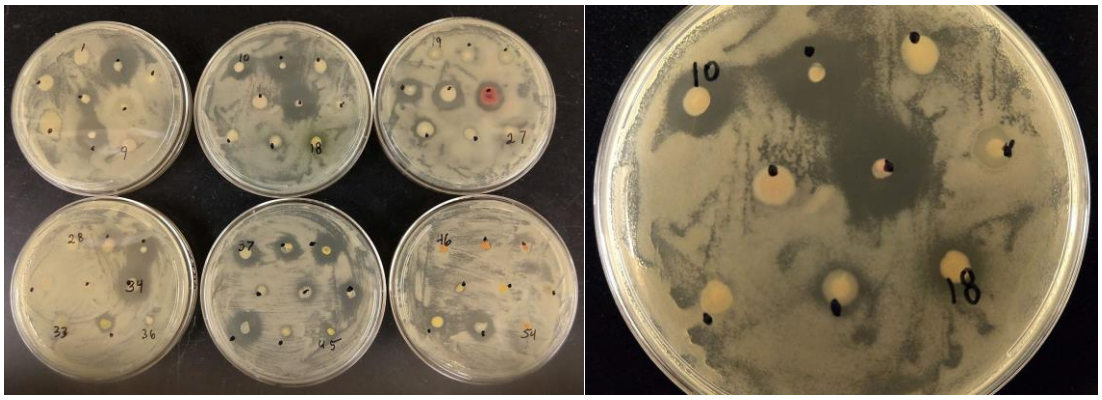


Table 1. Apple biocontrol project screening results. Endophyte strains with any activity against each of the pathogens. Pw *Phacidiopycnis washingtonensis*, Np *Neofabraea perennans*, Bc *Botrytis cinerea*, Pe *Penicillium expansum*, and Ea *Erwinia amylovora*. Strain names preceded by a number indicate the site from which they were isolated: 1 Wenatchee area, 2 Entiat/Okanagan area, 3 Yakima River area near Ellensburg, and 4 Methow area. Bold font indicates the strain was chosen for genomic sequencing (see Table 2).

Pw	Np	Bc	Pe	Ea
1SS-L-D	**1SS-L-D**	**1SS-L-D**	1SS-L-H	1 SS-L-C
ISS-L-E	1SS-L-F	4_2_2	1Cv-L-C	4RDLD
1SS-L-F	1SS-L-J	WP 40	WP 40	2RDLC
1SS-L-H	4_5_3	WP 41	WP 41	**2RDLD**
1SS-L-I	4_4_2	WP 42	WP 42	3Pop12L1
1SS-L-J	WP 40	AFE 4A	AFE 4A	**3YPLB**
1Cv-L-C	WP 41	AFE 21B	WPB	2ALE2
1 SS-S-A	WP 42	AFE 5	AFE 3	2PtLE
4_2_2	AFE 4A	1 SS-A	**2PtLD**	2OPSB
4_5_3	AFE 21B	1 SS-B	**3YPLB**	3RS1
4_3_2	1 SS-S-B	1 Cv-S-A	4ASD	**3RS3**
4_4_2	WW7B	AFE 8	4RDLI	3Pop12L4
WP 40	AFE5	WPB	4RDLJ	3YPS2
WP 41	AFE9	4RLD	4RDLG	3YPS3
WP 42	AFE14	4RDLD	3YPS3	2 OPSB
PTD1	**2PtLD**	**3ThS2**		2PtLC2
AFE 4A	2SASA	2OPSA		2PTLF1
AFE 21B	2ALE2	**2PtLD**		2SASD

4RDLD	2RDSA	2SASA		2RDSB
3ThS2	2PtLE	2RDLC		2RDLA
2PtLD	2OPSB	**2RDLD**		**2ALA1**
2SASA		4SBLB-		2ALB
2RDLC		**3WL2**		4RLA
2RDLD		3WL3		**4RLE**
3WL2		3Pop12L1		4RFA
3WL3		**3YPLB**		4RFB
3Pop12L1		**3YPLD**		4RSC
3YPLB				4ASA
3YPLD				4ALB
3RS1				4ALC
4ASD				**4RDLA**
3RS3				4RDLE
3ThS1				4RDLF
3Pop12S3				4HNLA
3Pop12L3				4HNLB
3Pop12L4				4SBLA
3YPS2				3RF1
3YPS3				3RL2
				3ThS3
				3ThL1

Table 2. Endophyte isolates chosen for sequencing after rRNA preliminary identification.

Endophyte Strain Name	**16S rDNA Identification**
1SSLD	*Erwinia* sp.
2PtLD	*Serratia* sp.
2ALA1	*Pseudomonas* sp.
2RDLD	*Serratia* sp.
3YPLB	*Pseudomonas* sp.
3YPLD	*Pseudomonas* sp.
3ThS2	*Pseudomonas* sp.
3WL2	*Acinetobacter* sp.
3RS3	*Enterobacter* sp.
4RDLA	*Erwinia* sp.
4RLE	*Pantoea* sp.

Genomic DNA Sequencing. Eleven strains were chosen for full genomic sequencing based on the number of pathogens towards which the strain was inhibitory, the strength of the inhibitory activity, and uniqueness of the strain compared to the other top-performing strains (**Table 2**). We prepared genomic DNA and sent the samples to GeneWiz for sequencing. Sequence data analysis was performed in our "Phase 2" grant that had funding allocated to a bioinformatics postdoctoral researcher.

EXECUTIVE SUMMARY

Project Title: Development of New Biocontrol Strains from Washington Native Trees

Keywords: Fire blight; *Erwinia amylovora*, post-harvest decay; *Penicillium expansum*, *Botrytis cinerea*, *Neofabraea perennans*, *Phacidiopycnis washingtonensis*

A largely untapped resource for new biocontrol strains is the natural plant microbiome of Washington native trees and shrubs. In high-stress environments, plants use partnerships with beneficial bacteria to defend themselves against fungal pathogens. Natural selection for host protection through microbial interactions provides a potential pool of beneficial microorganisms for use in agriculture. Our laboratory previously identified and characterized over a dozen endophyte strains from wild poplar trees that inhibited the growth of the agriculturally important plant pathogens *Rhizoctonia solani* AG-8, *Fusarium culmorum, Gaeumannomyces graminis* var. *tritici*, and *Pythium ultimum*. By focusing on endophytes, the microorganisms within plants, they could inhibit pathogens from within the trees, as well as on the plant and fruit surfaces, ultimately reducing application costs and improving long-term effectiveness.

Through this Phase 1 grant, new microbial endophyte strains were isolated from native plants in natural areas near apple tree growing areas of Wenatchee, Entiat/Okanagan, Yakima, and Methow. A total of 119 strains (15 previously characterized and 104 new isolates) were screened using *in vitro* assays for inhibition of the post-harvest decay pathogens, *Penicillium expansum*, *Botrytis cinerea*, *Neofabraea perennans*, and *Phacidiopycnis washingtonensis*, as well as the causal agent of fire blight, *Erwinia amylovora*. Two to three dozen inhibitory strains for each pathogen were identified. Some of the strains inhibited overall fungal growth, likely through the production of volatile antimicrobial compounds. Fifteen strains grew in the presence of *Penicillium expansum*, a fungus known to produce the antimicrobial compound, patulin, which also has human impacts and is of concern in apple products. Growth of these strains suggests that they may be able to degrade patulin. Forty strains inhibited the bacteria, *Erwinia amylovora*, providing a strong pool of candidate biocontrol strains for this important pathogen. Preliminary rRNA sequence characterization was performed on all of the most active strains, and a subset of the isolates with the strongest or broadest activities was selected for full genomic sequencing. The subsequent genomic analyses were performed in our “Phase 2” grant.

PROJECT REPORT **YEAR:** 2021

Project Title: Phase 2: New biocontrol strains from Washington native plants

PI: Sharon L. Doty
Organization: University of Washington

Telephone: 206-616-6255
Email: sldoty@uw.edu
Address: Environmental & Forest Sciences

Address 2: University of Washington
City/State/Zip: Seattle, WA 98195-2100

Co-PI (2): Tianna DuPont
Organization: Washington State University

Telephone: (509) 293-8758
Email: tianna.dupont@wsu.edu
Address: Tree Fruit Research & Extension Center
Address 2:1100 N Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Dr. Ashour Amiri
WSU Tree Fruit Research and Extension Center
509-663-8181 ext 268
a.amiri@wsu.edu

Total Project Request: **Year 1:** $34,219 **Year 2:** **Year 3:**

WTFRC Budget: None

Budget 1
Organization Name: University of Washington
Contract Administrator: Carol Rhodes, Director, Office of Sponsored Programs
Telephone: (206) 543-4043
Email address: osp@uw.edu

Item	2021-2022	(type additional year if relevant)	(type additional year if relevant)
Salaries	$19,882		
Benefits	$5,643		
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$3,394		
Travel			
Plot Fees	$2,500		
Miscellaneous	$2,800		
Total	$34,219	0	0

Footnotes: Misc category: UW Mass Spectrometry Center

Budget 1
Organization Name: UW **Contract Administrator: Carol Rhodes**
Telephone: 206-543-4043 **Email address: osp@uw.edu**
Station Manager/Supervisor: Optional **Email Address: Optional**

Item	2021-2022	(type additional year if relevant)	(type additional year if relevant)
Salaries	$16,132		
Benefits	$4,344		
Wages			
Benefits			
Equipment			
Supplies	$3,394		
Travel			
Miscellaneous	$2,800		
Plot Fees			
Total	$26,670	Total year 2	Total year 3

Footnotes: Misc category: UW Mass Spectrometry Center

Budget 2
Organization Name: WSU **Contract Administrator: Shelli Tompkins**
Telephone: 509-293-8800 **Email address: shelli.tompkins@wsu.edu**
Station Manager/Supervisor: Optional **Email Address: Optional**

Item	2021-2022	(type additional year if relevant)	(type additional year if relevant)
Salaries	$3,750		
Benefits	$1,299		
Wages			
Benefits			
Equipment			
Supplies			
Travel			
Plot Fees	$2,500		
Miscellaneous			
Total	$7,549	Total year 2	Total year 3

Footnotes:

OBJECTIVES

Specific goals and objectives:

1) Bioinformatics analysis of the genomes of the best-performing strains. A goal of this objective is to determine if any of the active strains contain genes associated with human pathogenesis that would render them unsuitable for direct agricultural applications. Such strains would be reserved as potential sources for purified bio-active compounds instead of as biocontrol strains. Another goal of this objective is to determine if the inhibitory strains harbor known anti-microbial genes that would inform potential biocontrol mechanisms. (Doty Lab)
2) Field testing of 4 of the top-performing Phase 1 strains for biocontrol of the fire blight pathogen, *Erwinia amylovora*. Space and personnel limitations restricted plant testing to only three of the active strains at this time; however, we will expand the testing to a few more strains in 2022 using DuPont's current funding, and in a follow-up Phase 3 proposal for 2023. (DuPont Lab)
3) Testing if the microbial strains that inhibit *Penicillium expansum* can also significantly reduce the concentration of patulin. The goal of this assay is to test if the 15 of our isolates that grew in the presence of *P. expansum* are able to degrade the anti-microbial compound, patulin. (Doty Lab)
4) Identifying the volatile inhibitory chemicals produced by some of the strains. The goal of this objective is to begin analysis of the antimicrobial volatile compounds from several of the strains as a potential novel protection during storage to prevent post-harvest decay. (Doty Lab)

SIGNIFICANT FINDINGS

- Eleven strains were fully sequenced, and the genomes were assembled, annotated, and analyzed. Seven of the strains were determined to be novel species
- Genomic analysis for genes related to human pathogenicity that would exclude them from direct use as biocontrol strains was performed. Five of the strains were tagged as potential human pathogens: 2PtLD, 2RDLD, 2WL2, 1SSLD, and 3YPLD. The other six strains were not: 2ALA, 3ThS2, 3YPLB, 3RSE, 4RDLA, and 4RLE.
- These 6 strains were then further screened for genetic potential to be plant pathogens. The *Erwinia* sp. strain, **4RDLA**, does not have the pathogenicity genes of the plant pathogen, *Erwinia amylovora*. *Pseudomonas* sp. strain **2ALA1**, *Pseudomonas koreensis* strain **3YPLB**, and *Pantoea* sp. strain **3RS3** also lack the known plant pathogenicity genes.
- The genomes of the 6 strains were screened for evidence of genes involved in antimicrobial or plant growth promoting traits. Each of the six strains contained the biosynthetic genes for at least one known antimicrobial compound
- Of the three strains tested for *in vivo* activity against the fire blight agent, *Erwinia amylovora*, **4RDLA** had activity equal to that of the commercial biocontrol product, Blossom Protect, providing a locally-sourced, new biocontrol strain. The other two (**2ALA1** and 4RLE) tested strains had inhibitory activity levels between the commercial products and the water-treated check
- Resistance to the antimicrobial compound, patulin, that is produced by *Penicillium expansum* was verified by both growth in spiked media and by patulin degradation in 5 of the strains
- A total of 9 strains showed some degradation of patulin
- Production of a volatile compounds was confirmed in 6 of 7 tested strains by gas chromatography mass spectrometry

METHODS

Objective 1: Bioinformatics (Doty Lab). Library preparation, sequencing and genome assembly. The draft genome was generated by GENEWIZ® (Seattle, WA) using a MiSeq Illumina sequencing platform. The paired-end libraries were constructed using the Nextera DNA Flex Library preparation kit and each library was sequenced using a 2 × 250-bp format. The MiSeq run was performed using the MiSeq Reagent Kit v3 (600 cycles). The assemblies were then performed using the Geneious Prime (GP) platform (v2021.1.1, https://www.geneious.com). For QC, the paired end reads were trimmed and normalized using BBDuk and BBNorm (v38.84), respectively. Only the trimmed reads with phred scores ≥30 and ≥20 bp in length were retained, and the normalization target depth of coverage (DOC) was set at 40 (minimum 6 DOC). Assembly was performed in GP using the Geneious Assembler, and only contigs > 1000 bp were retained.

Assembly and annotation. Open reading frame (ORF) prediction was performed using the Rapid Annotations using Subsystem Technology platform (RAST, Aziz *et al.* 2008), and completeness was calculated based on the presence of lineage-specific single copy marker genes using CheckM (v1.0.18, Parks *et al.* 2015). In this regard, a minimum completeness >99 % was achieved for all strains.

