PennState
College of Agricultural Sciences

PROPOSAL TRANSMITTAL

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Title: Continuing the quest for fire blight management alternatives: Optimizing native bacterial antagonists and plant immune stimulation

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Proposed Project Period: **04/01/2018 - 03/31/2019** **Total Project Request:** **$15,824**

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2018 RESEARCH GRANT PROPOSAL
STATE HORTICULTURAL ASSOCIATION OF PENNSLYVANIA PROGRAM

Title: **Continuing the quest for fire blight management alternatives: Optimizing native bacterial antagonists and plant immune stimulation**

Personnel:

PI: Dr. Kari Peter, Assistant Professor or Tree Fruit Pathology, Department of Plant Pathology & Environmental Microbiology, The Pennsylvania State University Fruit Research and Extension Center, Biglerville, Pennsylvania Phone: (717) 677-6116 Ext. 223 Email: kap22@psu.edu

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Project Duration: One year (April 1, 2018 – March 31, 2019)

Amount requested: $**15,824**

Justification

Few methods exist for fire blight disease control in apple. The main available controls are removal of infected portions of trees, antibiotic sprays at bloom time, and use of less susceptible varieties and rootstocks. However, many of the newer, highly-popular apple varieties, including 'Gala,' 'Ginger Gold,' and 'Fuji', are very susceptible to fire blight. These susceptible varieties can have the effect of increasing fire blight disease problems when they are used extensively. Also, antibiotic use in agriculture is coming under increasing scrutiny out of concerns about the development of antibiotic-resistance among bacteria. Another complicating factor influencing fire blight susceptibility is the increasing adoption of the tall spindle system, which involves planting a high density of trees on dwarfing rootstocks spaced closely together while supported with a trellis. This production system requires a different training and pruning system compared to free standing trees grown on semi-dwarf rootstocks. For pruning, it is recommended to remove larger branches while leaving a small stub to ensure the development of a replacement branch. Termed "renewal pruning," this style encourages new growth from the stub that is left. Unfortunately, this new growth is highly susceptible to fire blight and, once infected, the bacteria can quickly move into the central leader of the tree due to proximity and the young age of the growth.

Fire blight, caused by *Erwinia amylovora*, is a continuing problem faced by apple growers, so the development of novel control measures could be very useful. This project builds on previous SHAP-supported research and is in line with the 2018 SHAP Plant Pathology research priorities: Alternative Disease Control Strategies and Disease Resistant Management for Key Diseases **by aiming to develop novel strategies to control fire blight disease in apple trees using bacterial antagonists and plant immune stimulation**, with the objective of providing growers with novel options for disease control.

Plant immune stimulation

Plants have a well-developed, innate immune system that makes most plant species resistant to most potential pathogens. One strategy for safe and effective pest and disease control is through controlled activation of the plant immune system by foliar sprays containing plant defense activating compounds. The most recognized plant immune system inducing chemical is Actigard (acibenzolar-S-methyl), which has been available for use for several years on vegetables and became available for use on apples in 2015. However, Actigard is an expensive product making multiple applications during one season cost prohibitive. Over the last several years, more products (LifeGard, Regalia, Vacciplant) have come onto the market labeled to improve plant health by preventing diseases. As a result of 2016 and

2017 SHAP support, we evaluated several of these commercial products, as well as glycerol (which stimulates the immune system), both in the greenhouse (2016 and 2017) and in the field (2017) for their ability to limit the fire blight severity, specifically shoot/trauma blight, which is often unpredictable and difficult to control, especially on very young trees. The most interesting trend we observed for these products was an influence of tree age: the younger the tree, the more the treatment limited disease severity. Actigard mitigated shoot blight by affording 79% control in the greenhouse; in the field on 10 year old dwarf Gala trees, we observed 45% control. For Regalia, we observed approximately 21% control in the greenhouse, whereas only 1% control was observed in the field. For LifeGard, we observed 13% control in the greenhouse, whereas 0% in the field. However, the opposite was true for Vacciplant (8% control in the greenhouse vs. 28% in the field) and glycerol (6.4% in the greenhouse vs. 15% in the field). This was our first year evaluating these products under field conditions to limit shoot/trauma blight and another year is necessary. An important unknown with these products is the duration of the immune system signal for limiting disease and how many applications are necessary while the tree has susceptible tissue (green, succulent shoots). **We are proposing to evaluate the above mentioned products for their duration of protection against disease on potted nursery trees in the greenhouse, as well as repeating the evaluations in the field, while looking more closely at the influence of tree age (newly planted vs. established trees)**.

