Effects of *Pseudomonas fluorescens*-produced antibiotic 2,4-Diacetylphloroglucinol on Chestnut Blight fungus *Cryphonectria parasitica*

By
Emilie Bell
April 2017



Allegheny College

Department of Biology
and
Department of Environmental Science

Effects of *Pseudomonas fluorescens*-produced antibiotic 2,4-Diacetylphloroglucinol on Chestnut Blight fungus *Cryphonectria parasitica*

By
Emilie Bell
April 2017

A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

_____

Pledge

Approval by the Senior Thesis Committee:

_____

Dr. Catharina Coenen, First Reader

_____

Casey Bradshaw-Wilson, Second Reader

Table of Contents

Abstract……1

Introduction……..2

Methods……9

Results……14

Discussion……..19

Works Cited……22

Abstract

Before the 1900s, the American chestnut tree, *Castanea dentata*, was a major component in the United States' eastern deciduous and hardwood forests. It was a keystone species that many species of animals as well as humans depended on. When the chestnut blight, a fungus called *Cryphonectria parasitica*, was introduced from China, virtually all American chestnut trees were wiped out. Roots continue to survive in the soil and send up sprouts, but these sprouts do not survive to maturity. Attempts to bring back the chestnut tree have been largely unsuccesful. The purpose of this study was to determine if the root-associated bacteria *Pseudomonas fluorescens* and the antibiotic it produces, 2,4-diacetylphloroglucinol (DAPG), could be used as an effective treatment against *C. parasitica*. Chestnut trees were treated with a *P. fluorescens* inoculum, which was applied directly to cankers. Change in canker size was measured before and after treatment. Antibiosis experiments were performed with *P. fluorescens* and the *P. fluorescens* mutant lacking the *phlD* gene and plugs of *C. parasitica* in order to see if zones of inhibition were produced. Well diffusion assays were performed with DAPG and *C. parasitica* to also observe any zones of inhibitions. All treatments were found to be ineffective. Although this method was unsuccessful in treating chestnut blight, it is still important to find a way to restore the American chestnut tree.

Introduction

More than a century ago, the American chestnut tree, *Castanea dentata*, was a major component in the United States' eastern deciduous and hardwood forests (Anagnostakis 1987; Powell 2014). Prior to the early 1900s, 25% of hardwood trees in the eastern deciduous forest of the U.S. were American chestnut trees (Powell 2014). It was a keystone species that supported human populations as well as many different species of animals. The trees had a stable and high nut crop and the large nutritious chestnuts supported many animals such as squirrels, Carolina parakeets, passenger pigeons, raccoons, deer, bear, turkeys, blue jays, as well as domestic livestock. People also depended on the chestnuts; they harvested, sold, and traded them (Anagnostakis 1987; Powell 2014). They roasted and candied them, made them into flour for soups and breads, and made gluten free beer out of them. Oak trees replaced the chestnut tree populations but the Oak trees' nut crop isn't as high or stable as the chestnuts'. The chestnut trees generated a larger quantity of nuts partly because they could flower even after frosts. Acorns are only half as nutritious as chestnuts, thus, wildlife populations declined and became less diverse (Powell 2014). Additionally, the wood was sturdy, straight-grained, fast growing, and highly rot resistant due to tannins in the bark and the wood (Powell 2014). It was used in construction, furniture, telegraph poles, and musical instruments. The tannins that were extracted made huge contributions to the leather tanning industry (Anagnostakis 1987). Lacking this sort of wood today, lumber is treated with chemicals that often have heavy metals and preservatives that can harm the environment and human health when they leach into soil and food. Additionally, the chestnuts could be used for ethanol fuel (Powell 2014).

Unfortunately, when *Castanea mollissima*, Chinese chestnut trees, and their seeds were brought over from China, they brought a pathogen, *Cryphonectria parasitica*, commonly known

as the chestnut blight (Powell 2014). The blight was first found in the US in early 1900. In the New York Zoological Garden, American chestnut trees were reported to be dying in 1905 (Anagnostakis 1987). By 1950, the chestnut blight had spread throughout the American chestnut's entire natural range. Virtually all of the chestnut trees were wiped out (approximately three to five billion trees killed) hurting humans and animals alike. Although the roots of these trees continue to survive and spend up sprouts, these sprouts also get infected after a few years and cannot reach sexual maturity and thus cannot flower and produce seeds (Powell 2014).

Cryphonectria parasitica, the cause of the chestnut blight, is a haploid ascomycete fungus, meaning it is a sac fungus that produces spores. *Cryphonectria parasitica* destroyed American as well as European chestnut tree, *Castanea sativa*, populations. *Cryphonectria parasitica* has an alternating life cycle. It is equipped with perithecia, sexual structures that produce ascospores, and pycnidia, asexual structures that produce conidia. Ascospores (sexually produced spores) and conidia (asexually produced spores) are transported from tree to tree by wind, rain, and animals (Anagnostakis 1982). *Cryphonectria parasitica* is a wound parasite, which means it needs to penetrate inner bark through injuries, which are often caused by insects, birds, and cracks that occur naturally (McCarroll and Thor 1978; Anagnostakis 1982). It grows through filamentous thread-like structures called hyphae which form feathery lattices called mycelial fans. The mycelium produces oxalic acid that eats through the tree's bark (Powell 2014). It reaches the xylem and phloem, preventing nutrient and water transport, girdling and killing the tree. The infection is identifiable by the appearance of cankers (McCarroll and Thor 1978; Anagnostakis 1982); the bark swells, rises, and cracks. Sometimes an orange color appears due to orange-pigmented stomata that encase the spore-bearing structures.Today, only a few adult chestnuts and millions of roots stumps remain. Sometimes these stumps produce a few

shoots that can survive 10 years or longer. They rarely live long enough to produce seeds (Powell 2014).