Identification of species. The taxonomic classification of the strains was completed according to the method described in Meier-Kokthoff & Goker (2019). Briefly, the Type (Strain) Genome Server (TYGS) infers species/subspecies identification and phylogenies using digital DNA:DNA hybridization (dDDH) of whole genomes against a type-strain database of >15k species and subspecies. A dDDH (d_4, Table 2) score of >70% is the threshold for classification to the species level. Four of the strains, 2RDLD, 3WL2, 3YPLB, and 4RLE, were identified to the species level, while the remaining 7 strains, 1SSLD, 2ALA1, 2PtLD, 3RS3, 3ThS2, 3YPLD, and 4RDLA, were only matched to the genus level, indicating that they may represent novel species.

Virulence and Pathogenicity. Bacterial virulence and pathogenicity are complex and highly variable mechanisms, and these traits can vary within and between closely related strains, under different environmental conditions, or host specificity. Most current protocols investigate the annotated genomes for the presence of genes and pathogenic islands (PAIs) associated with the synthesis of enterotoxins and secretion systems (e.g., T3SS, T4SS, and T6SS). While not conclusive, these methods can be used to predict whether a given strain is more or less likely to be virulent to humans and/or plants and are useful for informing or complimenting experiments. The annotated genomes of the strains were screened for human pathogenic genes using the PathogenFinder (PF) pipeline (v1.0, Cosentino *et al.* 2013). PF screens the genomes for the presence of gene families commonly associated with either pathogens or non-pathogens, and then returns a probability score for a given strain being a human pathogen. PF does not predict for plant pathogens. The PF results were compared with the output of the IslandViewer (IV4) pipeline (v4.0, Bertelli *et al.* 2017) which screens the annotated genomes for the presence of genomic islands via alignments with closely related strains, either human or plant associated.

Antimicrobial traits. The annotated sequences of the endophytes were screened for plant beneficial traits using various bioinformatic pipelines. BAGEL4 (v 4.0) and antiSmash (v 6.0.1) mine assembled and annotated genomes for gene clusters associated with the biosynthesis of secondary metabolites including those associated with antimicrobial properties (van Heel *et al.* 2018, Blin *et al.* 2021). Additional reviews were done on biosynthesis pathways using the KEGG reference maps (Kanehisa & Goto 2000).

Objective 2: *Erwinia amylovora in vivo* inhibition assay (DuPont Lab). A two-acre research block of mature Red Delicious apples at WSU Columbia View Orchard 48 Longview Rd. East Wenatchee, WA was used for this trial. The experiment was arranged in a randomized complete block with five single tree replications. Products were applied at a dilution of 400 ml per 100 gallons of water to the lower 8 ft to wet, near dripping at 0.4 gal/tree. At 100% bloom (of the king blooms), 19 Apr 2021, *Erwinia amylovora* was applied at 1 x10^6 CFU ml^{-1} (verified at 40-94 x10^6 CFU ml^{-1}) to lightly wet

each cluster. Trees were visually evaluated for flower cluster infection weekly from when symptoms became visible 10 days after treatment for 4 weeks and infection counts summed across all dates. Fruit was evaluated for fruit skin marking before fruit colored over (8-16 Jun 2021). *E. amylovora* was enumerated at full bloom, petal fall and one week post petal fall from a bulk sample of 5 flower clusters per tree. Clusters were sonicated in sterile 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). Statistical analysis was performed using general linear mixed models (GLIMMIX) analysis of variance ANOVA, and multiple means comparison Fisher's T test (LSD) SAS v 9.4. Environmental conditions during bloom (14-26 Apr 2021) ranged from an average maximum temperature of 72 °F and minimum of 43 °F with 36% average humidity. A precipitation event (0.04 in) occurred on 24 Apr the evening after petal fall sprays were applied. All applications were made under fast drying conditions.

Objective 3: Testing for patulin reduction (Doty Lab). Due to their ability to inhibit the growth of *Penicillium expansum,* 15 endophyte strains were chosen for this study. An additional four were chosen as controls because they did not inhibit *Penicillium expansum,* and therefore were thought not to degrade patulin. Because the Phase 1 inhibition assay was carried out using potato dextrose agar, potato dextrose broth (PDB) was used for the patulin degradation assay. After preparation of PDB, half was reserved for control cultures, while the other half was spiked with 50 ug/mL of patulin. The 15 endophyte strains were grown on rich media (MGL), suspended, and used to inoculate 2.5 mL of both PDB and PDB with patulin (PDB+PAT) to a 0.1 OD_{600}. Cultures were incubated in sealed 14 mL tubes at 86°F for 3 days. Cultures were then passed through a 0.2 um filter to remove cells. The concentration of patulin remaining in the filtered culture media was analyzed by liquid chromatography mass spectrometry (LC-MS/MS) using a Waters Xevo-XS TQ-S by the UW Mass Spectrometry Center. The quantification method was designed using a 100 ug/mL patulin standard with a dilution of 1:100 in H_2O for both standards and samples.

Objective 4: Testing for volatile inhibitory compounds (Doty Lab). Fungal strains (*Penicillium expansum, Phacidiopycnis washingtonensis,* and *Borytris cinerea*) were cultured on potato dextrose agar (PDA). A spatula was then used to cut small squares of agar from the outside edge of the fungal cultures, allowing for a standardized propagation of these squares onto many PDA plates. Plates were incubated at room temperature, and once fungal growth was apparent, bacterial strains were streaked in one continuous line across the whole surface of a plate of MGL agar. The fungal plate was then inverted and placed on top of this bacterial plate, both without lids, allowing for shared headspace. The gap between the plates was sealed with Parafilm and the sandwiched plates were incubated in darkness at room temperature. Twenty strains were tested for volatile inhibition in this way, compared against control sandwiched plates containing no bacteria. Inhibition was evaluated quantitatively based on three factors: decreased plate coverage, decreased fungal growth density, and less mature fungal colonies (reduced spore production, reduced colony wrinkling in *Pw*).

The 7 best-performing strains of bacteria (3ThS1, 3ThS2, 3WL2A, 3WL2B, 3WS2, 3YPS2, and 3YPS3) were selected for volatile production analysis via gas chromatography mass spectrometry (GCMS). For optimal growth, 6 ml slants were prepared by pouring MGL agar into sterile 17 ml amber septa vials. An overnight culture of each of the 7 strains was then diluted to an OD_{600} of 0.4, and 100uL was pipetted onto the surface of the agar. These vials were then incubated at 86°F. A 10 ml headspace volume was removed by syringe and bubbled through methyl tert-butyl ether (MTBE) in GCMS auto-sampler vials and left under pressure for 3 days. The MTBE headspace extracts were then analyzed by GCMS using an Agilent 5975 GCMS through the UW Department of Chemistry Mass Spectrometry Facility. Chromatograms were analyzed manually in comparison to the NIST database of mass spectra.

RESULTS AND DISCUSSION

Objective 1. Genomic analysis (Doty Lab). The results of our Phase 1 grant to isolate microbial endophyte strains with inhibitory activities against key pathogens were analyzed for the strongest or most significant activities. Strain 2PtLD had the broadest range, inhibiting *Phacidiopycnis washingtonensis* (Pw), *Neofabraea* (Np), *Botrytis cinerea* (Bc), and *Penicillium expansum* (Pe). Strain 3YPLB was the strongest against Bc but also had activity against Pw, Pe, and *Erwinia amylovora* (Ea). Strain 2RDLD was strong against Pw and Ea with some activity against Bc. Strain 1SSLD was the strongest against Np and also had activity against Pw and Bc. Strain 3ThS2 was very strong against Pw and Bc. 3WL2 and 3YPLD had volatile inhibitory action against Bc. 2ALA1, 4RLE, 3RS3, and 4RDLA were all strongly inhibitory against Ea.

The genomes of 11 top-performing strains were fully sequenced, and the genomes were assembled, annotated, and analyzed. Seven of the strains were determined to be novel species: *Erwinia* sp. strains 1SSLD and **4RDLA**, *Serratia* sp. strain 2PtLD, *Pseudomonas* sp. strain 2ALA1 and 3ThS2, and *Pantoea* sp. strains 3RS3 and 3YPLD. Each of the 11 strains was then analyzed for the potential to be human pathogens based on the presence of specific genes known to be associated with human pathogenicity. Five of the strains scored as potential human pathogens. Though these strains could likely not be used as direct biocontrol strains, they could serve as sources of novel antimicrobial compounds: 2PtLD, 2RDLD, 2WL2, 1SSLD, and 3YPLD. These 5 strains were dropped from the further genomic analyses during this Phase 2 project but will be analyzed in early 2022. The other six strains did not score as potential human pathogens and so were further screened: 2ALA, 3ThS2, 3YPLB, 3RSE, 4RDLA, and 4RLE. While powerful bioinformatics programs exist for testing for human pathogenicity, it is more challenging to predict plant pathogens based on genomics analysis. The genomes of the 6 strains were screened for any "pathogenic islands" associated with known plant pathogens. Though these gene clusters are associated, they do not necessarily mean the strains are plant pathogens but simply predict if a strain is potentially pathogenic and should be further tested. The *Erwinia* sp. strain, **4RDLA**, does not have the pathogenicity genes of the plant pathogen, *Erwinia amylovora*. *Pseudomonas* sp. strain **2ALA1** and *Pseudomonas koreensis* strain **3YPLB** lack the T3SS associated with plant pathogens. Pantoea strain **3RS3** does not have the complete Type 3 or Type 6 secretion systems. However, *Pseudomonas* sp. strain 3ThS2 has two T6SS associated with plant virulence effectors. *Pantoea agglomerans* strain 4RLE has T3SS and T6SS-3 clusters associated with plant pathogenicity. Since these secretion systems are common in bacteria and therefore are not necessarily for exporting virulence compounds that would affect agricultural crops, these strains should be further tested on plants with precautions.

The annotated sequences of the six strains were screened for several known plant beneficial traits and antimicrobials. None of the strains had genes known for nitrogen fixation, indole acetic acid (auxin) production, or ACC deaminase (associated with conferring plant stress tolerance). Each had genes involved in phosphate solubilization that can provide this important macronutrient to plants. All six strains had siderophores used for scavenging iron, a limiting resource. Siderophore production is often regarded as a competitive mechanism excluding other microorganisms but it can also be seen as a plant growth promoting mechanism since endophytes migrate from the soil environment to the plant interior. Each of the strains contained biosynthetic genes for at least one known antimicrobial compound. Strains 3RS3 and 3YPLB have genes for amonabactin. 2ALA1 has genes for lankacidin C and chitinase; 3ThS2 for pyroverdine and fragin, 4RDLA for desferrioxamine E, and 4RLE for turnerbactin and desferrioxamine E. This information will guide further studies to determine the mechanisms by which these strains inhibit the growth of the pathogens.

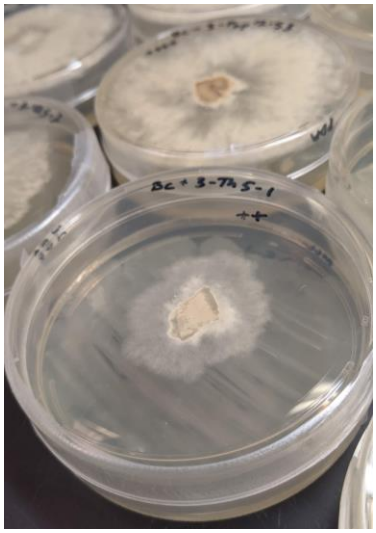
Objective 2. Biocontrol of fire blight on Red Delicious apple blossoms in Wenatchee, WA in 2021. Of the potential biological controls, **4RDLA**, performed comparably (17.0 infections per 100 clusters) to commercial organic (Blossom Protect, 17.8) and conventional (Firewall 17, 16.1-17.0) standards. Strain 2ALA1 and 4RLE performed better (23.4 and 30.4, respectively) than the water-treated check that had 38.6 infections per 100 clusters. The active component of Blossom Protect is the yeast *Aureobasidium pullulans* and is thought to act only by exclusion. Based on our in vitro assays, our strains have a more direct inhibitory mechanism. They also have the advantage of being locally sourced strains. The best-performing strain, 4RDLA, was cleared through the bioinformatics investigations (Objective 1), and can continue to proceed for further characterization and plant testing towards commercialization as a new biocontrol strain for fire blight.

Objective 3. Degradation of patulin by endophytes. Many of the strains tested demonstrated some ability to reduce the concentration of patulin in spiked media, however there were notable standouts. The WP series (WPB, WP40, WP41, WP42) were all able to degrade patulin below quantifiable concentrations. Along with AFE3, these strains demonstrate that endophytes have the capacity for extremely effective patulin degradation. Though all strains grew less in the presence of patulin, those strains with the strongest ability to degrade patulin also increased the most in optical density (OD). One outlier to this was strain 2PtLD, which had undetectable growth with patulin in the media. In this case it may be possible that the strain was able to degrade patulin but was not tolerant to it, meaning most cells succumbed to patulin but survived long enough to degrade some patulin. A more likely explanation however is that the lysed cells of this strain released a compound that inhibited our detection of patulin, perhaps through disrupting ionization during mass spectrometry. Four strains that did not inhibit *Penicillium expansum* were included (2RDLA, 2RDLC, 2RDSC, 3YPLD) in this test. Two of these strains seem to have the ability to degrade patulin, but only doubled the initial 0.1 OD. These two strains could also have inhibited ionization, or over time these strains may be able to degrade patulin to levels where they will begin to increase in OD. Because different bacteria can have different concentrations at which they are affected by patulin, it is not surprising that many strains had trouble growing in PDB+PAT. Three strains (4AsD, 4RDLG, 4RDLI) had trouble growing in PDB alone. This would likely be a problem with the initial inoculation because all strains had been tested for their ability to grow on PDA. These strains could also prefer adherent colony growth to planktonic growth in liquid. The increase in patulin seen in 4AsD seems to either indicate contamination by *P. expansum* or a related fungus, or could be dilution error prior to analysis by mass spectrometry since all PDB+PAT cultures were started with a concentration 50 ug/mL.