During 2017, we also evaluated copper and bacterial-based products on potted nursery trees in the greenhouse as part of a small efficacy trial for limiting fire blight severity. The products we tested included: Cueva, Kocide 3000, Serenade ASO, and Stargus (a new Marrone BioInnovations product, not-yet released). We have observed favorable results using Cueva in the field on older semi-dwarf trees in limiting shoot blight incidence; however, we have not measured severity. In the greenhouse, we treat trees first then follow with shoot-tip inoculations two days later. Similar to the immune system inducer experiments, we measure severity by how much necrosis occurs after shoot tips are inoculated, such that the less shoot necrosis means more control of the disease. Surprisingly, Cueva provided 29% control (compared to 79% with Actigard), whereas Kocide, Serenade ASO, and Stargus provided 13%, 9%, and 14% control, respectively. We did not anticipate copper having the ability to limit disease severity since is typically used as a protectant; however, the formulation of Cueva is unique compared to other copper products. **Consequently, we are eager to re-evaluate these products in the greenhouse and in the field (as described above), thereby providing growers with additional tools for young trees.**

Bacterial antagonists

As a result of 2016 and 2017 SHAP support, it was discovered and confirmed that an avirulent mutant of the fire blight pathogen *Erwinia amylovora (*Δ*argD*), which is unable to synthesize the essential amino acid arginine, effectively blocked the growth of virulent *E. amylovora* on apple flowers. We pre-treated detached 'Gala' apple flowers with suspensions of the Δ*argD E. amylovora* strain and then inoculated with 100,000 virulent *E. amylovora* bacteria. The growth of the virulent *E. amylovora* in flowers pre-treated with Δ*argD* was 2-3 orders of magnitude lower than in control flowers that had not received Δ*argD* pre-treatment. These tests were repeated in 2017 and confirmed the effect. We hypothesize that this blockage of growth of the virulent *E. amylovora* cells is due to the Δ*argD* occupying spaces on the apple flowers and consuming resources on the plant surfaces that the virulent *E. amylovora* requires to survive and grow. This substantial blocking of virulent *E. amylovora* growth by Δ*argD* on apple flowers could be useful for prevention or amelioration of blossom blight under orchard conditions in Pennsylvania. **We propose to test effectiveness of the avirulent Δ*argD E. amylovora* strain for fire blight disease reduction in Pennsylvania orchard conditions during the 2018 season.** (We have been in contact with USDA APHIS and are in the process of obtaining a USDA APHIS permit to perform this work in a PSU FREC research apple block.)

With SHAP support in 2016, several isolates of *Bacillus subtilis* were obtained from peach flower samples that effectively block the growth of *E. amylovora* on culture plates. Several hundred bacterial isolates from peach, cherry, and apple flower samples were tested for ability to block *E. amylovora* growth *in vitro*, several were identified with at least some ability to block *E. amylovora* growth, and the

B. subtilis isolates from peach flowers were the most effective of these. Based on zones of inhibition on microbiological plates, our *B. subtilis* strain is substantially and significantly more inhibitory to *E. amylovora* growth than the *B. subtilis* strain from the Seranade® product. While antibiosis results in culture do not guarantee that a microbe will be an effective biopesticide, the results are promising and the *B. subtilis* strain should be tested for ability to block blossom blight disease development.

