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Graduate Studies

BIOLOGICAL CONTROL OF *CRYPHONECTRIA PARASITICA* WITH *STREPTOMYCES* AND AN ANALYSIS OF VEGETATIVE COMPATIBILITY DIVERSITY OF *CRYPHONECTRIA PARASITICA* IN WISCONSIN, USA.

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BIOLOGICAL CONTROL OF *CRYPHONECTRIA PARASITICA* WITH *STREPTOMYCES* AND AN ANALYSIS OF VEGETATIVE COMPATIBILITY DIVERSITY OF *CRYPHONECTRIA PARASITICA* IN WISCONSIN, USA.

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ABSTRACT

Smith, A.S. Biological control of *Cryphonectria parasitica* with *Streptomyces* and an analysis of vegetative compatibility diversity of *Cryphonectria parasitica* in Wisconsin, USA. MS in Biology, December 2013, 52pp. (A. Baines)

The American chestnut tree (*Castanea dentata*) has been plagued by the fungal pathogen *Cryphonectria parasitica*. While the primary biological control treatment has relied upon the use of hypovirus, a mycovirus that reduces the virulence of *C. parasitica,* here the potential for a *Streptomyces* inoculum as a biological control is explored. Two Wisconsin stands of infected chestnut in Galesville and Rockland were inoculated with hypovirus and *Streptomyces* using a randomized block design. At these stands the *Streptomyces* treatment reduced canker length expansion rates more than the hypovirus treatments and control. The *Streptomyces* treatment had significantly lower canker width expansion rates compared to the control. In addition to having reduced canker expansion rates, the trees inoculated with *Streptomyces* had the lowest mortality rate. The diversity of the fungus was low at the study sites and consisted of only two known vegetative compatibility types at each stand. This low level of diversity made it ideal for hypovirus dispersal, and for limiting canker expansion rates. This research supports the hypothesis that *Streptomyces* treatment is an effective alternative to hypovirus treatment that may prove beneficial in areas where hypovirus efforts have failed.

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INTRODUCTION

Disease History

American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) once dominated the forests of the eastern United States, growing upwards of 37 meters tall and 1.5 meters in diameter (Anagnostakis 1987). The growth rates of the trees exceed that of other forest species like black walnut *(Juglans nigra* L.) and northern red oak (*Quercus rubra* L.) (Jacobs and Severeid 2004). In addition to its fast growth rates, the chestnut tree was prized for its rot resistant lumber. With high tannin levels the lumber for *C. dentata* is as resistant to rot as redwood and was highly sought for use in railroad ties, telephone poles, and fences (Burnham 1988), however, the United States saw its first case of chestnut blight in 1904 in New York City (Merkel 1905). The causal agent of chestnut blight was later determined to be the fungus, *Cryphonectria parasitica* (Murr). Barr. (Previously *Endothia parasitica*). *Cryphonectria parasitica* spread at an exponential rate and had infected nearly all the eastern trees by 1940 (Figure 1) (Gravatt 1949, Karban 1978).