Strain	OD in PDB	OD + PAT	PAT final conc. (ug/mL)
1-Ss-L-H	0.2	0.1	39.98
1-Cv-L-C	0.9	0.1	40.87
***2-Pt-L-D**	**1.6**	**0.1**	**3.01**
3-YP-L-B	1.0	--	45.07
3-YP-S-3	1.1	0.1	13.02
4-As-D	0.1	0.1	58.21
4-RD-L-G	0.1	0.1	47.17
4-RD-L-I	0.1	0.1	47.04
***AFE 3**	**1.3**	**0.8**	**<1.0**
AFE 4A	1.0	0.1	33.58
***WPB**	**3.6**	**1.3**	**<1.0**
***WP40**	**4.3**	**1.2**	**<1.0**
***WP41**	**3.5**	**1.2**	**<1.0**
***WP42**	**3.7**	**1.2**	**<1.0**
***2-RD-L-A**	**1.2**	**0.2**	**7.81**
***2-RD-L-C**	**1.6**	**0.2**	**2.83**
2-RD-S-C	0.7	0.1	38.74
3-YP-L-D	1.0	--	38.20
blank	--	--	49.03

*superior patulin degrading strains

Objective 4. GCMS Identification of Volatiles.



Above: 3ThS1 shows volatile inhibition of *B.c.* compared to other plates.

Though qualitative, the sandwiched plate assay worked well in identifying endophyte strains which produce inhibitory volatiles. Of the strains tested by GCMS, 6 of the 7 strains produced a volatile matching the formula $C_6H_{14}O$. The exact structure of the molecule could not be determined due to the structural similarity of related molecules containing the same diagnostic peaks. 3YPS3 produced an additional compound with the formula $C_{15}H_{28}O_4$, which can be narrowed down to an esterified form of succinic acid. Only strain 3YPLB contained no volatile peaks above controls. Inhibition by strains could also be metabolic, for example CO_2, which would not be detected using this method. It was clear by looking at the bubbles in the media, however, that all the tested strains were producing gasses. Unfortunately siloxanes of different sizes dominated all samples, including controls. These siloxanes were likely extracted from the silicone septa of the GCMS auto-sampler vials through processing and storage. Due to the large amount of siloxane contamination, it would be beneficial to repeat these experiments using direct headspace analysis, avoiding the need for MTBE.

STRAIN	DIAGNOSTIC PEAKS	CLOSEST COMPOUND MATCH	FORMULA	RT (min)
3THS1	45, 70	Butane, 1-methoxy-3-methyl-	C6H14O	3.17
3THS2	45, 70	Butane, 1-methoxy-3-methyl-	C6H14O	3.17
3WL2B	45, 70	Butane, 1-methoxy-3-methyl-	C6H14O	3.19
3WS2	45, 70	Pentane, 1-methoxy-	C6H14O	3.16
3YPS2	45, 70	Pentane, 1-methoxy-	C6H14O	3.18
3YPS3	45, 70	Butane, 1-methoxy-3-methyl-	C6H14O	3.18
3YPS3	85, 101	Succinic acid, 3-methylbut-2-yl 3-hexyl ester	C15H28O4	2.97

EXECUTIVE SUMMARY

Project Title: Phase 2: New biocontrol strains from Washington native plants

Keywords: Biocontrol; Fire blight; *Erwinia amylovora*, post-harvest decay; patulin; *Penicillium expansum, Botrytis cinerea, Neofabraea perennans, Phacidiopycnis washingtonensis*

In the phase 1 of this project, new microbial endophyte strains were isolated from native plants in natural areas near apple tree growing areas of Wenatchee, Entiat/Okanagan, Yakima, and Methow. These were screened for inhibition of *Erwinia amylovora* (Ea), *Penicillium expansum* (Pe), *Botrytis cinerea* (Bc), *Neofabraea perennans* (Np),and *Phacidiopycnis washingtonensis* (Pw). Genomic and biochemical analyses of the top-performing strains were conducted in this phase 2 of the project as well as a small field test for activity against apple fire blight.

Bioinformatics is a necessary process to identify potential human pathogens that would preclude the direct use of the microorganism in agricultural settings. Strain 2PtLD had the broadest range, inhibiting Pw, Np, Bc, and Pe. Strain 3YPLB was the strongest against Bc but also had activity against Pw, Pe, and Ea. Strain 2RDLD was strong against Pw and Ea with some activity against Bc. Strain 1SSLD was the strongest against Np and also had activity against Pw and Bc. Unfortunately, genomic analysis of these strains indicated that they are potential human pathogens so they could not be used directly in agriculture; however, they may provide sources for novel antimicrobial compounds. Strain 3ThS2 was strong against Pw and Bc. Strains 2ALA1, 4RLE, 3RS3, and 4RDLA were all strongly inhibitory against Ea. None of these strains were tagged as potential human pathogens, and thus could be used directly in agriculture. Furthermore, the genomes of strains 4RDLA, 2ALA1, 3YPLB, and 3RS3 also lack known plant pathogenicity genes.

We conducted a field test of three strains for protective activity against fire blight. Strain 4RDLA had activity equal to that of the commercial biocontrol product, Blossom Protect, and conventional treatments. The other two strains, 2ALA1 and 4RLE, had inhibitory activity levels between the commercial products and the water-treated check. All three strains could be used as locally-sourced, new biocontrol strains for apple fire blight.

Patulin, a known anti-microbial mycotoxin produced by *Penicillium expansum*, can also have adverse human impacts. Of our collection of 119 endophyte strains, 15 grew in the presence of the fungus, and therefore may be resistant to patulin and could have the ability to degrade it. Purified patulin was added to media inoculated with each of the strains, and liquid chromatography mass spectrometry was used to monitor degradation of patulin. The wild poplar endophytes from our previous collection (WPB, WP40, WP41, WP42, and AFE3) were all able to degrade patulin below quantifiable concentrations, demonstrating that endophytes have the capacity for extremely effective patulin degradation. Overall, 9 strains had some ability to degrade patulin; however, 7 of them were tagged as potential human pathogens based on genomic sequencing. Two of the strong patulin-degraders, 2RDLA and 2RDLC, were included in the test as probable non-degraders since they had not blocked the pathogen growth in our original screens. These had not been sequenced and so now should be further studied. Since resistance to the pathogen did not fully correlate with the ability to degrade patulin, a follow-up study could use patulin itself as a screening tool.

Volatiles (air-borne molecules) were suggested by the activity seen by some of our endophyte strains in the Phase 1 of our project because it was noted that they inhibited overall growth of the fungus rather than only near the point of contact. In this Phase 2 grant, sandwich assays were used to directly screen for this air-borne activity. Of the 6 best-performing strains in this assay, two had been fully sequenced (3WL2 and 3ThS2). While 3WL2 was tagged as a potential human pathogen and the other 4 strains have not yet been sequenced, the volatile inhibitory compounds may still provide potential anti-spoilage products. Production of volatile compounds was confirmed by gas chromatography mass spectrometry, and this analysis will continue in early 2022.

This phase 2 grant enabled a fire blight control field test with positive results and advanced genomic and biochemical characterizations to be performed. These studies will continue toward the development of potential biocontrol strains for use in the apple industry.

CONTINUING REPORT: YEAR 1

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

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

Co-PI: Dr. James Mattheis
Organization: USDA ARS Tree Fruit Research Laboratory
Telephone: 509-664-2280, Ext 249
Email: james.mattheis@usda.gov
Address: 1104 N. Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Dr. Mark Mazzola and Dr. David Granatstein

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

Total Project Request: **Year 1: $60,180** **Year 2:** $39,781 **Year 3:** $0

Other funding sources: **None**
WTFRC Collaborative Expenses: None

Budget 1
Organization Name: USDA ARS Tree Fruit Research Laboratory
Contract Administrator: Sharon Blanchard
Telephone: 509-664-2280, Ext 257 **Email address:** sharon.blanchard@usda.gov
Station Manager name and email address: James Mattheis; james.mattheis@usda.gov

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

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

OBJECTIVES

1. To evaluate select soil amendments for the ability to recruit and support a rhizosphere microbiome in fumigated soil that is resilient to pathogen invasion and also improve other characteristics of healthy soil (nutrient availability, water holding capacity, etc.) (YEAR 1)

2. To determine the role of select amendment-modified soil microbial communities in limiting pathogen re-infestation and reducing potential post-harvest pathogens (YEAR 2)

SIGNIFICANT FINDINGS

- Liquid chitin (LC) and composted chicken manure (CCM) soil amendments had the greatest effect on the chemical and physical properties of fumigated replant soil.

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

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

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

METHODS:

Preliminary evaluation of composted materials: Prior to use in experiments as soil amendments, the quality, stability and maturity of composted materials was evaluated by Soiltest Farm Consultants (Moses Lake, WA) for both shitake mushroom compost (derived from mushrooms cultivated on 20% grain/80% hardwood sawdust) and composted chicken manure (non-pelleted, broiler-based, OMRI listed). The analyses conducted include moisture and solids content, pH, electrical conductivity (EC), total N, organic C, organic matter, ash, ammonium-N, nitrate-N, C:N ratio, CO_2 evolution, and cucumber growth bioassay (Table 2).

Site Description: All soil used in this experiment was collected from Sunrise Research Orchard, Rock Island, WA. This location is known to possess the consortia of soilborne pathogens defined as causal agents of apple replant disease (Mazzola 1998; Mazzola et al. 2015). The soil type at SR orchard is Pogue fine sandy loam, with a pH of 6.9 and an organic matter content of 1.2% (Soil Test Farm Consultants). The overall nitrogen status of the soil has been described as deficient with levels generally ranging from 2.5 to 3.5 ppm (Cascade Analytical, Wenatchee, WA).

Collection of soil: WSU Sunrise Orchard block 14b was fumigated on April 01, 2021 (pers comm Cameron Burt). The old orchard block, previously planted to apple, had been removed in 2017. Telone II (1,3-dichloropropene) was applied at a rate of 122 lbs. per acre; injected at 18 inches depth. Sectagon K-54 (metam potassium) was also applied at a rate of 318 lbs. per acre; injected into the top 6 inches. Fumigated soil was collected on April 26th, 2021 (orchard manager advised a 2-3 week waiting period). Approximately 90 gallons of fumigated soil was removed from the plot by shoveling soil from the top 12” into 5 gallon buckets, which were placed throughout the plot area at regular

intervals. Buckets and shovels were disinfected with 10% bleach prior to use to minimize cross-contamination. In addition, approximately 30 gallons of unfumigated replant soil was collected from a nearby block containing apple trees (SRO block 12b). The top stubble within tree rows was removed using a shovel; soil was then *carefully* collected from the top 12", so as not to damage existing tree roots. Lids were placed on all buckets to minimize moisture loss and soil was transported back to the lab. Soil was then stored in 30 gallon bins with lids in a cool, dry place until use.

Soil preparation: On month after collection, soil was mixed thoroughly in a cement mixer. The cement mixture was cleaned between soil treatments (fumigated vs. replant soil) by sterilizing with 2% bleach and rinsing with water three times. Mixed soil was used in the bioassay with apple seedlings (as described below) and in the main experiment with apple rootstocks (Obj. 1).

Prerequisite bioassay: This experiment was conducted to check for evidence of disease control in the fumigated soil. Surface-sterilized Gala apple seedlings, germinated and prepared as previously described, (Mazzola and Gu, 2000) were grown in pasteurized potting soil for 5-weeks. Plants of similar size were then carefully transplanted into plastic cone-tainers (cell diameter of 6.35 cm and a depth of 25.4 cm) containing either the fumigated orchard soil, the unfumigated replant soil or pasteurized replant soil. All soil pasteurization was performed at 160°F following established lab protocols. Cone-tainers were placed in a standing rack in a randomized complete block design with 10 plants per treatment. Plants were grown in the greenhouse for an additional 4 weeks. Upon harvest, total seedling biomass, root biomass, shoot biomass and root infection by the nematode *P.penetrans* were measured.

Soil Amendments: Seed meal formulations used in Experiment 1 (*B.juncea* + *S.alba* and *B.napus*) were ground, passed through a 1 mm^2 sieve, weighed out and portioned into individual bags for addition to fumigated or unfumigated SR orchard soil according to the application rates listed in Table 1. The *B.juncea* + *S.alba* SM formulation was prepared by blending *B. juncea* and *S. alba* at a ratio of 1:1. Pre-weighed packets of seed meal were added and thoroughly incorporated into the soil by hand. 2.5L of seed meal-amended soil was placed into each pot, moistened with 300 ml autoclaved water and sealed in gas impermeable bags (Bitran) to retain seed meal-generated volatile compounds (e.g. allyl isothiocyanate). The bags were removed after 1 week, and pots were maintained in the greenhouse for an additional 6-weeks prior to planting to allow for degradation of potentially phytotoxic compounds. All other soil amendments were thoroughly mixed into the fumigated soil 2-weeks prior to planting, according to the application rates listed in Table 2. The amount of material added to each pot was calculated according to an "applied" volume of 6340 cubic feet of soil per acre, based on a high density orchard system with a layout of 12' between tree rows, 3.5 ft wide weed-free strips, and tilling 6" deep. The amendment rate for the composted chicken manure treatment was determined as described below.

Table 1. Application rates of organic amendments used in Experiment 1

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

*Amendment not included in original proposal

Amendment rate for composted chicken manure: . The application rate for the CCM used in this experiment was determined based on a soil EC threshold value of 1.7 mmho/cm (DuPont and Granatstein, 2020). Fruit trees are relatively salt sensitive, suffering decreased growth and yield when EC values in the root zone > 2 mmhos/cm (https://www.bctfpg.ca/horticulture/fruit-tree-nutrition). The amount of compost that could be added to 2.5L SR orchard soil or 3500g dry weight (based on a bulk density mass of 1.4g for Pogue fine sandy loam) without exceeding an EC value of 1.7 mmho/cm was calculated. It was predicted that 250 g *dry* compost would give a final EC value of 1.6 mmho/cm (we did not want to exceed 1.7 mmho/cm). After adjusting for compost moisture (29%), 322.5g of "wet" compost was identified as a suitable "per pot" amendment rate.