Several efforts have been made to bring back the American chestnut tree. One method has been to use hypovirulent strains of *C. parasitica,* which are weaker, allowing the tree to fight back and recover. Hypovirulent strains are *C. parasitica* strains that have been infected with a fungal virus. Viruses from these strains can be passed to the fungus inhabiting a tree that is already infected if the strain in that tree is of the same vegetative compatibility (VC) group (Milgroom and Cortesi 1999; Milgroom and Cortesi 2004). Vegetative incompatibility is a self/nonself recognition system that restricts virus transmission (Milgroom and Cortesi 1999). Virus-infected trees with *C. parasitica* have swollen, superficial cankers, commonly called "healing cankers" because they cause little to no harm (Milgroom and Cortesi 2004). In Europe, hypoviruses were released as a means of biocontrol and chestnut populations recovered, due to less fungal diversity. Using hypovirulent strains was unsuccessful in reducing the infection in the U.S. due to the large diversity of fungal strains, and thus, a greater diversity of VC groups, resulting in an inability for the hypovirulent strains to be passed from tree to tree. Thus, hypovirulent strains of *C. parasitica* were used as biocontrol agents effectively in Europe and not in the U.S. because in order for hypovirulence to passed from tree to tree, fungus must have vegetative compatibility (Milgroom and Cortesi 2004; Milgroon and Corstesi 1999).

Another method to re-create viable American chestnut populations has been hybridization by cross breeding American chestnuts with Chinese chestnuts that are naturally resistant. Offspring trees are then backcrossed with an American chestnut in order to get all the desired American traits, while also keeping the desired resistance of Chinese trees. This method is imprecise and requires breeding out all the unwanted traits over generations and thousands of

trees. Another method has been the use of transgenics. Twenty-seven genes that might be involved in the Chinese chestnut's, *C. mollissima*, resistance to blight have been identified and tested for use in transgenics, hoping to transfer resistance to *C. parasitica*. Some have been successful. Wheat contains a gene (the *OXO* gene) for an enzyme called oxalate oxidase that detoxifies or breaks down oxalic acid, the acid *C. parasitica* uses to attack the tree. Transgenic American chestnuts containing the *OXO* gene are showing promising survival rates, and some appear to be more resistant than the Chinese chestnuts (Powell 2014; Welch 2007). Despite these results, this approach will also take generations to create a new line of trees to restore the chestnuts to the forests. Additionally, genetic engineering such as hybridization uses only one parent tree, thus only capturing a very small amount of the genetic diversity of the chestnut trees. This leads to a genetic bottleneck and requires that other trees be bred in later in order to restore genetic diversity. Therefore, additional methods are needed to help restore and preserve genetic diversity of the chestnut stumps currently alive in forests. In order to bring back the genetic diversity needed for a sustainable population, it is necessary to develop a way to allow the shoots to survive to sexual maturity so the trees would flower and create seeds. Some fungicides have been proven to be effective against *C. parasitica*, however, they are only recommended for use in nurseries or other enclosed areas. The use of fungicides in forests is risky because they are contaminants that have relatively long term effects (Trapiello 2015). Mud-packing is another method that has seen some success. Mud-packing involves packing mud made with soil from the area by the tree against the canker and wrapping it with a dark plastic (Hebard 2017). The fact that this has been somewhat successful as well as the fact the chestnut roots survive suggests that there might be soil-borne anti-fungals.

The genus *Pseudomonas*, especially fluorescent pseudomonads, produces secondary metabolites that have antagonistic effects and/or antibiotic activity against certain pathogens (Haggag and Soud 2012). The ubiquitous root-associated bacterium *Pseudomonas fluorescens* produces secondary metabolites that have antagonistic effects on certain plant pathogens. *Pseudomonas fluorescens* isolates containing the *phlD* gene produce the secondary metabolite 2,4-diacetylphloroglucinol (DAPG), an antibiotic, and have been used to protect crops from root pathogens. The biocontrol activity of many *P. fluorescens* isolates has been linked to DAPG production (Brazelton et al. 2008; Weller et al. 2002).

DAPG exhibits broad spectrum antifungal, antibacterial, anthelmintic (harmful to worms and other parasites), and phytotoxic activity and thus it is toxic to a broad spectrum of organisms including bacteria, fungi, protists, and nematodes (Schnider-Keel et al. 2000). This suggests that it targets basic cellular processes, but the mechanisms of DAPG's toxicity and how it affects root systems are largely unknown (Brazelton et al. 2008; de Souza et al. 2003). DAPG leads to increased crop yields because it suppresses take-all of wheat, black root rot of tobacco, tomato wilt, damping-off of sugarbeet, bacterial soft rot of potato, and potato cyst nematodes as shown in studies using DAPG negative mutants of *P. fluorescens* compared with non-mutants (Schnider-Keel 2000). Four genes are involved in making DAPG. *phlD*$^{-}$ strains of *P. fluorescens* are created by inhibiting the first gene, thus rendering the pathway no longer functional. DAPG could be used as a biocontrol with fewer concerns for the ecosystem and it's inhabitants than fungicides, for example, because it has a short half life and it is naturally produced by the soil dwelling bacteria *P. fluorescens*.