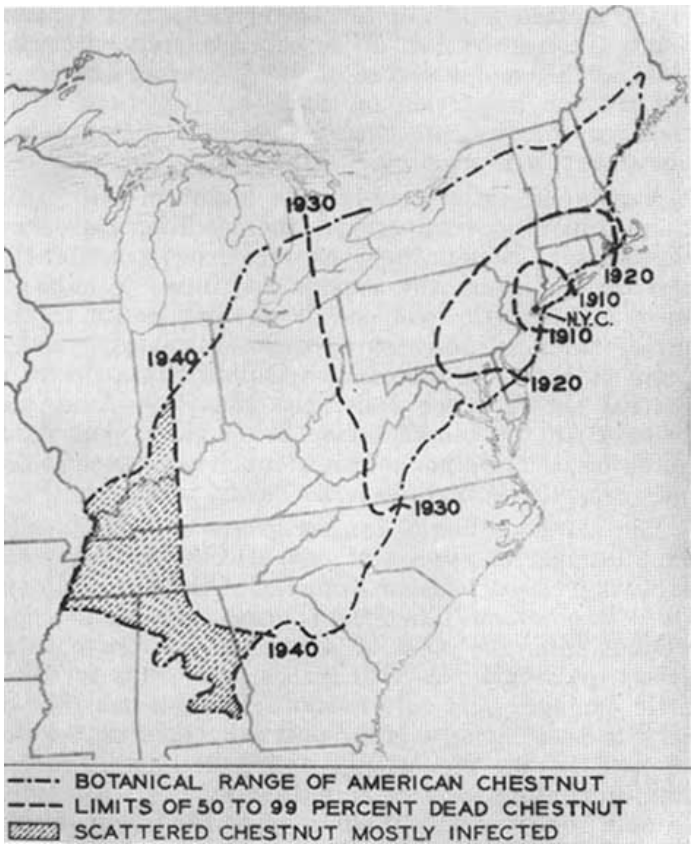

Figure 1. The spread of *Cryphonectria parasitica* across the natural range of *Castanea dentata* in the Eastern forests of the United States (Gravatt 1949).

Some stands in the upland forest communities of Virginia continue to persist as sprouts (Stephenson *et al.* 1991) while others within the same region have seen the complete loss of the American chestnut and the replacement of the chestnut by different species like the red oak (*Quercus rubra L.*) (Agrawal and Stephenson 1995). Since the introduction of *C. parasitica* most remaining chestnuts have been reduced to sprouts that emerge from the root collar. Most sprouts do not live to sexual maturity, furthering the devastation caused by this disease (Paillet 2002).

Within the genus *Castanea*, the disease is able to develop much faster in *Castanea dentata* because of less resistance in the American chestnut compared to their European and Asian relatives. Asian trees in particular are much more resistant to the fungus; one

reason for this resistance is increased genetic variability in these trees in comparison to American chestnuts (Dane *et al.* 2003, Griffin *et al.* 1983). The range of genetic diversity between Asia, Europe and North America is most likely caused by the westward expansion of the pathogen from Asia through Europe to North America. The phylogeny of the *Castanea* genus was estimated based on DNA sequence data, showing that the Chinese chestnut (*Castanea mollissima*) and the Japanese chestnut (*Castanea crenata*), which have the greatest resistance, have been around for millions of years longer than the European (*C. sativa*) and North American species (*C. dentata*) (Lang *et al.* 2007). The Chinese and Japanese chestnut trees evolved alongside the *C. parasitica* allowing it to have a natural resistance to the pathogen. This natural resistance is not seen in the European and American varieties and the outbreak of *C. parasitica* quickly lead to the loss of nearly all the mature American chestnut trees within the native range, as they had never before encountered this pathogen. European stands initially lost great numbers of trees but were able to survive the disease outbreak due to naturally occurring hypovirus and a slight increase in genetic resistance in comparison to the American chestnuts (Heiniger and Rigling 1994, Robin and Heiniger 2001).

Within North America, another factor that is important in the differential survival of *C. dentata* in the presence of disease is the genetic diversity of the trees. The stands in this study (discussed later) were founded by only a few individual trees which subsequently have given rise to hundreds of offspring at the Rockland, WI and Galesville, WI stands (Personal communication Larry Severeid, Philip Lunde). These stands represent an interesting scenario where the trees likely have limited genetic diversity due to founder effect. Other studies have observed similar phenomena; for

example, a group of Japanese beech trees (*Fagus crenata*) was isolated following the cutting of trees in the stand and the resulting decreased genetic variability was attributed to founder effect (Takahashi *et al.* 2000). Planted stands of a tree species in general contain less genetic diversity than the trees of that same species in a natural stand. Planted stands of the South American fruit tree *Inga edulis* have been found to be less genetically diverse than naturally occurring stands with an allelic richness that decreased from 39.3 to 31.3 with an approximate loss of 20% of alleles (Hollingsworth *et al.* 2005). Decreases in diversity can greatly limit the ability of the populations to survive following the introduction of a pathogen, as they may have lost the alleles to fight off these invaders by random chance. If resistance alleles are lost in a planted stand, the likelihood that the population will be able to survive the outbreak of a pathogen will be greatly decreased.

Life Cycle of *Cryphonectria parasitica*

The chestnut blight fungus is a member of the Phylum Ascomycota, the largest phylum of Fungi with approximately 64,000 described species. Ascomycetes have diverse roles as decomposers, mutualists, and pathogens of plants, other fungi, and animals (Schoh 2009). *Cryphonectria parasitica* is dispersed by the release of spores from stroma. The stroma is a structure on the fungus that contains both a perithecium where the sexual ascospores are produced and a pycnidium where the asexual conidia are produced.

