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Soluble Material Secreted from *Penicillium chrysogenum* Isolate Exhibits Antifungal Activity against *Cryphonectria parasitica*: The Causative Agent of the American Chestnut Blight

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Abstract

The American chestnut (*Castanea dentata*) was once the dominant canopy tree along the eastern region of the United States. *Cryphonectria parasitica*, the causative agent of chestnut blight, was introduced from Asia in the early 1900's, and obliterated the chestnut population within 50 years. We sought to identify environmental microbes capable of producing factors that were fungicidal or inhibited growth of *C. parasitica* in the hopes developing a biological control of chestnut blight. We isolated a filamentous fungus that significantly inhibited the growth of *C. parasitica* upon co-cultivation. Extracellular fractions of this fungal isolate prevented *C. parasitica* growth, indicating that a potential fungicide was produced by the novel isolate. Sequence analysis of 18S rRNA identified this inhibitory fungus as *Penicillium chrysogenum*. Furthermore, these extracellular fractions were tested as treatments for blight *in vivo* using chestnut saplings. Scarred saplings that were treated with the *P. chrysogenum* extracellular fractions healed subjectively better than those without treatment when inoculated with *C. parasitica*. These data suggest that material secreted by *P. chrysogenum* could be used as a treatment for the American chestnut blight. This work may assist the reclamation of the American chestnut in association with breeding programs and blight attenuation. Specifically, treatment of small groves under the right conditions may allow them to remain blight free. Future work will explore the mechanism of action and specific target of the extracellular fraction.

Keywords: American chestnut; Chestnut blight; Biological control; Inhibition; *Penicillium*

Introduction

The American Chestnut (*Castanea dentata* [Marsh.] Borkh.) was a prevalent canopy species in the eastern region of the United States of America, with an estimated 4 billion trees up to the 1900's [1-4]. Known as a foundation species, chestnut trees were co-dominant with oak trees in the Appalachian Mountain region for an estimated 4000 years [1,4-7]. The American chestnut germinates quickly, has a rapid growth rate, and the wood has high levels of a family of compounds called tannins that impede decomposition [1,8,9]. The capture and slow release of carbon by the decay-resistant Chestnuts makes these trees very competitive in carbon sequestration capabilities compared to other species of tree, and if reclaimed the chestnut could play a major role in combating climate change [1,4,10]. In addition to the promising carbon sequestration, the chestnut has the ability to provide consistent nutrition for wildlife and livestock, as well as timber for humans [3,4,11]. Because of the American Chestnut's dominance, the decline of this tree is devastating for both the ecosystem and economics, and the tree currently survives an understory shrub which rarely flowers [1,2,12,13].

The American Chestnut blight (causative agent - *Cryphonectria parasitica* (Murr.) Barr.) was first documented in North America in 1905 at the New York Zoological Garden by Merkel [2,14]. In a time span of 50 years, this fungus led to the near eradication of the American Chestnut [2,4,15,16]. There have been at least five *C. parasitica* populations discovered in the eastern part of North America [17]. The chestnut blight fungus is a filamentous ascomycete fungus capable of dikaryon formation when the fungal conidia and hyphae from different populations fuse [17]. The resulting fruiting body generates spores, which are spread by wind to other trees [17]. The spores enter open wounds in stem tissue, and subsequent fungal growth is accompanied by the secretion of oxalic acid (OA) which results in a decrease in the pH of the chestnut tissue [2]. The reduced pH levels promote blight growth while simultaneously inhibiting chestnut tissue function, and has been shown to be essential to canker formation [2,18]. Hyphal growth then eventually cuts off the flow of nutrients throughout the tissues, and the tree dies as a result of the process known as girdling [2,19,20].

Since the initial decimation of the American Chestnut, many endeavors have been pursued in attempts to reclaim this once dominant tree [2,4]. These attempts have primarily focused on genetically bolstering American chestnutsts [5,21-23]. Genetic manipulation by crossing American Chestnuts with other blight resistant species (Chinese chestnut- *Castanea mollissima* [Blume]) has proven difficult [4,24]. This is likely because the blight resistant hybrids are not as robust as the American Chestnut, or because these crosses cannot sustain blight resistance in the population [4,5,24,25]. One of the most advanced hybrids, BC3F3, is a third backcross generation [4,5,25,26]. This hybrid demonstrates desired phenotypic traits, such as a rapid growth rate, but has lower blight resistance than the Chinese chestnut [5,25,26].