Preliminary experiments performed by students in Biology 580 during the fall of 2015 at Allegheny College suggest that the *P. fluorescens* strain *Pf*-5 can inhibit growth of *C. parasitica*

mycelia through the production of DAPG. Antibiosis experiments were performed between *C. parasitica* and *P. fluorescens*. Well diffusion assays were done with *P. fluorescens* in the wells and plugs of *C. parasitica* on potato dextrose agar (PDA) plates. Four wells were made on each plate with one type of modified *P. fluorescens* in each well. One *C. parasitica* plug was placed in the middle of the plates. Additional PDA plates were streaked with *P. fluorescens* with plugs of *C. parasitica* on either side. In both experiments, zones of inhibition were measured. The supernatant of *P. fluorescens* strains that produced 2,4-diacetylphloroglucinol were found to inhibit mycelial growth of *C. parasitica* more effectively than supernatants from strains lacking the *phlD* gene. Both supernatant and pelleted bacteria that lacked the *phlD* gene lost the ability to inhibit hyphal (hyphae are branching, filamentous structures) growth, but pelleted bacteria were still able to inhibit mycelial (a mat made up of hyphae) growth. This suggests that DAPG is important for the inhibition of individual hyphae but not for the mycelium. This could be due the fact that DAPG becomes inactivated over time due to its sensitivity to light, and wasn't able to kill all the fungus before it broke down. Specific data for the well diffusion assays are included in Table 1. For the streaked plates, it was just noted that there was an obvious zone of inhibition. (Biology 580 Section 01 Allegheny College 2015 unpublished results, methods from Haggag and Soud 2012).

	Pf-5		Phl-D	
	Hyphae	Mycelium	Hyphae	Mycelium
SC	0.4	0.8	0	0.4
ST	0.7	1.1	0.1	1.0
PC	0.4	0.4	0	0.5
PT	0	0.6	0.2	1.1

Table 1. *Cryphonectria parasitica* growth inhibition by *Pseudomonas fluorescens*. *Pseudomonas fluorescens* (*Pf-*5) and the *P. fluorescens* mutant lacking the *phlD* gene were used in well diffusion assays with plugs of *Cryphonectria parasitica*. The bacteria were centrifuged in order to separate the supernatant from the pellet to

determine if the bacteria themselves inhibit growth or if their secondary metabolites do. The wells contained either supernatants from bacterial cultures (S) grown in either Castric broth (minimal media, C) or 1/2 TSB (rich media, T) or pellet bacteria (P) grown in Castric broth or 1/2 TSB. Distances were measured in centimeters from the well to the edge of the approaching mycelium of *C. parasitica*.

Smith (2013) treated American chestnut trees for chestnut blight using a streptomyces inoculum. Stroptomyces was chosen because it is a soil-borne bacterium that produces antibiotics as well as chitinolytic enzymes which can degrade chitin in fungal cell walls (Smith 2013). *P. fluorescens* was chosen for this study instead because it is also a soil-borne bacterium that produces an antibiotic, because of the results of the preliminary study described above, and to investigate if more than one species of bacteria contribute to the anti-fungal properties seen in soil.

There is a lot of evidence showing that *P. fluorescens* can be used as an effective treatment for root pathogens. The goal of this study, however, is to investigate the effects of the antibiotic, DAPG, produced by *P. fluorescens* on *Cryphonectria parasitica* in the field and in the lab, treating an above ground pathogen. If a method of applying *P. fluorescens* directly to the tree, thus inhibiting *C. parasitica*, is developed, the tree could defeat the pathogen and survive to maturity. This study investigates the effects of strains *P. fluorescens Pf*-5 and *phlD*$^{-}$ on *C. parasitica* isolates in antibiosis experiments, the effects different concentrations of DAPG on *C. parasitica* isolates via well diffusion assays (finding an effective concentration could be useful information in determining an effective and efficient way of treating the chestnut trees), and the effects of applying a *P. fluorescens* inoculum directly to chestnut trees. Results were analyzed by measuring the zones of inhibition (areas between DAPG and *C. parasitica* where no growth of

the fungus occurred) in the well diffusion assays and antibiosis experiments, and by measuring canker size of the treated trees.

Methods

In the first experiment, American chestnut trees were treated directly in the forest surrounding the Robertson Sports Complex of Allegheny College in Meadville, PA. In order to directly treat the trees, a *P. fluorescens* inoculum was prepared. To prepare the inoculum (modified from Manikandan et al. 2010) one colony of *Pf*-5 from a 1/2 TSB plate was inoculated into five mL nutrient broth and allowed to grow overnight at 27ºC on a shaking platform at 200 rpm. One mL of this log phase culture was placed into one liter of sterile nutrient broth (eight grams Difco nutrient broth in one liter of water) with 2% glycerol and incubated for three days at 25°C on a shaking platform at 135 rpm. To prepare for application (modified from Smith 2013), the inoculum was blended for three minutes with one liter of fragmented 2.5% water agar in order to obtain a consistency that would allow the inoculum to be placed into the trees. Root sprouts selected for treatment were living, had present cankers, and had not received any previous treatments. Forty trees were originally selected and measured. The circumference was measured as well as the longest and widest active portion of a chosen canker before treatment and again four weeks later to determine the baseline growth of the canker. Control trees were not treated but their second measurements are described as post treatment because the measurements was taken at the same time the treated trees' post treatment measurements were taken. At this point, three trees were eliminated because the specific canker originally measured could not be re-identified. Thus, 18 trees were placed in the experimental group and 18 were placed in the control group. Trees were split into groups in a way that each tree in one group had a similar

