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College of Agricultural Sciences

PROPOSAL TRANSMITTAL

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Title: Understanding why biocontrol fails to protect against fire blight in the Eastern US

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2018 Research Grant Proposal
State Horticultural Society of Pennsylvania

Title:
Understanding why biocontrol fails to protect against fire blight in the Eastern US

Personnel:
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Duration of Project:
One year

Justification:
Biological control agents (BCAs), that is microorganisms that can antagonize plant pests, have been researched and developed to control important diseases of fruit trees for several decades. Despite this long history of research and development, most BCAs do not provide the same level disease control or reliability that is provided by conventional chemical controls, such as antibiotics. The problem of poor BCA performance is particularly acute in the Eastern US, where commercially available products provide less protection than they do in the Western growing regions[1]. Although significant efforts have focused on isolating and testing new and different microorganisms with the hope of finding an ideal biocontrol strain, that is one that will provide consistent and significant disease protection, there has been far less effort invested in the equally important question: why do biocontrols fail? Without answering this fundamental question, significant progress in developing effective alternative methods for controlling fruit tree diseases will be slow to progress, if at all. The general lack of biocontrol effectiveness in controlling fruit tree pathogens presents a barrier to adoption of organic production practices, as it forces growers to rely on antibiotics that are incompatible with current organic standards. If biological controls could be more effectively integrated into production practices, this would lower the barrier for growers to adopt organic practices, which are generally viewed favorably by the public and yields fruit that demands a higher market premium.

There are several reasons why biocontrol may fail. The BCA may not adequately colonize or persist long enough to protect against the pathogen[2]. The BCA may colonize and persist, but not express traits necessary to antagonize the pathogen[2]. The BCA may colonize, persist and express antagonizing traits, but the pathogen none-the-less is able to proliferate to levels sufficient to cause disease [2]. Despite our general understanding of the potential reasons that a BCA may fail, rarely is there an attempt to understand the underlying causes of control failure. To address this gap in understanding, we propose to answer the following questions in field experiments: is poor BCA colonization a significant contributor to poor disease control? If so, then is this poor colonization related to being excluded by the natural microbial community?

Pseudomonas fluorescens A506 (*Pf* A506), the active agent in the commercial preparation BlightBan® A506 (sold by Nufarm Ltd.), is an ideal BCA with which to investigate these questions. *Pf* A506 has been one tool used to control fire blight of pear and apple trees in the Western US (Washington, Oregon, California) for several decades, though it has had far less success in controlling this disease in the East (Michigan, Pennsylvania, New York). *Pf* A506 is thought to control the fire blight pathogen, *Erwinia amylovora*, through multiple activities, including direct competition for nutrients and space on stigmatic surfaces[3], as well as the production of an iron-inducible antibiotic that is toxic toward *E. amylovora*[4]. Field trials performed in Oregon indicate that *Pf* A506 can reduce fire blight disease incidence by ~35% (~20-40% interquartile range of disease incidence reduction). Inclusion of an iron amendment to the *Pf* A506 application, or application of this strain along with another registered BCA (*Panteoa agglomerans* C9-1) can improve performance to ~50%. Of particular note is the importance of the relative timing between *Pf* A506 and pathogen inoculation for control effectiveness. If *Pf* A506 and the pathogen are co-inoculated, then there is little effect on the pathogen population (and potentially a positive effect on the pathogen population[3]). If, however, *Pf* A506 is applied first and allowed 72 hours to proliferate prior to pathogen inoculation, then there is a significant reduction in the pathogen population, which would presumably result in reduced disease incidence. Unfortunately, however, often in tests only the pathogen population or disease outcome are monitored, not both. Rarely is the *Pf* A506 population monitored. Additionally, the bulk of research on *Pf* A506 has been performed in geographic regions where this organism is relatively effective. Little is known regarding why this organism is less effective in the Eastern half of the US. The result of previous study designs and geographically limited testing is a fragmented and poor understanding of what makes *Pf* A506 successful in certain instances and unsuccessful in others.

Research directed at understanding the underlying cause of poor fire blight control by *Pf* A506 in Pennsylvania will potentially provide multiple benefits to growers. First, by working with this organism, which is already an EPA registered product, any insight gained that improves its efficacy can immediately be leveraged by Pennsylvania growers to improve fire blight control. Second, by working with a well-studied organism[1,2], where our results can be compared to a wealth of previous research, we aim to gain key insights into how the activity of biocontrols may generally differ between the Western warmer, drier climates and the cooler, wetter climates of the East. By making these comparisons, we may be able to develop a better understanding of how to better accommodate preexisting BCAs for this region or develop new BCAs specifically adapted for this region. For example, if we find that the natural microbial community excludes *Pf* A506, then future research efforts could be directed toward developing additional microbial strains that can exclude the inhibitory natural microbes (acting like 'blocking linemen') to promote *Pf* A506 colonization. This research will also complement current research conducted at Michigan State University, where researchers are asking similar questions regarding why BCAs do not work well to control fire blight in Michigan orchards[5]. Our approach is different and compliments theirs, however, in that they have examined how an initial application of broad spectrum biocide might assist the subsequent colonization a BCA.