The type of reproduction is determined by environmental factors like the nutrition available, temperature, and light. Sexual reproduction can be induced by different factors such as nitrogen starvation, light, and cold temperatures. Under these conditions and when both mating types, MAT-1 and MAT-2, are present a conidium with one mating

type, which acts as a male, fertilizes a receptive hypha of the other mating type. The cytoplasms of the conidium and hypha undergo plasmogamy to form a dikaryon. An ascus is the cell where sexual spores are produced. The ascus is formed as a result of crozier formation (Casselton 2002). Within the ascus, the haploid nuclei from the two fungi will fuse via karyogamy. This diploid nucleus then undergoes meiosis resulting in the formation of four haploid nuclei. These nuclei then divide by mitosis forming 8 haploid nuclei. These nuclei then separate in the cytoplasm and spore walls form around the nuclei and get incorporated into the ascospores within the developing ascus by free cell formation. These ascospores are expelled from an opening in the neck of the perithecium during periods of rain in spring, summer, and autumn. It is generally accepted that these spores are carried by wind and dispersed to nearby trees where they can infect and begin the cycle again (Guerin *et al.* 2001, Marra and Milgroom 2001, Nelson 1996).

In addition to sexual reproduction, which is very important for creating diversity within a population, *Cryphonectria parasitica* also disperses via spores produced by asexual reproduction inside of flask shaped structures called pycnidia. These conidia are asexual spores that are a product of mitosis and are genetically identical to the mycelium. The spores are borne on specialized structures called conidiophores, inside the pycnidia. Conidia are released from the opening of the pycnidia within the stroma. The conidia are then generally dispersed to nearby trees by carriers such as insects and birds as well as rain (Anagnostakis 1987, Archer 1924, Studhalter and Ruggles 1915). Both sexual and asexual spores of the fungus enter through natural openings as well as wounds in the chestnut bark, grow, and destroy the tissues down to the cambial layer of the tree. The

cambial layer is essential for production of the xylem and phloem. Once a tree branch or trunk has become girdled, the production of these layers and therefore the transport of water and nutrients are greatly reduced. The fungus does not infect the roots and often the tree will send up many sprouts when the main stem has been infected or damaged. These sprouts are usually re-infected and killed by the fungus as the bark splits during secondary growth, before they can reach sexual maturity (Karban 1978). While all trees are susceptible to infection, smaller branches are more likely to become girdled and die (Davelos Baines *et al.* 2013).

The growth rate of *C. parasitca* differs between the American Japanese and Chinese chestnut. In American chestnut trees the average growth of cankers has been quantified at approximately 1 mm/day in diameter while in the Chinese chestnut (*Castanea mollissima*) and Japanese chestnut (*Castanea crenata*) cankers expanded at 0.2 to 0.6 and 0.08 to 0.2 mm/day in diameter respectively (Anagnostakis 1992).

Treatment and Control Efforts

Many attempts have been made to stop or slow the spread of this fungus. Breeding programs have been designed to breed resistance of Asian chestnut trees into American chestnut trees. The breeding is performed by hybridizing American and Chinese chestnuts and then backcrossing three or more times to the American chestnut. The goal of the backcrossing is to re-establish the American chestnut phenotypic characteristics like its small sweet nut, or tall straight stature that had been lost or decreased from the initial hybridization. While maintaining the American chestnut phenotype is part of the struggle, the main goal is to establish the resistance associated with Chinese chestnut trees (Griffin 2000, Jacobs 2007).

The most common treatment to combat *C. parasitica* is the use of hypovirus as a biological control. Hypovirulence, which results in the reduced pathogenicity of *C. parasitica*, can be an effective control because the virulence of *C. parasitica* is weakened. The virulence is weakened by viruses from the *Hypoviridae* family that infect the fungus and can slow growth and reduce sporulation (Liu and Milgroom 1996, Milgroom and Cortesi 2004). Hypovirus is spread between isolates of *C. parasitica* of the same vegetative compatibility group (see below) by anastomosis. Following the fusion of the hyphae, cytoplasm is exchanged and virulent isolates of *C. parasitica* can be transformed from virulent to hypovirulent strains (Choi and Nuss 1992).

