Research Grant Proposal for 2022
State Horticultural Association of Pennsylvania
Due: January 14, 2022

Title Auxotrophic *Erwinia amylovora* biopesticide characterization and efficacy testing

Personnel Tim McNellis, lead researcher
Department of Plant Pathology and Environmental Microbiology
The Pennsylvania State University
Phone #: (814) 863-7646
Address: 318 Buckhout Laboratory
Department of Plant Pathology and Environmental Microbiology
The Pennsylvania State University
University Park, PA 16802

Kari Peter, co-PI
Penn State Fruit Research and Extension Center (FREC), and
Department of Plant Pathology and Environmental Microbiology
The Pennsylvania State University

Graduate student, researcher, and Ph.D. candidate in Plant Biology
Huck Institutes of the Life Sciences, and
Department of Plant Pathology and Environmental Microbiology
The Pennsylvania State University

Project Duration One year

Justification

Fire blight is an ongoing challenge for apple producers in Pennsylvania and worldwide. While antibiotics provide a good measure of protection from the disease, concerns about antibiotic resistance development and environmental impact of antibiotic sprays mean that alternative fire blight management tools are desirable. In addition, antibiotics are no longer permitted in organic fruit production. Novel, organic-compatible fire blight management alternatives would be advantageous. This proposal addresses the **alternative disease control strategies** topic that is included in the SHAP Research Committee 2022 Topical Priority List.

With previous SHAP funding, we discovered that certain auxotrophic, non-pathogenic mutants of the fire blight pathogen *Erwinia amylovora* can dramatically reduce the growth of virulent *E. amylovora* on apple flowers. Auxotrophic mutants are defined as requiring a supplement for growth that the normal bacteria do not require. Typically, auxotrophic mutants are defective in the biosynthesis of an essential amino acid or a nucleotide, and they cannot survive and grow unless the missing metabolite is available to them in their environment. Some of these auxotrophic *E. amylovora* mutants are not able to cause fire blight (non-pathogenic), presumably because they cannot obtain the missing metabolite from the host plant during infection.

We do not yet know how the auxotrophic *E. amylovora* mutants block growth of virulent *E. amylovora* on flower surfaces. The mechanism could involve use of host resources needed by the virulent bacteria for growth, physically blocking locations needed by the virulent bacteria to establish infection, activation of plant immune responses, or some combination of these factors.

However, the ability of auxotrophic mutant *E. amylovora* strains to inhibit growth of virulent *E. amylovora* on apple flowers suggested that the auxotrophic mutants might be able to inhibit fire blight disease development on blossoms.

With SHAP support, we tested this possibility at two orchard locations in 2021. We prepared formulations of promising auxotrophic *E. amylovora* strains and applied these to blooming apple trees. These auxotrophic mutants were generated by ultraviolet (UV) light treatments and are therefore not regulated as genetically modified organisms. These tests were done both at FREC by Kari Peter's group and at Washington State University (WSU) by collaborator Tianna DuPont's group. The results were very promising. At FREC, the auxotroph reduced fire blight incidence from 95% to 60%, outperforming a commercial copper product. At WSU, the auxotroph reduced fire blight incidence as effectively as the antibiotics streptomycin and oxytetracycline (Figure 1).

