

This proposal aims to further develop and understand the approach of using non-pathogenic *E. amylovora* strains to compete with virulent *E. amylovora* cells in the flower environment to slow bacterial growth and potentially reduce fire blight disease incidence or development as an alternative for blossom blight management. Because of SHAP funding in 2016, 2017, and 2018, we have tested and confirmed that application of high concentrations of *E. amylovora* to apple, crabapple, and hawthorn flowers will inhibit the growth of virulent *E. amylovora* cells by two or three orders of magnitude. So far, this activity has been specific to the flower tissue environment, since we found in 2018 that this inhibitory effect does not occur in wounded immature apple fruit tissues. It is likely that the non-pathogenic *E. amylovora* strains compete for limited resources on the apple flower exterior, thereby limiting the growth of the virulent *E. amylovora* cells. However, it is also possible that the non-pathogenic *E. amylovora* cells elicit plant immunity within the flower tissues and inhibit the growth of the virulent *E. amylovora* cells indirectly. In any case, we now wish to determine whether the inhibition of growth of the virulent *E. amylovora* cells is sufficient to reduce fire blight disease incidence or severity during blossom blight. In 2018, we applied for a USDA-APHIS permit to apply the non-virulent *E. amylovora* strain as a biocontrol at a PSU FREC orchard block. However, the application was not approved, with a request to determine whether the non-pathogenic *E. amylovora* strains could cause disease in apple flowers and pear fruits. Better characterization of the non-pathogenic *E. amylovora* strain behavior on host plants will improve the likelihood of approval by the USDA, and they were supportive of re-application for a permit. The present proposal aims to provide more information about non-pathogenic *E. amylovora* strain behavior on apple flowers and pear fruits, as well as to better understand the mechanism underlying the inhibition of virulent *E. amylovora* growth by high concentrations of non-pathogenic *E. amylovora*. This information will improve chances for approval of field testing. It is also possible that high concentrations of natural isolates of non-pathogenic, epiphytic bacteria, such as *Pantoea agglomerans*, can also inhibit the multiplication of virulent *E. amylovora* cells on apple flowers. Testing for this will provide additional insight into the potential mechanism of action of inhibition of virulent *E. amylovora* growth by floral epiphytes, and if successful, may provide an alternative fire blight management approach.

Objectives:

This project builds on previous SHAP funded projects with the aim of the objectives to address the 2019 SHAP priorities under the Plant Pathology section: **Alternative Disease Control Strategies and Disease Resistant Management for Key Diseases:**

Objective #1: Evaluate applications of glycerol, Vacciplant, and Regalia on potted trees in the greenhouse to understand optimal timing for usage to achieve effective fire blight disease control.

Objective #2: Evaluate applications on different aged trees in the field of Actigard, Apogee, Cueva, Regalia, and Vacciplant, as standalone and in combination, to understand potential additive effects to manage shoot blight severity.

Objective #3: Test whether non-pathogenic *Erwinia amylovora* proposed biocontrol strains can block fire blight disease symptom development caused by normal *E. amylovora* on flowers on potted apple trees in the greenhouse.

Objective #4: Test whether non-pathogenic *Erwinia amylovora* proposed biocontrol strains can cause disease on pear fruits.

Objective #5: Test whether high concentrations of natural, epiphytic bacterial strain *Pantoea agglomerans* can block *E. amylovora* growth and fire blight disease development on apple flowers, both detached flower and flowers on trees growing in the greenhouse.

Objective #6: In the case of favorable results from Objectives 1 & 2, Dr. McNellis will reapply for a USDA-APHIS permit for field testing of non-pathogenic *E. amylovora* strains for biocontrol of fire blight blossom blight.

Procedures:

Objective #1 Procedures (Peter Program): In the greenhouse, applications of glycerol, Regalia, and Vacciplant will be tested alone at different timings to determine which application timing would be most effective at reducing shoot blight. Treatments will be applied 14 days pre-inoculation, 10 days pre-, 7 days pre-, 4 days pre-, 4 and 2 days pre-, 2 pre- and 2 days pre- and post-inoculation, and 2 days post-inoculation. Four shoots per tree will be inoculated by wounding shoot tips with a scalpel dipped in a suspension of *Erwinia amylovora*, strain Ea273, adjusted to 10^7 cfu/mL (Fig 2). Shoots will be evaluated for severity of fire blight approximately 7 and 14 days after inoculation. Severity will be assessed as a percent of blight symptoms down vs total shoot length as well as with a rating scale of 0-7, with 0 being no infection and 7 being the most severe.

Objective #2 Procedures (Peter Program): In the field, applications of Actigard, Apogee (2 oz/A), Cueva, Regalia, and Vacciplant will be tested alone or in combination to determine what would be most effective at reducing shoot blight in a field setting. Treatments will be applied 4 and 2 days pre-inoculation and shoot inoculations will be carried out with the same method as the greenhouse study. To determine effectiveness of the products on a newly established block vs. an established block, treatments will be applied in a 2018 planted Gala/M.9 block; 2015 planted Gala/M.7 block; and a 2011 Gala/M.9 block. Shoot inoculations will be carried out two to three weeks after bloom. Programs: 1) Untreated; 2) Actigard (2 oz/A); 3) Apogee (2 oz/A); Regalia; Apogee + Regalia; 4) Glycerol; 5) Glycerol + Cueva; 8) Vacciplant.