An alternative strategy involves the use of hypovirulent strains of *C. parasitica* that have been attenuated by a virus for the biological control of European Chestnut blight [2,21,22]. In this strategy, a dsRNA hypovirus is used to infect *C. parasitica* [27]. Healthy *C. parasitica* is orange to yellow in color whereas the hypovirulent

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strains have a white phenotype associate with slower development and reduced sporulation [21]. Additionally, hypovirulent strains have an 18-fold decrease in OA concentrations as compared to wild type [2,28]. Upon hyphal fusion, hypovirulent strains have been shown to convert wild type strains into hypovirulent strains [21]. While viral-infected *C. parasitica* strains show pronounced changes, the diverse blight fungus populations combined with the restriction of virus population in the United makes effective spread of the hypovirus difficult [2,27]. While these individual strategies are making promising progress in the effort to reclaim the American Chestnut, they are not completely protective against the blight. Therefore, the development of new and alternative strategies is warranted.

Microbes are utilized to produce therapeutics in many fields. Chestnut roots are protected from blight, in part, due to the soil flora making an inhospitable environment for *C. parasitica* [2,4,13,29]. It is likely that environmental microbes may exist that antagonize the growth of *C. parasitica.* The goal of this study was to identify and isolate a microbe capable of inhibiting the American chestnut blight fungus, *C. parasitica.* A fungal soil isolate (AF6), determined to be *P. chrysogenum*, produced a secreted factor that completely inhibited the growth of *C. parasitica* in vitro. Moreover, American chestnut saplings treated with *P. chrysogenum* AF6 secreted fraction were resistant to the *C. parasitica* infection. Utilization of *P. chrysogenum* AF6 may have potential for preventing American Chestnut blight and returning this canopy species to prominence in the Eastern United States.

Materials and Methods

Acquisition of isolates

Soil samples were obtained from West Liberty University campus and near a naturally grown American Chestnut tree near Wheeling, West Virginia, USA. Soil samples were diluted in sterile distilled water, vortexed, and serially diluted in phosphate buffered saline (PBS). Serial dilutions were spread plated onto both tryptic soy agar (TSA) and potato dextrose agar (PDA). Plates were incubated at room temperature (~25°C) for 2-3 days.

In addition to soil isolates, PDA and TSA plates were exposed to indoor air inside Arnett Hall of Sciences, West Liberty University for one minute. Plates were incubated at room temperature for 2-3 days.

Cultivation of fungi/ isolates

Individual isolates with a bacterial colony morphology were streak plated on TSA and incubated at room temperature for 2-3 days. Frozen stocks were made by mixing glycerol with overnight tryptic soy broth cultures of isolates (3:1 ratio by volume, culture: glycerol) which were stored at -80°C. Additionally, the advancing margins of fungal hyphae were transplanted onto PDA, and incubated at room temperature for up to 10 days. To make stocks, plugs of fungal isolates were placed into cryovials containing PDA, and were incubated at room temperature for up to 10 days. The tubes were then stored at-80°C.

Competition assays

To determine whether environmental isolates were inhibitory against *C. parasitica*, competition assays were performed (Figure 1A). On both PDA and TSA, a plug of *C. parasitica* (Murr. (Barr.)) was placed in the center of the plate (Figure 1A) (ATCC® 38755). An isolate from above was streaked or a plug was placed around the edge of the plate (Figure 1A). Plates were incubated at room temperature for up to 10 days, and then evaluated.

Candidates that qualitatively inhibited *C. parasitica* were further tested individually in subsequent competition assays. *C. parasitica* was placed in the center of PDA and TSA plates (Figure 1B). The isolate with observed inhibitory action was then streaked or transplanted to each quadrant from the original stock (Figure 1B). The plates were incubated at room temperature for up to 10 days, and then growth was evaluated.

Disc diffusion assay

A fungal isolate that exhibited robust inhibitory action toward *C. parasitica* (*P. chrysogenum* AF6) was grown in tryptic soy broth (TSB) for one week (BD). The supernatant was passed through a 0.2 µm filter producing a cell-free filtrate. A plug of *C. parasitica* was placed in the center of a TSA plate from the frozen stock. Whatman discs (0.6 cm) placed in each quadrant of this place were infused with 15 µl of cell-free filtrate. The plates were incubated for up to a week at room temperature, and then the zones of inhibition were measured using a metric ruler.