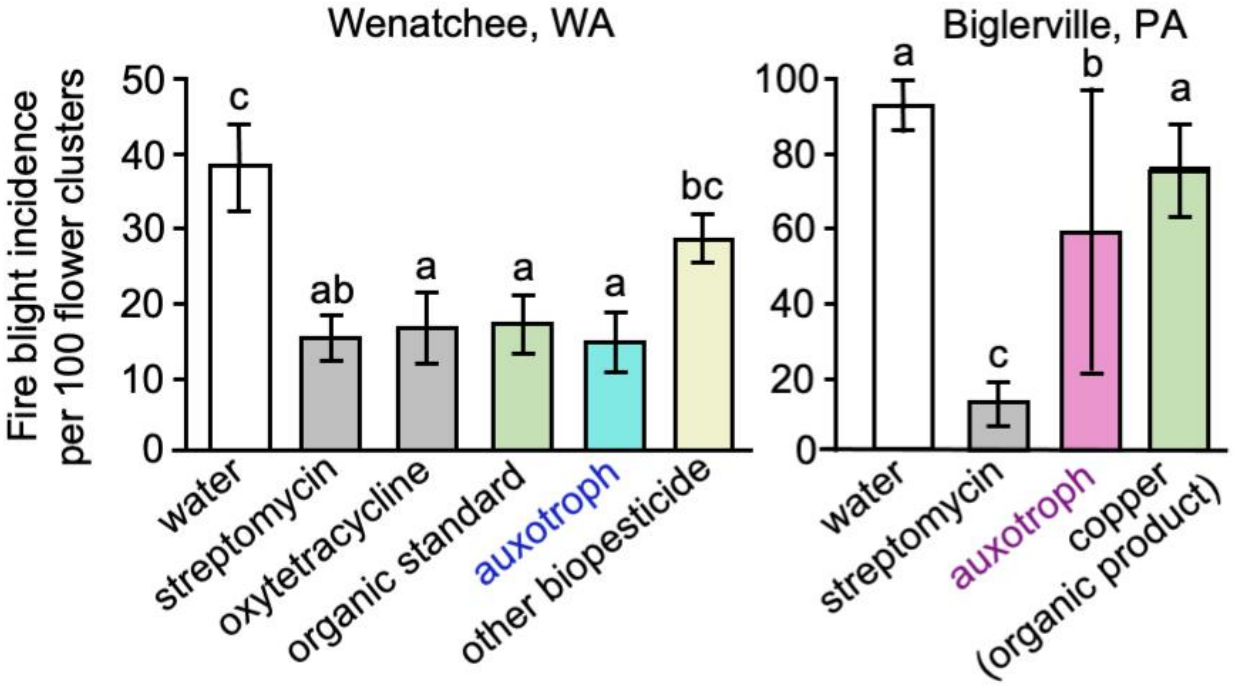

Figure 1. Non-pathogenic, auxotrophic mutant *Erwinia amylovora* strains provided significant protection from fire blight infections in blooming apple trees at two locations in spring of 2021. Bars sharing the same letter have no statistically significant difference.

The Wenatchee and Biglerville trials had several differences in methodology and conditions. The auxotrophs used at each site were derivatives of local strains of virulent *E. amylovora*. That is, the auxotrophs used in each test were different. Two applications of auxotroph were made at Wenatachee, while one auxotroph application was made at Biglerville. The auxotrophic mutant cell concentration used at Biglerville was about twice that used in Wenatchee. The auxotrophic mutant was applied in water at Wenatchee, while in Biglerville an adjuvant was included. The trees at Wenatchee were 'Red Delicious', while the trees at FREC were 'Pink Lady'. Finally, the fire blight disease pressure was much higher at Biglerville than at Wenatchee, which is typical of these two different environments. Despite these differences, both tests showed that auxotrophic mutant *E. amylovora* strains provided significant fire blight protection to blooming

apple trees and have the potential to be developed into novel fire blight biopesticide products. The current proposal aims to further develop, test, and refine this technology.

This project will further test the efficacy of auxotrophic *E. amylovora* mutant strains for fire blight disease management and put this approach into context with other available fire blight mitigation options. This project promotes interdisciplinary cooperation between labs with basic (McNellis) and applied (Peter) research emphases and facilitates translation of basic plant pathology and microbiology research into field application. We have found that auxotrophic *E. amylovora* strains do not persist for long on plant tissues, at least in the greenhouse environment, with the bacteria becoming undetectable after about two weeks. This means that use of auxotrophic mutants for fire blight management would likely have minimal environmental impact and would therefore contribute to long-term sustainability of tree fruit production. Furthermore, it is unlikely that pathogenic *E. amylovora* could evolve resistance to auxotrophic *E. amylovora* mutant treatments to reduce fire blight disease. Most likely, *E. amylovora* auxotrophic mutants counteract the growth and virulence of pathogenic *E. amylovora* through resource depletion or infection site exclusion or induction of plant general immunity, or some combination of these mechanisms. It would be unlikely that virulent *E. amylovora* could ever evolve resistance to these mechanisms. This also would contribute to long-term sustainability within the tree fruit industry and be an advantage over treatments like antibiotics and copper, where resistance development and environmental impact are ongoing considerations.

Objectives

1. Determine the mutation causing auxotrophy for five candidate *E. amylovora* biopesticide strains by genome sequencing and analysis.

2. Produce auxotrophic mutant culture at a scale sufficient for orchard testing.

3. Test the auxotrophic *E. amylovora* mutant formulation for fire blight disease inhibition at FREC experimental orchards.

Procedures

Objective 1 – A series of UV light-induced *E. amylovora* auxotrophs with various metabolic defects have already been isolated in the McNellis lab with previous SHAP funding. Unlike the genetically engineered bacteria used for the laboratory and growth chamber studies leading up to the present study, we do not know the precise nature of the auxotrophic defects in the UV-induced mutants. In the present study, we will isolate genomic DNA from five candidate auxotrophic strains with various deficiencies, including the strain used at FREC in 2021 (Figure 1). Bacteria will be grown in culture and total genomic DNA will be isolated using the Bacterial Genomic DNA Isolation Kit from Norgen Biotech Corp., or a similar kit. Auxotroph genomes will be sequenced at the Penn State Genomics Core Facility at University Park. Genomes will be assembled and the mutations leading to auxotrophy will be identified by comparison to the *E. amylovora* reference genome using routine techniques in the McNellis lab. This will provide important information about the potential mode of action of the auxotroph in fire blight disease inhibition and will allow us to put the UV-induced mutants into context with the engineered

laboratory mutants. This work will be done by the graduate student, who has prior experience with genome sequence analysis and mutation detection.