Objective #3 Procedures (McNellis Program): Third leaf ‘Fuji’ trees on M.9-Nic29 rootstocks will be potted and grown in the greenhouse at Penn State University Park. Flowers will be sprayed with 10^{10} cfu/ml of non-pathogenic *E. amylovora*, either the $\Delta argD$ mutant, which is unable to synthesize the essential amino acid arginine, or a $\Delta leuB$ mutant, which is unable to synthesize the essential amino acid leucine. After 4 hours, all flowers in an individual cluster will be inoculated with about 2,000 cells of virulent *E. amylovora* by applying a droplet of 20 μ L of 10^5 cfu/ml of the bacteria to the base of the flower hypanthium or to the stigma using a micropipette. Control flowers will include mock-inoculated flowers, flowers inoculated with virulent *E. amylovora* without pre-treatment, and untouched flowers. Flowers in one cluster will receive only one treatment type. Incidence of blossom blight within each flower cluster will be monitored for two weeks after inoculation. This will include observation and rating for degree of necrosis in the flowers. This experiment will tell us whether the non-pathogenic *E. amylovora* strains selected as potential biocontrols will produce any blossom blight disease on intact trees, which will be important information for application for field testing of this as a potential fire blight biocontrol.

Objective #4 Procedures (McNellis Program): Immature pear fruitlets will be collected from FREC trees and inoculated using standard procedures. Pear fruitlets will be inoculated with both the $\Delta argD$ and $\Delta leuB$ mutants, since preliminary data suggests that the $\Delta leuB$ mutant may be more completely compromised in virulence on pear than the $\Delta argD$ mutant. Starting bacterial concentrations will be approximately 2,000 cells per fruit. Fruits inoculated with virulent, wild-type *E. amylovora* and mock-inoculated fruits will be used for controls. Disease severity will be monitored over the course of 7 days, and disease severity will be measured using a quantitative disease rating scale that we developed for apple fruits.

Objective #5 Procedures (McNellis Program): Detached apple and crabapple flowers collected at University Park and at Rock Springs orchards nearby to University Park will be spray-coated with 10^{10}

cfu/ml concentration of a known, non-virulent, epiphytic *P. agglomerans* strain available in Dr. McNellis' lab. After 4 hours, the flowers will be inoculated with about 2,000 cells of virulent *E. amylovora* by applying a droplet of 20 μ L of 10^5 cfu/ml of the bacteria to the base of the flower hypanthium or to the stigma using a micropipette. Growth of the virulent *E. amylovora* cells will be quantitatively measured at 2 days post inoculation by grinding up the apple flowers and serial dilution plating of the extracts on LB medium plates. Controls will include flowers inoculated with virulent *E. amylovora*, but without *P. agglomerans* pre-treatment as a negative control, and flowers inoculated with non-virulent *E. amylovora* as a positive control.

Objective #6 Procedures (McNellis Program): If results are favorable from Objectives 1 & 2, that is, if the $\Delta argD$ and $\Delta leuB$ mutants do not cause significant or detectable disease in pears or apple flowers, Dr. McNellis will submit an application to the USDA-APHIS for approval of field testing of non-pathogenic *E. amylovora* strains for fire blight biocontrol. This objective does not depend on whether fire blight disease inhibition is observed in Objective #1; the only way to really know whether biocontrol is effective in the field will be to do a field test under natural infection conditions.

Peter Budget:

Wages for part-time seasonal employee (\$9.23/hr, 20 h/wk, 13 weeks):	\$2,400
Wages fringe benefits (7.81%)	\$188
Materials and supplies:	\$500
Travel	\$1,000
Total requested:	\$4,088

McNellis Budget:

Summer 2019 graduate student stipend (1/2 time, grade 12)	\$6,730
Graduate student fringe benefits (7.81%):	\$526
Laboratory supplies:	\$1,000
Total requested:	\$8,256

Total amount requested: \$12,344

Budget Justification:

Salaries/Wages - \$9,130: Funds are requested for 2019 summer support for a graduate student, who will conduct the McNellis Lab portion of the project; and a seasonal technician to assist with the Peter Lab portion of the project.

Fringe Benefits - \$714: Fringe benefits are computed using the fixed rates of 38.97% applicable to Category I Salaries, 14.74% applicable to Category II Graduate Assistants, 7.81% applicable to Category III Salaries and Wages, 0.18% applicable to Category IV Student Wages, and 25.34% for Category V, Postdoctoral Scholars and Fellows, for fiscal year 2018 (July 1, 2018, through June 30, 2019). 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, 2019, 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.

Materials and Supplies - \$1,500: Funds are requested for microbiological media, plates, laboratory disposables, and antibiotics for the flower biocontrol research. Expenses for materials were determined from catalog prices using Penn State's eBuy. These funds will also cover the costs of trees, potting mix, pots, fertilizer, and greenhouse other greenhouse related costs.

Travel - \$1,000: Funds are requested to support travel (transportation, hotel, meals) to present this research at regional and national meetings. All travel will be in accordance with University travel regulations and mileage will be charged at the current rate on the date of travel.