BIOCONTROL POTENTIAL OF ENDOPHYTES OF HEALTHY \textit{CASTANEA DENTATA} FOR APPLICATION AGAINST \textit{CRYPHONECTRIA PARASITICA}

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

Brandon Potter

College of Science and Health
Biology

August, 2017
BIOCONTROL POTENTIAL OF ENDOPHYTES OF HEALTHY *CASTANEA DENTATA* FOR APPLICATION AGAINST *CRYPHONECTRIA PARASITICA*

By Brandon Potter

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology.

The candidate has completed the oral defense of the thesis.

Anita Baines, Ph.D.  Date  8/2/17
Anita Baines, Ph.D.
Thesis Committee Chairperson

Thomas Volk, Ph.D.  Date  8/2/2017
Thomas Volk, Ph.D.
Thesis Committee Member

Todd Osmundson, Ph.D.  Date  8/2/17
Todd Osmundson, Ph.D.
Thesis Committee Member

Marc Rott, Ph.D.  Date  8/2/17
Marc Rott, Ph.D.
Thesis Committee Member

Thesis accepted

Meredith Thomsen, Ph.D.  Date  9-14-17
Meredith Thomsen, Ph.D.
Graduate Studies Director
ABSTRACT

Potter, B.R. Biocontrol potential of endophytes of healthy *Castanea dentata* tissue for application against *Cryphonectria parasitica*. MS in Biology, August 2017, 78pp. (A. Baines)

The American chestnut, *Castanea dentata* (Marsh.) Borkh., was devastated by an exotic fungal pathogen, *Cryphonectria parasitica* (Murr.) Barr., introduced in the early 1900’s. Restoration of *C. dentata* is desirable for ecological and economic reasons. Endophytes, organisms that reside within host plant tissue without causing symptoms, may be a reservoir for sources of biological control agents (BCAs) active against *C. parasitica*. To evaluate this potential resource, endophytes were isolated from *C. dentata* stem sections. These were each screened in *in vitro*, dual-culture competition tests against four virulent *C. parasitica* isolates and objectively assigned to antagonism classes. Of 109 endophytes screened, 84 (77%) showed some degree of antagonism against *C. parasitica*. Of the top ranked endophytes 10 of 24 tested were shown to excrete antibiotic substances active against *C. parasitica*. Microscopy failed to reveal any direct mycoparasitism among these top endophytes. Four of the top 18 endophytes were capable of colonizing a pre-established lawn of *C. parasitica*. The internal transcribed spacer (ITS) of these top performing endophytes was sequenced and each species putatively identified with Basic Local Alignment Search Tool (BLAST). The results of these experiments suggest several endophytes, particularly NC3K (Sordariomycetes), are candidates for further research and potential application as BCAs active against *C. parasitica*. 
ACKNOWLEDGEMENTS

I would like to thank the following people for their support and assistance throughout my research and writing process without whom I could not have completed this thesis: my graduate advisor, Anita Baines; Tom Volk for teaching me so much about life and fungi; Todd Osmundson, who dedicated so much of his own time showing me the techniques, issues, and troubleshooting of sequencing; Marc Rott for his thorough feedback and constructive criticisms along the way (even though I am sure he hates how many semicolons I have fit into this sentence); my mother, who has read various versions of this thesis and was invaluable in editing (and probably agrees with Marc about the semi-colons); Jaqui Adams for collecting many chestnut stem samples from North Carolina; Kyle Kaszinski, a colleague and friend who’s drive was a valued motivating factor to me; and lastly Justinn, who —along with mushrooms— made Wisconsin so much better.

I also owe gratitude to William MacDonald and Mark Double of West Virginia University for providing lab strains of *Cryphonectria* for use in my experiments. Lastly, I would like to acknowledge financial assistance received for this work provided by the University of Wisconsin –La Crosse Office of University Graduate Studies in the form of a Research Service and Educational Leadership Grant.
TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................................ vii
LIST OF FIGURES ..................................................................................................................... viii
INTRODUCTION .......................................................................................................................... 9
   History and Significance ............................................................................................................. 9
   The American Chestnut, *Castanea dentata* ............................................................................. 9
   The Chestnut Blight Fungus, *Cryphonectria parasitica* ........................................................ 10
      Life cycle ........................................................................................................................... 11
      Dispersal ............................................................................................................................ 12
      Ecology .............................................................................................................................. 12
   Epidemiology of Chestnut Blight .......................................................................................... 13
      Introduction of the pathogen. ............................................................................................. 13
      Disease progression through the forest. ............................................................................. 15
      Current status of the forest................................................................................................. 17
   Other Threats to American Chestnut Survival ...................................................................... 19
   Efforts to Combat Blight ....................................................................................................... 19
      *Castanea* breeding programs ............................................................................................. 20
      Biocontrol: *Cryphonectria hypovirus* .............................................................................. 22
      Biocontrol: mud-packs and microflora .............................................................................. 24
      Transgenic *Castanea dentata* ............................................................................................. 25
   Introduction to Endophytes ................................................................................................... 26
      Ecology of endophytes. ...................................................................................................... 28
      Exploitation of a resource .................................................................................................. 28
      Ecological implications ...................................................................................................... 30
CURRENT RESEARCH .............................................................................................................. 34
   Hypothesis and Research Aims ............................................................................................... 34
   Methods ..................................................................................................................................... 34
      Overview ............................................................................................................................... 34
   Endophyte Isolation from Healthy Chestnut Stems .............................................................. 35
   Competition Experiments ..................................................................................................... 35
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Blight susceptibility of common <em>Castanea</em> species worldwide.</td>
<td>9</td>
</tr>
<tr>
<td>2. Graphic explanation of The American Chestnut Foundation’s backcross breeding program based on three resistance genes originating from <em>C. mollissima</em>.</td>
<td>21</td>
</tr>
<tr>
<td>3. <em>Cryphonectria parasitica</em> isolates used in the current research.</td>
<td>28</td>
</tr>
<tr>
<td>4. Primers used during PCR for amplification of target genes.</td>
<td>42</td>
</tr>
<tr>
<td>5. Endophyte rankings following in vitro competition experiments.</td>
<td>40</td>
</tr>
<tr>
<td>6. Endophyte rankings following the in vitro competition, antibiosis, and invasion experiments.</td>
<td>43</td>
</tr>
<tr>
<td>7. Putative identification of sequenced isolates (ITS) from the top matched BLAST results.</td>
<td>53</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. A native-range map of <em>C. dentata</em> overlaid with lines indicating the progression of <em>C. parasitica</em> through the environment over time from its introduction point in New York</td>
<td>8</td>
</tr>
<tr>
<td>2. <em>Castanea dentata</em> root sprouts, like this one in Asheville, North Carolina, are common throughout its range.</td>
<td>10</td>
</tr>
<tr>
<td>3. The disease triangle illustrates the required variables for disease to develop in a plant.</td>
<td>31</td>
</tr>
<tr>
<td>4. A: 9 mm diameter olive pitter removes a plug of apple for inoculation with colonized agar. B: Inoculated apples. C: Lesion mensuration.</td>
<td>37</td>
</tr>
<tr>
<td>5. The ITS1-F and ITS4 primers amplify the ITS1, 5.8S, and ITS2 region.</td>
<td>42</td>
</tr>
<tr>
<td>6. Endophytes emerging from stem sections on PDA. Squares can be seen where individual endophytes were removed and re-plated as they emerged in order to achieve axenic cultures.</td>
<td>45</td>
</tr>
<tr>
<td>7. Lesion size at 7 (grey) and 14 (black) days following inoculation (standard error of the mean shown shown).</td>
<td>46</td>
</tr>
<tr>
<td>8. Representative examples of each ranking at 10 days of growth. Each panel shows the same endophyte (left) matched against each of the four <em>C. parastica</em> strains (right) used in the competition experiments.</td>
<td>41</td>
</tr>
<tr>
<td>9. A=Endophyte NC3K invading a pre-established lawn of <em>C. parasitica</em> (Schomberg isolate). B=Endophyte MI11A failing to grow beyond inoculation plug.</td>
<td>42</td>
</tr>
<tr>
<td>10. Conidiophores of NC3K readily form in culture on PDA, providing an ideal source of conidia for the production of standardized inoculant.</td>
<td>54</td>
</tr>
</tbody>
</table>
INTRODUCTION

History and Significance

The American Chestnut, *Castanea dentata*

The American chestnut, *Castanea dentata* (Marsh.) Borkh., is a high-value tree species, both for wildlife and anthropogenic use. Its nuts once served as a staple food source, and it served as the most valuable timber species throughout its native range (Anagnostakis, 2001; Wang et al., 2013). As a keystone species in North America’s eastern-broadleaf forests, the loss of American chestnut to blight was devastating to people and wildlife throughout the region.

*Castanea dentata* is in the family Fagaceae. *Castanea* is monecious, with male and female flowers borne on the same tree. Despite this, *C. dentata* is self-infertile, instead being a strict outcrosser (Davis, 1983). *Castanea dentata* is wind pollinated, though pollen dispersion is limited; for a tree to successfully reproduce it needs to be within about 100 meters of another sexually mature tree (Paillet, 1988). When released of any competition, *C. dentata* is able to set fruit at just 4 years of age, though in forest settings masting may not occur until 20 or more years (Paillet and Rutter, 1989; Wang et al., 2013). Chestnut fruit is a one-seeded nut, up to three of which may be borne within each spiny, cup-shaped involucre.

*Castanea dentata* was once a dominant tree of the Eastern North American deciduous forests, with its native range broadly following the Appalachian Mountains from Maine and Quebec to Mississippi and Florida (Woods, 1953). *Castanea* was
established in refugia throughout the southern part of its range for over 15,000 years. Around 10,500 BCE, as ice retreated with the end of the last glacial period, *Castanea dentata* slowly extended its range northward, reaching its maximum range as recently as 2000 years ago (Davis, 1983). Following landscape disturbances by European settlers, which likely favored *C. dentata* regeneration, American chestnut was estimated to comprise a quarter to one half of the canopy trees in hardwood forests throughout the Oak-Chestnut region (Burnham, 1988; Paillet, 1988; Wang et al., 2013).

*Castanea dentata* is a heavy and consistent mast producer, able to provide a reliable food source year-to-year. This differs from the inconsistent masting qualities of the oak-hickory dominated forests that replaced chestnut-dominated forests, thus greatly reducing the carrying capacity for wildlife throughout chestnut’s range (Diamond et al., 2000; Gilland et al., 2012). The nuts are smaller than the European Chestnut, *Castanea sativa*, but are said to be sweeter with superior taste (Wang et al., 2013). American chestnut was also a prized timber tree. Its habit is monopodial, forming a dominant, straight bole, much preferred in the logging industry. Chestnut wood is highly rot resistant and sturdy, yet lighter and more easily worked than oak. The species was able expand in diameter up to 2.5 cm a year, rapidly growing up to 37 meters tall with a diameter exceeding 1.5 meters (Buttrick, 1925). Restoration of the American chestnut to its former position in the ecosystem has long been a goal of foresters, wildlife managers, nature lovers, and chestnut farmers.

**The Chestnut Blight Fungus, *Cryphonectria parasitica***

Restoration of the American chestnut requires solutions that address the devastating disease *C. parasitica* causes in *C. dentata*. In order to do so, a thorough
understanding of *C. parasitica*’s biology is necessary, including its lifecycle, dispersal strategies, and ecology.

**Life cycle.** *Cryphonectria parasitica* (Murr.) Barr is an ascomycetous fungus in the order Diaporthales. In the wild *C. parasitica* readily produces both sexual and asexual fruiting bodies. Meiotically produced ascospores are borne in asci within long-necked perithecia, characteristic of the Diaporthales, from which they are forcibly ejected. Mitotically produced conidia are borne directly from conidiophores that arise from mycelium or, more commonly, in pycnidia. Conidia borne in pycnidia occur embedded in a gelatinous matrix that is exuded from the ostiole.

If either an ascospore or a conidium lands on an appropriate substrate, they may germinate and become new, monokaryotic individuals. These individuals may anastomose with other *C. parasitica* of the same vegetative compatibility group. Vegetative compatibility is controlled in a self/non-self-recognition system by six, unlinked vegetative incompatibility (*vic*) genes; two alleles of each gene exist, resulting in 64 possible vegetative compatibility (VC) groups (Cortesi and Milgroom, 1998). Vegetative anastomosis among compatible individuals does not affect sexual mating and mating type is not involved in the vegetative compatibility recognition system in *C. parasitica* (Anagnostakis, 1988). Heterokaryons produced via vegetative compatibility are distinct from those produced as a result of sexual mating (Leslie, 1993).