With additional SHAP funding in 2017, we tested the ability of this antagonistic *B. subtilis* strain to block fire blight infection in the field. 20 liters of the *B. subtilis* strain were grown in culture to high density, spun down from the culture, and sprayed onto open apple blossoms at the FREC orchards using standard equipment. *B. subtiltis* concentrations were very high, in excess of 10^{10} cells/ml in the spray application. Flowers were then challenged a few hours later with 10^7 cells/ml of virulent *E. amylovora* several hours after application. Blossom blight incidence was recorded approximately two weeks post-inoculation. Unfortunately, no statistically significant reduction in fire blight blossom blight incidence was detected in trees pre-treated with antagonistic *B. subtilis* compared to controls not receiving *B. subtilis* pre-treatment. There could be several reasons for this result, including that the antagonistic activity of the *B. subtilis* strain is due to secreted antibiotics or quorum-sensing inhibitory compounds, which would have been diluted and mostly lost during our preparation procedure, and the *B. subtilis* sprayed onto the flowers might not have had sufficient time to regenerate these compounds prior to *E. amylovora* inoculation for the antibiosis effects to manifest fire blight disease reduction. **Therefore, this year, we propose to determine whether the antibiosis activity of our *B. subtilis* strain is present in liquid culture supernatants and whether this material can be extracted, concentrated, and retain *E. amylovora* inhibitory activity.** This would bring us closer to being able to apply the material to flowers for effective fire blight management.

Our objectives for this proposed research are as follows:

Objective #1: Evaluate the duration of the induced immune response for controlling fire blight after applications of Actigard, LifeGard, Regalia, and Vacciplant.

Objective #2: Evaluate copper- and bacterial-based products on potted greenhouse trees in limiting fire blight severity.

Objective #3: Evaluate the most promising products from Objectives 1 and 2 for limiting shoot/trauma blight in the field on newly planted and established apple trees.

Objective #4: Test whether non-virulent *Erwinia amylovora* Δ*argD* strain application to open apple flowers in field tests inhibits natural and inoculated fire blight floral infections and shoot trauma blight.

Objective #5: Test antagonistic *B. subtilis* culture supernatants and culture supernatant extracts for *E. amylovora* inhibition in culture.

Objective #1 Procedures (Peter Program): Actigard, LifeGard, Regalia, and Vacciplant will be evaluated in separate experiments on potted nursery trees in the greenhouse. We want to determine the number of foliar applications necessary for a response (1 vs. 2 applications) and the amount of time the induced response lasts in limiting fire blight. For each, we will evaluate the following: 1) 2 applications (4 days and 2 days) vs. 1 application (2 days) before inoculation; and 2) using either 1 or 2 applications, we will inoculate 2, 5, 10, and 15 days post-treatment to evaluate the length of the induced immune response and its control for fire blight severity. Each treatment will have five replicates and four shoots per tree will be inoculated where possible. Shoot tips will be inoculated by dipping scissors in a suspension containing 10^8 cfu/ml of the *Erwinia amylovora* strain Ea581a. Fire blight will be evaluated based on how fast and how far symptoms (necrosis) progress down the shoot. Shoot blight evaluations will be made after approximately ten days.

Objective #2 Procedures (Peter Program): Copper (Cueva, Kocide, and Previsto) and bacterial-based (Serenade ASO, Double Nickel, and Stargus) products will be evaluated for their efficacy of limiting fire blight severity on potted nursery trees in the greenhouse. These results will contribute aid in determining which products to further evaluate for our field evaluations in Objective #3. Two foliar applications will be made prior to inoculations and trees will be inoculated 48 hours after the last application. Inoculations and evaluations will be as described in Objective #1.

Objective #3 Procedures (Peter Program): The products that show the most promising results from Objectives 1 and 2 will be further evaluated in the field. Since our research to date has shown tree age may influence how well these products perform in the field, we will evaluate the products in two separate fire blight susceptible apple blocks based on tree age: a newly planted variety on M.9 (planted April-early May) and a 7 yr old Crimson Crisp/M.9 337. The older trees will be evaluated early in the season (approximately May), whereas the newly planted trees will be evaluated later in the season (August) when there is sufficient growth. Treatments will be arranged in a randomized complete block with five replicates/treatment and four shoots per tree will be inoculate, where possible, as previously described. Products will be applied using a backpack mistblower and trees will be inoculated 48 hours after treatment. Taking environmental conditions into consideration, we will use less inoculum in the field compared to our greenhouse tests to not overwhelm the trees. Fire blight incidence and severity will be evaluated approximately 1 week post inoculation.

