PROJECT REPORT **YEAR:** 2021

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

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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.

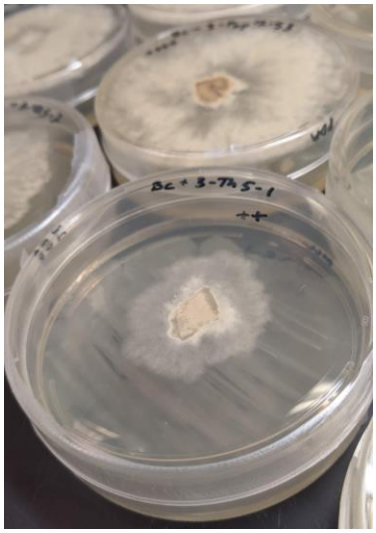
Objective 2. Biocontrol of fire blight on Red Delicious apple blossoms in Wenatchee, WA in 2021. Of the potential biological controls, **4RDLA**, performed comparably (17.0 infections per 100 clusters) to commercial organic (Blossom Protect, 17.8) and conventional (Firewall 17, 16.1-17.0) standards. 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.