*Cryphonectria parasitica* prefers a bipolar, heterothallic mating system, indicating it has two mating types, MAT1-1 and MAT1-2 (Anagnostakis, 1988; Zhang et al., 1998). Despite this, self-fertilization also occurs and is likely dependent on a genetic proclivity to do so (Marra and Milgroom, 2001). Sexual mating in *C. parasitica* begins when a
conidium fuses with a trichogyne, a specialized receptive structure, on an individual of
the opposite mating type (Casselton, 2002). When this occurs a perithecium is formed at
the site. Inside the developing perithecium, many dikaryotic cells are produced destined
to develop into asci. Within these cells, plasmogamy and meiosis occur. Following the
meiotic production of four haploid nuclei within the ascus, these undergo one mitotic
division. Each of the eight resulting haploid cells develop into a septate, bicellular
ascospore.

**Dispersal.** Dispersal of *C. parasitica* ascospores and conidia occurs abiotically
via wind and rain and biotically by animal vectors. Both perithecia and pycnidia form
embedded in orange fungal stroma that emerges in abundance from infected wood.
Ascospores are forcibly ejected from perithecia and dissemination is thought to be
primarily airborne (Anagnostakis, 2001). Airborne dispersal of ascospores has been
confirmed from perithecia following periods of sufficient moisture for spore ejection to
occur (Heald et al., 1915). Animals also disperse *C. parasitica*, particularly via conidia.
Conidia are exuded from pycnidia embedded in a gelatinous matrix, appearing as yellow-
orange tendrils called cirri, able to adhere to passing insects, birds, and mammals
(Anagnostakis, 2001; Griffen et al., 2009; Nannelli et al., 1998; Russin et al., 1984;
Scibilia and Shain, 1989). Conidia that wash into the soil during rains may also be
subsequently dispersed by wind following desiccation; in such scenarios, conidia may
remain viable for up to several months (Heald and Gardner, 1914; Russin and Shain
1984).

**Ecology.** *Cryphonectria parasitica* usually grows saprobically on bark and wood,
or pathogenically on phloem and cambium, of all species of *Castanea*, related trees in the
Fagaceae, as well as others, including red maple (*Acer rubrum*) (Baird, 1991). Nash and Stambaugh (1987) confirmed that *C. parasitica* isolated in North Carolina from alternate hosts (*Quercus* sp.) were capable of causing severe disease in *C. dentata*. Similarly, Davis and Torsello (1999) identified scarlet oak in Pennsylvania (*Quercus coccinea*) as reservoirs for virulent *C. parasitica*, however, they also found one isolate (of 102 studied) with hypovirulent characteristics, suggesting alternate hosts may also harbor curative strains of the blight.

On alternate host genera, *C. parasitica* exists as a saprobe and weak pathogen. In addition to saprobic and pathogenic trophic strategies, *C. parasitica* may exist as an endophyte within asymptomatic chestnut tissue (Bissegger and Sieber, 1994); in this capacity, *C. parasitica* is probably acting as a latent pathogen. The ability of *C. parasitica* to persist as a pathogen, saprobe, or endophyte of a diversity of trees throughout the range of American chestnut ensures a persistent supply of *C. parasitica* inocula in the environment.

**Epidemiology of Chestnut Blight**

**Introduction of the pathogen.** Human travel and trade has transported many species, intentionally and unintentionally, far from their native ranges. Asian and European chestnut trees were brought to North America for ornamental use and food production. Thomas Jefferson grafted European chestnut (*Castanea sativa*) to American rootstock at his Virginia home in 1773. Mature Japanese chestnuts (*Castanea crenata*) were also documented to be well established by the mid 1800’s on the east coast of the United States (Anagnostakis, 2001). Infected *C. crenata* or another Asian species brought to North America harbored a minor fungal pathogen. This pathogen, *Cryphonectria*
parasitica, escaped in North America and quickly established itself among a dense population of blight-naive American chestnut (C. dentata).

Chestnut blight was first documented in 1904, on American chestnuts (C. dentata) grown as shade trees at the Bronx Zoo (Merkel, 1905). At the time, the causal agent was unknown. Symptoms progressed from cankers, showing orange stroma, to flagging in branches distal to the canker, epicormic shoots spouting beneath the canker, and finally death of above ground stems. In 1913 United States Department of Agriculture botanist David Fairchild asked Frank Meyer, a Dutch botanist working on a collecting trip in China, to look for the disease (Anagnostakis, 1989; Arnold Arboretum, 2015). Meyers found the blight in China in 1913 and in Japan in 1915. He noted that it caused much less of a problem on Chinese trees (C. mollissima). He targeted this species, along with Japanese chestnuts (C. crenata), as candidates for hybridization to create “immune or nearly immune strains of chestnut” (Anagnostakis, 1989).

Samples of blight sent by Meyers were used to confirm the Chinese blight was the same species, Cryphonectria parasitica, infecting chestnuts in the United States. Cultures were grown in the laboratory for morphological comparisons, as well as inoculated into Castanea dentata growing in the vicinity of Washington D.C. (Shear and Stevens, 1913). These trees quickly became diseased, showing orange stroma and reproductive structures of C. parasitica. This confirmed that the Chinese species was the same that was causing blight in the United States, supporting the hypothesis that the species was Oriental in origin. This experiment also represents an additional introduction of C. parasitica to eastern forests.
**Disease progression through the forest.** The trees at the Bronx Zoo were at the epicenter of a fungal infection that would reduce *C. dentata* from the dominant canopy tree to an understory shrub. From the epicenter in New York, the epidemic expanded at about 37 kilometers a year, infecting the entire native range within 50 years (Anagnostakis, 1987) (Figure 1). *Cryphonectria parasitica* is efficient at spreading across the environment. Its spores have evolved to utilize wind (ascospores) and animal vectors (conidia) to disseminate far distances. Additionally, the economic status of *C. dentata* timber enlisted humans to transport the fungus. Communities scrambled to harvest blight-killed and infected trees before the standing timber lost value. Logging activity within infected forests and extraction of infected wood to lumber mills and beyond also must have played a role in spreading the blight.

*Castanea dentata*, lacking a co-evolutionary history with this foreign species of *Cryphonectria*, proved to be unprepared to respond to infection. Asian chestnuts, on the other hand, had been able to evolve resistance factors in response to each avirulence factor of *Cryphonectria parasitica* individually as they arose. This is in agreement with the gene-for-gene concept introduced by Harold Henry Flor (Flor, 1942; Flor, 1955; Flor, 1971). Over generations of pathogen-host interactions, *C. parasitica* ratcheted up its virulence factors with chestnut species evolving resistance in kind; each avirulence gene of the pathogen was neutralized by a corresponding resistance gene among the host species. When faced with *C. parasitica* possessing a toolbox of avirulence genes, *C. dentata* populations, lacking corresponding resistance genes, were overwhelmed. There was little chance that an individual would randomly possess genes able to respond to these many, new avirulence factors from the foreign pathogen.
Figure 1. A native-range map of *C. dentata* (Little, 1977) overlaid with lines indicating the progression of *C. parasitica* through the environment over time from its introduction point in New York. Blight had spread through the entire native population by 1950 (adapted from Gravatt, 1949).

Susceptibility to *C. parasitica* varies greatly among host species. Asian chestnut species that share a native range with *C. parasitica* experience greatly reduced disease
compared to American and European species. In turn, disease symptoms are lower still when \textit{C. parasitica} infects oaks compared to Asian chestnuts (Table 1).

Table 1. Blight susceptibility of common \textit{Castanea} species worldwide.

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Origin</th>
<th>Degree of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{C. dentata} (Marsh.) Borkh.</td>
<td>American chestnut</td>
<td>Eastern North, America</td>
<td>No resistance$^{a,b}$</td>
</tr>
<tr>
<td>\textit{C. sativa} Mill.</td>
<td>European chestnut</td>
<td>Asia minor</td>
<td>Low resistance$^{a,b}$</td>
</tr>
<tr>
<td>\textit{C. ozarkensis} (Ashe) Tucker</td>
<td>Ozark chestnut</td>
<td>North America, Ozarks</td>
<td>Low to some resistance$^b$</td>
</tr>
<tr>
<td>\textit{C. pumila} var. \ \textit{pumila} Mill.</td>
<td>Chinquapin</td>
<td>Eastern North, America</td>
<td>Some resistance$^{a,b}$</td>
</tr>
<tr>
<td>\textit{C. mollissima} × \textit{C. dentata} C.dentata</td>
<td>Hybrid</td>
<td>Cultivar</td>
<td>Some resistance$^b$</td>
</tr>
<tr>
<td>\textit{C. crenata} × \textit{C. dentata}</td>
<td>Hybrid</td>
<td>Cultivar</td>
<td>Some resistance$^b$</td>
</tr>
<tr>
<td>\textit{C. seguinii} Dode.</td>
<td>Dwarf Asian chestnut</td>
<td>China</td>
<td>Some resistance to resistant$^{a,b}$</td>
</tr>
<tr>
<td>\textit{C. crenata} Sieb. &amp; Zucc.</td>
<td>Japanese chestnut</td>
<td>Japan, Korea</td>
<td>Resistant$^{a,b}$</td>
</tr>
<tr>
<td>\textit{C. mollissima} Blume</td>
<td>Chinese chestnut</td>
<td>China</td>
<td>Very resistant$^{a,b}$</td>
</tr>
<tr>
<td>Transgenic \textit{C. dentata} (Marsh.) Borkh.</td>
<td>American chestnut</td>
<td>GMO</td>
<td>Not susceptible$^c$</td>
</tr>
</tbody>
</table>

$^a$ = Fei et al., 2012. $^b$ = Graves, 1950. $^c$ = Zhang et al., 2013

**Current status of the forest.** Following the introduction of \textit{C. parasitica} in the early twentieth century, \textit{C. dentata} has been effectively removed from its role in the ecosystem (Anagnostakis, 2001). While little-to-no resistance existed in American chestnut populations, the fungus does not attack and kill the rootstock or young coppice shoots (Figure 2). Survival of the root system is not due to resistance among these tissues on the part of the tree, or the inability of \textit{C. parasitica} to infect and degrade this tissue.
Rather, it is the microbial inhabitants of the soil in which the roots are submerged that protect the root system and permit the tree’s survival via continued production of new stems via root-sprouting (see “Biocontrol: mud-packs and microflora” section below).

Figure 2. *Castanea dentata* root sprouts, like this one in Asheville, North Carolina, are common throughout its range.

The bark of young stems provides adequate protection from invasion by *Cryphonectria*. This physical barrier has allowed the tree to persist throughout its range as a small sterile shrub. Unfortunately, as the tree matures and secondary growth progresses, cracks appear in the smooth, cuticle-covered bark that expose inner bark tissue. Through these cracks, those that occur at the crotch of branch nodes, and wounds, *C. parasitica* is able to invade dead, mature bark and living vascular tissue beyond it.

Trees surviving to sexual maturity are rare, and very few older, mature trees survive. Under such conditions, sexual reproduction is hampered further by the obligate
outcrossing requirement of *C. dentata*. While it remains a common understory bush, this species has slim chances to evolve blight resistance on its own since it seldom reproduces sexually in the wild.

**Other Threats to American Chestnut Survival**

In addition to the dominant blight pathogen, two other introduced species threaten the survival of *C. dentata*. These include the Oomycete, *Phytophthora cinnamomi*, and the Oriental chestnut gall wasp, *Dryocosmus kuriphilus* (Anagnostakis, 2001).

*Phytophthora cinnamomi* is an exotic invasive that attacks the roots of woody plants among a large range of hosts worldwide. This Oomycete is a very destructive pathogen that causes plant diseases known as “dieback” and “root rot.” It is most common throughout the southern portion of *C. dentata*’s native range, causing the most damage in moist soils and during wet years. This disease is fatal to *C. dentata*, killing the entire plant at the roots. Like *C. parasitica, P. cinnamomi* is also a non-native invasive, likely originated near Indonesia (Zentmyer, 1977). Japanese (*C. crenata*) and some Chinese trees (*C. mollissima*) have good resistance to *P. cinnamomi* (Anagnostakis, 2001).

*Dryocosmus kuriphilus*, the oriental chestnut gall wasp, is the most recent introduced threat affecting *C. dentata*, arriving in Georgia in 1974. This wasp utilizes chestnut buds as egg-laying sites. The wasp larvae develop within galls that reduce tree shoot elongation, fruit production, and survival (Anagnostakis, 2001).

**Efforts to Combat Blight**

A variety of novel methods are being utilized in the attempt to restore *C. dentata* as a forest species; breeding resistant trees, introducing hypovirulent blight strains, and
Castanea breeding programs. Breeding chestnut trees resistant to the blight began almost immediately. These original attempts were interspecific hybrids of C. dentata crossed with various Asian species. These hybrid trees proved useful for farmers interested in nut production and ornamental uses. However, their phenotype lacked many favorable characteristics unique to pure C. dentata, such as its tall, straight growth habit and superior nut flavor. More sophisticated attempts at breeding followed.