This research will address the critical need to understand whether BCA failure is related to poor colonization or if it is related to ineffective inhibition of the pathogen despite adequate colonization.

Objectives:

Our objectives support the 2018 SHAP Research Priority "Alternative Disease Control Strategies" list under Plant Pathology. The proposed research will seek to understand whether poor colonization, resulting from poor competitive ability with the native microflora, by *Pf* A506 is the cause of inefficient control of fire blight, or whether *Pf* A506 can colonize and persist well, but is none-the-less unable to inhibit *E. amylovora* populations.

Objective 1: Assess whether the population of *Pf* A506 negatively correlates with the population of *E. amylovora* or with the incidence of shoot blight.

If there is a negative correlation between *Pf* A506 colonization and either disease or *E. amylovora* colonization, then this would indicate that increasing Pf A506 colonization will likely improve fire blight control.

Objective 2: Assess whether the natural bacteria present in apple blossoms express competitive traits toward *Pf* A506, and whether the abundance competitive bacteria negatively correlates with the ability of *Pf* A506 to colonize a given blossom.

If a significant proportion of the natural microbial community is able to suppress *Pf* A506, this would indicate that disruption of the native microbial community may increase colonization ability of Pf A506 and thereby increase control efficiency.

Procedures:

Field trial (application)

All applications will be performed at the Penn State Fruit Research Extension Center (FREC) in Biglerville, by Kari Peter and a student assistant.

We will use a plant pathology research block of 10 yr old Gala on B.9 rootstock and treatments will be arranged in a randomized complete block design with 4 replicates per treatment. The following treatments will be compared: 1) *Pf* A506 only, 2) *Pf* A506 followed by *E. amylovora* (pathogen), 3) water followed by pathogen (control), and 4) water only (control for background levels of fire blight disease). *Pf* A506 will be applied 3x during bloom time (as a concentrate according to the manufacturer's specifications, 150 g BlightBan® A506 per 50 gallons of water sprayed until dripping). Applications will occur at 20% bloom, the second at 70% bloom and the final application at full bloom. The pathogen will be spray inoculated at ~10^5 CFU/ml until dripping 24-48 hours after the final *Pf* A506 application. We will aim to inoculate at least 25 blossom clusters per replicate.

Field trial (sampling)

Initial sampling and plating will be performed at FREC by Kari Peter and a student assistant, after which the plates will be passed to Kevin Hockett who will incubate and evaluate the plates, with the assistance of a student, in his laboratory in 315 Buckhout on the University Park campus.

Immediately following the third application of *Pf* A506, five flowers per replicate tree (20 flowers total per treatment) will be sampled to assess the initial *Pf* A506 population. As we do not anticipate any *Pf* A506 colonization of flowers from treatments 3 and 4 (controls), we will only sample two flowers per tree (8 flowers total per treatment) for these treatments to

confirm lack of colonization. Immediately following application of the pathogen, five flowers per replicate tree will be sampled to assess its initial population, as well as the populations of *Pf* A506. As we do not anticipate any *Pf* A506 or pathogen colonization of treatment 4, only two flowers per tree will be sampled for this treatment. All treatments will be sampled again 48-72 hours following pathogen inoculation (five flowers per replicate tree).

A final sampling will be performed at the onset of symptoms. Depending on environmental conditions, the time to observe symptoms may vary from 5 – 21 days. Consequently, blossoms will be monitored closely starting approximately 5 days post inoculation. For this sampling, five blossoms exhibiting blight and five non-blight blossoms that were inoculated with the pathogen will be recovered from each replicate tree for treatment 2. Additionally, five blossoms exhibiting blight and up to five non-blight blossoms (if any are observed) from treatment 3 (pathogen only inoculation) will be sampled for the pathogen.

For all samplings, pistil and nectary bacterial populations will be enumerated by cutting away the sepals and anthers and other excess tissues from each flower. Trimmed flowers will be placed into 2 ml of 10 mM potassium phosphate buffer (pH 7.1) and sonicated for 5 minutes to dislodge cells adhering to the plant surfaces. Following sonication, each sample will be serially diluted (1/10) in buffer to a final dilution of 10^{-6}. From each dilution, 10 µl will be spread onto sectored plates selective either for *Pf* A506, the pathogen, or non-selective to recover the broader bacterial community. *Pf* A506 will be selected for on 1/3 strength *Pseudomonas* agar F (Difco Laboratories) amended with rifampicin at 50 µg/ml. The pathogen will be selected on CCT medium[6] with streptomycin amended at 100 µg/ml. The non-selective for the broader bacterial community will be 1/3 strength *Pseudomonas* agar F without rifampicin amendment. Cyclohexamide will be amended to all media at 50 µg/ml to inhibit fungal growth. Plates selective for *Pf* A506 will be incubated at 28°C for 48-72 hours prior to enumeration. Plates selective for *E. amylovora* will be incubated at 37°C for 48-72 hours prior to enumeration. Non-selective plates will be incubated at room temperature for up to five days prior to enumeration and sampling.