The amount of available N in 250g dry compost was also determined based on the Soiltest results: 7.54g available N per 1 kg dry compost (or 1.89g per 0.25kg). Test results also indicated that the dry compost contained 22.5g/kg of organically bound N. Using a mineralization rate of 15%, we predicted 0.84g of mineralizable N per 0.25kg dry compost; 1.89g available N + 0.84g mineralizable N = 2.73 g N. Soiltest results showed that there was 18.1 mg of available N per kg dry SR orchard soil. 18.1 mg/kg x 3.5 kg soil per pot = 63.5 mg N or 0.63g N. This amount of Nitrogen was then added to the amount of N predicted to be coming from the compost: 2.73g + 0.63g = 2.8g N per pot. Using the BC Tree Fruit Production Guide cited above, a recommended nitrogen fertilizer application rate of 15g/tree was identified. 15 g/tree – 2.8 g/pot = 12.2 g N. Therefore, we assumed that 12.2 grams of N would need to be added to each pot. This was applied in the form of ammonium sulfate (21-0-0; N-P-K), split into 3 applications (1-month apart). At planting, 19g ammonium sulfate was dissolved in water and added to each pot containing CCM (58g ammonium sulfate x 0.21 = 12.2g Nitrogen).

Planting/harvest: G.11 rootstocks were used in this experiment. Prior to planting, root volume, trunk diameter (16-18 cm above soil line), and total biomass were recorded. For each treatment type (including fumigated, unfumigated, and unfumigated + seed meal controls), there were 7 replicate pots. For each pot, soil was dumped into a sterilized grey bin and hand mixed to break up hardened clumps and homogenize any bio-crusts that had formed. Bulk soil samples were collected, and soil was placed back into the pot with a single rootstock. Pots were set up in a completely randomized block design in the greenhouse and maintained under standard light and temperature regimes (Somera et al., 2020). At the end of the experiment (3 months post planting), the effect of soil treatments on rootstock growth was assessed by measuring increases in trunk diameter, total rootstock weight, root mass, and leader-shoot length. Upon harvest, bulk soil was pooled from all replicate pots within each treatment. These samples were immediately sent to Soiltest Farm Consultants for testing. A variety of chemical and physical properties were measured to assess the influence of the above soil amendments on overall soil health. The measured properties are listed in Table 3.

Microbial Community Composition (Note: Analyses are currently in process.) Rhizosphere soils were collected from all soil treatments 4 weeks after planting and at harvest (3 months after planting) and DNA was extracted. DNA from 4-week rhizosphere soil samples (five replicates per treatment) has been sent to Molecular Research (Shallowater, TX, USA) for bacterial 16S rRNA and fungal ITS amplicon sequencing; however, this data has not yet been received. Sequencing is being conducted as previously described in Somera et al. (2020). DNA from 12-week rhizosphere soil samples is expected to be sent out for sequencing by the end of the month (January 2022) and composition of the bacterial and fungal communities will be determined shortly thereafter. Together, these two datasets (4 and 12 week) will be used to evaluate the trajectories taken by bacterial and fungal rhizosphere communities following rootstock growth in fumigated soil treated with a variety of soil amendments. This will complete Objective 1. The ability of select amendments to improve the ability of soil to defend against pathogen reinvasion following fumigation will be tested in Experiment 2.

RESULTS AND DISCUSSION:

Compost Quality Testing: Data received in the Soiltest analysis reports was used to evaluate compost quality and stability based on the guidelines outlined in "Compost Use for Tree Fruit", WSU Extension Factsheet, FS337E (Dupont and Granatstein, 2020) and "Interpreting Compost Analysis", OSU Extension, EM 9217 (Sullivan, et al., 2018).

Table 2. Results of analyses conducted for evaluation of composted chicken manure (CCM) and shitake mushroom compost (SMC) prior to use as soil amendments in the post-fumigation microbial "steering" experiment (Obj. 1).

	Composted Chicken Manure		Shitake Mushroom Compost		Characteristics of "High-quality" Compost	
	As Received	*Dry Weight*	*As Received*	*Dry Weight*	Recommended[#]	Within Range (CMC/SMS)
Moisture (%)	29	NA	57	NA	40 - 60%	Y/Y
Solids (%)	71	NA	43	NA		
pH	8.5	NA	3.8	NA		
E.C. (mmhos/cm)	14.2	20	1.0	2.3		
Total N (%)	2.67	3.76	0.48	1.1	>2%	Y/N
Organic C (%)	26.6	37.4	15.9	36.9		
Organic Matter (%)	52.5	73.9	39.7	92.1	>40% (dry weight)	Y/Y
Ash (%)	18.6	26.1	3.4	7.9		
NH_4-N (mg/kg*)	5234	7369	941	2184		
NO_3-N (mg/kg)	122	172	12.4	28.8		
Plant-available N (NH_4+NO_3)	5356	7541	953.4	2212.8		
C:N ratio	NA	10:1	NA	33:1	25-40	Y/Y
NH_4:NO_3 ratio	42.9	42.8	75.9	75.8	<10	N/N
CO_2 evolution (mg CO_2-C/g OM/day)	6.3		4.1		<8	Y/Y
Cucumber emergence (%)	0		27		80-100	N/N
Cucumber vigor (%)	0		52		80-100	N/N

* mg/kg = ppm

[#]Values listed are from WSU Extension Factsheet FS337E, OSU Extension Factsheet EM 9217, or Soiltest reports

"High-quality" compost should have a moisture content between 40-60% (Granatstein and Dupont, 2020; Sullivan et al., 2018). Moisture contents of CCM and SMC were 29 and 57, respectively (Table 2). Although SMC had a higher moisture content than CCM, it also contained a higher percentage of organic matter (92% of dry wright). This result is surprising; in general, the higher the moisture content, the lower the amount of organic matter per ton of compost. Therefore, SMC is likely to be a good option for building soil organic matter.

Electrical conductivity (EC) is an indicator of the salt level in the compost (Na + nutrient salts) and is a critical property of compost quality. CCM had an EC value of 20 mmho/cm, compared with SMC of only 2 mmho/cm. High EC values, which are typical of chicken manure-based composts, are not

desirable because high salt levels can harm plants (Granatstein and Dupont, 2020). The damage threshold for apple/pear is at or above 1.7 mmho/cm (Granatstein and Dupont, 2020). Consequently, CCM needs to be applied at a low rate when incorporated into soil. Like chicken manure-based composts, spent mushroom composts typically contain high levels of salts (Franke-Whittle et al, 2019); the low EC value of the particular mushroom compost used here is surprising.

Compost pH values indicated that SMC was highly acidic (pH=3.8), while CCM was moderately alkaline (pH = 8.5), which is typical of manure-based composts. In general, high pH compost should be avoided on soils which are already above the optimum pH for tree fruit (optimum pH = 6-6.5) (Granatstein and Dupont, 2020).

Compost stability: Composting is generally defined as "the controlled aerobic decomposition of organic matter by microorganisms into a stable, humus-like soil amendment" (Dupont and Granatstein D. 2020). Because microorganisms respire CO_2 during aerobic decomposition, CO_2 evolution is an indication of the resistance of the material to further biological decomposition (i.e. stability). "Highly stable" and "stable" composts have CO_2 evolution rates below 2 and 8 mg CO_2-C/g OM/day, respectively (Sullivan et al., 2018). Results indicated a higher level of microbial activity in the composted chicken manure than in the shitake mushroom compost. In the report, CCM was described as being "moderately unstable" (6.3 CO_2-C/g OM/day), while SMC was characterized as "stable" (4.1 CO_2-C/g OM/day). This result suggests that more of the organic carbon in CCM would be lost as CO_2 gas after field application than that of SMC. Therefore, SMC is more likely to benefit soil health by building soil organic matter.

Compost maturity: Immature composts are often harmful to plants due to high levels of intermediate decomposition by-products, including ammonia. The term "compost maturity" refers to a material that does not cause adverse effects when applied to plant-growing media and is generally determined via plant bioassays. It should be noted that, for this experiment, "fresh", heat-treated SMC, which is almost completely dominated by shiitake mycelium, was selected over "aged" material because it contains a high level of chitin and is more consistent between batches at earlier stages. The data from the cucumber bioassay clearly indicated that the CCM was an extremely "immature" material, as none of the plants germinated. This result (0% plant emergence) is likely the due to the high EC value (as described above). The SMC resulted in 27% emergence and 52% vigor; indicating that this material is also relatively "immature". It should be noted that, in the cucumber bioassay, test materials were applied in extremely high amounts (1:1 compost/vermiculite). "Field" application rates used in the microbial "steering" experiment were much lower.

The ratio of NH_4 to NO_3 can also be used as an indicator of compost maturity. As composts mature, the ratio of NH_4:NO_3 generally decreases due to microbial nitrification (conversion of NH_4 to NO_3) (Sullivan et al., 2018). NH_4:NO_3 ratios >10 typically indicate less mature material. In accordance with the results of the cucumber bioassay, the high NH_4:NO_3 ratios in chicken manure (43:1) and shitake mushroom (76:1) composts indicate immaturity. It should also be noted that NH_4-N made up approximately 20% of the total N (dry weight) in both materials, which is a relatively high percentage. In most composts, inorganic N (NH_4 + NO_3) is less than 5% of the total N (Sullivan et al., 2018). However, the total N (%) was 3X higher in the CCM than in the SMC. The actual amounts of NH_4 (mg/kg) and NO_3 in CCM were 3 and 6X higher than that of SMC, respectively. Although SMC would be less desirable if N fertilization was the goal, the aim of this experiment was to identify materials which could be used to "steer" the apple rhizosphere in favor of a more prophylactic or disease-suppressive state, post-fumigation. Moreover, because the CCM is moderately alkaline (pH > 7.5), a greater proportion of NH_4 may be in the ammonia form (NH_3 gas), leading to loss of N to the atmosphere and negative effects on plant growth (Sullivan et al., 2018). This may have been another factor contributing to 0% plant emergence in the cucumber bioassay with CCM.

The total carbon to total nitrogen ratio (C:N) is another metric which is sometimes used to assess compost stability/maturity. It has been suggested that well-composted materials should typically reach a C:N ratio similar to that of stable soil organic matter (i.e. between 10-15) (Wichuck and McCartney, 2010). C:N ratios between 15-25 have been recommended for compost use with tree fruit on the grounds that N immobilization is more likely to occur at C:N ratios > 25. C:N ratios < 15 may indicate a high probability for N mineralization but also leaching/volatization (Granatstein and DuPont, 2020). This ratio, however, is not necessarily a good indicator of compost maturity. For example, composts containing large amounts of manure will have a low C:N ratio whether they are fully composted or not (Sullivan et al., 2018). The chicken manure compost (CCM) had a C:N ratio of 10:1, indicating the potential for N leaching/loss (although the product is marketed as a "slow nitrogen release plant food"). In comparison, the shitake mushroom compost (SMC) had a much higher C:N ratio (33:1), which is typical of more "woody" composts with more recalcitrant (i.e. less easily degradable) carbon. In this case, C:N ratio is not necessarily a reliable indicator of compost stability or maturity.

Taken together, the results of the compost-specific analyses indicate that, although neither material is fully-composted, "fresh" SMC is more stable and mature than the CCM. In addition, the high EC and pH of CCM are likely to negatively affect plant root growth unless the application rate is considerably low. Finally, test results also pointed to the potential for carbon and nitrogen loss with CCM. In comparison, although the total N content of SMC is low, SMC is likely to benefit soil health by building soil organic matter.

Experiment 1 (for Objective 1)

Prerequisite bioassay: It was expected that apple seedlings grown in recently fumigated orchard soil and in pasteurized replant soil would demonstrate increased plant growth relative to those cultivated in unfumigated replant soil from the same location. No significant differences in total seedling biomass were identified between any of the treatments; however, root to shoot ratios (R/S) of plants cultivated in pasteurized replant soil (a) were significantly higher than those cultivated in unfumigated replant soil (b) ($p = 0.01$) (Fig. 1). The fact that R/S ratios were lower in seedlings planted into "live" unfumigated replant soil (b) suggests that soil microbes were negatively affecting root growth in this treatment. Although no statistically significant differences existed between fumigated soil (ab) and unfumigated soil (b), mean root biomass (data not shown) and R/S ratios were *slightly* higher in fumigated soil (ab), providing some evidence of disease control in the fumigated soil. However, our ability to detect growth differences may have been limited by the relatively short duration of the experiment.

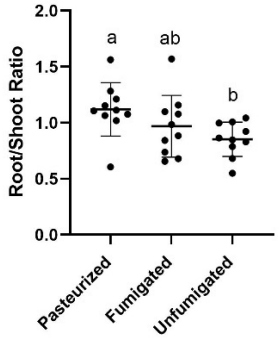


Figure 1. Effect of soil treatment on growth of 4-week old Gala apple seedlings as measured by root to shoot ratios (R/S). Fumigated and unfumigated replant soil was collected from SRO in April of 2021. Different letters indicate significantly different means, and represent comparisons of all three treatments (Kruskal-Wallis followed by Dunn's test).

Upon harvest, fine root tissue was also assessed for *P.penetrans* abundance. The replant pathogen complex resident to this site includes this root lesion nematode; however, not a single *P.penetrans* was identified in the roots of apple seedlings cultivated in any of the soil treatments. This may be another reason why we did not detect larger growth differences between fumigated and unfumigated treatments. The lack of *P.penetrans* infecting apple seedlings cultivated in unfumigated replant soil may be related to when the soil was collected (April 2021). During early spring, when soil temperatures generally remain below 70°C, nematode populations in soil may be less active due to overwintering.