Breeding programs have focused on identifying rare, resistant American chestnuts to breed into cultivars able to tolerate the now omnipresent C. parasitica (Clark et al., 2012). Some programs focus on pure American chestnut seed stock, such as the American Chestnut Cooperatives Foundation. They work only with surviving, sexually mature American trees (C. dentata) that show resistance to the blight, with the hope of breeding a pure American variety able to be reintroduced as a wild forest tree.

An alternative approach has been to use interspecific, American-hybrid crosses which have been created to breed resistance into surviving, pure-American tree lineages. The American Chestnut Foundation (ACF) began a breeding program with a goal to produce phenotypically American chestnut trees that possessed Asian resistance genes. To do so they began with some of the hybrids (C. dentata × C. mollissima) from original breeding attempts. These were continually backcrossed to surviving, pure American chestnuts (C. dentata). At each generation, trees would be selected for American characteristics and blight resistance. Today the ACF has trees that are over 15/16ths C.
dentata genetically, of American phenotype, and are blight resistant (Table 2). In 2009 the first of these trees to be field tested were out-planted within its native range (Clark et al., 2012). Preliminary results show good resistance to C. parasitica, but high damage and mortality due to deer browse and Phytophora cinnamomomi root rot (Clark et al., 2014; Clark et al., 2012).

The oldest of the ongoing breeding program, undertaken by the Connecticut Agriculture Experiment Station, is attempting to breed blight-resistant varieties that may resist P. cinnamomomi root rot and infestation by the Asian gall wasp, D. kuriphilus, as well (Anagnostakis, 2012). This effort is also based around breeding resistance genes from Asian varieties into American lineages.

Table 2. Graphic explanation of The American Chestnut Foundation’s backcross breeding program based on three resistance genes originating from C. mollissima, the Chinese chestnut (from “The Path to Most Resistance,” n.d.).

<table>
<thead>
<tr>
<th>Average percent American parentage</th>
<th>Parents</th>
<th>Degree resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Chinese</td>
<td>100% American</td>
<td></td>
</tr>
<tr>
<td>(blight resistant/orchard type)</td>
<td>(blight susceptible/timber type)</td>
<td>Low</td>
</tr>
<tr>
<td>50</td>
<td>(F₁) First generation hybrid</td>
<td>100</td>
</tr>
<tr>
<td>75</td>
<td>Backcross: American×F₁=BC₁</td>
<td>87.5</td>
</tr>
<tr>
<td>87.5</td>
<td>Backcross: American×BC₁=BC₂</td>
<td>87.5</td>
</tr>
<tr>
<td>93.75</td>
<td>Backcross: American×BC₂=BC₃</td>
<td>87.5</td>
</tr>
<tr>
<td>93.75</td>
<td>Intercross: BC₃×BC₃=BC₃F₂</td>
<td>34.4</td>
</tr>
<tr>
<td>93.75</td>
<td>Intercross: BC₃F₂×BC₃F₂=BC₂F₃</td>
<td>0</td>
</tr>
</tbody>
</table>
Biocontrol: *Cryphonectria hypovirus*. In Italy in the late 1950’s, healing cankers were noticed on blight-infected chestnuts, (*C. sativa*). Isolates of *C. parasitica* from these healing cankers showed reduced ability to cause disease and were able to transfer this ability to virulent blight strains through anastomosis (Grente, 1969; Van Alfen et al. 1975). The agent responsible was a double stranded, unencapsidated RNA virus (Choi and Nuss, 1992). Four species of related viruses, placed in the family Hypoviridae, have been found to infect *C. parasitica*. These viruses vary in their ability to induce a hypovirulent phenotype of *C. parasitica*. *Cryphonectria hypovirus-1* (CHV-1) is found throughout Europe and is often used for biocontrol of blight, with several introduction attempts made in the Unites States (Milgroom & Cortesi, 2004). A hypovirulent *C. parasitica* isolate from New Jersey yielded CHV-2 (Hillman et al., 1994). *Cryphonectria parasitica* isolated from the recovering cankers of trees in Michigan were shown to be infected with another naturally occurring hypovirus, CHV-3 (Fullbright et al., 1983). The most common hypovirus among *C. parasitica* populations in the eastern United States, CHV-4, does not induce any hypovirulent traits in its host (Liu et al., 2002).

A consequence of not having a protective capsid is that members of the Hypoviridae are not able to persist in the environment very long. Therefore, virus transfer cannot occur extracellularly. Instead new individuals can only become infected when the dsRNA virus is passed from one mycelium to another via cytoplasmic transfer following anastomosis. The self/non-self-recognition system of *C. parasitica* is a major deterrent to the natural spread of hypovirus (Anagnostakis, 1983). Stability of the cells at the junction following anastomosis is crucial to viral transfer. Anastomosis between incompatible *C. parasitica* results in a transient hyphal bridge, the cells of which undergo degeneration.
before appreciable cytoplasmic exchange (Newhouse and MacDonald, 1991). The more vegetative compatibility loci that differ between the hyphae of meeting *C. parasitica*, the less likely it is that virus transfer will occur (Liu and Milgroom, 1996). In the laboratory, hypovirus transmission was 100 percent between strains homoallelic at all six *vic* loci (Cortesi et al., 2001). In the field, natural spread and persistence of hypovirus – requirements for successful biocontrol - necessitates a population of *C. parasitica* with low diversity of vegetative compatibility groups (Milgroom & Cortesi, 2004).

Spread and persistence of hypovirus in a population of fungi is also affected by the vertical transmission of the virus to progeny via conidia or ascospores. Hypovirus-infected *C. parasitica* typically produce sexual ascospores devoid of the virus (Anagnostakis, 1982). Additionally, sexual reproduction among *C. parasitica* may further limit the proliferation of hypovirus as novel combinations of *vic* alleles results in new VC types within the population. Even among conidia produced mitotically from infected mycelium, virus-free conidia range from 10 to above 90 percent (Russin and Shain, 1984). Transgenic *C. parasitica* strains that contained a genomically integrated, hypovirus cDNA have been produced in the hopes that vertical hypovirus transmission would be stabilized and, therefore, be present in new VC types created in the population by sexual recombination (Anagnostakis et al., 1998). This strategy has not proven to be a persistent method of hypovirus introduction in the field, possibly because the hypovirus cDNA is strongly selected against among a blight host population in a natural setting (Milgroom & Cortesi, 2004). Recently “super donor” *C. parasitica* strains, capable of transmitting CHVs to a wide range of VC types, have been produced through disruption
of vic genes, offering new hope for virus mediated biocontrol among blight populations with diverse VC types (Zhang and Nuss, 2016).

Virus-mediated tree recovery has improved the health of chestnut stands (Castanea sativa) in Europe, but hypovirus is limited in its ability to spread through the more diverse American population of blight fungus (Milgroom & Cortesi, 2004). Even when hypovirus treatment is successful in maintaining the life of a C. dentata tree or stand, there is significant disfiguration caused to the tree, greatly reducing any value as timber (Anagnostakis, 2001). However, it should be noted that coppice stands of C. sativa used for timber in Europe are successfully controlled by hypovirus. Such disfiguration is also less of a concern among nut producers when treatment is able to maintain nut production.

**Biocontrol: mud-packs and microflora.** Application of mud-packs onto blight cankers is a proven biocontrol method (Weidlich, 1978). Mud-packs must be applied to each individual canker, be kept moist for several months, and extend well beyond the growing edge of the canker (Hebard). For these reasons, this method is limited in its applicability. Mud-packs are utilized by nut growers and breeders to prolong the life of individual trees. In this respect, they can play a crucial role in assisting surviving root-sprout trees reach sexual maturity for use in germplasm preservation.

It is likely that antagonistic microorganisms within the soil are responsible for the success of the mud-pack method. This notion has been supported by studies that have tested the biocontrol potential of soil bacteria against C. parasitica. Groome et al. (2001) identified a Bacillus megaterium isolate antagonistic to C. parasitica, and suggested it may be involved in the success of mud-packs. Streptomyces isolates from
soil have also been shown to reduce canker growth more effectively than hypovirus in treated trees (Schultz, 2013).

Mud-packing and derivative methods may be useful alternatives in situations where high vegetative compatibility diversity among the blight population limits horizontal transmission of hypovirus. However, these options should not be used where hypovirus is effectively controlling the blight epidemic; a population of hypovirus-infected cankers is necessary for the long-term success of hypovirus-based biocontrol (Milgroom and Cortesi, 2004). Mud-packs also may risk aggravating _P. cinnamomi_ root-rot, or spreading the pathogen to new trees; applying damp soil to trees may not only favor _P. cinnamomi_ infection, but healthy trees may be inoculated with the pathogen if infected soil is inadvertently used.

In addition to these soil-borne antagonists, organisms isolated from tree cankers have yielded potential BCA’s. _Penicillum_ and _Trichoderma_ isolates collected from cankers of _C. sativa_ growing in the Black Sea region have shown biocontrol potential in living chestnut stems (Akilli, et al., 2011). Researchers have also observed an increase in isolation of both hypovirulent _Cryphonectria_, as well as other species of fungi, from aging cankers in the West Salem stand compared to young cankers (Double et al., 2014). The most common fungal species invading aging cancers belonged to the genus _Trichoderma_, of which many are well characterized mycoparasites with utility as biocontrol agents.

**Transgenic _Castanea dentata_.** The most recent strategy to combat the blight has been to produce transgenic trees capable of resisting _C. parasitica_. Researchers have implanted a gene from wheat, _Triticum aestivum_, into _C. dentata_. The chosen gene codes
for the production of oxalate oxidase, an enzyme that degrades oxalate. Oxalate production plays a role in the virulence and substrate colonization of many plant pathogenic fungi (Dutton and Evans, 1996). Havir and Anagnostakis (1983) showed that virulent isolates of *C. parasitica* produced high levels of oxalate, whereas hypovirulent isolates did not. Oxalate is also known to be produced in planta at the edge of expanding cankers on American chestnut trees (Roane et al., 1986). These transgenic trees have shown very high blight resistance and retain a complete and pure *C. dentata* genome, with the exception of the transgenic gene (Polin, et al. 2006; Welch et al., 2007; Zhang et al., 2013).

**Introduction to Endophytes**

The term ‘endophyte’ has been variously defined in the literature. First introduced in 1866 by Anton de Bary, he broadly defined them as “any organisms occurring within plant tissue” (in Hyde and Soytong, 2008). The most frequently used definition -and the one used throughout the present paper- is that endophytes comprise “all organisms inhabiting plant organs that, at some point in their life, can colonize internal plant tissue without causing apparent harm to the host” (Petrini, 1991). This definition is widely accepted -with the assumed caveat that mycorrhizae and nitrogen-fixing, root-associated bacteria are tactically excluded. This exclusion is supported by a lack of mutualistic nutrient transfer with the host among true endophytes, as well as distinct evolutionary origins (Brundrett, 2004; Sieber, 2007). Fungal endophyte communities tend to be dominated by Ascomycota (Arnold, 2007; De Errasti et al., 2010; Higgans et al., 2007; Rubini et al., 2005), though Basidiomycota are also isolated less
frequently, and may be underrepresented in many studies due to culture-based sampling bias (Arnold and Lutzoni, 2007; Rodriguez et al., 2009).

Endophytes occur ubiquitously within plants (Petrini, 1991; Hyde and Soytong, 2008). Fungal, bacterial, and archael organisms are all found in this \textit{in planta} niche (Chelius and Triplett, 2001; Ma et al., 2013; Müller et al., 2015; Steinrucken et al., 2015). Furthermore, fungal endophytes may harbor prokaryotic endosymbionts themselves (Hoffman and Arnold, 2010; Hoffman, 2010) and all of these players may host viruses. Each of these levels of symbiosis has a role in altering the phenotype of the host plant (Márquez et al., 2007). Endophytes are capable of colonizing all regions of a plant: inter- or intra-cellularly within roots, stems, bark, leaves, and seeds (Rodrigues et al., 2009).

Fungi that qualify as ‘endophytes’ have historically been separated into two major groups: the clavicipitaceious endophytes, specific to some grasses; and the non-clavicipitaceious endophytes, a broad group of fungi that infect all plant lineages (including grasses) (Hyde and Soytong, 2008; Sieber, 2007). Rodriguez et al. (2009) introduced a new classification system that retained the clavicipitaceious endophytes together as Class 1 endophytes, and delineated the non-clavicipitaceious endophytes into three functional groups (Class 2-4). Class 2 endophytes are similar to Class 1 in that both occur as extensive, systemic infections within all parts to a plant (roots, stems, leaves), and may be vertically transmitted via the plant seed. Class 3 endophytes are exclusive to the above ground portions of the plant (stems, leaves); are horizontally transmitted; form localized infections; and occur as highly diverse assemblages within individual plants. Lastly, Class 4 endophytes comprise the dark septate endophytes. Dark septate
endophytes are restricted to plant roots and are so named because of their formation of darkly melanized hyphae with frequent septa (Rodriguez et al., 2009).