Objective #4 Procedures (McNellis Program): The avirulent Δ*argD E. amylovora* mutant strain will be grown in culture to high cell densities and centrifuged down to obtain the cells. Cells will be resuspended to a concentration of 10^{10} cells/ml in dilute, mild detergent (Tween 80) as a wetting agent. Blooming apple trees at the FREC orchard will be sprayed with the Δ*argD E. amylovora* mutant strain, and control trees will be sprayed with detergent only, or left untreated. Approximately 24 hrs later, blossoms will be inoculated with a bacterial suspension of 10^7 *E. amylovora* cells/ml using a spray bottle. All applications will be performed using standard biopesticide application equipment and procedures. In addition, we will have two additional treatments, which will include another control and trees treated with the Δ*argD E. amylovora* mutant strain and will not include *E. amylovora* blossom inoculation. For these treatments, we will rely on natural fire blight infections. Fire blight blossom blight development will be monitored and compared to control trees. The Δ*argD E. amylovora* mutant strain will also be prepared for inclusion in the trauma blight experiments described in Objective #2 Procedures above.

Objective #5 Procedures (McNellis Program): The *B. subtilis* isolate will be grown in culture in the laboratory, and cell-free supernatants will be obtained by sequential centrifugation followed by filtration through a 0.2 micron filter. These supernatants will be tested in culture for *E. amylovora* growth inhibition, on plates, using the zone of inhibition assay that is standard in Dr. McNellis' lab. Several growth media will be tested to determine in which medium the *B. subtilis* produces the most inhibitory activity, including LB, King's B and minimal M9 medium. In addition, cell-free culture supernatants will be extracted with ethyl acetate to determine if the inhibitory substance(s) can be extracted from the culture supernatants, using standard procedures.

Peter Budget:

Seasonal assistant (50% time)	$2,200
Wages fringe benefits (7.9%)	$174
Trees and soil:	$2,500
FREC Greenhouse fees:	$400
Travel	$2,000
Total requested:	**$7,274**

McNellis Budget:

Summer 2018 graduate student stipend (Sara Klee, Ph.D. Candidate – PPEM):	$6,534
Graduate student fringe benefits (7.9%):	$516
Laboratory supplies:	$1,500
Total requested:	**$8,550**

Total amount requested: $15,824

Budget Justification:
Salaries/Wages - $8,734
Funds are requested for summer support ($6,534) for one graduate student in the Department of Plant Pathology and Environmental Microbiology in Dr. McNellis' program (Ms. Sara Klee), who will be contributing 50% of her time on this project from May 12 – August 15, 2018. She will prepare and test flower biocontrol inoculum suspensions and analyze field samples, as well as test and optimize the new bacillus biocontrol strain and analyze its antagonist production. A seasonal wage employee will be hired for the summer season to work in Dr. Peter' at the PSU Fruit Research and Extension Center and will contribute 50% of their time during their 12 week summer employment ($9.25hr/20 h/wk for 12 weeks) assisting with the greenhouse and field experiments. Both the summer graduate support and wage employee are considered Category III Salaries and Wages.

Fringe Benefits - $690
Fringe benefits are computed using the fixed rates of 41.6% applicable to Category I Salaries, 15.40% applicable to Category II Graduate Assistants, 7.90% applicable to Category III Salaries and Wages, 0.10% applicable to Category IV Student Wages, and 26.30% for Category V, Postdoctoral Scholars and Fellows, for fiscal year 2018 (July 1, 2017, through June 30, 2018). 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, 2018, 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 - $4,000
Funds ($1500) are requested for Dr. McNellis' program to purchase 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. Funds ($2000) are requested for Dr. Peter's program to purchase apple trees (70) that will be used in experiments the greenhouse and trees (150) that will be planted in the field to field test the best treatments observed in the greenhouse on newly planted trees. Funds ($500) are also requested for purchase of potting soil needed for planting of greenhouse grown trees.

Travel - $2,000
Funds are requested to support Mr. Brian Lehman, to attend the Annual American Phytopathological Society meeting in Boston, MA July 29 – August 3, 2018. He will be presenting a poster about the greenhouse and field fire blight research he has conducted from 2017 – 2018 resulting from SHAP support. Funds will be used to support transportation, hotel, and meal costs for the trip.

Other costs - $400
Funds are requested to support the costs of using greenhouse space at the Fruit Research and Extension Center for 6 months ($200/quarter).