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

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

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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
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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

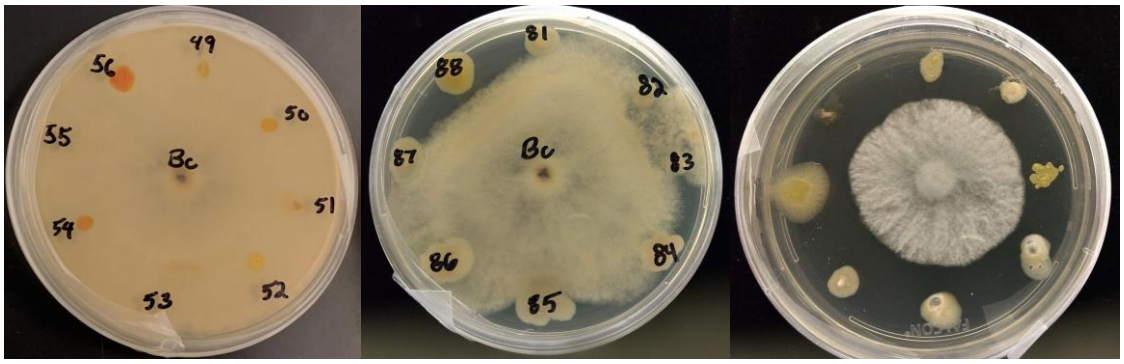

Figure 2. Inhibition of *Neofabraea perennans*. A) Example of four spotted endophyte isolates. B) Close up of disrupted hyphae growth near sample #4, with the leading edge of fungal growth becoming filiform as opposed to the smooth edge seen near sample #1.

A B

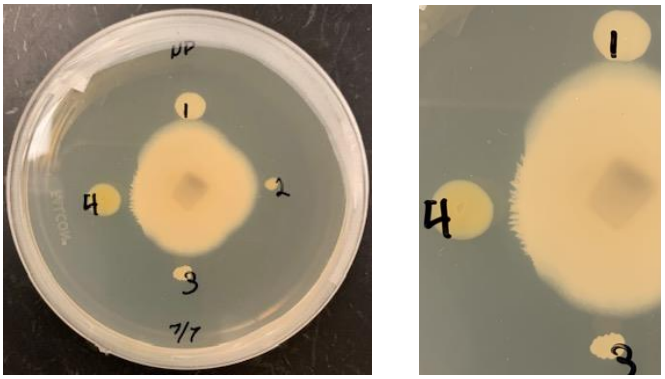

Figure 3. Inhibition of *Erwinia amylovora*. The pathogen was inhibited by several of the endophyte strains as indicated by clearing zones on the lawn of *Erwinia* growth. A. Overall screening results. B. Close up of one of the assay plates showing the strong inhibition of *Erwinia* growth.

A B

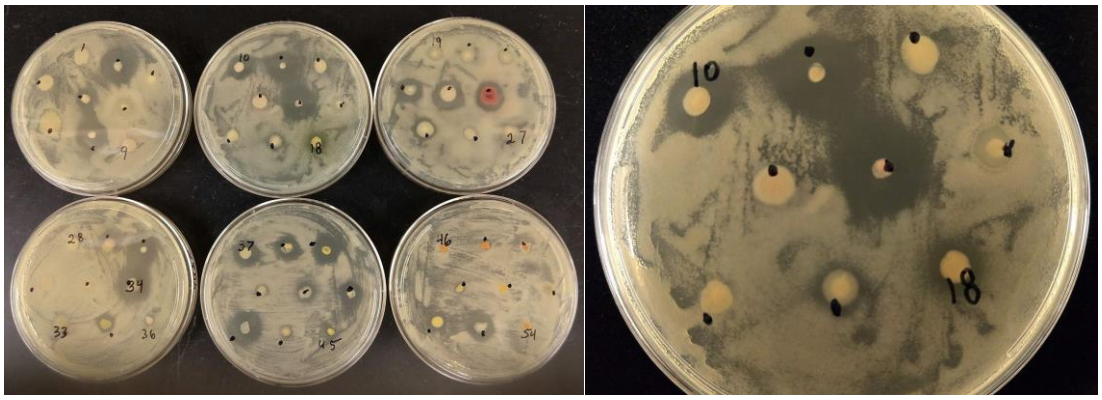

Table 1. Apple biocontrol project screening results. Endophyte strains with any activity against each of the pathogens. Pw *Phacidiopycnis washingtonensis*, Np *Neofabraea perennans*, Bc *Botrytis cinerea*, Pe *Penicillium expansum*, and Ea *Erwinia amylovora*. Strain names preceded by a number indicate 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.