**Ecology of endophytes.** Fossil evidence confirms that relationships with endophytic fungi arose early among land plants (Krings et al., 2007). The correlation between the phylogenies of extant endophyte lineages with that of their hosts further supports that these symbiotic relationships are ancient. While this long evolutionary history among dominant endophyte lineages and their hosts precludes them from being devastating pathogens (Sieber, 2007), endophytes exist on a continuum from pathogen to mutualist (Carroll, 1988). Furthermore, many species recognized as pathogens are closely related to commensal or mutualist endophytes, with role switching having occurred over evolutionary time (Sieber, 2007).

The nature of the host-endophyte relationship is not only dependent on the species involved, but a range of abiotic and biotic factors (Partida-Martínez and Heil, 2011). For instance, an endophyte that enhances host fitness by reducing predation or pathogen damage may become a net cost to the host in the absence of such stressors. Many endophytes may exist as latent saprobes that persist to produce reproductive propagules following host senescence (Parfitt et al., 2010).

**Exploitation of a resource.** Clavicipitaceous endophytes have a proven history of usage in turf grass as a means to reduce insect herbivory and improve stress tolerance (Clay, 1991). Horizontally transmitted, class 3 endophytes have been shown to prevent insect herbivory (Miller and Adams, 2007; Wilson and Carroll, 1997). The ability of putative class 3 endophytes to limit pathogen damage in woody trees has been most extensively studied among the chocolate tree, *Theobroma cacao*, with positive results
Beneficial endophytes may act to protect their host from pathogens by a variety of mechanisms. These include modulating their host’s immune system, promoting host plant growth, production of antimicrobial secondary metabolites, occupation of a pathogen’s niche, or direct physical antagonism of a pathogen (Gao et al., 2010). Endophytes also may benefit their host in unexpected and indirect ways; Lehtonen et al., (2006) showed reduced virus infection among endophyte infected plants due to reduced herbivory by the virus’s aphid vector.

Endophytes must necessarily have some antagonistic abilities against their host plant in order to infect and persist within its tissues (Schulz and Boyle, 2005). Plants respond to the presence of invaders through systemic or induced acquired resistance via microbial/pathogen-associated molecular pattern-triggered immunity and effector triggered immunity. The endophyte and host actions must remain balanced for an endophyte to persist without causing its host harm (Schulz et al., 1999). A deadlock between the endophyte and its host may nonetheless benefit the plant by improving its immunity to subsequent pathogen assault. For instance, one mechanism for this would be the upregulation of the production of secondary metabolites by the host plant (Mucciarelli et al., 2007). Endophytes are also capable of directly increasing host nutrient acquisition and growth by producing various enzymes, chelating compounds, and phytohormones (Yuan et al., 2010). White and Torres (2010) have suggested that endophytes may increase host stress responses by improving oxidative stress tolerance. This may occur
either by increasing host production of antioxidant metabolites (phenolics) or by direct
production of antioxidant compounds by endophytes.

Secondary metabolites produced by endophytes also may improve host plants
fitness. Most noted among endophyte secondary metabolites are those that prevent
herbivory. One reason the clavicipitaceous endophytes play such an outsized role in the
literature on endophytes is their prolific production of ergot alkaloids, which have
gained wide attention due to their toxicity to mammals (Zabalgogeazcoa, 2008).
Endophyte secondary metabolites are often antimicrobial against bacteria and fungi and
may help control pathogens directly when they are produced in planta (Yuan et al., 2010).

Competition for, and physical occupation of, a pathogens niche by an endophyte
also serves to exclude disease-causing agents (Gao et al., 2010). Blumenstein et al.
(2015) confirmed extensive niche overlap among the Dutch elm disease pathogen,
Ophiostoma novo-ulmi, and endophytes of elm (Ulmus spp.). In circumstances where an
endophyte and a pathogen are in direct contact the endophyte may be directly
antagonistic, further enhancing its protective abilities for its host. Trichoderma spp. for
instance, which commonly occur as endophytes, are capable of coiling around and
directly lysing the hyphae of other fungi.

Endophytes, particularly the taxonomically diverse class 3 endophytes that are the
focus of this research, are highly variable in their roles and interactions with their host
plant. Despite this, there is promising evidence and high potential for a minority of these
species to be developed as applicable biocontrol agents.

**Ecological implications.** The potential ability of chestnut endophytes present in
North America to protect their host tree from blight raises questions about the extent of
host protection provided by woody endophytes. Since endophytes are highly diverse, even among small samples of tissue, the occurrence of antagonistic endophytes in North America may not have been biologically significant in terms of blight protection on a population—or even an individual—scale during the initial blight invasion.

Alternatively, in the absence of inherent genetic resistance, perhaps the ability of the rare mature *C. dentata* individual to survive virulent *C. parasitica* infection is influenced by the endophytes it harbors.

In what is commonly recognized as “the disease triangle,” plant illness results from an interplay requiring a susceptible host, a virulent pathogen, and a conducive environment (Figure 3). The environmental influences resulting in disease suppression may be abiotic or biotic.

If individual trees in a forest survive while the broader population suffers disease under the same abiotic environmental conditions, there is likely a genetic basis for resistance. However, if it is found there is no genetic basis for resistance and abiotic environmental influences fail to explain tree survival, only the tree’s biotic interactions...
remain as the possible source for disease protection. Among these interactions, it is the microbiome of the plant that may provide disease protection, including mycorrhizal fungi, endophytes, epiphytes, and organisms in the rhizosphere.

There is some disagreement whether any natural blight resistance in American chestnuts exists. Chestnut researcher Dennis Fulbright as well as the American Chestnut Foundation both state that there has not been any inherent resistance discovered in American trees (Bailey, 2013). On the other hand, researchers at The American Chestnut Cooperative Foundation are staking their breeding attempts on the progeny of “resistant” pure-American trees, the healthiest of which were the Gault tree in Ohio and the Floyd tree in Virginia (ACCF, 2016). Even if it is assumed that there is some limited genetic resistance in these trees, it may nonetheless be their ecological partners that have prevented stem mortality.

The possibility also exists that among endophytes that have persisted in American chestnut forests, where *C. parasitica* has now been ubiquitous for over 100 years, evolution has driven antagonisms to develop or strengthen. Endophytes of chestnut must have been subject to selective pressures exerted by the blight-driven alteration of *C. dentata* ecology, as well as direct competition with the invasive *C. parasitica*, which proved a voracious colonizer of chestnut tissue. If such interactions may drive the evolution of antagonism against blight among endophytes, Asian chestnut populations may harbor endophytes with even more promise as BACs given the long history of coevolution of endophytes of Asian chestnuts and *C. parasitica*. However, introducing exotic endophytes as BACs introduces new concerns.
Native endophytes that already exist in an ecosystem without attracting attention by causing negative outcomes—from a human perspective—are preferable to exotic organism. Exotic endophytes, even if they were to prove more capable of blight control, may act as pathogens on newly encountered plant species. It is conceivable, for instance, that *C. parasitica* can exist as a beneficial endophyte in non-*Castanea* plant species in Asia and/or that some variants are functionally endophytic and do not cause disease among Asian *Castanea* species. For this reason, caution should be taken when transporting any BAC to new environments. Knowledge of the natural history of species of interest as a BAC would be necessary to evaluate such risks. Sieber (2007) goes as far as suggesting pathogen risks posed by endophytes should be evaluated during plant quarantine procedures; a daunting task that is impractical, and likely impossible, for endophytes not yet known to be pathogenic in alternate host species.
CURRENT RESEARCH

Hypothesis and Research Aims

The hypothesis tested was that endophytic fungi isolated from healthy chestnut tissue are able to control pathogen growth in vitro. This work was intended to identify endophyte species with potential uses as biological control agents (BCAs) against *C. parasitica* to assist in the recovery of *C. dentata* in Eastern North American forests. The development of endophytes as biocontrol agents would provide a blight control option complementary to the ongoing efforts based on plant resistance and hypovirus-mediated recovery.

Methods

Overview

Endophytes isolated from healthy chestnut tissue were tested against *C. parasitica* for antagonism in vitro. The endophytes that showed the most promising antagonism were putatively identified to species and targeted for additional testing. Additional experiments included microscopic observation of potential mycoparasatism against *C. parasitica*, evaluation of the excretion of antibiotic compounds, and a test of the ability to colonize a pre-established lawn of *C. parasitica*. For each positive result observed in these follow-up experiments, a value of 1 was added to the numerical score assigned to each endophyte following the original competition experiments.
**Endophyte Isolation from Healthy Chestnut Stems**

Field collection of stem samples focused on trees that appeared healthy, without any symptoms of blight. Samples were collected during the summer of 2014 from sites throughout western North Carolina and from the ‘county line’ property in Michigan. Healthy stems under 1 cm in diameter were clipped so that two to four years of growth were represented from each sample (up to 100 cm in length). Stem samples were kept frozen in sealed Tyvek envelops until endophyte isolation.

Endophytes were isolated from surface-sterilized stem sections of *C. dentata* following established culture protocol by plating surface sterilized sections onto Difco potato-dextrose agar (PDA) (Schulz et al., 1993). Stem sections were cut into manageable pieces before being serially submerged in 95% ethanol for 30 seconds, a sodium hypochlorite solution (5.25% v/v) for 3 minutes, 75% ethanol for 30 seconds, followed by sterile, distilled water for 30 seconds. Each section was imprinted on PDA and the aliquots of the rinse water were plated to confirm surface sterilization. Isolations were only performed from batches of sterilized stems that had no contamination on the imprint and aliquot plates. Transverse and longitudinal sections of stems were plated on potato dextrose agar. Emerging endophytes were isolated in individual axenic cultures, resulting in a library of 109 cultivable endophytes.

**Competition Experiments**

**Characterization of *C. parasitica* test-strain pathogenicity.** Four hypo-virus free *Cryphonectria parasitica* isolates generously supplied by Dr. William MacDonald and Mark Double of West Virginia University were used throughout the competition studies. Additionally, one hypo-virus infected strain from Dr. Anita Baines lab at UW-La
Crosse was used during strain pathogenicity testing as an outgroup to confirm the pathogenicity of the virus-free test strains. One uncharacterized strain from West Salem, Wisconsin (WS-15) was also included, though it was not used throughout the remainder of this research since its virulence was uncharacterized at the time (Table 3).

Table 3. *Cryphonectria parasitica* isolates used in the current research. a = isolates provided by West Virginia University, b = isolates from University of Wisconsin –La Crosse.

<table>
<thead>
<tr>
<th>C. parasitica isolate</th>
<th>Hypovirus</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bockenauer(^a)</td>
<td>uninfected</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>Schomberg(^a)</td>
<td>uninfected</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>Rhyme(^a)</td>
<td>uninfected</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>EP-155(^a)</td>
<td>uninfected</td>
<td>Connecticut</td>
</tr>
<tr>
<td>WS-15(^b)</td>
<td>unknown</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>EP-713(^b)</td>
<td>CHV-1</td>
<td>France</td>
</tr>
</tbody>
</table>

The virulence of each *C. parasitica* strain was characterized with the Golden Delicious apple assay. Pathogenicity tests using Golden Delicious apples have been shown to correlate with in planta tests using chestnut stem sections or living trees (Fullbright, 1984; Elliston, 1985; De Lange, Wingfield, and Wingfield, 1998). Fifteen, fresh, unblemished Golden Delicious apples were wiped clean with 100% ethanol and inoculated at equidistance with each of the four, hypovirus-free *C. parasitica* test strains used in the current study. In addition, 5 apples were inoculated with a confirmed, hypovirus-infected *C. parasitica* as well as an uncharacterized lab strain (two inoculations of each isolate in each apple).
Apples were inoculated by removing a 9 mm diameter, 5-7 mm deep plug of tissue with an olive stuffer and a spatula (both sterilized). Two 9 mm diameter plugs of PDA taken from the growing edge of a *C. parasitica* test strain were placed into the resulting hole with aerial hypha pointing towards the apple tissue. Any excess agar that protruded from the hole was removed with a spatula so as to be flush with the surface of the apple. A small piece of masking tape was used to seal the hole to prevent desiccation during the test and provide a labeling location. Apples were stored individually in open plastic sandwich bags. The size of the resulting cankers was measured each day for the first 7 days, and again at day 14 and 21 (Figure 4). The longest distance across each canker (a) was measured, along with the perpendicular maximum (b); an area estimate was then calculated for each canker using the formula:

\[
\text{Area} = \frac{a}{2} \times \frac{b}{2} \times \pi
\]

Figure 4. A: 9 mm diameter olive pitter removes a plug of apple for inoculation with colonized agar. B: Inoculated apples. C: Lesion mensuration.
**Statistical analysis.** IBM SPSS version 23 was used to perform analysis of variance (ANOVA) to determine if the canker size induced on the apples varies significantly among different *C. parasitica* test strains. A planned contrast was used to test EP-713, the hypovirulent strain, against all others. An additional planned contrast compared each hypovirus-free strain against all other hypovirus-free strains (EP-713 was excluded from this analysis).