Amendment impacts on soil health properties: In order to explore amendment-based changes in the abiotic soil health characteristics of fumigated orchard soil, all metrics listed in Table 3 (with the exception of the individual cations influencing cation exchange capacity) were used as input for a Principal Coordinates Analysis (PCA) (Fig. 2). PCA is an exploratory statistical method used to reduce the dimensionality of datasets containing a large number of variables in order to visualize patterns in the data. This is achieved by identifying the two most important or "principal" components (PCs), which explain the majority of variation in the data. The two new PCs represent the sum of variances from a combination of variables (22 different soil characteristics in this case). The samples (8 different experimental treatments) are then "projected" onto a 2-dimensional scatter plot (Fig. 2). Since PC1 and PC2 accounted for approximately 87% of the total variation in the dataset, this 2-dimensional scatter plot of the 8 experimental samples (Fig. 2) is a very good approximation of the original scatter-plot in 22-dimensional space. PC1 accounts for the largest proportion of variation (approximately 74%). Therefore, the more separation among samples along this axis, the more different they are. Fumigated soil amended with LC and CCM led to the greatest overall differences in abiotic soil health characteristics, relative to the fumigated control, as they both appear on the right side of PC1, while all other samples (including the fumigated control) appear on the left (Fig. 2). In addition, a large number of abiotic characteristics were similarly altered by the LC and CCM soil amendments. The green lines in Fig. 2 indicate which factors influenced the variation (explained by each axis) and show correlations between the factors. For example, nitrate and EC are so tightly correlated along PC1 (x-axis) that their individual vector lines are hard to distinguish. This suggests that nitrates make up most of the soluble salts in the EC readings. Both Fum + LC and Fum + CCM had high EC readings. Most particularly, the Fum + CCM treatment resulted in an EC value of 3.3 mmho/cm, which is above the damage threshold for apple/pear (1.7 mmho/cm). The high level of nitrate (277 mg/kg) in this treatment clearly had a negative impact on plant growth as none of the trees survived. Although the trees were able to tolerate the extremely high nitrate-N concentrations (148 mg/kg) resulting from the LC amendment, this treatment would be expected to lead to increased production of nitrous oxide (N_2O) from microbial denitrification, a greenhouse gas which is approximately 300 times more potent than CO_2.

The data indicates seed meal, shitake mushroom compost, and insect frass soil amendments all alter the chemical and physical properties of fumigated replant soil in similar ways, forming a relatively compact cluster of points in the upper left quadrant of the plot. As compared to the fumigated control soil, these samples evidenced increased water holding capacity, pH levels and C:N ratios. A recent study found that the C:N ratio and pH are key predictors of microbial-community composition and enzymatic activities in agroecosystems (Xu et al., 2020). Therefore, these 5 treatments are likely to be similar in terms of microbial community composition as well (data analysis in progress). Interestingly, the Fum +BjSa (2t) resulted in the greatest increase in C:N ratio and soil water holding capacity (the amount of water that a given soil can physically hold against the force of gravity), with the lowest cation exchange capacity (CEC) value. In fact, the PCA analysis indicated that soil C:N ratios and CEC values were generally negatively correlated with each other (as they extended in nearly opposite directions). This result was not expected and the reason for this correlation is unknown. CEC refers to the relative ability of a soil to store exchangeable cations (many of which are

essential nutrients) and buffer against rapid changes in pH. The most dominant soil cations were Calcium (Ca^{2+}) and Magnesium (Mg^{2+}) in 7 out of 8 treatments as shown in Table 3. The dominant cations in Fum + CCM were potassium (K^{+}) and Ca^{2+} were.

Table 3. The effect of the different soil amendments on the chemical and physical properties of fumigated replant soil. These metrics are for bulk soil collected 3 months post planting. All analyses were conducted by Soiltest Farm Consultants (Moses Lake, WA).

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

*Additional treatments not included in original proposal
millequivalents per 100 grams of soil

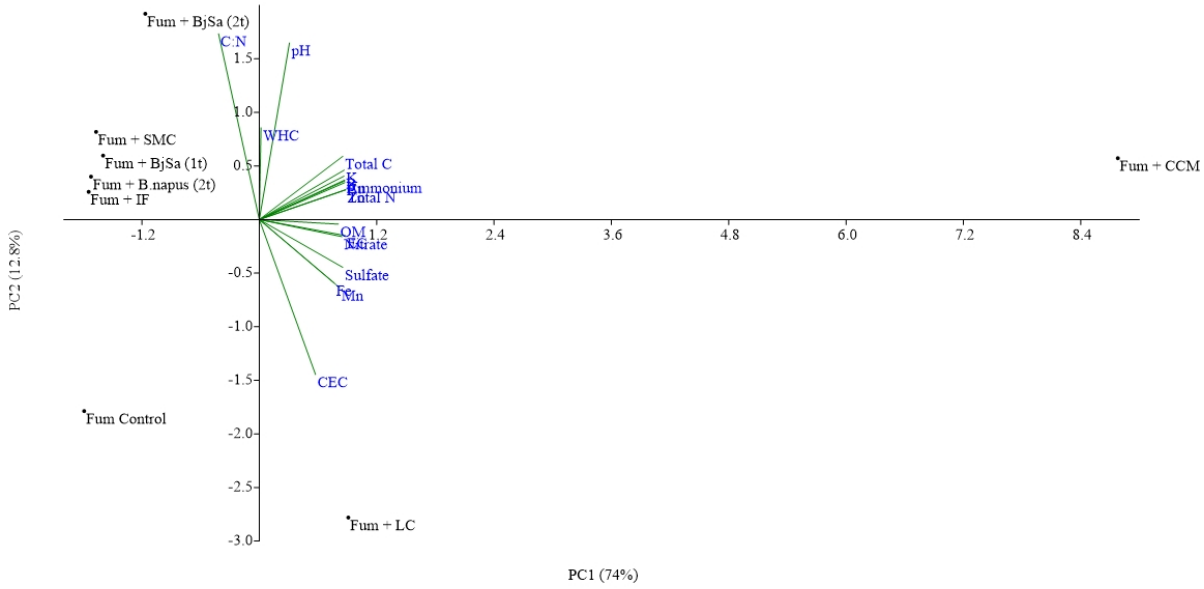


Figure 2. Principal components analysis of 22 different abiotic soil health characteristics in fumigated orchard replant soil (Fum control), and fumigated replant soil amended with Bj/Sa seed meal (1 and 2 tons per acre), shitake mushroom compost (SMC), composted chicken manure (CCM), liquid chitin (LC), insect frass (IF), or *B.napus* seed meal (2 tons per acre) at 3 months post-planting (derived using PAST statistical program).

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

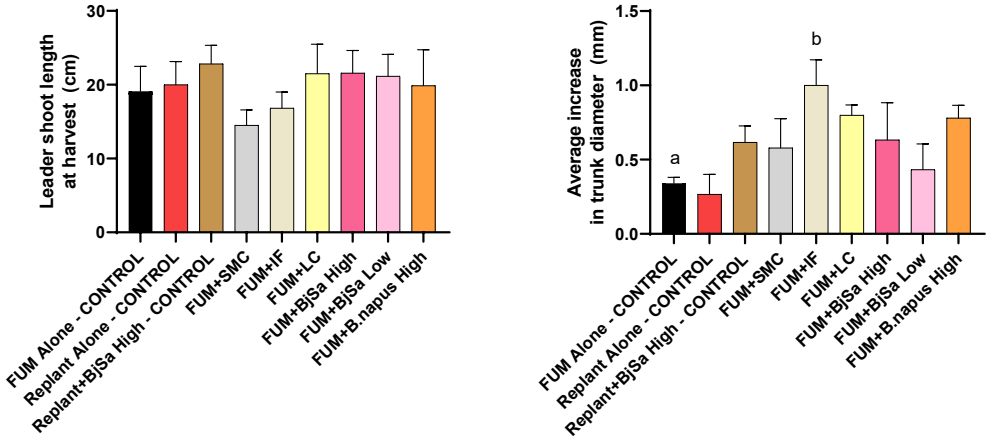


Figure 3. (A) Leader shoot length and (B) Increase in trunk diameter at harvest. Different lowercase letters indicate significantly different means and represent statistical comparisons between the unamended fumigated control (FUM Alone) and all other soil treatments. BjSa High and *B.napus* High = 2 tons seed meal per acre, BjSa Low = 1 ton seed meal per acre, SMC = shitake mushroom compost, LC = liquid chitin, IF = insect frass. Bars represent standard error of the mean.

No significant differences were identified in leader shoot length between the unamended fumigated soil control treatment and all other soil treatments (Fig. 3A; Kruskal-Wallis Test followed by Dunn's Multiple Comparisons Test, $p = 0.64$). Although not significant, the shitake mushroom compost (FUM + SMC) and the insect frass (FUM+IF) resulted in reductions in leader shoot length relative to all other treatments. It should be noted however that plants cultivated in the SMC treatment allocated more energy to root biomass than any other treatment (data not shown). A more robust root system may be beneficial by increasing plant tolerance to drought and/or replant pathogens. Insect frass was the only treatment which resulted in a significant increase in trunk diameter (from planting to harvest) relative to the fumigated control ($p=0.027$) (Fig. 3B). The amount of wood produced during the growing period (trunk diameter) is an indicator of overall tree health. In general, with the exception of the unfumigated replant soil control, all amendments resulted in an increase (albeit non-significant) in mean trunk diameter relative to the unamended fumigated control soil.

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Project Title: Understand the epidemiology of *Botrytis* to curb gray mold postharvest
WTFRC Project Number: CP-18-102

PI: Achour Amiri
Organization: WSU-TFREC
Telephone: 509-633-8181 ext.268
Email: a.amiri@wsu.edu
Address: 1100 N. Western Ave.
City/State/Zip: Wenatchee/WA/98801

Co-PI: Tobin Peever
Organization: WSU-Pullman
Telephone: 509-335-3754
Email: tpeever@wsu.edu
Address: Johnson Hall 357
City/State/Zip: Pullman/WA/99164-6230

Cooperators: Chelan Fruit, Stemilt

Report Type: Final Project Report

Project Duration: 3-Year

Total Project Request for Year 1 Funding: $32,360
Total Project Request for Year 2 Funding: $34,943
Total Project Request for Year 3 Funding: $33,371

Other related/associated funding sources

Agency Name: WSDA-Specialty Crop Block Grant program
Amt. awarded: $170,195
Notes: "Strategies to enhance pre- and postharvest management of gray mold in pome fruit" PI: Amiri, co-PI: Tobin Peever

WTFRC Collaborative Costs: None

Budget 1

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

Item	2018	2019	2020	2021
Salaries[1]	14,400	14,976	15,575	0
Benefits[1]	6,385	6,640	6,906	0
Wages[2]	5,760	5,990	6,230	0
Benefits[2]	545	567	590	0
Equipment	0	0	0	0
Supplies[3]	1,500	3,000	3,000	0
Travel[4]	1,070	1,070	1,070	0
Miscellaneous	0	0	0	0
Plot Fees[5]	2,700	2,700	0	0
Total	32,360	34,943	33,371	0

Footnotes:

[1] Salaries are for a Research Intern (0.3 FTE) at 44.3% benefit rate.

[2] Wages are for non-student temporary employee for summer help at 9.5% benefit rate.

3 Supplies include reagents for PCR and qPCR, agar media, plates and sampling materials.

[4] Travel to commercial and experimental orchards and packinghouses in WA for trials set -up, sampling and data collection.

[5] Plot fees for a block to be used for preharvest trial on gray mold in years 1 and 2.

OBJECTIVES:

Objective 1. Investigate infections timing of fruit by *Botrytis* in Washington apple orchard to determine critical timing of infections and subsequent disease expression in storage.

Objective 2. Evaluate the effect of preharvest weather conditions (rain and temperature) on infections and gray mold development in organic and conventional orchards.

Objective 3. Develop a timely preharvest spray management program to abate gray mold postharvest.

SIGNIFICANT FINDINGS:

- *Botrytis* was detected on flowers and fruit collected throughout the season from bloom to harvest.
- *Botrytis* was detected in the of orchard atmospheres throughout the season from bloom to harvest at low frequencies and variable among locations.
- The size of *Botrytis* inoculum was greater in organic orchards compared to conventional orchards.
- The inoculum size decreased from bloom to fruit set in conventional but then increased toward maturity and harvest. In organic orchards, the inoculum size increased throughout the season.
- Variabilities in inoculum size and dynamic throughout the season has been observed between orchards located in different districts.
- Effect of temperatures seems to be the factor that drives preharvest infection as summer infection tend to be lower as temperatures rise above 85°F.
- The impact of leaf wetness duration was hard to accurately assess across locations, but some microclimates and overhead cooling may increase infection risks.
- A spray program consisting of at least three sprays a season was effective in reducing gray mold development postharvest

RESULTS AND DISCUSSION

Objective 1. Infection timing of *Botrytis* preharvest and postharvest

As shown on Figure 1 below, *Botrytis* was detected in orchards at almost all sampling times. There seem to be a carry-over from bloom to fruit and increases as the fruit mature. Fungicide spray programs for each orchard were obtained and are being analyzed to correlate with potential fungicide effect on reduction of Botrytis load on fruit as this can be explained by the slight reduction observed before harvest (Figure 1) following the preharvest spray. However, the incidence of fruit infected (not decayed) with *Botrytis* increased significantly to 66% in Orondo and 58% in Mesa after 6 months of storage in CA. It is important to note that the fruits used in this study were not treated postharvest.

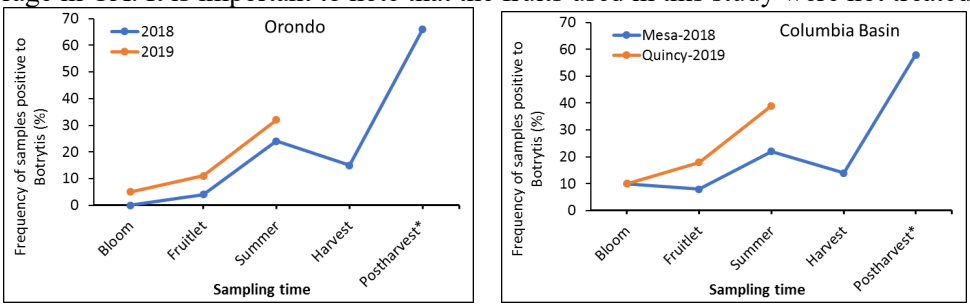


Figure 1. Evolution of *Botrytis* incidence throughout the preharvest growing season as detected by qPCR. 2019 Sampling after harvest are being analyzed.

Infections by *Botrytis* were observed in all parts of the fruit (cuticle, stem-end, calyx and inner flesh) at harvest at variable frequencies between orchards (Figure 2). This observation indicates that not only the external parts (calyx, cuticle and stem-end) of the fruit contains *Botrytis* inoculum at harvest, but also the flesh which indicates latent (dormant) infections from previous infections in the orchard. The frequency of samples carrying Botrytis remained steady or increased slightly in storage.