counterpart in the other group that had a similar amount and size of cankers. In an attempt to treat one tree, it was discovered that the drill bit was too small. This tree was eliminated from the experiment, thus there were 17 experimental and 17 control trees. Due to harsh weather conditions in December 2016, treatment was delayed. Smith (2013) inoculated chestnut trees with a streptomycete inoculum over winter months, so cold weather should not be a problem in terms of the effectiveness of treatment. A series of holes were drilled using a 9/64 drill bit on the outer edge of the canker approximately one centimeter apart and half a centimeter deep. The number of holes depended on the size of the canker. The *P. fluorescens* inoculum was placed into the holes using a 20 ml syringe. Trees used as controls were not drilled into or treated. Post-treatment measurements were taken in March, eight weeks after treatment occurred. A t-test was used to determine whether or not the change in canker size was significant.

Samples of infected chestnut tree bark were collected from trees in the forest surrounding the Robertson Sports Complex in February 2017. Samples were taken from cankers on American chestnut trees, using a utility knife. They were placed in small envelopes that were labeled. Samples were taken from four trees; tree 18, tree 20, tree 23, which were not used in the first experiment, and an additional tree labeled tree 41. Four bark pieces were taken from each tree, thus pieces were labeled T18-1, T18-2, T18-3, T18-4, T20-1, T20-2, T20-3, T20-4, T23-1, T23-2, T23-3, T24-4, T41-1, T41-2, T41-3, T41-4. Samples T41-1 and T41-2 came from hypovirulent cankers (hypovirulent cankers can be identified by a swelling of the bark around the edges of the canker). In the lab, samples were surface-sterilized individually for one minute in 70% ethanol and then for two minutes in 10% bleach in order to kill any bacteria and fungi on the outside of the bark (*C. parasitica* resides inside the bark). Each sample was then placed in a sterile petri dish (100 x 15 mm) containing water agar. The plates were sealed with parafilm and

placed on a shelf 80 cm under two fluorescent light bulbs at room temperature. A second set of samples were collected and surface-sterilized as above, however, after the bleach, the bark pieces were rinsed with sterile DI water. This was performed incase growth didn't occur in the original plates, however, mycelia developed from both rinsed and un-rinsed bark pieces. After approximately 10 days, mycelial plugs from each of the original plates were transferred to individual petri dishes containing Yeast Malt (YM) extract agar (three grams yeast extract, three grams malt extract, five grams peptone, 10 grams dextrose, 20 grams Bacto agar) using a sterilized cork borer. Two plugs were taken from each isolate and plated individually. Thus, plates were labeled T18-1-1, T18-1-2, T18-2-1, T18-2-2, etc. The plates were again sealed with parafilm and placed under the same light at room temperature. Plugs were passaged to new YM plates roughly every five days in order to keep fungal isolates alive and growing. Old isolates were stored at 4°C (Zakel 2000).

The second experiment of the study was an antibiosis experiment. Strains *Pf*-5 and the *phlD*$^-$ mutant were streaked down the middle of separate PDA plates with a plug of *C. parasitica* from freshly grown mycelium on either side. Both bacterial strains were streaked for isolation from freezer stocks. Unfortunately, no bacterial growth occurred on these plates. Different freezer stocks were used to streak again. Only a few colonies grew on these plates. In an attempt to get more growth, different freezer stocks were thawed, vortexed, then streaked for isolation. Once, again, only a few colonies grew on these plates. Colonies were taken from the plates of the second and third attempts and were streaked for isolation. Bacterial growth occurred on these plates, however, they did not look like *P. fluorescens*. In order to determine if the bacteria on the plates was *P. fluorescens*, colonies of each strain were placed individually in one mL artificial urine media (AUM; 2.427 g of urea, 0.034 g of uric acid, 0.090 g of creatinine, 0.297 g of

$Na_3C_6H_5O_7$ $2H_2O$, 0.634 g of NaCl, 0.450 g of KCl, 0.161 g of NH_4Cl, 0.089 g of $CaCl_2$ $2H_2O$, 0.100 g of $MgSO_4$ $7H_2O$, 0.034 g of $NaHCO_3$, 0.003 g of NaC_2O_4, 0.258 g of Na_2SO_4, 0.100 g of NaH_2PO_4 H_2O, and 0.011 g of Na_2HPO_4 in 200 mL of deionized water) and one mL of M63 (a nutrient broth; supplemented with magnesium sulfate and casamino acids) overnight at 28°C on a shaking platform at 220 rpm. If the bacteria was *P. fluorescens*, they should have produced secondary metabolites that would appear green. Unfortunately, this did not occur and thus it is doubtful that the bacteria was *P. fluorescens*. New bacteria could not be ordered in a timely fashion so the experiment was continued anyway. Colonies of both strains were placed in individual test tubes containing three mL 1/2 TSB and were left overnight at 28°C on a shaking platform at 220 rpm. The fungal isolates used in this experiment were T18-1-2, T18-2-2, T18-3-2, T20-1-2, T20-2-1, T20-3-1, T20-4-1, T20-4-2, T41-1-1, T41-2-1, T41-2-2, T41-3-1, T41-4-1, and T41-4-2. These isolates were chosen because they appeared to be *C. parasitica*; they were white or orange and not green or black. Isolates from the same bark piece were used if they looked different thus appearing to have different morphology. Using a sterilized cork borer, two plugs of each isolate were placed on opposite sides of PDA plates with the fungal side of the plug facing the middle of the plate. If the fungus had covered the whole YM plate, the plugs were placed at the edge of the PDA plates. If their was less fungal growth, the plugs were placed closer to the middle of the PDA plates. After the plugs were placed on the agar, the *P. fluorescens* inoculum was spread down the middle of the plate in between the plugs using a sterile cotton swab. Each fungal isolate was on two plates; one had the *Pf*-5 inoculum and the other had the *phlD*$^-$ inoculum. Plates were sealed with parafilm and left under the light. After three to four days, measurements of the zones of inhibition were taken. Measurements were taken from the bottom of the plate from the bacteria to the end of the mycelium mat, though a