**Preliminary screening.** Preliminary trials were carried out on potato-dextrose agar (PDA) in 9 cm diameter petri dishes. *Cryphonectria parasitica* was plated along with an isolated endophyte 3 cm apart on PDA, and allowed to interact in order to ascertain antagonism potential. Control plates of endophytes and *C. parasitica* varieties plated alone were included for comparison.

Preliminary screening utilized qualitative categorization of the endophyte-pathogen interaction adapted from previously described methods (Badalyan et al., 2002). This qualitative method allowed a large number of endophytes to be screened for activity. Endophytes were scored based on their ability to (A=1) deadlock in culture, (B=2) deadlock at a distance, (C=3) partially overgrow the blight fungus, or (D=4) completely overgrow the blight with replacement by the endophyte. The same values were assigned as negatives in the cases where *C. parasitica* outcompeted the candidate endophyte. Positive values in any of these categories indicated antagonism, with A-type considered the least strong, B-type intermediate (indicative of possible excretion of antimicrobials), and C- and D-types the strongest. A single numerical antagonism index (AI) was assigned to each endophyte, calculated with formula:

\[
AI = \frac{[A \times 1 + B \times 2 + C \times 3 + D \times 4]}{N}
\]
Where \( n \) = the frequency of each interaction type between a particular endophyte and \( C. parasitica \), and \( N \) = the total number of replicates performed for each pairing. Four replicates were performed for each endophyte using a different \( C. parasitica \) isolate for each. Each replicate was analyzed at 2 and 4 weeks following plating, with the final AI consisting of an average of the values from each time-point.

**Confirmatory competition experiments.** Following preliminary screening, the top 15 C- and D-type (overgrew \( C. parasitica \)) and three B-type (inhibited \( C. parasitica \) at a distance) endophytes were subjected to additional replicates to ensure validity of the preliminary results (\( n=12 \)). Though the B-type isolates used in further testing did not rank as high as some excluded C-type endophytes, their ability to prevent \( C. parasitica \) growth at a distance suggested they may be secreting antibiotic substances into the media. For this reason, the three highest ranked of the B-type endophytes were also included. The same competition design used in the preliminary screening was repeated.

**Characterization of the Endophyte-Pathogen Interaction**

**Physical mycoparasitism observation.** Hyphal interactions between the top 15 C- and D-type antagonistic isolates and \( C. parasitica \) were observed by phase contrast microscopy. Samples were prepared by producing dual slide-cultures, allowing interactions between an antagonistic endophyte isolate and \( C. parasitica \) to occur on the observation slide itself. Slides were prepared by pouring a thin layer of agar directly onto a glass slide and placing a broken segment cover glass across the agar’s center. The two opposing sides of the agar were inoculated with either the candidate endophyte or \( C. parasitica \). Each slide was then incubated in a moist chamber, at room temperature, until
the two species had adequately interacted. At this time, each slide was observed under high magnification to elucidate the nature of antagonism.

**Antibiosis test.** To determine if endophytes were excreting antifungal compounds antagonistic to *C. parasitica*, the top performing endophytes from previous experiments (15 top-ranked and 3 top B-type), as well as 6 A-type endophytes that produced exudate and/or pigmentation in dual-culture experiments, were grown as liquid cultures in potato-dextrose broth (PDB). Agar plugs (9 mm dia.) of each endophyte were taken from dual culture plates of the endophyte and *C. parasitica*. A single plug of each endophyte was used to inoculate 200 ml of PDB in an Erlenmeyer flask. Resulting liquid cultures were incubated at ambient room temperature on a shaker table set to 140 rotations per minute. After 10 days of growth, samples were transferred to 10 ml centrifuge tubes. These were centrifuged for 10 minutes at ~1690 g (3000 rpm, ThermoScientific, CL2 centrifuge, 16.8 cm radius rotor) and just under 2 ml of supernatant liquid was pipetted off and transferred to 2 ml centrifuge tubes. The ~2 ml samples were then centrifuged for 20 minutes at 17,000 g (13,300 rpm, Fisher Scientific, accuSpin Micro17). From each of these prepared supernates, 100 µl was pipetted from the top and loaded into a well of a 96 well plate (n=4). The positive control was supernate from a *C. parasitica* liquid culture. The negative control was ethanol. A sample of each supernate was observed under magnification to ensure no hyphae were present. Additionally, a 100 µl aliquot of each supernate was plated and spread on agar to ensure sterility of the supernate.

A suspension of *C. parasitica* conidia was produced by transferring conidia from sporulating isolates into sterile water. Three of the four WVU isolates (Rhyme, EP-155,
and Bockenauer) readily produce conidia in droplets of exudate on PDA. One isolate (Schomberg) also produced prolific conidia, but did so dispersed along its mycelium and did not form droplets. Conidial solutions were produced from each of the three exudate producing isolates by transferring exudate into sterile, distilled water using an inoculation loop. Conidia were quantified using a Petroff-Hausser counting chamber for each solution. A master solution was then produced containing approximately equal quantities of conidia from each of the three exudate producing isolates. This concentrated conidial solution was diluted with PDB to a standard concentration of approximately $1 \times 10^5$ spores per milliliter for use as inocula. Into each well 100 µL of inocula was added, resulting in 200 µL total solution. Plates were observed at 40x magnification for five days, and again at day 10, to confirm hyphal growth or inhibition.

For endophytes that showed inhibition the test was repeated following adapted protocol for establishing minimum inhibitory concentrations (MIC) of compounds for filamentous fungi. The first well was loaded with 200 µL supernate. The last well was loaded with 100 µL ethanol as control. The remaining 10 wells were filled with 100 µL PDB. From the first well 100 µL of supernate was pipetted and mixed into the second well. This was repeated serially, and the last 100 µL aliquot discarded, so that each well resulting in a gradation from half strength supernate to a 1024-fold dilution. Following addition of 100 µL of inocula, each well volume totaled 200 µL. Plates were monitored for growth for 5 days.

**Invasion test.** The top 15 C- and D-type endophytes were also subjected to a competition test intended to evaluate the ability to compete on a substrate already fully colonized by *C. parasitica*. A 9 mm plug of each endophyte was placed, aerial hypha
downwards, onto a 9 cm agar plate that was completely colonized by *C. parasitica*. Only the Schomberg *C. parasitica* isolate was used initially due to its even growth in culture (n=3). Endophytes that had positive results were also tested against the Rhyme, EP-155 and Bockenauer *C. parasitica* isolates (n=12, where three replicates were performed for each of the four *C. parasitica* isolates).

**DNA Identification of Endophyte Species**

A Qiagen Plant Mini Kit was used to isolate DNA from cultured fungal samples. Replication and sequencing were performed for the ribosomal-DNA internal transcribed spacer (ITS) region through use of the ITS1F and ITS4 primers (Table 4, Figure 5).

Table 4. Primers used during PCR for amplification of target genes.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Direction</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS-1</td>
<td>ITS1F</td>
<td>5'-CTTGGTCATTAGAGGAAGTAA-3'</td>
<td>Forward</td>
<td>Gardes and Bruus, 1993</td>
</tr>
<tr>
<td>ITS-2</td>
<td>ITS4</td>
<td>5'-TCCTCCGCTTATGATATGC-3'</td>
<td>Reverse</td>
<td>White <em>et al.</em>, 1990</td>
</tr>
</tbody>
</table>

Figure 5. The ITS1-F and ITS4 primers amplify the ITS1, 5.8S, and ITS2 region. Graphic adapted from Vancov and Keen, 2009

Extracted DNA was combined with Illustra PuReTaq Ready-To-Go polymerase chain reaction (PCR) beads and DNA primers corresponding to the target loci. PuReTaq Ready-To-Go PCR beads contain all reagents required for PCR except template DNA, primers, and water (contents: Taq DNA polymerase, dNTPs, Bovine Serum Albumin,
stabilizers and reaction buffer). This solution underwent PCR in a thermalcycler following cycling conditions set forth by Taylor et al., (2008): 96°C for 2 minutes, followed by 25 cycles at 94°C for 30 seconds, 57°C for 40 seconds and 72°C for 3 minutes, finishing at 72°C for 10 minutes. The quality of the resulting PCR products was confirmed by running agarose gel electrophoresis with a molecular size standard. Successfully amplified samples were sent to the UW Biotechnology Center in Madison for Sanger sequencing. Individual sequence results were run through the National Center for Biotechnology Information’s Basic Local Alignment Search Tool (BLAST) to find likely species identities for each candidate fungus.
RESULTS

Most endophytes isolated for this research showed antagonism against *C. parasitica* in dual culture on PDA. Among the 15 rated most antagonistic, none showed direct mycoparasitism though four successfully colonized a pre-established lawn of *C. parasitica* on PDA. The supernate of 10 of 24 tested endophytes showed inhibitory activity against a *C. parasitica* spore solution. Of the 24 endophytes for which DNA identification was attempted, all were Ascomycetes and most were within the order Xylariales.

**Endophyte Isolation from Healthy Chestnut Stems**

From stem samples, 109 endophytes were isolated into axenic culture. Of these, 61 originated from North Carolina and 48 from Michigan. Nearly all of the isolated endophytes were filamentous fungi. All stem sections had endophyte growth, irrespective of the age or origin of the sample (Figure 6).
Figure 6. Endophytes emerging from stem sections on PDA. Squares can be seen where individual endophytes were removed and re-plated as they emerged in order to achieve axenic cultures.

**Competition Experiments**

**Characterization of *C. parasitica* Test-Strain Pathogenicity**

Comparison of lesion size in Golden Delicious apple assay confirmed that the four strains from West Virginia University used throughout the rest of the experiment represent virulent *C. parasitica* isolates compared to a known hypovirus-infected isolate, EP-713; additionally, the previously uncharacterized isolate, UWL- Baines lab strain WS-15, grouped with the virulent isolates (F=48.740, df=29.311, p<0.001 at day 7; F=48.686, df=37.831, p<0.001 at day 14). Among only the virulent isolates the West Salem isolate, WS-15, showed significantly more virulence than the others (F=48.686, df=14.806, p<0.001 at day 7; F=48.686, df=11.885, p=0.002 at day 14) and Bockenauer isolate showed significantly less virulence (F=48.686, df=44.457, p<0.001 at day 7;
F=48.686, df=31.306, p=0.004 at day 14) as indicated by relative lesion size. The effect sizes among virulent isolates were much smaller than comparisons between any hypovirus-free strain and EP-713 (Figure 7).

**Preliminary screening and Confirmatory Competition Experiments**

Of the 109 endophytes screened, 77 (70%) showed some antagonism to *C. parasitica* (Table 5). This includes endophytes that deadlocked on contact, deadlocked at a distance, or overgrew *C. parasitica* (Figure 8). Alternatively, 32 of 109 (29%) endophytes were unable to prevent overgrowth by *C. parasitica*. In confirmatory tests, all interactions were consistent with preliminary trials.

Figure 7. Lesion size at 7 (grey) and 14 (black) days following inoculation (standard error of the mean shown shown). Isolate labels are as follows: B= Bockenauer, S= Schomberg, R=Rhyme E= EP-155, WS=WS-15, EP=EP-713. Isolates Bockenauer, Schomberg, Rhyme, and EP-155 are known hypovirus-free isolates; WS-15 is an uncharacterized isolate that exhibited white colony morphology; EP-713 is a known hypovirus-infected isolate.
Characterization of the Endophyte-Pathogen Interaction

Physical Mycoparasitism Observation

Observation of slide cultures of each interaction (of the 15 endophytes tested) failed to elucidate any specific physical mechanisms by which antagonism occurs. Most notably, there were no cases where hyphae from an antagonistic endophyte could be seen coiling, penetrating, or otherwise interacting directly with \textit{C. parasitica} hyphae. The failure to find these features does not preclude the possibility that some of the endophytes were able to directly parasitize \textit{C. parasitica}. The hyphal morphology of \textit{C. parasitica} tended to clump into multi-hyphal cords, form reticulate networks of fused hyphae, and form coiled mycelia. This morphology made it more difficult to differentiate true mycorparasitism at the blight-endophyte interaction zone.