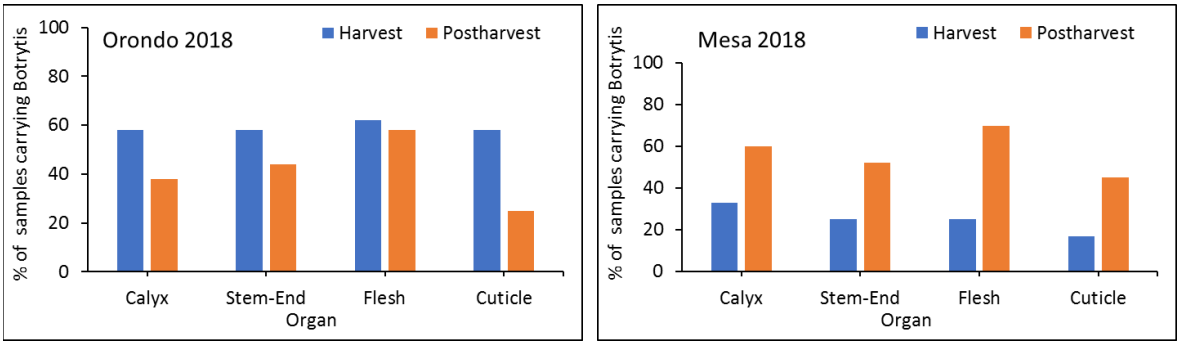


Figure 2. Incidence of *Botrytis cinerea* on different organs of the fruit at commercial maturity (harvest time) in organic and conventional orchards in 2018. Samples from 2019 Are being analyzed currently.

<u>Correlation between infection levels at harvest and gray mold incidence in storage:</u> There was a positive correlation between the incidence of Botrytis infections at harvest and gray mold incidence after 8 months of storage (Figure 3). Between 18 and 25% of fruit infected at harvest developed gray mold in storage but is was not proportional. Data of the 2020-21 season showed the same trend (data not shown).

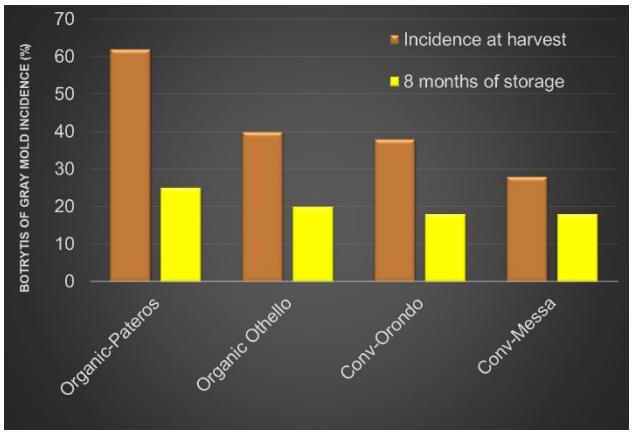


Figure 3. Botrytis incidence at harvest (brown bars) and gray mold incidence after 8 months if storage at 34°F in regular atmosphere on Fuji apples collected from four orchards in central Washington in 2019.

Objective 2. Impact of weather conditions on Botrytis incidence preharvest

Temperatures, rainfall, and leaf wetness duration (LWD) data have been collected from the experimental orchards for two growing seasons using the WSU AgWeatherNet platform. The weather stations were located within 2 miles radius from the experimental sites. Data were collected a week before and a week after our sampling to be able to make a close correlation. *Botrytis cinerea* infection risk is the highest at temperatures ranging from 68°F to 77°F and LWD of 8 to 14 hours. Our data showed variability between daily temperatures and LWD values between orchards (Figure 4). However, we see that daily average temperatures in 2019, were below 80°F except for the sampling done during the summer when temperatures were above 80°F. This would explain the slight decrease in Botrytis infection incidence that we saw during the summer across orchards (Figure 1). Except for the Mesa experimental orchard, LWD duration in 2019, were below 0.2 U (2 hours) during the periods between end of May and early September. The figure 3 clearly shows LWD values >6 hours for periods that correspond to our early (April-May) and late (preharvest-October) sampling when the infection by Botrytis was high. Temperatures were significantly higher in 2020 whereas LWDs values were slightly higher compared to 2019 (data not shown). In the 2020-21 season, more packers reported increased

gray mold incidence in cold storage. Whether this is weather-related only, will require more in-depth analyses of the weather data not just for one season but multiple seasons. The long-term objective behind this study is to make such clear correlation and develop potential risk models for Botrytis infections in the PNW.

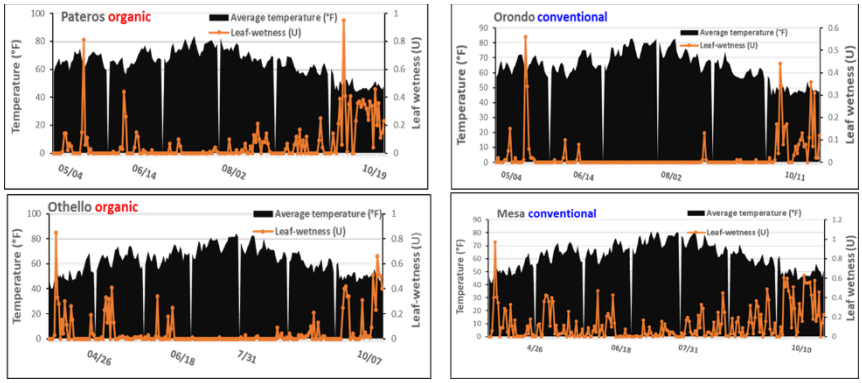


Figure 4. Temperatures (black lines) and leaf wetness duration (orange lines) values in four experimental orchards in 2019.

Objective 3. Develop a timely preharvest spray program to abate gray mold in storage

Spray programs consisting of Luna Sensation, Inspire Super, Fontelis, Topsin-M and Pristine were applied alone or in rotation duration two growing seasons on a Fuji block at the Sunrise orchard. Sprays were conducted at petal fall, fruit set (1-1.5" diameter), late-summer (August), and 7 days preharvest. We compared a conservative spray (1 spray/season), moderate-low (2 sprays), moderate (3 sprays) and extensive (4 sprays) to a non-treated control. Fuji apples were harvested at commercial maturity, stored at 35°F in regular atmosphere, and inspected for decay every 2 months. A summary of overall decay incidence and gray mold incidence are shown in Table 1. Our results indicate that fruit set spray combined with preharvest spray may be the best to reduce gray mold decay in storage. The one 7 day preharvest fungicide spray was not highly effective against gray mold compared to the other treatments. While a decision about the number of sprays a season may be economic, our future studies will aim at estimating the cost/benefits of moderate high and aggressive sprays versus conservative and moderate-low sprays considering cost of sprays and packout.

Table 1. Overall and gray mold decay incidence on Fuji apples sprayed with multiple fungicides at different phenological stages in 2019 and 2020.

		Decay incidence							
		Fungicide sprayed at				2019-20		2020-21	
Treatment type		Petall fall	Fruit Set	Late summ	7 DPH	Overall	Gray mold	Overall	Gray mold
	Untreated control	-	-	-	-	35	12	42	18
Conservative	1 spray-early	LS	-	-	-	11	5.0	13	6.0
	1 spray-early	-	LS	-	-	18	9.0	22	8.0
	1 spray-late	-	-	-	P	8.0	3.0	10	4.0
Moderate-Low	2 sprays-early	LS	IS	-	-	18	11	21	7.0
	2 sprays-mid/late	-	-	IS	P	7.0	3.0	9.0	3.0
Moderate-high	3 sprays-early/mid	LS	IS	F	-	8.0	3.0	10	2.0
	3 sprays-mid/late	-	F	IS	P	13	5.0	11	3.0
Aggressive	4 sprays	LS	TM	IS	P	6.0	1.0	8.0	1.5

LS= Luna Sensation, F = Fontelis, P = Pristine, IS = Insper Super, TM = Topsin-M

Executive summary

Project Title: Understand the epidemiology of *Botrytis* to curb gray mold postharvest
Key words: Gray mold, infection timing, weather, management

Abstract:

In statewide surveys conducted in 2016 and 2017 in Washington State, gray mold caused by *Botrytis* sp., was widespread and accounted for 30% to 40% of total decay on apple. Gray mold was found in 88% of grower lots surveyed across the state with incidences ranging from 5% to 75% of total decay across regions and lots. While Botrytis is known as a wet-region pathogen, it is surprising to see such relatively high gray mold incidences in the Pacific Northwest. This study aimed to define key infection timing of fruit by Botrytis, and we found an infection peak at petal fall and preharvest. Albeit infections seem to slow-down during the summer, they continue at a lower rate. We have also seen some effect of temperatures on Botrytis infections whereas the role of wetness in the regions still need future investigation. However, given the rate infection we saw at all sampling stage, indicate that *Botrytis* of pome fruit in Central Washington may be adjusting to shorter wetness periods if appropriate temperatures occur to infect fruit at a level that is enough to be challenging to packers in the region. Because of the semi-arid weather in central Washington, there may have been a tendence in the industry to wait until 14 to 0 days before harvest to make a spray. Our findings on key infection timing have been used to design and implement multiple spray programs at different phenological stages. Our data indicate that a program consisting of 3 sprays applied at fruit set, mid-summer, and 7 days preharvest (7 dph), reduces gray mold the best. The two first sprays may help in reducing latent infections that may develop early and mid-season while the last spay (7 dph) help reducing the newest infections that may occur closer to harvest when fruit are the most susceptible to infections.

OTHER OUTCOMES

Grants

Funds from this grant were leveraged to secure tow extra-mural grants, one from the Specialty Crop Block and another from the USDA-Crop Protection and Pest Management program to continue research on Botrytis epidemiology, role of weather conditions, fungicide resistance, *Botrytis* population genetics and pre- and postharvest management.

1. Epidemiology-based tactics to abate gray mold of pome fruit in the Pacific Northwest. USDA-NIFA CPPM. $199,805. P.I.: A. Amiri, Co-PI.: Karina Gallardo. 2020-2023.

2. Strategies to enhance pre- and postharvest management of gray mold in pome fruit. Specialty Crop Block Grant program (SCBG), WSDA-USDA. $230,155. P.I.: A. Amiri, Co-P.I.: T. Peever. 2019-2022

Abstracts and Manuscripts:

1. Amiri A., Mulvaney K.A., Pandit L.K., De Angelis R.D. **2017***. First report of resistance to fluxapyroxad and fluopyram in *Botrytis cinerea* from commercial apple orchards in Washington State. *Plant Disease* 101: 508.

2. Amiri A., Ali MD.E., De Angelis D.R., Mulvaney K.A., Pandit L.K. **2019***. Prevalence and distribution of *Penicillium expansum* and *Botrytis cinerea* in apple packinghouses across Washington State and their sensitivity to the postharvest fungicide-pyrimethanil. Proceedings of IV International symposium on Postharvest Pathology. Pp 1-4.

3. Amiri A., Acosta W. **2020**. Gray mold factsheet. http://treefruit.wsu.edu/crop-protection/disease-management/gray-mold/

4. Amiri et al. 2022. Determination of key infection timings of apple fruits by Botrytis cinerea in the Pacific Northwest. Plant Disease (Submitted)

5. Amiri et al. 2022. Optimization of preharvest spray programs in pome fruit orchards to abate gray mold decay in cold storage. Plant Disease (In preparation).

Talks

1. Wilson A., Amiri A. Management of gray mold pre and postharvest. *Northwest Apple Day*. Jan 21st, **2020**.

2. Amiri A. Management of Botrytis and Phacidiopycnis rots: field and storage. *Chelan Horticultural Meeting*. January 21st, **2019**.

3. Amiri, A. Epidemiology of Botrytis of pome fruit in the Pacific Northwest. *Western Pest and Disease Conference*, Portland, January 10th, **2019**

4. Bengyella L., Amiri A. Dynamic variation of Botrytis populations in pear orchards of the PNW. *WA Tree Fruit Association Annual meeting*, Yakima, December 4th, **2018**.

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

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

Co-PI (2): **Yanmin Zhu**
Organization: USDA-ARS
Telephone: 509-664-2280 ext. 215
Email: yanmin.zhu@ars.usda.gov
Address: 1104 N. Western Ave
City/State/Zip: Wenatchee,WA,98801

Cooperators: Dr. Wojciech Janisiewicz (USDA-ARS, Kearneysville); Certis, Sym-Agro, Marrone Bio, Wilbur-Ellis, Several growers and packers in Washington State.

Report Type: Continuing Project Report

Project Duration: 3-Years

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

Other funding sources: None

WTFRC Budget:

Item	2019	2020	2021	2022
RCA Room Rental	6,300*	6,300	6,300	0
Total	6,300	6,300	6,300	0

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

Budget 1

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

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

Footnotes:

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

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

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

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

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

OBJECTIVES

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

SIGNIFICANT FINDINGS:

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

METHODS

OBJECTIVE 1. Evaluate the adequacy and efficacy of current and novel preharvest management organic strategies (Year 1-3)

Activity 1.3: Develop effective spray programs based on optimal timing and number of applications of combined treatments: Based on the efficacy of different treatments from trials conducted as outlined in activities 1 and 2, the best treatments at bloom and preharvest will be combined for up to 5 applications from bloom to harvest. Aggressive (up to 5 applications/season) and conservative (1-2 applications/season) spray programs will be designed and tested for two successive seasons as described in Activities 1 & 2 above. Adjustments, in timing and numbers of sprays, may be made after Year 2 to enhance efficacy in Year 3.

Progress: *Field trials are conducted. Fruits from a 3 season are in storage. Decay incidence and types data will be available by June 2022.*

OBJECTIVE 2. Evaluate the benefits of using dynamic control atmosphere (DCA), GRAS products and biocontrol agents to control rots in storage. (Year 1-3)

Activity 2.1. Efficacy of Dynamic Controlled Atmosphere. Work to assess efficacy of DCA and CA inreducing incidence of 4 key postharvest pathogens, i.e. *Botrytis cinerea*, *Neofabraea perennans, Penicillium expansum* and *Mucor piriformis* has been concluded.