Fungal isolate phenotypic and genotypic analysis

Macroscopic and microscopic identification: PDA plates were inoculated with the isolate inhibitory toward *C. parasitica* (*P. chrysogenum* AF6) and incubated at room temperature until the plate was completely covered with fungal mycelium. Fungal plugs were transferred to PDA, and sterile glass slide covers were inserted into the agar. The plate was incubated at room temperature until the fungal mycelium completely covered the medium. The slides were then stained with Lactophenol Blue and were observed with an Olympus IX73 microscope [30]. The microscope was connected to a Windows computer with CellSens Standard software version 1.7.

For fluorescence imaging, the inhibitory fungus (*P. chrysogenum* AF6) was grown on PDA with 1-5% Direct Yellow 96, and sterile slide covers were placed into agar. Fungus was incubated at room temperature in the dark until the plate was completely covered with mycelium. Epifluorescence microscopy images were obtained using an Olympus IX73 microscope in the green channel.

Genetic identification: To extract chromosomal DNA from the inhibitory fungus (*P. chrysogenum* AF6), a plug from stock was placed onto PDA and incubated at room temperature until mycelium completely covered the surface of this plate. The PDA with complete growth, and a mortar and pestle were frozen overnight at -80°C. The fungus was scraped into the mortar, with care to leave as much agar behind as possible. The fungus was liquefied with the pestle, and 500 µL of lysis buffer (40 mMol/L Tris-acetate, 20 mMol/L sodium acetate, 1 mMol/L EDTA, and 1% w/v SDS pH7-8) was added. The mixture was mixed via pipetting for about 30 cycles or until frothy. 2 µL of 10 mg/ml RNase A was added and the mixture was incubated at 37°C for

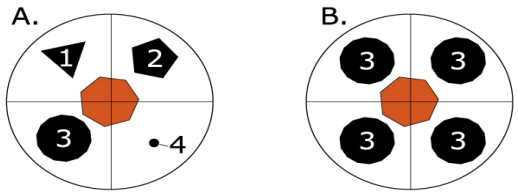

Figure 1: Diagram of the method used to identify environmental microbes that inhibit *C. parasitica*. Environmental microbes were isolated, plated with plugs of *Cryphonectrica*, and plates were observed for the presence of zones of inhibition (A). Isolates that significantly inhibited the chestnut blight fungus were then re-plated with plugs of *Cryphonectrica* to confirm the inhibitory capability (B). In the scope of this study, other fungi were the best competitors of *C. parasitica*.

5 minutes. 165 µL of 5M NaCl was added and the tube was inverted several times. The tube was centrifuged at 13,000 rev/min for 20 minutes at 4°C. The supernatant was moved to a fresh tube, and 800 µL of Phenol:chloroform was added and the tube was gently inverted. The mixture was microfuged at max speed for 5 mins. The aqueous portion of the solution was removed, and equal volume of Phenol:chloroform was added, and centrifugation and the extraction was repeated. DNA was precipitated with 0.75X isopropanol, and then washed with 70% ice cold ethanol. The DNA pellet was dried and then dissolved in 50 µL TE buffer, and stored at -20°C.

The primers nu-SSU-0817 (5′-TTAGCATGGAATAATRRAATAGGA) was paired with nu-SSU-1196-3′ (5′ – TCTGGACCTGGTGAGTTTCC) or nu-SSU-1536-3′ (5′ - ATTGCAATGCYCTATCCCCA) to amplify a portion of the fungal 18S rRNA gene using phusion polymerase (NEB) according to the instructions of the manufacturer [31]. The amplicons were analyzed via agarose gel electrophoreses (data not shown) and were sequenced (Beckman Coulter Genomics). The initial sequence (data not shown) was used to generate the following primer set: 5'-CGAAAGTTAGGGGATCGAAGACG and 5'-TAAGAAGCCAGCGGCCCGCA. These primers were used to amplify a portion of *P. chrysogenum* 18S rRNA gene via PCR using phusion polymerase (NEB) according to the instructions of the manufacturer. The purified amplicon DNA was sequenced by GeneWiz.