The absence of evidence of mycoparasitism suggests indirect interactions resulting from antibiotic secretions and/or nutrient competition are more likely explanations for observed antagonisms. At least two endophytes (NC3K, NC1B1) initiated areas of highly vacuolated \textit{C. parasitica} hyphae throughout the interaction zone, indicative of stress. This is likely a reaction to antibiotic compounds released by the endophytes or hyphal interference induced by another mechanism.
Table 5. Endophyte rankings following \textit{in vitro} competition experiments. The antagonism score represents the average of their ranking score from observations at the second and fourth week.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Isolate ID</th>
<th>Antagonism Score</th>
<th>Rank</th>
<th>Isolate ID</th>
<th>Antagonism Score</th>
<th>Rank</th>
<th>Isolate ID</th>
<th>Antagonism Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MAMSD3B</td>
<td>4.00</td>
<td>38</td>
<td>NC1O</td>
<td>1.38</td>
<td>75</td>
<td>LOST3</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>M11A</td>
<td>4.00</td>
<td>39</td>
<td>M17E</td>
<td>1.38</td>
<td>76</td>
<td>LOST4</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>NC3A</td>
<td>4.00</td>
<td>40</td>
<td>M17B</td>
<td>1.25</td>
<td>77</td>
<td>NC3S</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>M16A</td>
<td>4.00</td>
<td>41</td>
<td>NC5W(A)</td>
<td>1.25</td>
<td>78</td>
<td>NC4E</td>
<td>0.63</td>
</tr>
<tr>
<td>5</td>
<td>NC1K</td>
<td>3.88</td>
<td>42</td>
<td>M12A</td>
<td>1.25</td>
<td>79</td>
<td>NC1C</td>
<td>0.63</td>
</tr>
<tr>
<td>6</td>
<td>M10A</td>
<td>3.50</td>
<td>43</td>
<td>M1BD2</td>
<td>1.25</td>
<td>80</td>
<td>M1MSD7</td>
<td>0.50</td>
</tr>
<tr>
<td>7</td>
<td>NC3K</td>
<td>3.50</td>
<td>44</td>
<td>NC3H</td>
<td>1.25</td>
<td>81</td>
<td>M1ma5</td>
<td>0.50</td>
</tr>
<tr>
<td>8</td>
<td>aM12B</td>
<td>3.38</td>
<td>45</td>
<td>NC5F</td>
<td>1.25</td>
<td>82</td>
<td>NC1G</td>
<td>0.50</td>
</tr>
<tr>
<td>9</td>
<td>M19B</td>
<td>3.25</td>
<td>46</td>
<td>NC1I</td>
<td>1.25</td>
<td>83</td>
<td>NC5G</td>
<td>0.50</td>
</tr>
<tr>
<td>10</td>
<td>NC1B1</td>
<td>3.25</td>
<td>47</td>
<td>NC5J</td>
<td>1.25</td>
<td>84</td>
<td>M1MSD5C</td>
<td>0.13</td>
</tr>
<tr>
<td>11</td>
<td>NC3P</td>
<td>3.25</td>
<td>48</td>
<td>NC3M</td>
<td>1.25</td>
<td>85</td>
<td>NC4A</td>
<td>0.00</td>
</tr>
<tr>
<td>12</td>
<td>NC3D</td>
<td>3.13</td>
<td>49</td>
<td>NC3E</td>
<td>1.25</td>
<td>86</td>
<td>M112B</td>
<td>-0.04</td>
</tr>
<tr>
<td>13</td>
<td>NC5W</td>
<td>3.00</td>
<td>50</td>
<td>M19A</td>
<td>1.13</td>
<td>87</td>
<td>NCBD3C</td>
<td>-0.17</td>
</tr>
<tr>
<td>14</td>
<td>NC3V</td>
<td>3.00</td>
<td>51</td>
<td>NC4D</td>
<td>1.13</td>
<td>88</td>
<td>M18A</td>
<td>-0.38</td>
</tr>
<tr>
<td>15</td>
<td>NC1P</td>
<td>3.00</td>
<td>52</td>
<td>NC5C</td>
<td>1.00</td>
<td>89</td>
<td>M1I1A</td>
<td>-0.63</td>
</tr>
<tr>
<td>16</td>
<td>NC3B</td>
<td>3.00</td>
<td>53</td>
<td>NC4C</td>
<td>1.00</td>
<td>90</td>
<td>M1MSD4A</td>
<td>-0.75</td>
</tr>
<tr>
<td>17</td>
<td>NC1E</td>
<td>3.00</td>
<td>54</td>
<td>M1MSD6A</td>
<td>1.00</td>
<td>91</td>
<td>M18B</td>
<td>-0.88</td>
</tr>
<tr>
<td>18</td>
<td>M10C</td>
<td>3.00</td>
<td>55</td>
<td>M1MSD5B</td>
<td>1.00</td>
<td>92</td>
<td>al133C</td>
<td>-1.00</td>
</tr>
<tr>
<td>19</td>
<td>NC1B2</td>
<td>3.00</td>
<td>56</td>
<td>NC5K</td>
<td>1.00</td>
<td>93</td>
<td>NCBD1F</td>
<td>-1.00</td>
</tr>
<tr>
<td>20</td>
<td>M1ma5A</td>
<td>2.50</td>
<td>57</td>
<td>NC3Q</td>
<td>1.00</td>
<td>94</td>
<td>M1MSD3A</td>
<td>-1.00</td>
</tr>
<tr>
<td>21</td>
<td>M4A</td>
<td>2.50</td>
<td>58</td>
<td>NC5T</td>
<td>1.00</td>
<td>95</td>
<td>M1MSD1A</td>
<td>-1.00</td>
</tr>
<tr>
<td>22</td>
<td>M3A</td>
<td>2.25</td>
<td>59</td>
<td>M17D</td>
<td>1.00</td>
<td>96</td>
<td>M13B</td>
<td>-1.00</td>
</tr>
<tr>
<td>23</td>
<td>M111C</td>
<td>2.00</td>
<td>60</td>
<td>NC3Y</td>
<td>1.00</td>
<td>97</td>
<td>M15B</td>
<td>-1.00</td>
</tr>
<tr>
<td>24</td>
<td>NC1N</td>
<td>2.00</td>
<td>61</td>
<td>NC5M</td>
<td>1.00</td>
<td>98</td>
<td>NC5A</td>
<td>-1.00</td>
</tr>
<tr>
<td>25</td>
<td>M1C</td>
<td>2.00</td>
<td>62</td>
<td>M17B</td>
<td>1.00</td>
<td>99</td>
<td>M1MSD6B</td>
<td>-1.25</td>
</tr>
<tr>
<td>26</td>
<td>NC1PA</td>
<td>2.00</td>
<td>63</td>
<td>NC5P</td>
<td>1.00</td>
<td>100</td>
<td>NCBD2A</td>
<td>-1.25</td>
</tr>
<tr>
<td>27</td>
<td>NC1M</td>
<td>1.88</td>
<td>64</td>
<td>NC4B</td>
<td>1.00</td>
<td>101</td>
<td>M1MSD2A</td>
<td>-1.50</td>
</tr>
<tr>
<td>28</td>
<td>NC3T</td>
<td>1.75</td>
<td>65</td>
<td>M11B</td>
<td>1.00</td>
<td>102</td>
<td>M112C</td>
<td>-1.75</td>
</tr>
<tr>
<td>29</td>
<td>M12A</td>
<td>1.75</td>
<td>66</td>
<td>NCBD1A</td>
<td>1.00</td>
<td>103</td>
<td>NC5V</td>
<td>-2.00</td>
</tr>
<tr>
<td>30</td>
<td>M1ma5A</td>
<td>1.75</td>
<td>67</td>
<td>NC4F</td>
<td>1.00</td>
<td>104</td>
<td>NC3C</td>
<td>-2.50</td>
</tr>
<tr>
<td>31</td>
<td>NC1F</td>
<td>1.75</td>
<td>68</td>
<td>NCBD1B</td>
<td>1.00</td>
<td>105</td>
<td>M1ma3A</td>
<td>-2.63</td>
</tr>
<tr>
<td>32</td>
<td>M17C</td>
<td>1.75</td>
<td>69</td>
<td>M18C</td>
<td>1.00</td>
<td>106</td>
<td>M15C</td>
<td>-3.00</td>
</tr>
<tr>
<td>33</td>
<td>aM12A</td>
<td>1.75</td>
<td>70</td>
<td>NCBD3A</td>
<td>1.00</td>
<td>107</td>
<td>M110B</td>
<td>-3.00</td>
</tr>
<tr>
<td>34</td>
<td>M111B</td>
<td>1.75</td>
<td>71</td>
<td>NC5D</td>
<td>1.00</td>
<td>108</td>
<td>NC5CA</td>
<td>-3.50</td>
</tr>
<tr>
<td>35</td>
<td>NC5A</td>
<td>1.75</td>
<td>72</td>
<td>NCBD3A</td>
<td>1.00</td>
<td>109</td>
<td>NC3J</td>
<td>-3.50</td>
</tr>
<tr>
<td>36</td>
<td>NC1A</td>
<td>1.50</td>
<td>73</td>
<td>LOST1</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>NC5R</td>
<td>1.38</td>
<td>74</td>
<td>LOST2</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 8. Representative examples of each ranking at 10 days of growth. Each panel shows the same endophyte (left) matched against each of the four *C. parastica* strains (right) used in the competition experiments: clockwise from the top left, Schomberg, Rhyme, EP155, and Bockenauer. Scores for each ranking example are listed in parenthesis following its description: A=complete overgrowth of the endophyte by *C. parastica* (-4); B=partial overgrowth of the endophyte by *C. parastica* (-3); C=deadlock on contact (1); D=deadlock at a distance (2); E=partial overgrowth of *C. parastica* by the endophyte (3); F=complete overgrowth of *C. parastica* by the endophyte (4).
**Antibiosis Test**

The supernate from the liquid cultures of ten of the 24 endophytes tested (15 C- and D-type, 3 B-type, and 6 A-type) showed strong growth inhibition of the *C. parasitica* conidial solution (Table 6). Among these, there was a complete absence of growth after 5 days. After an additional 5 days (day 10), all wells of the endophytes that had inhibited growth at 5 days showed some growth, though this remained visibly less than endophytes that showed no inhibition at day 5. In all cases, the supernate of these ten endophytes failed to show inhibitory activity against *C. parasitica* when diluted just 4-fold or more at 5 days, being indistinguishable from control wells.

**Invasion Test**

Of the 15 top ranked endophytes tested, four successfully colonized petri dishes of PDA with pre-established lawns of *C. parasitica*. Of these NC3K, (unknown Sordariomycetes), was the most aggressive and fast growing; it completely overgrew the plates over the course of the 14-day experiment in all cases (Figure 9). Of the three other endophytes that were scored positive for this test -NC1B1, NC3P, and NC1D- none completely overgrew the *C. parasitica* lawn completely in any replicate.

![Figure 8. A=Endophyte NC3K invading a pre-established lawn of *C. parasitica* (Schomberg isolate). B=Endophyte MI11A failing to grow beyond inoculation plug.](image-url)
Table 6. Endophyte rankings following the in vitro competition, antibiosis, and invasion experiments. A value of 1 was added to the original competition scores if an endophyte was positive for antibiosis and/or invasion ability. Red lines show endophytes that dropped in ranking following a negative antibiosis or invasion test, green lines show endophytes that increased, and grey lines indicate no change.

<table>
<thead>
<tr>
<th>Endophytes Ranked by Antagonism Scores Following Each Experiment:</th>
<th>Competition</th>
<th>Competition + Antibiosis</th>
<th>Competition + Antibiosis + Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 MAMSD3B</td>
<td>4.5</td>
<td>NC3K</td>
<td>5.5</td>
</tr>
<tr>
<td>4 MI11A</td>
<td>4.5</td>
<td>MI10A</td>
<td>5.25</td>
</tr>
<tr>
<td>4 NC3A</td>
<td>4.375</td>
<td>aMI2B</td>
<td>5.25</td>
</tr>
<tr>
<td>4 MI6A</td>
<td>4.25</td>
<td>MI9B</td>
<td>4.5</td>
</tr>
<tr>
<td>3.875 NC1K</td>
<td>4.25</td>
<td>NC1B1</td>
<td>4.375</td>
</tr>
<tr>
<td>3.5 NC3K</td>
<td>4.25</td>
<td>NC3P</td>
<td>4.25</td>
</tr>
<tr>
<td>3.5 MI10A</td>
<td>4</td>
<td>MAMSD3B</td>
<td>4.125</td>
</tr>
<tr>
<td>3.375 aMI2B</td>
<td>4</td>
<td>MI11A</td>
<td>4</td>
</tr>
<tr>
<td>3.25 MI9B</td>
<td>4</td>
<td>NC3A</td>
<td>4</td>
</tr>
<tr>
<td>3.25 NC1B1</td>
<td>4</td>
<td>MI6A</td>
<td>4</td>
</tr>
<tr>
<td>3.25 NC3P</td>
<td>4</td>
<td>NC3V</td>
<td>4</td>
</tr>
<tr>
<td>3.125 NC1D</td>
<td>3.875</td>
<td>NC1K</td>
<td>3.875</td>
</tr>
<tr>
<td>3 NC5W</td>
<td>3.125</td>
<td>NC1D</td>
<td>3</td>
</tr>
<tr>
<td>3 NC3V</td>
<td>3</td>
<td>NC5W</td>
<td>3</td>
</tr>
<tr>
<td>3 NC1P</td>
<td>3</td>
<td>NC1P</td>
<td>3</td>
</tr>
<tr>
<td>2.25 MI3A</td>
<td>3</td>
<td>NC1N</td>
<td>3</td>
</tr>
<tr>
<td>2 MI11C</td>
<td>2.25</td>
<td>MI3A</td>
<td>2.25</td>
</tr>
<tr>
<td>2 NC1N</td>
<td>2.25</td>
<td>NC5F</td>
<td>2.25</td>
</tr>
<tr>
<td>1.75 NC3T</td>
<td>2</td>
<td>MI11C</td>
<td>2</td>
</tr>
<tr>
<td>1.25 NC5F</td>
<td>2</td>
<td>MI7B</td>
<td>2</td>
</tr>
<tr>
<td>1 MI7B</td>
<td>1.75</td>
<td>NC3T</td>
<td>1.75</td>
</tr>
<tr>
<td>1 NC4C</td>
<td>1</td>
<td>NC4C</td>
<td>1</td>
</tr>
<tr>
<td>1 NC3Q</td>
<td>1</td>
<td>NC3Q</td>
<td>1</td>
</tr>
<tr>
<td>1 NC5K</td>
<td>1</td>
<td>NC5K</td>
<td>1</td>
</tr>
</tbody>
</table>
DNA Identification of Endophyte Species

Of the 109 endophytes 24 were chosen for sequencing, comprising the 15 top ranked endophytes, as well as three B-type and six A-type endophytes subjectively selected for their ability to antagonize at a distance or produce exudate/pigment, respectively. The six A-type endophytes were the same as used in the antibiosis test. All species sequenced were shown to be Ascomycota, with the majority belonging within the order Xylariales (Table 7).