Activity 2.2. Efficacy of organic postharvest treatments. Currently, there are no known effective products that can provide an acceptable level of efficacy against major postharvest diseases in commercial packinghouses. Like the conventional systems, the possibility of adding a postharvest fungicide application would add to the level of efficacy provided by the preharvest treatments. Herein,

we suggest testing some GRAS products and rare biopesticides (Table 3) that may be labeled in the future is their efficacy if proven. We will conduct trials using artificially-inoculated experiments, focused on 4 major pathogens *B. cinerea*, *N. perennans*, *P. expansum*, and *M. piriformis*, and also using naturally infected fruits. Experiments and disease evaluations will be conducted as explained in Activities 1.1 and 1.2.

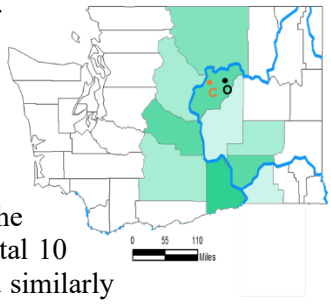
Table 3. Suggested number of fruits, atmosphere types, and storage duration to test on artificially and naturally-infected fruit

#	Trade name	Hypothesized Mode of action	Product Type
1	**Non-treated control**	-	-
2	Scholar SC[a]	FRAC 12	Fungicide
3	Bioferm	Competition	Biological
4	Cinnerate	Contact	Plant extract
5	SB. PSS 5.6	Sanitizer/Contact	Anions
6	Glyceryl palmitate(s)	Fruit Enhancer/Contact?	Inducer
7	Natamycin	Antibiotic	Biofungicide
8	OSO™ 5%SC[c]	FRAC 19	Biofungicide[b]

[a] is a conventional fungicide to be used for comparison with other treatments. [b] Organic label for pre and potentially postharvest application is pending for OSO™.

Progress: *Storage trials are ongoing and Decay incidence data will be available by June 2022.*

OBJECTIVE 3. Acquire novel knowledge about the impact of different spray regimes and storage conditions on fruit microbiomes pre- and postharvest (Year 2-3, Amiri & Zhu).



Activity 3.1. Sites and sampling: One conventional 'Fuji' (C) and another organic (O) orchard located in Chelan or Grant counties will be used for sampling (Figure on right). To avoid any interference of the rootstock, blocks established on the same rootstock will be used. In each orchard, 10 non-adjacent trees (with 10 buffer-rows) will be randomly selected and tagged before bloom. Samples will be collected as detailed in Figure 1 (below). At full bloom, 10 flowers/tree will be sampled from the circumference of the tree and pooled to make one biological replicate (total 10 biological replicates/orchard). The same number of fruits will be sampled similarly at each sampling time. All samples will be collected 3 days before and 3 days after pesticides treatments have been made. Samples will be transported in separate Ziploc bags to TFREC and will be immediately processed for DNA extractions or frozen at -80°C until further usage.

	Full bloom	Immature fruit	Mature fruit	1 month storage	6 months storage	Total
Organic	20 (10+10)[a]	20	20	20	20	**100**
Conventional	20	20	20	20	20	**100**

Figure2. Scheme of the sampling protocol to be used for microbial analyses. [a]10 samples will be collected 3 days before fungicide treatments and 10 others will be 3 days after the treatments.

Activity 3.2. DNA extraction, sequencing and analyses: Microbial and fungal DNA will be extracted from flowers and fruits immediately or soon after sampling using the MP-FastPrep DNA extraction kit following the manufacturer' protocol. The verification of DNA quality and dilution will be done as described in Objective 4. The ITS (ITS1 and ITS4) rDNA and 16S rRNA genes will be used to investigate the fungal and microbial communities, respectively, as described previously (Manter and Vivanco, 2007). The amplified products will be sequenced using an Illumina MiSeq instrument at the Institute of Biotechnology at the University of Idaho, United States.

Raw sequence reads will de-multiplexed, low quality read ends will be trimmed using Trimmomatic (Bolger et al. 2014), and low-quality sequences will be removed. QIIME2 (https://qiime2.org/) was used to perform the downstream diversity and taxonomy composition analysis. Corresponding paired end reads will be merged, and un-joined reads will be discarded. The remaining sequences will be used to determine differences in bacterial and fungal communities between flowers and fruit samples from the organic and conventional orchards at each sampling time, and to calculate the Shannon diversity index to obtain alpha and beta diversity statistics. Sequences will be grouped to obtain operational taxonomic units (OTUs) with 97% similarity. The resulting OTUs will be compared against the trained full-length Greengenes 13_8 OTUs database (http://greengenes.secondgenome.com/) for bacterial taxonomic classification. For ITS taxonomy analysis, a database will be trained using the fungus sequences in UNITE (Fungal ITS) (https://unite.ut.ee/). The output files will be visualized in QIIME2 and underlying data will be extracted to perform a principal component analysis and plotting in R (https://www.r-project.org/).

The hypotheses that microbiomes will differ (i) between organic and conventional orchards (ii) between growth stages and (iii) before and after fungicide treatments will be tested. Each sample's richness and diversity will be evaluated using the Chao1 and the Shannon indices (Chiu and Chao, 2016), respectively. To evaluate the effect of different environmental and management factors on microbiome composition, multivariate mixed regression models will be fitted to the normalized counts using the GLMM or GLMIMX (i.e., combination of general linear and mixed modes) approach. Non-Metric Multidimensional Scaling (NMDS) analysis will be performed to visualize the dissimilarity matrix based on the (i) presence of cultured pathogens and (ii) use of specific practices. To test the association of the composition of microbial community with these covariates of interest, we will use the Permutational Multivariate Analysis of Variance (PERMANOVA) and the recently developed PERMANOVA-S (Tang et al., 2016). Statistical modeling will be conducted in R and Statistical Analysis Software (SAS).

Progress: *Sampling from the field and DNA extractions are concluded and final sampling from storage is expected in Jan. 2022. Final microbiome analyses are expected by May 2022.*

OUTREACH ACTIVITIES: (Amiri, Year 3)

Amiri will summarize the most important and major findings from the work accomplished in Years 1 & 2 to be presented at an extension event planned in early spring of 2021 (if the other extension proposal submitted is funded) and/or at regular meetings, such as Northcentral Apple days, organized by Extension Specialist Dr. DuPont, at Pom Club and Apple Review Days. Additional work from Year 3 will be presented at workshop planned on March 9th 2022. Data will also be summarized for publication in the WSU Fruit Matter newsletter.

Results and Discussion

Activity 1.1. Efficacy of preharvest materials in reducing postharvest decays

Season 2019-20

Nine treatments, including a control (non-treated), a conventional fungicide (Merivon) and seven organic materials sprayed 7 days preharvest on a Fuji block in East Wenatchee in 2019.

After 8 months of storage at 34F in a regular atmosphere, overall decay (all decay types) incidence was 99% in the control and was reduced to below 50% by 5 organic products with Serenade Pro, Double Nickel and Regalia being the most effective (Figure 3). While OSO, provided a good efficacy against gray mold and blue mold when data were analyzed for each pathogen (Data not shown), it reduced overall decay incidence by 30%.

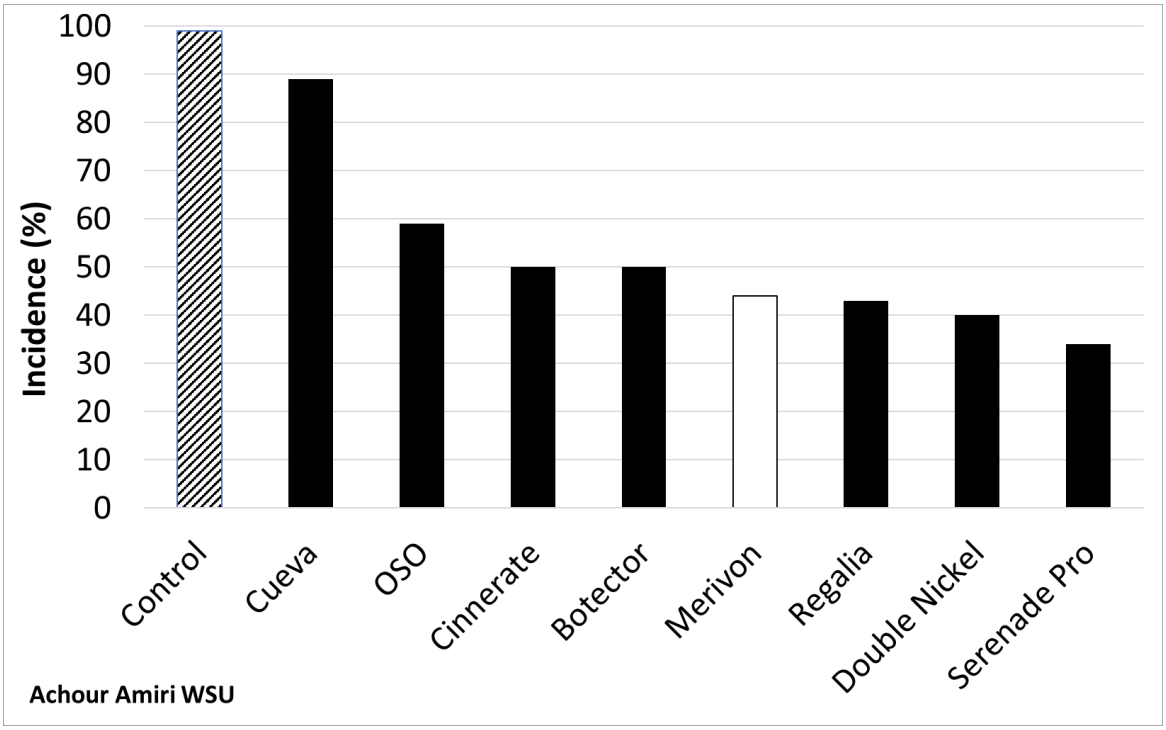


Figure 3. Overall decay (all decays combined) incidence on Fuji treated with the materials 7 days preharvest and stored at 34°F in a regular atmosphere for 8 months. Season 2019-20.

Season 2020-21

In 2020, 12 organic materials were sprayed 7 days preharvest and compared to the conventional Merivon and a non-treated control. The efficacy of most materials tested in 2019 was consistent and the 2020 trial showed that 8 organic materials significantly reduced overall decay incidence after 9 months of RA storage (Figure 4)

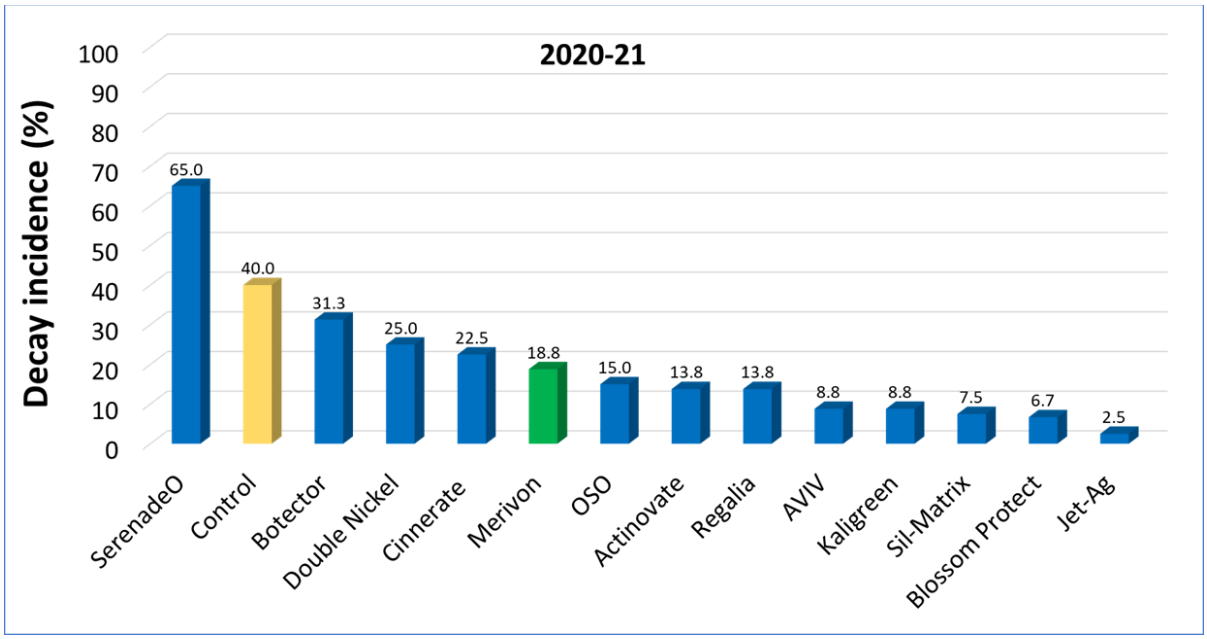


Figure 4. Overall decay (all decays combined) incidence on Fuji treated with the materials 7 days preharvest and stored at 34°F in a regular atmosphere for 8 months. Season 2020-21.