Field trial (disease assessment)

The overall effectiveness of the *Pf* A506 treatment compared to the water control (treatment 2 vs. treatment 3) will be assessed using a previously described approach[6]. The number of diseased and non-diseased blossom clusters per tree will be assessed to determine the disease incidence for each treatment. The relative disease incidence will be calculated by dividing the disease incidence of treatment 2 by the disease incidence of treatment 3 within each block. To assess the significance of the difference between the two treatments, Fisher's protected LSD test at $P = 0.05$ will be used to separate the means of arcsine square root-transformed relative disease incidence data.

Field trial (correlation between Pf *A506 and pathogen populations)*

The correlation between the enumerated *Pf* A506 and pathogen populations will be compared using an ordinary least-squares regression with the *Pf* A506 population as the independent variable and the pathogen population as the dependent variable. Statistical significance will be assessed at a value of $P = 0.05$.

Field trial (effect of disease on Pf *A506 population)*

To assess whether poor colonization or persistence of *Pf* A506 contributes to disease outcome, the *Pf* A506 population will be enumerated from both diseased and non-diseased buds. A standard paired t-test will be performed between the mean populations of diseased and non-diseased buds with significance assessed at a value of $P = 0.05$. Population distributions will be tested for normality, and if found to be non-normally distributed, either data transformation or a non-parametric t-test variant (such as the Wilcoxon signed-rank test) will be employed.

Assessment of bacterial competitors

Bacterial community members recovered on non-selective *Pseudomonas* agar F from treatments 1-3 will be tested for their ability to antagonize *Pf* A506 in culture. Twenty to thirty random colonies recovered from each blossom will be tested for the ability to antagonize *Pf* A506 growth in a soft-agar overlay similar to previously described methods[7]. Each colony will be tested to confirm it is not rifampicin or streptomycin resistant (and thus is neither *Pf* A506 nor the pathogen). Colonies will be spotted directly onto a lawn of *Pf* A506 and allowed to incubate for 24-48 hours. Zones of visibly reduced growth surrounding colonies will be considered indicative of antagonism toward *Pf* A506.

Correlation between number of antagonizing colonies and Pf A506 blossom populations

To assess whether a higher proportion of antagonizing bacteria correlates with poor colonization or persistence of *Pf* A506 on apple blossoms, the two variables will be compared by an ordinary least-squares regression. In this analysis, the proportion of antagonizing bacteria will serve as the independent variable and the *Pf* A506 population as the dependent variable. Statistical significance will be assessed at a value of $P = 0.05$.

Budget:

Funds are requested for the following categories:

Hourly wages: $12,800 for two student assistants to assist with field and laboratory aspects at FREC and the University Park campus.

Fringe benefits: $512 (6,400 x 7.9%; 6,400 x 0.1%) fringe for the two student employees.

Materials and supplies: $3,250 for media and buffer components, antibiotics, pipette tips, plates and costs associated with field application of both *Pf* A506 and the pathogen.

No funds are sought for salaries, travel, or miscellaneous.

Total: $16,562

Amount requested for Hockett Lab: $7,734

Amount requested for Peter Lab: $8,828

Other support:

Start-up funds to the Hockett laboratory will be used to cover all unexpected or unaccounted costs associated with this research.

References:

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2. Johnson, K. B. Pathogen Refuge: A Key to Understanding Biological Control. *Annual Review of Phytopathology* **48** (1), 141–160, doi:10.1146/annurev.phyto.112408.132643 (2010).
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4. Temple, T. N., Stockwell, V. O., Loper, J. E. & Johnson, K. B. Bioavailability of Iron to *Pseudomonas fluorescens* Strain A506 on Flowers of Pear and Apple. *Phytopathology* **94** (12), 1286–1294, doi:10.1094/PHYTO.2004.94.12.1286 (2004).
5. Herrick, C. Tackling the Challenges of Organic Apple Production Head-on. *www.growingproduce.com/fruits/apples-pears/tackling-the-challenges-of-organic-apple-production-head-on*
6. Stockwell, V. O., Johnson, K. B., Sugar, D. & Loper, J. E. Mechanistically Compatible Mixtures of Bacterial Antagonists Improve Biological Control of Fire Blight of Pear. *Phytopathology* **101** (1), 113–123, doi:10.1094/PHYTO-03-10-0098 (2011).
7. Humphrey, P. T., Nguyen, T. T., Villalobos, M. M. & Whiteman, N. K. Diversity and abundance of phyllosphere bacteria are linked to insect herbivory. *Molecular Ecology* **23** (6), 1497–1515, doi:10.1111/mec.12657 (2014).