On three occasions the closest BLAST match for top ranked endophytes showed them to be the same, or extremely closely related, species. MI9B, aMI2B, and MI10A closely matched an uncultured fungus clone (accession KJ572257.1) that is likely *Daldinia childiae* (accession HM192904.1). MAMSD3B, MI11A, NC1K, NC3A, and NC3T all closely matched the same Sordariomycetes (accession JF773622.1) that is most similar to *Biscogniauxia mediterranea* (accession EF026134.1). Lastly, NC1B1 and NC3V both matched an unknown Sordariomycetes, (accession EU680529.1) (Table 7).

Morphologically the three species whose sequencing failed (NC3K, NC3P, and NC1D) were very similar to NC1B1 and NV3V in culture, having white, banded growth; irregular areas of black pigment; and production of conidiophores that released abundant conidia (Figure 10). These five endophytes are likely very closely related, and may be the same species.
Table 7. Putative identification of sequenced isolates (ITS) from the top matched BLAST results. For endophytes that failed sequencing attempts identification is based on morphological similarity to isolates that were successfully sequenced. Rank number on this table does not account for intermediate endophytes (Table 5) not chosen for additional testing.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Code</th>
<th>Query cover (%)</th>
<th>Identity (%)</th>
<th>Description</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>1^4</td>
<td>NC3K*</td>
<td></td>
<td></td>
<td>Uncultured Xylariales</td>
<td></td>
</tr>
<tr>
<td>2^4</td>
<td>NC1B1</td>
<td>100</td>
<td>100</td>
<td>Uncultured Xylariales</td>
<td>EU680529.1</td>
</tr>
<tr>
<td>3^4</td>
<td>NC3P*</td>
<td></td>
<td></td>
<td>Uncultured Xylariales</td>
<td></td>
</tr>
<tr>
<td>4^4</td>
<td>M10A</td>
<td>99</td>
<td>100</td>
<td>Daldinia aff. chilidae</td>
<td>KJ572257.1</td>
</tr>
<tr>
<td>5^4</td>
<td>aM12B</td>
<td>99</td>
<td>99</td>
<td>Daldinia aff. chilidae</td>
<td>KJ572257.1</td>
</tr>
<tr>
<td>6^4</td>
<td>M19B</td>
<td>99</td>
<td>99</td>
<td>Daldinia aff. chilidae</td>
<td>KJ572257.1</td>
</tr>
<tr>
<td>7^4</td>
<td>NC1D*</td>
<td></td>
<td></td>
<td>Uncultured Xylariales</td>
<td></td>
</tr>
<tr>
<td>8^4</td>
<td>MI1SD3B</td>
<td>97</td>
<td>100</td>
<td>Biscogniauxia aff. mediterranea</td>
<td>JF773622.1</td>
</tr>
<tr>
<td>9^4</td>
<td>M11A</td>
<td>95</td>
<td>100</td>
<td>Biscogniauxia aff. mediterranea</td>
<td>JF773622.1</td>
</tr>
<tr>
<td>10^4</td>
<td>NC3A</td>
<td>96</td>
<td>100</td>
<td>Biscogniauxia mediterranea</td>
<td>JF773622.1</td>
</tr>
<tr>
<td>11^4</td>
<td>M16A</td>
<td>99</td>
<td>100</td>
<td>Nigrospora aff. oryzae</td>
<td>HQ630982.1</td>
</tr>
<tr>
<td>12^4</td>
<td>NC3V</td>
<td>100</td>
<td>100</td>
<td>Uncultured Xylariales</td>
<td>EU680529.1</td>
</tr>
<tr>
<td>13^4</td>
<td>NC1K</td>
<td>96</td>
<td>100</td>
<td>Biscogniauxia aff. mediterranea</td>
<td>JF773622.1</td>
</tr>
<tr>
<td>14^4</td>
<td>NC5W</td>
<td>100</td>
<td>100</td>
<td>Pestalotiopsis sp.</td>
<td>KM507773.1</td>
</tr>
<tr>
<td>15^4</td>
<td>NC1P</td>
<td>100</td>
<td>99</td>
<td>Beech leaf mycelium</td>
<td>AB041994.1</td>
</tr>
<tr>
<td>16^4</td>
<td>NCIN</td>
<td>99</td>
<td>100</td>
<td>Pezicula sporulosa</td>
<td>AF141166.1</td>
</tr>
<tr>
<td>17^4</td>
<td>M3A</td>
<td>100</td>
<td>99</td>
<td>Aspergillus tubingensis</td>
<td>HQ728255.1</td>
</tr>
<tr>
<td>18^4</td>
<td>NC5F</td>
<td>99</td>
<td>95</td>
<td>Pestalotiopsis coeculi</td>
<td>EU273507.1</td>
</tr>
<tr>
<td>19^4</td>
<td>M11C</td>
<td>97</td>
<td>96</td>
<td>Amphiporthella leiphasia</td>
<td>AJ293882.1</td>
</tr>
<tr>
<td>20^4</td>
<td>M17B</td>
<td>100</td>
<td>100</td>
<td>Epicoccum nigrum</td>
<td>JN689342.1</td>
</tr>
<tr>
<td>21^4</td>
<td>NC3T</td>
<td>93</td>
<td>100</td>
<td>Biscogniauxia aff. mediterranea</td>
<td>JF773622.1</td>
</tr>
<tr>
<td>22^4</td>
<td>NC4C</td>
<td>99</td>
<td>99</td>
<td>Botryosphaeriaceae sp.</td>
<td>JF439465.1</td>
</tr>
<tr>
<td>23^4</td>
<td>NC3Q</td>
<td>100</td>
<td>99</td>
<td>Dicarpella dryina</td>
<td>KC145909.1</td>
</tr>
<tr>
<td>24^4</td>
<td>NC5K</td>
<td>100</td>
<td>99</td>
<td>Pestalotiopsis sp.</td>
<td>KP689121.1</td>
</tr>
</tbody>
</table>

^* = endophyte failed sequencing attempts; =~ D-type (complete overgrowth over C. parasitica); =~ C-type (partial overgrowth over C. parasitica); =~ B-type (deadlock at a distance); =~ A-type (deadlock on contact).
Figure 10. Conidiophores of NC3K readily form in culture on PDA, providing an ideal source of conidia for the production of standardized inoculant.
DISCUSSION

Endophyte Isolation

Woody endophytes are usually horizontally transmitted. Endophyte assemblages within the sampled chestnut tissue were dependent on exposure to endophyte inocula from the immediate environment during tree growth. High quality woodlands in the North Carolina with large persistent populations of root-sprout *C. dentata* were chosen as sampling sites; these areas are likely to be good sources of general endophyte inocula, and also may still harbor *Castanea*-specific endophyte species. Healthy stems were chosen based on the notion these may be more likely to contain endophytes antagonistic to *C. parasitica*; alternatively, it was likely that stems with active infection did not host endophytes capable of protecting the tree tissue from blight.

Additionally, one medium-aged, chestnut-dominated site from Michigan was also sampled. This site is well outside of the native range of the American chestnut and is also experiencing moderately successful, hypovirus-mediated stand recovery. It was thought that these circumstances may provide unique opportunities to collect different endophyte symbionts compared to trees in North Carolina.

No specific methods were used to exclude any endophyte group; however, it is likely that most of the endophytes isolated were Class 3 fungal endophytes, *sensu* Rodriguez (Rodriguez, et al., 2009). Class 2 fungal endophytes may also have been isolated, but this classification would require knowledge of such an endophyte’s lifecycle and is beyond the scope of the current research.
Study was necessarily limited to endophytes favorable to laboratory culture methods. This work is ultimately intended for the potential development of BCAs from the diversity of chestnut endophytes. For this reason, utilizing methods that fail to capture occult endophytes may have been practically beneficial; fungi that would be onerous to culture for mass inoculation were implicitly selected against.

**Competition Experiments**

**Characterization of *C. parasitica* Test-Strain Pathogenicity**

Each hypovirus-free test strain was inoculated into a common apple in order to minimize differences among apples. However, the hypovirus-infected control group was inoculated onto different apples for practical reasons concerning space and the slower cultivation time of this isolate. Variation among all apples was low, allowing valid statistical analysis of the data.

The difference in growth among the hypovirus-infected isolate and all others was large, confirming that the hypovirus induced a hypovirulent phenotype and that the uninfected isolates were virulent in comparison. Among the hypovirus-free isolates, the WVU isolates Schomberg, EP-155, and Rhyme showed no significant differences in growth rate. However, differences were found for the Bockenauer (significantly less virulent) and the WS-15 (significantly more virulent) isolates. The variation observed within virulent isolates confirms the assumption that basal virulence among hypovirus-free *C. parasitica* is naturally variable. These results emphasize the importance of characterizing the virulence of isolates used in antagonism research and supports the use of multiple isolates for such tests. Additionally, variation in virulence of virus free *C. parasitica* isolates may have implications when utilizing hypovirulence as a biocontrol.
Differences in virulence among isolates —when uninfected by hypovirus— may not seem like an immediately relevant factor when introducing a hypovirulent isolate for biocontrol. However, it must be remembered that conidia and ascospores produced from a virus-containing isolate are often free of the virus (Russin and Shain, 1984). If an isolate that would otherwise show high inherent-virulence is infected with hypovirus and introduced to a stand for the purposes of biocontrol, it may actually serve to introduce new avirulence genes of *C. parasitica* to the blight population, thus strengthening the pathogen population. By choosing isolates within the desired vegetative compatibility type (when multiple options exist) that show the least inherent virulence when virus-free, foresters may be able to limit the introduction of new virulent strains and their corresponding avirulence genes into the forest they are attempting to save. Alternatively, an isolate released as biocontrol must remain fit for survival in a natural environment following release to ensure its persistence and subsequent dissemination when population-scale control is the desired goal (Milgroom and Cortesi, 2004). In order to choose among potential *C. parasitica* donor strains, researchers should characterize the virulence of its virus-free offspring via single spore isolates, while also taking fitness for survival -in a natural setting when infected with hypovirus- into consideration.

**Preliminary Screening and Quantitative Competition Experiments**

*In vitro* screening allows many more potential BCAs to be screened under controlled conditions. However, it inevitably misses endophytes that require *in vivo* conditions to protect their host plant. For instance, an endophyte that benefits its host by activation of systemic required resistance will show negative results in an *in vitro* test. Despite this, *in vitro* screening remains widely used in the field due to practicality.
Recently Shehata et al. (2016) confirmed that \textit{in vitro} and \textit{in vivo} screening methods were highly correlated, indicating this method remains useful.

It was likely that at least some of the endophytes isolated from healthy \textit{C. dentata} shoots were capable of antagonism against \textit{C. parasitica} and may act to protect \textit{C. dentata} from blight invasion and disease \textit{in vivo}. As expected, competition studies showed a range of outcomes, from endophytes incapable of any control of \textit{C. parasitica}, to endophytes successfully antagonizing, and in some cases apparently extirpating, the blight fungus.