few hyphal strands may have stuck out past the mat. The mutant strain was used to confirm that any zones of inhibition were due to production of DAPG. Two isolates from each tree were chosen to be used in the next experiment. Isolates from the same tree that had different morphologies and the most growth were chosen; T18-2-2, T18-3-2, T20-2-1, T20-4-1, T41-1-1, T41-2-1.

In the final experiment, well diffusion assays were performed. They were conducted using varying concentrations of DAPG and a mycelial plug of *C. parasitica*. Twenty-four PDA plates were made. Four wells were made in each plate using a sterilized cork borer. One *C. parasitica* plug (from the isolates listed above) was placed in the center of the plate. Each fungal isolate was placed on four plates because four different concentrations were being tested; 0, 3, 30, and 300 nanograms of DAPG per milliliter (each treatment had 0.1% methanol so the DAPG would dissolve). Only one concentration of DAPG was used per plate. Roughly 7 drops of the designated DAPG solution was placed in each well using a 1000 μl pipette. The plates were sealed with parafilm and placed in a box in order to keep them in the dark due to the fact that DAPG is light sensitive, meaning it loses its effectiveness when exposed to light. The box was placed in a 27°C incubator. Some DAPG inevitably breaks down and thus high concentrations were being tested. Zones of inhibition were measured after three days.

Results

Forty-one chestnut trees were collected for use in the experiments. The location of the trees is shown in Figure 1.

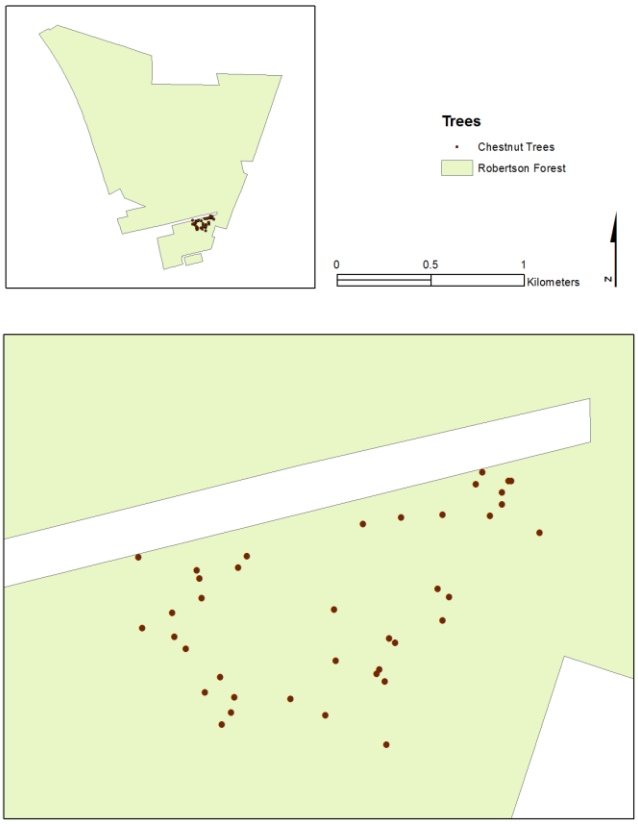
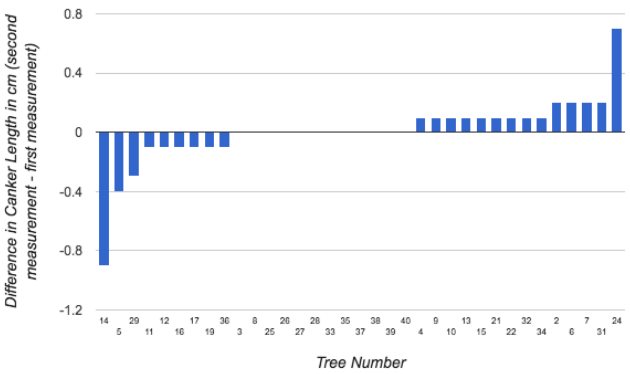


Figure 1. Chestnut trees at Robertson Complex. Forty-one American chestnut trees were identified and tagged. Their GPS coordinates were recorded using a mobile application and then used in the GIS mapping program ArcMap.

According to the measurements taken in order to establish baseline growth before treatment, some canker lengths and widths grew, some remained unchanged, and some shrunk. The difference in canker size is presented in Figure 2. Changes in canker length ranged from a decrease of 0.9 cm to an increase of an increase of 0.7 cm, changes in canker width ranged from

a decrease of 0.2 cm to an increase of 0.3 cm. A t-test was performed and showed that the change in length was not significant (p = 0.83). The p value for the change in width was 0.028 which is significant. However, the difference in the mean between the before and after measurements was only one millimeter.

(A).



(B).