Tree scar infection

Two scars were made on four American Chestnut saplings with a surface sterilized scalpel (Chief River Nursery). Two of the saplings were inoculated with a 1 cm^3 plug of *C. parasitica*, and were incubated for 8 days. After this time period, a new plug of *C. parasitica* was applied. Strips of paper towels were soaked in 500 µl of cell-free filtrate from the inhibitory *P. chrysogenum* AF6, and were applied to the scars every other day for a week.

Results

Obtaining isolates

Microbes capable of inhibiting the growth or killing *C. parasitica* were isolated from soil and air samples near a local healthy American Chestnut tree. Hundreds of isolates with differing bacterial and fungal morphologies were used in competition assays against *C. parasitica* (Figure 1).

Inhibitory isolates (Zones of Inhibition)

To determine whether isolated microbes were capable of inhibiting the chestnut blight fungus, environmental isolates were plated onto agar inoculated with *C. parasitica* in the center (Figure 1A). Stock cultures were generated for isolated microbes that exhibited robust inhibitory action against *C. parasitica.* These microbes were re-evaluated in a second competition assay containing only the desired isolate against *C. parasitica* (Figure 1B). This was done to confirm the inhibitory activity and to ensure that combinatorial effects from multiple isolates did not result in the observed inhibition of *C. parasitica.* A green filamentous fungus that we named AF6 (Figure 2) in Image 2A, qualitatively exhibited the most obvious inhibitory activity toward *C. parasitica.* Because of dramatic inhibition of *C. parasitica,* we further investigated AF6.

Further characterization of inhibitory fungus

Supernatant material inhibits *C. parasitica*: We wanted to test if the inhibitory action AF6 was due, at least in part, to a secreted molecule. Therefore, TSB was inoculated with AF6, and then this culture was filtered (0.2 µm pore size) to remove any cells. The resulting filtrate was dispensed onto Whatman discs which were placed on TSA inoculated with *C. parasitica*. After 10 days of incubation, the zones of inhibition were measured (Figure 3). The cell-free AF6 filtrate significantly impaired C. *parasitica* growth (Figure 3) suggesting that the inhibitory factor from AF6 is a secreted molecule.

Fungal isolate identification

To gain insight into the identification of AF6, we examined this strain microscopically. Brightfield microscopy revealed that this fungal isolate produced branched conidiophores containing chains of conidia emanated from flask-shaped phialides (Image 2B). We wanted to see if there were additional morphological details that became more evident with fluorescence microscopy. Epifluorescence microscopy of the AF6 fungal isolate cultivated with Direct Yellow 96 fluorescent dye revealed the presence of aseptic hyphae as well as conidiophores as we previously described (Figure 2C). Notably, the conidia were more difficult to see under fluorescence microscopy as they appeared to be less permeable to the Direct Yellow 96 than the hyphae (Figure 2C).

Macroscopic and microscopic morphologies of AF6 were consistent with *Penicillium* sp. To confirm this observation and to gain insight into the species identification of this fungus, we sequenced a fragment of this isolate's 18S rRNA gene [31]. BLAST analysis revealed that the portion of the 18S rRNA gene sequenced (GenBank accession number: KX085202) was identical to *P. chrysogenum* suggesting that our isolate was also of this taxon. We therefore designated this isolate *P. chrysogenum* strain AF6.

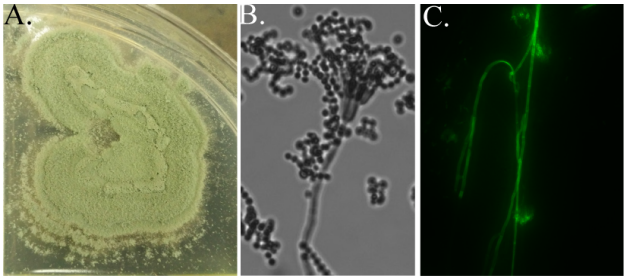

Figure 2: Macroscopic and microscopic morphology of the fungal isolate AF6. The inhibitory fungus, AF6, grown on PDA (A). Phase contrast (B) and fluorescent microscopy (C) of a conidium (B) and hyphae (C). For the fluorescence image, the fungus was grown on PDA containing Aldrich Direct Yellow 98 dye.