\textbf{Characterization of the Endophyte-Pathogen Interaction}

\textbf{Physical Mycoparasitism Observation}

Several known and commercially available BCAs act to inhibit pathogens by physical mycoparasitism. For instance, \textit{Trichoderma} sp., an established BCA against fungal pathogens in general agriculture, is known to interact by hyphal coiling followed by lysing (Cheng et al., 2012). Additionally, the patent for the commercial biological fungicide Prestop, which contains the fungus \textit{Gliocladium catenulatum}, includes descriptions of hyphal coiling that supports its role as an effective antifungal agent since it indicates direct mycoparasitism (Tahvonen et al., 1999). It should be noted that mycoparasites may also act to limit pathogens by additional mechanisms. For instance \textit{Trichoderma} sp. also act to limit pathogens by releasing volatile compounds before physical interaction (Ajith and Lakshmidevi, 2010) as well as indirectly through upregulating the plants systemic acquired resistance (Elad et al., 1998).

Microscopy of the interaction between \textit{C. parasitica} and each of the 15 highest ranked endophytes in this study failed to reveal any direct mycoparasitism, indicating that
antagonism in these cases may be due to an alternative mechanism. This could include exudation of substances with direct antibiotic effects against *C. parasitica*, hyphal interference, or indirect competition for resources including food and space. The nature of the interaction between a potential BCA and its target can have implications for the method of application and range of use. The microscopy results also indicate that, since they probably indirectly attack *C. parasitica*, the usefulness of these endophytes for biocontrol may be most successful if the plant is inoculated with the endophyte prior to *C. parasitica* infection. This prospect inspired the invasion test in this research, of which most of the top-ranked endophytes failed.

**Antibiosis Test**

Having failed to observe any direct mycoparasitism microscopically, an experiment was designed to test the possibility that antagonistic endophytes produced antibiotic substances. This test, in addition to the invasion test, also ensures that the highest ranked endophytes were not simply the fastest growing, as may occur in traditional dual-culture competition tests such as those used in this research for original endophyte sorting. In light of this, it should be noted that the top performing endophyte following the original completion trials, MAMSD3B, which was among the fastest growing in culture, failed the antibiosis and invasion tests (Table 6).

However, among the top performing endophytes, some were in fact excreting active substances into the growth media. Of the supernates that inhibited *C. parasitica* inocula, seven were from C- and D- type endophytes (with an ability to overgrow *C. parasitica*), one was from a B-type endophyte, and two were from A-type endophytes. The lack of inhibition from the supernate of two of the three B-type endophytes may
indicate that *C. parasitica* was inhibiting these endophytes at a distance during the competition experiment, rather than the other way around as originally interpreted. Alternatively, these B-type endophytes might emit volatile, airborne antibiotic compound(s) within the parafilm sealed petri plates that do not remain in the supernate of a liquid culture.

The supernates used in this experiment were from axenic liquid cultures. Therefore, false negatives also may have resulted if the endophyte requires the presence of a competitor (*C. parasitica*) to induce the production of antibiotic compounds. In an attempt to account for this, the agar plug used to inoculate each liquid culture was taken from a dual-culture plate from the competition experiments; though this effort may have been futile if such induction was highly localized to the interaction zone or transient following removal from a dual-culture environment.

**Invasion Test**

The invasion test was developed to test each endophyte’s ability to compete for substrate already colonized by *C. parasitica*. Again, endophytes that ranked at the top following the competition trials (MAMSD3B, M111A, NC3A, M16A, and NC1K) failed to invade a pre-established lawn of *C. parasitica*. Rather, NC3K, which ranked seventh in the original competition trials, rose to the top position following positive results for both antibiosis and invasion ability (Table 6). NC3K is slower growing than MAMSD3B; however, these data suggest NC3K has stronger potential to directly antagonize *C. parasitica*. 
DNA Identification of Endophyte Species

Morphological identification of endophytes is notoriously difficult; many species resist the formation of reproductive structures in culture, and most remain unidentified. Yet accurate identification to species is desirable if endophytes are to be utilized as biological control agents. For this reason, DNA identification was used to putatively identify individual, cultivated isolates that showed biocontrol potential during antagonism screening.

Using only DNA from cultured endophytes provides a limited picture of the actual ecological diversity in the stem’s samples, but it adequately identifies species of interest as biological control agents. Endophyte diversity may be high even within small sections of plant tissue from a single host (Bissegger and Sieber, 1994). Evaluation of the diversity among endophytes within sampled chestnut stems would require next-gen identification to elucidate non-cultivable species and was beyond the scope of this project. Uncultivable or difficult to culture endophytes also precludes the efficient production of inoculum.

The internal transcribed spacers, ITS1 and ITS2, are commonly used in fungal phylogeny (White et al., 1990). These regions of DNA separate the 18S and 5.8S, and the 5.8S and 28S, components respectively. The ITS regions do not code for any functional components of the ribosome. Thus, evolution of these sequences is not subject to selective pressure. These characteristics make ITS regions attractive targets for separating closely related species; the ITS regions will have more variability than sequences that code functional protein or RNA molecules. Ribosomal DNA is also a preferred target for practical reasons. Due to the repetition of rDNA genes in the genome, PCR can
successfully amplify ITS sequences from smaller quantity or lower quality DNA templates than would be possible for a single-copy gene. Through concerted evolution the repeated rDNA genes may remain more similar within individual species than related species, thus providing utility for species identification and phylogenetic analysis.

Sequencing was attempted for the ITS region of 24 of the 109 isolates in order to putatively identify them. This precludes the ability to make statements about the general ecology of the endophyte assemblages of cultivable endophytes isolated from the trees sampled for this research. However, of the endophytes sequenced, all were Ascomycota, which are known to dominate endophyte assemblages (Arnold, 2007; De Errasti et al., 2010; Higgans et al., 2007; Rubini et al., 2005). Among the top 15 ranked endophytes, all except one (MI6A, *Nigrospora* sp.) were within the order, Xylariales, a dominant lineage of endophytes among Fagaceae (Sieber, 2007). The relationship of these top-ranked endophytes is very close.

Interestingly, three groupings of sequenced endophytes had DNA results that matched the same species: MAMSD3B, MI11A, NC1K, NC3A, and NC3T as Sordariomycetes; MI9B, aMI2B, and MI10A as “uncultured fungus clone;” and NC1B1 and NC3V (possibly also NC3K, NC3P, and NC1D) as Sordariomycetes (Table 7). Each of these isolates emerged from different sections of *Castanea* stem tissue, ensuring they are not duplicates of individuals. In the case of the largest grouping, the stem sections originated from both Michigan (MAMSD3B and MI11A) and North Carolina (NC1K, NC3A, and NC3T). No endophytes were excluded at any point if it was suspected that they belonged to the same species.
While some members of these groupings ranked closely together, such as MAMSD3B, MI11A, and NC1K at 10, 11, and 12, respectively, and aMI2B and MI9A at six and seven, each grouping also had members among the sequenced endophytes, separated in rank from other isolates of the same species (Table 6). This variability in antagonism within a species may have occurred more commonly in this study than is apparent from the DNA data. For instance, NC3T, a member of the largest grouping, was ranked 28th overall, having only been selected for sequencing as a representative of the lower-ranked, A-type endophytes due to its strong pigmentation of the media during growth. In contrast, all other members of its grouping (MIMSD3B, MI11A, NC1K, and NC3A) ranked within the top 15 following the competition experiments. These differences in *in vitro* antagonism against *C. parasitica* suggest that, while the ability to overgrow *C. parasitica* may be a common trait among this species (putatively *Biscogniauxia mediterranea*), it is variable and not universal.

Variability among closely related species is certainly a common occurrence among endophytes. Variability within the same species may represent various stages of role switching along a gradient of symbiosis. Many mutualists have descended from lineages containing species pathogenic on the same host plants (Sieber, 2007). Freeman and Rodriguez (1993) showed that a single point mutation was responsible for a switch from pathogen to mutualist endophyte in *Colletotrichum magna*. This concept has also been described among the plant pathogenic bacterium, *Pseudomonas syringae*, which can be effectively controlled with non-virulent variants of the same species (Amijee et al., 1992). Also working with *P. syringae*, Mohr et al. (2008) showed that naturally occurring, non-pathogenic isolates resulted from the loss in ability to translocate effectors
into host plant cells; the isolates were monophyletic and had descended from pathogenic ancestors.

**Further Research**

This work has identified and characterized endophytic species of *Castanea* that are antagonistic to *C. parasitica*. In order to develop any of these candidates into an applicable BCA, more work must be done. Specifically, trials must confirm the candidate endophyte is antagonistic *in vivo* and improves health or survivability of the host tree. In order to carry out *in vivo* trials, effective inoculation methods must first be developed. Potential methods include spraying or injecting young trees with a solution of spores or liquid culture of mycelia.

Spraying aerial plant organs with a spore solution is an attractive option for species that readily produce spores in culture, as the top endophyte in this study, NC3K does. A spore solution can be easily standardized and applied. Also, depending on the life history of the endophyte, it may be the best method for inoculation. For instance, an endophyte that requires an appressorium in order to invade host tissue may be unable to gain entry if applied as a liquid culture of mycelia on the surface of the plant. Success of the chosen inoculation method must therefore be evaluated in a manner that fulfills Koch’s postulate that the organism may be re-isolated from host tissue—or alternatively its presence confirmed through DNA analysis. However, as is a common problem when studying endophytes, neither of these methods proves that the current infection resulted from inoculation or if the plant had already been naturally infected by the same species. Once an inoculation method is shown to effectively introduce the desirable endophyte into the host tree, an *in vivo* assay experiment may be designed.
An in vivo experiment should compare survival and/or disease severity among endophyte inoculated versus uninoculated, nursery grown seedlings or cuttings following an introduced challenge by *C. parasitica*. Alternatively a field study could be designed comparing treated verse untreated natural root sprouts in a wild setting. Any reduction in disease or increase in survival could be considered a positive result. Combinations of antagonistic endophytes may potentially show a synergistic effect (Carroll, 1995), particularly if they act through different mechanisms. The magnitude of any protective effect conferred to the host tree will determine the practicality of using the endophyte(s) as biocontrol. However, particularly among trees with moderate genetic resistance and among unestablished saplings, inoculation with an antagonistic endophyte may prove to be a decisive factor between death and survival.

Research Applications

Research into the biocontrol potential of endophytes of temperate plants has mostly been limited to reduction of insect herbivory (Calhoun et al., 1992; Sumarah et al., 2010; Sumarah et al., 2011; Wulf, 1990). Some of this research has been patented and is currently under commercial application; endophyte inoculated spruce seedlings are being planted with the hope that these stands will have less susceptibility to the devastating spruce budworm (J.D. Irving Limited, 2013; Miller and Adams, 2007).

Investigation of endophytes with the goal that they be developed for use as biocontrol of pathogens has largely been restricted to a few economically important tropical species, primarily the chocolate tree, *Cacao theobroma* (Arnold et al., 2003; Bailey et al., 2008; Hanada et al., 2008; Herre et al., 2007; Mejia et al., 2008), and the rubber tree, *Hevea brasiliensis* (Abraham, 2013; Gazis, 2010). One previous study has
identified an endophytic *Bacillus subtilis* of European chestnut, *C. sativa*, with antagonistic properties against blight (Wilhelm et al., 1998). The current research broadens the literature on this concept by investigating the biocontrol potential of endophytes of *C. dentata* for the first time.

Endophytes have been targeted as organisms with potential for the use in biocontrol of harmful plant pathogens and insect pests (Rodriguez et al., 2009). The successful identification of endophytes capable of biocontrol of *C. parasitica* offers new strategies to combat the invasive chestnut blight, ultimately helping to restore American chestnuts in Eastern forests and benefiting commercial chestnut farmers. Exploitation of endophytes may offer a strategy to improve blight resistance and tree survival during reintroduction efforts complementary to those already underway (conventional genetic resistance, hypovirulent *C. parasitica* isolates, and genetic modification). Specifically, application of antagonistic endophytes may improve survival among out-planted, nursery produced seedlings; improving nursery seedling quality has been implicated as primary target for improved success among reintroduction efforts (Clark et al., 2012).

As discussed previously, endophytes are highly diverse, even within small sections of stem. This diversity may prevent the host plant from experiencing a significant protective effect, even if an endophyte antagonistic to a pathogen is present. However, if trees or seedlings are saturated with a known, highly antagonistic endophyte a significant protective effect may be possible. This would likely be restricted to trees that have been heavily inoculated and would not persist and spread in a natural stand at a level necessary for population level protection. Persistence of BACs in a population is ultimately the goal of many biocontrol efforts, including hypovirus-mediated biocontrol.
Despite this limitation, endophytes have promise for the protection of desirable individual trees, chestnut orchards, and nursery grown seedlings used in reintroduction efforts.
REFERENCES


American Chestnut Cooperators’ Foundation. 2016. www.accf-online.org


———, 1995. Forest endophytes- pattern and process. Canadian journal of Botany. 73, S1316-S1324.


Hebard, F., no date. Soil compresses for curing individual chestnut blight cankers. The American Chestnut Foundation- MA Chapter.


Liu, Y., Milgroom M.G., 1996. Correlation between hypovirus transmission and the number of vegetative incompatibility (vic) genes different among isolates from a natural population of Cryphonectria parasitica. Phytopathology. 86(1), 79-86.