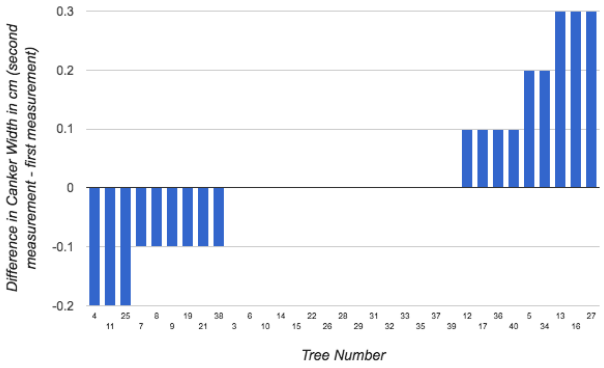


Figure 2. Change in canker size (cm) after four weeks. Measurements were taken of canker length and width and remeasured four weeks later. (A) Difference in canker length. (B) Difference in canker width.

Measurements taken after treatment also show that some cankers lengths and widths remained unchanged while some increased in size and others decreased in size. The difference in canker size for treated trees is presented in Figure 3 (A) and (B). Changes in canker length ranged from a decrease of 0.3 cm to an increase of 1.9 cm and changes in canker width ranged from a decrease of 0.3 cm to an increase of 0.2 cm. The difference in canker size for control trees is presented in Figure 3 (C) and (D). Changes in canker length ranged from a decrease of 0.4 cm to an increase of 1.3 cm and changes in canker width ranged from a decrease of 0.6 cm to an increase of 0.1 cm. A t-test indicated that the change in canker size that occurred was not significant for either treated or control trees (p = 0.66 for change in length between treated and control trees and p = 0.2 for change in width between treated and control trees). The difference is described as post treatment minus pretreatment in Figure 3.





(A).

(B).

(C).

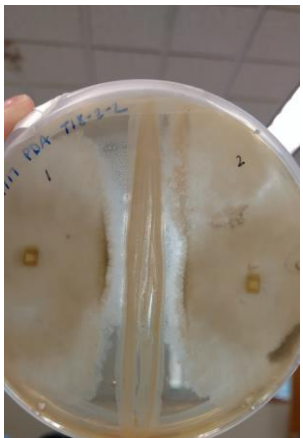
(D).

Figure 3. Difference in canker size. Measurements were taken of canker length and width after treatment or no treatment and compared to the pre-treatment measurements. (A) Difference in canker length in treated trees. (B) Difference in canker length in control trees. (C) Difference in canker width in treated trees. (D) Difference in canker length in control trees.

In the antibiosis experiment, there were some zones of inhibition on the *Pf*-5 plates; however, on a few of the plates, the fungus grew over the bacteria. Additionally, there were zones of inhibition on the *phlD*$^-$ plates that were bigger than those on the *Pf*-5 plates; the opposite of the expected results. The plates used in the experiment are shown in Figure 4; (A) shows all plates, (B) shows fungal growth and a zone of inhibition, and (C) shows dark fungus at the end of the mycelial mat.







(A). (B). (C).

Figure 4. PDA plates used in the antibiosis experiment. Plates were streaked down the middle with either *Pseudomonas fluorescens* strains *Pf*-5 or *phlD*$^-$. Plugs of *Cryphonectria parasitica* were placed on either side. (A) All plates used in the antibiosis experiment. (B) An example of the fungal growth and zones of inhibition. (C) Dark fungus at the edge of the mycelial mat.

When *phlD*$^-$ plates had larger zones of inhibition, the differences between the zones on the *Pf*-5 and *phlD*$^-$ plates were negative, as shown in Figure 4.

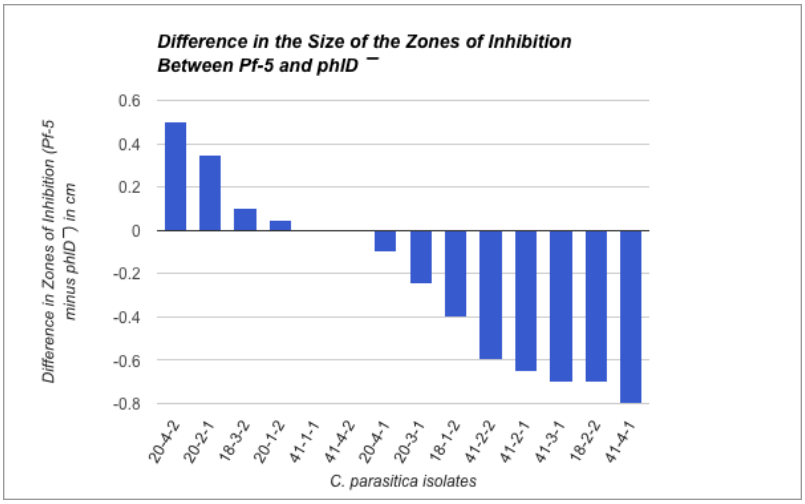


Figure 4. Differences in the size of the zones of inhibition between *Pf*-5 and *phlD*$^-$ plates. Plates were streaked down the middle with either *Pseudomonas fluorescens* strains *Pf*-5 or *phlD*$^-$. Plugs of *Cryphonectria parasitica* were placed on either side. Zones of inhibition were measured in centimeters.