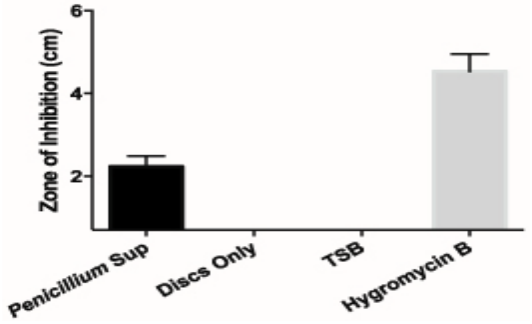

Figure 3: Supernatant material from a fungal isolate (AF6) inhibits growth of *C. parasitica.* A graphical representation of the zones of inhibition caused by the fungal supernatant. Fungal isolate AF6 was grown in TSB, and the cell-free filtrate was dispensed onto Whatman disks. Zones of inhibition surrounding the filtrate-infused disks were measured. Hygromycin B-infused (3.75 µg), TSB-infused, or untreated disks were used as controls. Data represent mean zones of inhibition ± SD. P<0.05.

Supernatant protects from *C. parasitica* in scar infection model

To determine whether *P. chrysogenum* AF6 filtrate could protect live American Chestnut trees from blight *in vivo*, we scarred saplings and inoculated these with *C. parasitica* (Figure 4). One group of scarred saplings was treated with AF6 filtrate, another was untreated, and the third was scarred and treated but not infected. The untreated scar demonstrated large amounts of *C. parasitica* colonization, and observed wound expansion (Figure 4B). However, the group of saplings containing scars treated with AF6 did not exhibit any observable infection and the wounds appeared to heal rapidly. The scarred, uninfected, treated saplings did not exhibit any signs of *C. parasitica* colonization or disease (Figure 4C). These data suggest that the material secreted by *P. chrysogenum* AF6 could have potential for preventing *C. parasitica* colonization of American Chestnut trees.

Discussion

The results presented here indicate that a novel fugal species produces a secreted factor that inhibits *C. parasitica.* Our findings further demonstrate that environmental microbes may show promise as a future biological control agent against chestnut blight. Current efforts to restore the American Chestnut involve utilization of a hypovirus that attenuates *C. parasitica* [17,18]. However, there is poor distribution of this virus in the United States [17,18]. Moreover, breeding programs to generate blight resistant trees have encountered hurdles concerning the inherently slow rate of this process [4,5,16,17]. The current findings represent an alternative to hypovirus-mediated attenuation and breeding resistance into chestnuts. Although here we uncovered only one type of fungus inhibitory to *C. parasitica*, there could be potentially more microbial agents able to inhibit Chestnut-blight fungus that have not yet been characterized.

The most plausible application for the direct use of the fungal factor to treat Chestnut Blight may be to utilize this antifungal in groves on a small scale. Such treatments are not uncommon forms of biological control. Fire Blight, which targets economically important apple trees, is partially controlled by streptomycin and other chemicals in orchards [32]. Under strict supervision, the Swiss government allows one treatment of the antibiotic to groves per year [32]. However, for both the fire blight and chestnut blight treatments, it is important to keep in mind the long term efficacy of treatments, the safety of people who come into contact with the treatments, the health of the target plant, and also the effects on the surrounding environment [32].

Figure 4: *Penicillium* sp. AF6 supernatant protects the American Chestnut from *Cryphonectrica* in a scar infection model. American Chestnut saplings were scarred and infected with *C. parasiticum* (A and B). As a control, a subset of saplings were scarred but not infected (C). The *Penicillium* sp. AF6 supernatant was applied infected (A) and to the non-infected sapling (C) every other day for a week and then evaluated. One subset of infected saplings were not treated with the *Penicillium* sp. AF6 supernatant (B).

Currently, breeding programs have made promising advances in producing blight-resistant chestnuts [5,25,26]. However, most of the breeding programs take decades before results manifest [5,25,26]. In the interim, additional advances could diversify our capacity to combat blight, bolstering the chances for success of the American Chestnut. In addition, if the genetic factors responsible for producing the inhibitory agent described here can be expressed in recombinant American Chestnut, these trees may be blight resistant and could be introduced into nature. However, due to the difficulties facing breeding programs for blight resistance, this strategy would undoubtedly experience similar hurdles as we have previously discussed [5,33].

Future directions of this work include isolating the active secreted compound from *P. chrysogenum* AF6 and characterizing the chemical structure. The secreted compound could possibly be a known anti-fungal agent. If indeed the compound is a novel agent, then the target and mechanism on fungi should be determined.

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