Project Title: Phase 3 New Biocontrol Strains Against Fire Blight

Report Type: Final Project Report

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Cooperators: None

Project Duration: 2-Year

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

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

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

Budget 1
Primary PI: Sharon L. Doty
Organization Name: University of Washington
Contract Administrator: Carol Rhodes
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Item	2023	2024
Salaries	$0.00	$10,768.00
Benefits	$0.00	$2,626.00
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$0.00	$915.00
Travel	$0.00	$200.00
Plot Fees		
Miscellaneous	$0.00	$464.00
Total	$0.00	$14,973.00

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

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

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

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

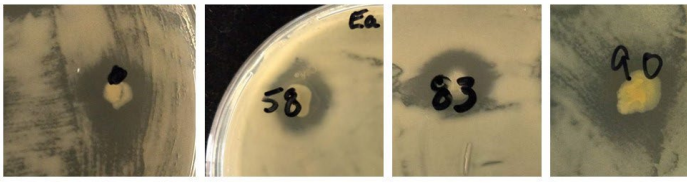
Significant Findings
UW- Dozens of bacterial strains with strong inhibition of *E. amylovora* growth *in vitro* were originally isolated in Phase 1 of this grant and confirmed in this Phase 3 grant.
Genomic analysis of the endophyte strains revealed a robust array of Biosynthetic Gene Clusters (BGCs).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

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

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

Results and Discussion

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



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

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

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

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

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

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

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

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

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

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

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

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

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

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

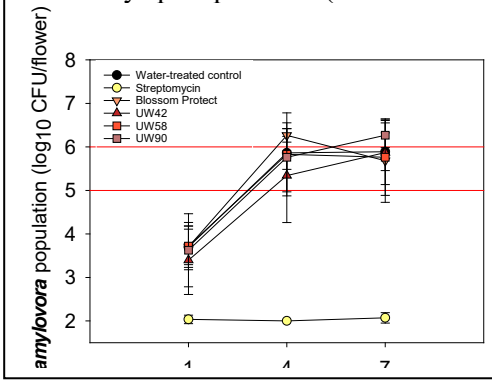
[w] Fruit skin marking is rated from an average of 25 fruit per tree. Rated on a 0 to 15 scale where ratings below 3 indicate no commercial downgrades.

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

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

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

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



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

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

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

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

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

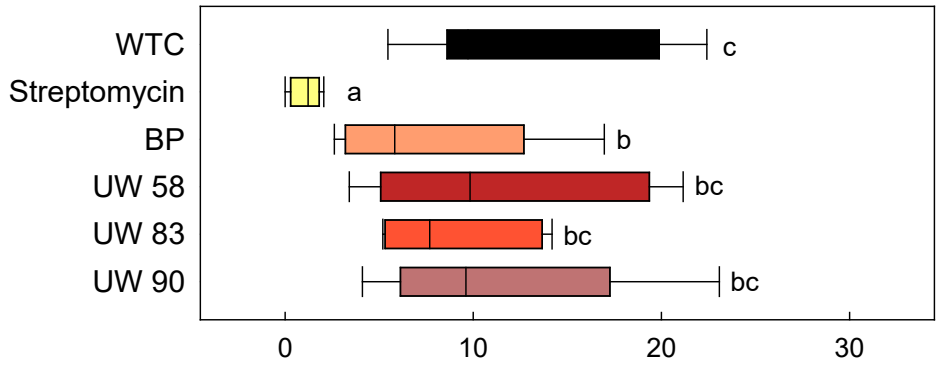
Treatment	Amount per 100 gal	Timing[z]	Infections per 100 clusters[y]				Fruit skin marking
Streptomycin standard (Firewall 50WP)[w]	8 oz	4,7	1.1	±	0.4	a[v]	0.05 ± 0.03
Blossom Protect + Buffer Protect	1.25 lb + 5 lb	2,3,6	7.5	±	2.6	b	0.06 ± 0.03
UW58	378.5 ml	1,3,5,8	11.7	±	3.3	bc	0.06 ± 0.02
UW83	378.5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

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

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

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

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



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

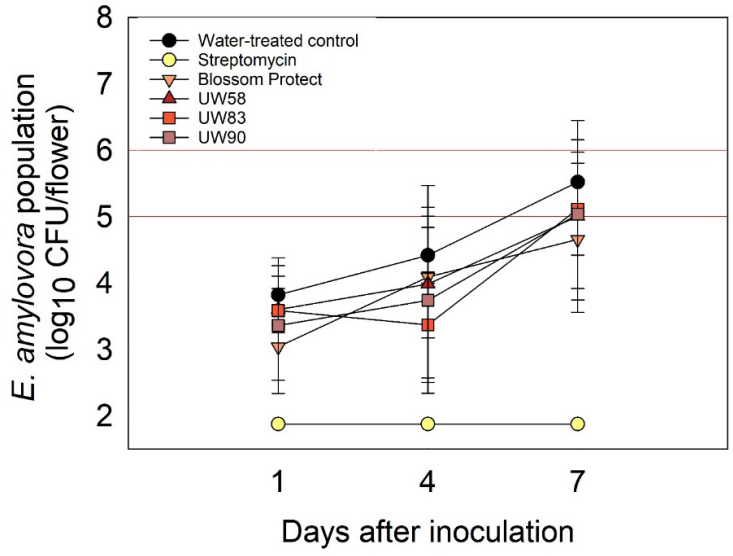


Figure 4. Effect of University of Washington treatments applied to WA 38 apple trees on the population size of *Erwinia amylovora* strain 153N on flowers 1 day after inoculation (full bloom + 1 day), at petal fall (full bloom + 4 days) and 3 days post petal fall (full bloom + 7 days).

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

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

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

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

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

Executive Summary

Project Title: Phase 3 New Biocontrol Strains Against Fire Blight

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

Abstract

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