Some of the plugs had what appeared to be black fungus on them, suggesting some contamination had occurred. The plugs that had black fungus were on plates with T18-3-2, T20-1-2, T20-2-1, T20-3-1, T20-4-1, and T41-4-1 for both *Pf*-5 and *phlD*$^-$ plates. T18-2-2 had some

dark fungus growing on the side of the plate which can be seen in Figure 1 (C). This also suggests contamination.

In the well diffusion assay, for each fungal isolate, the growth was the same across all four plates, and thus all four concentrations, as shown in Figure 3 (A). Thus, the varying concentrations of DAPG had no effect.

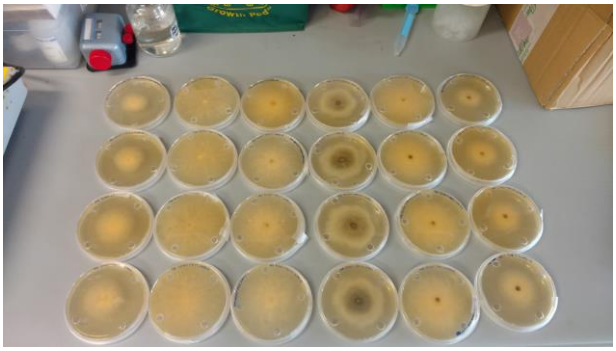
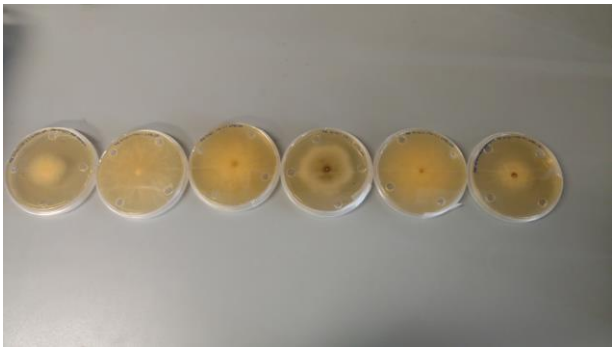
(A).  (B). 

Figure 3. PDA plates used in the well diffusion assay. Four wells were made around the edge of the plates. Different concentrations of DAPG were placed in the wells. Plugs of *Cryphonectria parasitica* were placed in the middle. (A) All 24 plates of the well diffusion assay. The top row is the 300 ng of DAPG per ml, followed by 30, 3 and 0. The columns represent different fungal isolates. Each fungal isolate has the same amount of growth for all DAPG concentrations. (B) Each fungal isolate on the 300 ng of DAPG per ml plates.

Discussion

American chestnut trees used to be a major component in in the United States' eastern deciduous and hardwood forests. When the chestnut blight, *Cryphonectria parasitica*, was brought over from Asia, virtually all chestnut trees were wiped out. The chestnut tree was a keystone species and it's disappearance hurt many different species of animals as well as humans. (Anagnostakis 1987; Powell 2014). Multiple different methods have been employed to try to bring back the American chestnut, but they have been met with limited to no success (Milgroom and Cortesi 2004; Powell 2014). This study was designed to test the effectiveness of

Psuedomonas fluorescens and *P. fluorescens* produced antibiotic 2,4-diacetylphloroglucinol (DAPG) as a biocontrol agent against *C. parasitica* through antibiosis experiments, by examining the effects different concentrations of DAPG on *C. parasitica* isolates via well diffusion assays, and by examining the effects of applying a *P. fluorescens* inoculum directly to chestnut trees.

A t-test showed that the change in canker size in both treated and control trees was not significant, indicating the treatment was not effective. This could be because *P. fluorescens* can not survive in the bark; perhaps it requires more moisture or less light. On the other hand, it could just be that DAPG is not effective against *C. parasitica*, which would support the findings from the other two experiments in this study. Additionally, a t-test was performed on the baseline growth of the cankers which showed that the change in cankers was also not significant. If the cankers weren't growing before treatment was given, then the treatment probably could not stop or reverse growth.

The antibiosis experiment produced inconclusive results. Although there were some zones of inhibitions on the *Pf-5* plates, the majority of the *phlD*$^-$ plates had larger zones of inhibition. Based on this experiment, it seems possible that some strains of *C. parasitica* are more sensitive to the DAPG produced by *P. fluorescens* and others might be more sensitive to other secondary metabolites whose production might be increased do the absence of DAPG production in the *phlD*$^-$ strain. It is also possible that *P. fluorescens* strain *Pf*-5 simply does not inhibit *C. parasitica* growth. These are speculations, however, and they could be inaccurate due to the fact that it seems unlikely that the bacteria used was actually *P. fluorescens*. Ultimately, *P. fluorescens* did not inhibit *C. parasitica* growth.

The well diffusion assay produced a more clear result; the DAPG did not inhibit fungal growth. This was evident due to the fact that the fungal growth was the same across all the concentrations of DAPG, from 0 to 300 ng/ml. This would support the findings in the antibiosis experiment (assuming the bacteria was *P. fluorescens*) since there was a lack of zones of inhibition of the *Pf*-5 plates. Haas and Keel 2003 stated that fluorescent pseudomonads produce about 0.5-3 nanograms DAPG on 1 gram of root material, which is why 3 ng of DAPG per ml was tested in this study, as well as 30 and 300 ng/ml to make up for any degradation by light. Nevertheless, 300 ng/ml is a very small amount, and the instability of DAPG in water may require that larger concentrations are applied in order to mimic the constant production of the compound by live bacteria. Thus, in future experiments, it could be beneficial to study a higher concentration.