Objective 2 – The most promising UV-induced auxotrophic mutant will be grown in a 100-liter culture at the Penn State CSL Behring Fermentation Facility at University Park. Culturing can be done overnight, and the bacteria are separated within 30 minutes through a continuous-flow centrifuge. We have found that the resulting bacterial preparation (bacterial wet-cell paste) remains viable in the refrigerator for at least three months after preparation, although we would plan to use the material shortly after production. This activity will be coordinated by the PI and the graduate student. The graduate student will also prepare pre-cultures needed by the fermentation facility and process the resulting wet-cell paste product.

Objective 3 – The auxotrophic mutant will be provided by the McNellis lab to the Peter lab in the form of wet-cell paste. Given the success of the Wenatchee experiment that simply used a suspension of auxotrophic mutant bacteria in water (Figure 1), we plan to use a water suspension at FREC in 2022. In addition, two applications of auxotroph will be made instead of one. Briefly, the protocol will be as follows. Trees will be marked as plots in randomized complete block designs, 4-5 trees per treatment. Additional treatment evaluations will include streptomycin (standard), Serenade ASO, and Blossom Protect (standard organic). Test and commercial products will be applied 24-36 hours before and again 12-24 hours after inoculation with virulent *E. amylovora* at 10^6 or 10^7 cfu/ml. Auxotrophic mutants will be applied at 10^9 - 10^{10} cful/ml in water. Virulent *E. amylovora* will be applied at 80-100% open king blooms. Beginning at one week post-inoculation, trees will be visually evaluated 1-3 times per week until approximately late June for blossom blight and for shoot blight in case the treatments provide some shoot blight protection as well. Fruit will also be evaluated for skin russet in mid to late summer. Statistically significant differences will be assessed using analysis of variance (ANOVA) and multiple *t*-test comparisons.

Budget

Salaries and wages - $7,486
Support is requested for Ph.D. candidate summer stipend for 2022 in the amount of $7,486. Student will be doing the *E. amylovora* auxotrophic mutant genomic DNA isolation and genome sequence analysis to detect mutations responsible for the auxotrophic phenotype and putting this information into context with the data from engineered auxotrophs used in prior laboratory studies. Student will also coordinate with the Fermentation Facility for production of large scale auxotrophic mutant culture for orchard testing and process the resulting wet-cell paste product.

Fringe Benefits - $597
Fringe benefits are computed using the fixed rates of 35.31% applicable to Category I Salaries, 11.26% applicable to Category II Graduate Assistants, 7.98% applicable to Category III Salaries and Wages, 0.35% applicable to Category IV Student Wages, and 24.78% for Category V, Postdoctoral Scholars and Fellows, for fiscal year 2022 (July 1, 2021, through June 30, 2022). If this proposal is funded, the rates quoted above shall, at the time of funding, be subject to adjustment for any period subsequent to June 30, 2022, if superseding Government approved rates have been established. Fringe benefit rates are negotiated and approved by the Office of Naval Research, Penn State's cognizant federal agency.

Supplies – $900
Support is requested for supplies needed to complete the laboratory work, microbial culturing, and field-testing objectives. Laboratory supplies include microbiological media reagents and a bacterial genomic DNA isolation kit ($400 – McNellis program). Field testing supplies include replacement apple trees for those lost due to fire blight test infections, tree ties and flags for identification, and buffer reagents for microbial suspensions ($500 – Peter program).

Purchased Services - $3,100
Support for a 100-liter culture fermentation and separation at the Penn State Fermentation Facility is requested based on past cost invoices ($1,000 – McNellis program). Support for sequencing five auxotroph genomes at the Penn State Genomics Core Facility is also requested based on a cost estimate from the facility director ($2,100 – McNellis program).

Indirect Costs - $0
F&A rates are negotiated and approved by the Office of Naval Research, Penn State's cognizant federal agency. Penn State's current provisional on-campus rate for research is 58.19% of MTDC from July 1, 2021, through June 30, 2022. New awards and new competitive segments with an effective date of July 1, 2022, or later shall be subject to adjustment when superseding Government approved rates are established. Per 2 CFR 200 (Appendix III, Section C.7), the actual F&A rates used will be fixed at the time of the initial award for the duration of the competitive segment.
Per sponsor guidelines, indirect costs are not included in the proposal budget.

Total Budget: $12,083

Other Support – none