Activity 1.3. Development of a timely preharvest organic spray program

Four organic materials which showed a good efficacy from Activity 1.1 were selected and sprayed sequentially from petal fall to 7 days preharvest. Results from the 2020-21 season showed that decay incidence after 9 months of RA storage was 5 times lower when 4 sprays were applied throughout the season compared to the untreated control (Figure 5)

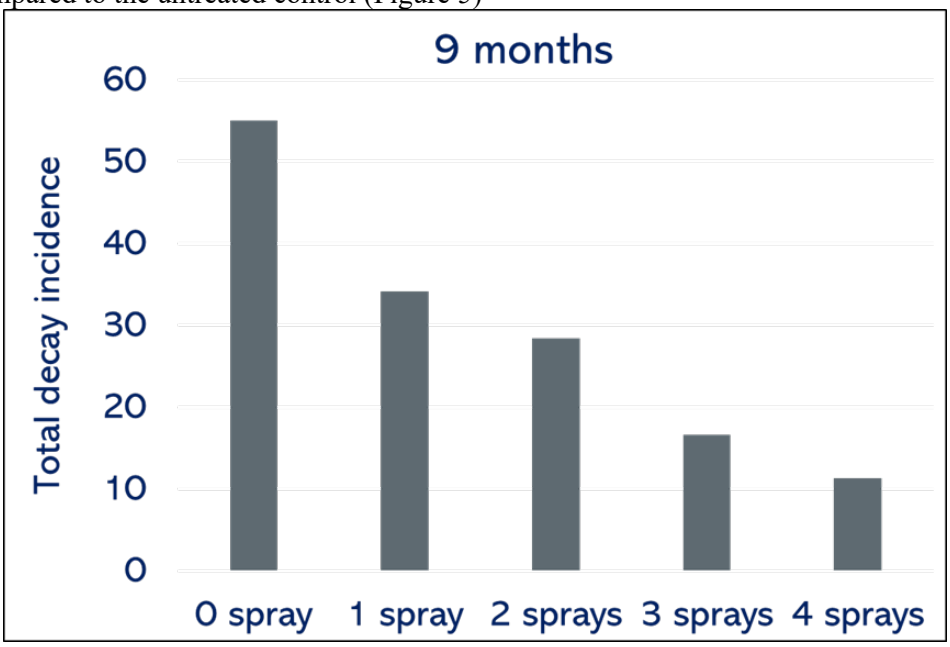
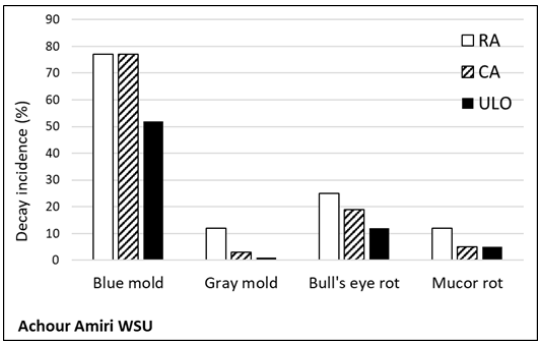


Figure 5. Overall decay (all decays combined) incidence on Fuji treated with different organic materials at different growth stage between petal fall and commercial maturity preharvest and stored at 34°F in a regular atmosphere for 9 months. Season 2020-21.

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



The trials were conducted on Fuji artificially inoculated with spore suspensions of *P. expansum* (blue mold), B. cinerea (gray mold), *N. perennans* (bull's eye rot) and *M. piriformis* (Mucor rot) and stored in 3 different atmospheres, regular (RA), CA (O_2 = 4% and CO_2 = 0.8%), and ULO (O_2 = 1.5% and CO_2 = 0.8%) and stored at 34°F for 5 months. Figure on right shows that ULO program tested this year had a slight reduction of incidence of blue mold, gray mold, and bull's eye rot but not mucor. Much additional research with different atmospheres and duration will be needed before making overall conclusions on the ULO/DCA benefits in fighting decays.

FUTURE WORK

- Collect final results from activities of Objectives 1 -3 by June 2022.
- Share final and preliminary data on organic decay management at Special Workshop planned on March 9th, 2022 at CTC or virtually.
- Submit final results in the final report by December 2022.

Project/Proposal Title: Outreach Program for Apple Decays Management in Washington

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

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

Cooperators: : Syngenta Crop Protection, Bernardita Sallato (WSU-Extension)

Report Type: Final Project Report

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $10,254
Total Project Request for Year 2 Funding: $10,425

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Achour Amiri
Organization Name: Washington State University
Contract Administrator: Anastasia (Stacy) Mondy
Telephone: 509-335-2587
Contract Administrator Email Address: arcgrants@wsu.edu

Item	2019	2020	2021
Salaries	2,950	3,068	0
Benefits	1,304	1,357	0
Wages	0	0	0
Benefits	0	0	0
Equipment	0	0	0
Supplies	5,000	5,000	0
Travel	1,000	1,000	0
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	**10,254**	**10,425**	**0**

Footnotes:

OBJECTIVES

1- Conduct statewide workshops to provide up-to date knowledge about disease infection timing, management, and fungicide resistance mitigation (Year 1).

2- Demonstrate visual approaches and portable field/packinghouses DNA-based devices for disease detection. Updates on organic management and disease management of 'WA 38' (Year 2).

SIGNIFICANT FINDINGS

Year 1 (2019-2020)

- A workshop was organized on March 4th, 2020 at the CTC in Wenatchee
- The workshop was attended by 110 stakeholders
- The participants were: packers (38%), growers/packers (29%), consultants (22%), industry reps (11%)
- 85 participants (77%) answered a survey at the end of the workshop
- 85% of surveyed participants said they learned a great/good deal
- The top areas the participants said they will do differently after the workshop are 1) pre and postharvest sanitation, 2) Re-evaluate fungicide programs/timing/, 3) fungicide rotation and 4) bin cleaning/sanitation
- The three top things liked by participants were: activities/hands-on (25%), presentations/flow (18%), and presentation on timeline/pre and postharvest management tactics (13%)
- 98% of participants increased their knowledge in infection timings, 84% increased their knowledge about most effective pre and postharvest fungicides, and 98% increased their knowledge about fungicide resistance risks and importance of rotating different FRAC groups.

Year 2 (2020-21)

- Because of the COVID-19 pandemic, the second year workshop was canceled and postponed to 2022.
- A workshop is scheduled on March 9th, 2021 at the CTC in Wenatchee
- If the COVID-19 pandemic still does not allow in person meetings by March 2022, the workshop will be conducted virtually.

RESULTS AND DISCUSSION

We present some statistics from the workshop and results from the survey conducted at the end of the workshop

1-Participants: 110 stakeholders from Washington and Oregon attended the full day workshop. The majority were identified as either packers (35%) or grower/packer (33%), whereas 20% and 13% of participants were consultants and industry representatives, respectively (**Figure 1**).

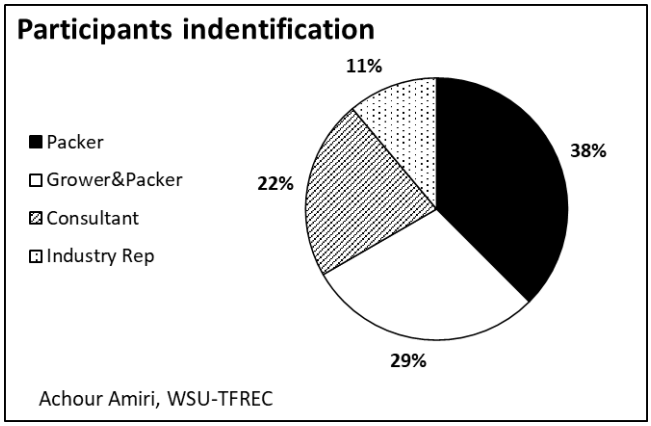


Figure 1. Percentages of different stakeholder categories who attended the 2020 disease workshop in Wenatchee, WA.

2- Practices the participants listed they will do differently as a result of the workshop. 85 participants (77%) filled a survey at the end of the workshop.

A majority of 34% of surveyed participants mentioned they would approach pre and postharvest sanitation differently after the workshop to enhance disease management (**Figure 2**). The three other top things that the participants said they would differently are: re-evaluate their fungicide program and timing (21%), rotate fungicide (15%) and clean/sanitize bins (10%).

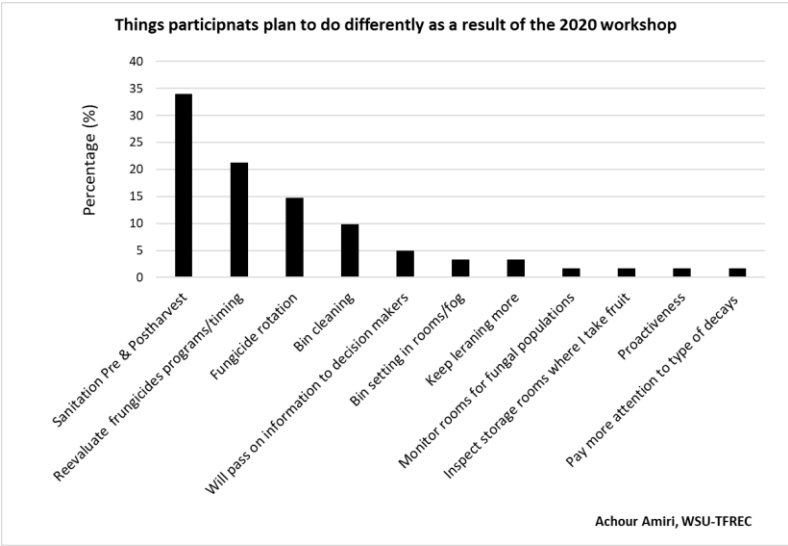


Figure 2. Practices that the 85 surveyed stakeholders would do differently as the result of the 2020 disease workshop in Wenatchee, WA.

3. Overall and specific knowledge learned by participants in the 2020 disease workshop

Overall knowledge: in all categories, a majority of participant learned a "great/good deal" as a result from the workshop (**Figure 3**). A majority of 81, 86, 87 and 87% of packers, packers/growers, consultants, and industry representatives, learned "great and good deal". None of the surveyed participant mentioned he learned "little" from this workshop

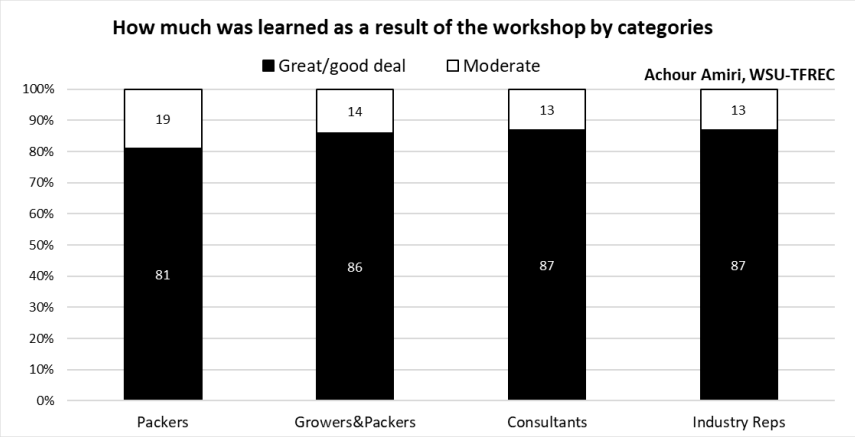


Figure 3. Overall knowledge acquired by different stakeholder categories (85 participants surveyed) who attended the 2020 disease workshop in Wenatchee, WA.

<u>Specific knowledge about important practices learned after the workshop</u>. The Participant were surveyed for their knowledge in five major topics before and after the workshop. The topics were 1) infections risks and timing in the orchard, 2) Effective preharvest materials, 3) most effective postharvest materials, 4) fungicide resistance risks/mitigation, and 5) importance of fungicide alternation. The % of participants with minimal knowledge before the workshop decreased very significantly after the workshop (Figure 4a). The % of participants with considerable knowledge before the workshop increased very significantly after the workshop in all topics (Figure 4B).

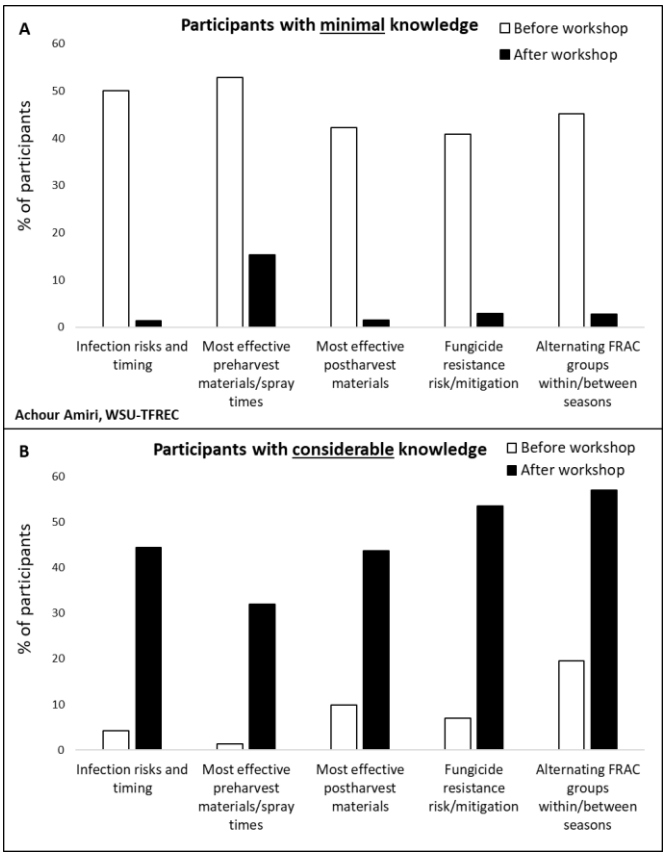


Figure 4. Percentage of surveyed participants with <u>minimal</u> (A) and <u>considerable</u> (B) knowledge on major disease management topics **<u>before</u>** and **AFTER** the 2020 disease workshop

Photographs from attendance and activities conducted during the disease workshop at CTC, Wenatchee, March 4th, 2020. Photo credit: Achour Amiri, WSU-FREC.

Executive summary

Project Title: Outreach Program for Apple Decays Management in Washington
Key words: Decay, Preharvest, Postharvest, Conventional, Organic

Abstract:
Diseases of pome fruits and the management practices used to abate them have evolved in recent years in Washington and the Pacific Northwest. New knowledge on the pathogens infection timeline, detection, and management in organic and conventional systems became available following recent research efforts on decays conducted by researchers in recent years with a strong support from the WA Tree Fruit Research Commission. This series of workshop intended to be part of the technology transfer of research findings to the pome fruit stakeholders in the region. The first-year workshop (2020) was focused on conventional decay management pre and postharvest and was attended by 110 stakeholders, i.e., growers, packers, consultants, and industry representatives. Pre and post-workshop surveys helped in determining urgent needs and how much attendees learned from the workshop and their plan to implement the acquired knowledge in their orchards and warehouses. Production and storage of organic apples have increased sharply in recent years in Washington and decays became a limiting factor for extended organic fruit storage. The workshop provided invaluable new knowledge on the most effective organic materials to spray preharvest and the best timing for material application to optimize decay management in organic storage rooms for more than 6 months.

Future Directions:

- Outreach efforts will continue in the mid- and long-term to insure timely technology transfer to stakeholders
- WTFRC funds have been leveraged to secure additional state and federal funding to continue future outreach efforts
- Recent research findings are being summarized and have either been formatted to produce extension bulletins or will be in the future as research makes progress
- A focus will be put on improving communication between growers, consultants and packers to optimize decay management pre- and postharvest.