A fair amount of research has been done on ways to bring back the chestnut. Based on this study, it seems that using *P. fluorescens* may not be the way to do so, but it is still important to find a way. Transgenic trees seem to be the most promising method so far, but it needs to be supplemented with other methods, such as mud-packing, in order to maintain the genetic diversity that exists in the chestnut tree roots and root sprouts. Bringing back the American chestnut tree would bring back an important food source, habitat, wood source, and possibly restore ecosystem productivity, function, and services that were lost when the trees were wiped out. Although many people are anxious when it comes to the topic of reintroduction due to the consequences of invasive species and accidental blights (such as the chestnut blight), the chestnut tree has only been gone for roughly 67 years, which is a relatively short period of time. Additionally, there is growing support that biodiversity conservation needs active restoration instead of just managing loss. Restoring the chestnut tree would help restore biodiversity which

is important for a healthy ecosystem. One study showed that restoration increased the provision of biodiversity by 44% as well as increasing ecosystem services by 25% (Macdonald and Willis 2013). Thus, bringing back the American chestnut tree would benefit wildlife as well as the ecosystem and the services that it provides humans.

Literature Cited

Anagnostakis, S. 1982. Biological Control of chestnut blight. Mycological Research. 215: 466-471.

Anagnostakis, S. 1987. Chestnut Blight: The Classical Problem of an Introduced Pathogen. Mycologia 79:23.

Brazelton JN, Pfeufer EE, Sweat TA, Gardener B, Coenen C (2008) 2,4-Diacetylphloroglucinol Alters Plant Root Development. Molecular Plant-Microbe Interactions 21: 1349-1358

Danisi J, Lynn D, Taylor R (2015) Displayed Antibiosis between P. Fluorescens and Cryphonectria.

de Souza, J., Arnould, C., Deulvot, C., Lemanceau, P., Gianinazzi-Pearson, V., & Raaijmakers, J. (2003). Effect of 2,4-Diacetylphloroglucinol on Pythium : Cellular Responses and Variation in Sensitivity Among Propagules and Species. Phytopathology, 93(8), 966-975.

Haggag WM, Abo El Soud M (2012) Production and Optimization of Pseudomonas fluorescens Biomass and Metabolites for Biocontrol of Strawberry Grey Mould. American Journal of Plant Sciences 3: 836-845

Haas, Dieter and Christoph Keel. "Regulation Of Antibiotic Production In Root-Colonizing Pseudomonas Spp. And Relevance For Biological Control Of Plant Disease". Annual Review of Phytopathology 41 (2003): 117-153. Print.

Hebard, F. (2017). *Mudpacking Cankers | The American Chestnut Foundation*. *The American Chestnut Foundation*. Retrieved April 2017, from https://www.acf.org/ma-ri/the-project/mudpacking-cankers/

Macdonald, D., & Willis, K. (2013). Key Topics in Conservation Biology 2 (1st ed.). UK: Wiley- Blackwell.

Manikandan, R, Saravanakumar D, Rajendran L, Raguchander T, Samiyappan R (2010) "Standardization Of Liquid Formulation Of Pseudomonas Fluorescens Pf1 For Its Efficacy Against Fusarium Wilt Of Tomato". Biological Control 54.2 (2010): 83-89. Web.

McCarroll, D. and E. Thor. 1978. Death of a Chestnut: The Host Pathogen Interaction. In: Proceedings of the American Chestnut Symposium. Morgantown, West Virginia. p 25.

Milgroom, Cortesi (1999) Analysis of population structure of the chestnut blight fungus based on vegetative incompatibility genotypes. Proceedings of the National Academy of Sciences 96: 10518-10523

Milgroom, M., and P. Cortesi. 2004. BIOLOGICAL CONTROL OF CHESTNUT BLIGHT WITH HYPOVIRULENCE: A Critical Analysis. Annual Review of Phytopathology 42:311-338.

Powell, W. (2014) The American Chestnut's Genetic Rebirth. Scientific American 310: 68-73

Schnider-Keel U, Seematter A, Maurhofer M, Blumer C, Duffy B, Gigot-Bonnefoy C, Reimmann C, Notz R, Defago G, Haas D et al. (2000) Autoinduction of 2,4-Diacetylphloroglucinol Biosynthesis in the Biocontrol Agent Pseudomonas fluorescens CHA0 and Repression by the Bacterial Metabolites Salicylate and Pyoluteorin. Journal of Bacteriology 182: 1215-1225

Smith A (2013) Biological Control of Cryphonectria Parasitica with *Streptomyces* and an Analysis of Vegetative Compatibility Diversity of *Cryphonectria parasitica* in Wisconsin, USA. Masters of Science in Biology. University of Wisconsin-La Crosse.

Trapiello, E., González-Varela, G., & González, A. (2015). Chestnut Blight Control by Agrochemicals in Castanea sativa under Managed Conditions. Journal Of Plant Diseases And Protection, 122(3), 120-124.

Welch, A., A. Stipanovic, C. Maynard, and W. Powell. 2007. The effects of oxalic acid on transgenic Castanea dentata callus tissue expressing oxalate oxidase. Plant Science 172:488-496.

Weller, D. M., Raaijmakers, J. M., McSpadden-Gardener, B. B., and Thomashow, L. S. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu. Rev. Phytopathol. 40:309-348

Zakel, K (2000) The VC group Characterization of the Chestnut Blight Fungus, *Cryphonectria parasitica*, at the Cussewago Chestnut Farm in Northwestern Pennsylvania. Senior Thesis, Biology Department, Allegheny College.