The hypovirus is a mycovirus that is a cytoplasmic double-stranded RNA (dsRNA) that can infect *C. parasitica*. Mycoviruses differ from most viruses as they do not have extracellular dispersal or produce a capsid. This infection can be detected in the field as hypovirulent cankers have swollen bark that appears calloused with minimal stromata. Virulent cankers have a different appearance, described as sunken bark with numerous stromata. This appearance is caused by the destruction of the cambial tissues. Hypovirulent cankers were first noted in 1964 when recovering chestnut trees were found in Italy (Anagnostakis 1987). This swollen appearance shows healing in the tree, as the trees are able to wall off the infection, but not remove it completely (Figure 2) (Anagnostakis 1987, McManus *et al.* 1989, Milgroom and Cortesi 2004).

Figure 2. *Cryphonectria parasitica* cankers both virulent (left) and hypovirulent (right) on American chestnut trees.

The alternate appearance of the hypovirulent cankers can be traced back to action of the hypovirus which reduces growth and sporulation in *C. parasitica*. The hypovirus and all mycoviruses differ from most viruses, as they do not have a capsid which greatly limits its dispersal because the virus cannot exist outside of its host. The hypovirus can only infect *C. parasitica* through the connection of the cytoplasm of and already infected *C. parasitica* hyphae (Hillman et. al 1994). There are four *Cryphonectria* hypovirus (CHV) types, CHV-1, CHV-2, CHV-3, and CHV-4 (Turina and Rostagno 2007). The *Cryphonectria* hypoviruses and their subtypes have varying genetic sequences that can be detected using sequencing techniques (Gobbin *et al.* 2003). CHV-1 is native to Europe and it is associated with pigment and sporulation reduction. Five subtypes of CHV-1 have been identified, and within these subtypes, CHV-1 subtype I is dominant and widespread throughout Europe, while the other four were found at much lower frequency

in France, Germany, and Spain (Allemann *et al.* 1999, Milgroom and Cortesi 2004, Turina and Rostagno 2007). CHV-2 has been identified in New Jersey and is characterized as the most debilitating in terms of its effects on virulence in the fungus but also with only slight reductions in pigment production and sporulation (Hillman *et al.* 1994, Liner-Basso *et al.* 2005). In the lower peninsula of Michigan, the CHV-3 is naturally occurring in the area and has allowed trees in local stands to survive (Fulbright *et al.* 1983, Milgroom and Cortesi 2004). CHV-4 is the most common in North America and most likely spread very early in the history of the epidemic when fewer vegetative compatibility (vc) types were present than today (see below). CHV-4 has undergone little genetic change since it spread decades ago and the virus exhibits little effect on the virulence of the fungus. As a result, it is not an effective agent for biological control (Linder-Basso *et al.* 2005, Milgroom and Cortesi 2004).

All *Cryphonectria* hypoviruses are characterized by the presence of ORF (open reading frame) B (3,165 codons), which contains the protease p48. This ORF is necessary for the establishment of the infection, and when mutated, infection cannot occur (Deng and Nuss 2008). Some types of virus, for example, the CHV-3 isolate Grand Haven 2 (GH2), only contain ORF B. ORF A encodes polyprotein p69, which is then processed into p29 and p40 (Suzuki and Nuss 2002). These two proteins are thought to be responsible for the reduced sporulation and conidiation seen in these fungi (Suzuki and Nuss 2002). The effects of GH2 inoculation are different and lessened in comparison to other hypoviruses, suggesting that ORF A has some effect on the fungi. Viruses that have both ORF A and ORF B, CHV-1 and CHV-2, for example, show a much greater reduction in sporulation and conidiation (Smart et. al 1999).

The Effect of Hypovirus Transmission by Vegetative Compatibility

Overall, hypovirus transmission into conidia can range from 1 to 99% (Nuss 2005). Transmission is critical to the success of the biocontrol of *C. parasitica,* because without spread of hypovirus to fungal isolates infecting new cankers on trees and to neighboring trees, each new canker would have to be individually treated (Milgroom and Cortesi 2004), which is impractical. Transmission of the hypovirus is limited by vegetative incompatibility within the fungus. Vegetative incompatibility is a self/non-self-recognition system, controlled by six unlinked two allele vegetative incompatibility (*vic*) loci (Cortesi and Milgroom 1998, Liu and Milgroom 1996). When one *vic* gene varied transmission was 48-50%. When two *vic* genes varied transmission was reduced to 13 to 14%. When more than two *vic* genes differed between fungal isolates the chance that successful transmission of the virus that occurred was further reduced to 3-4% (Liu and Milgroom 1996). This research suggests that populations with less diverse vegetative compatibility types will have the greatest potential success of establishing a successful biocontrol using hypovirus treatment.

The number of vegetative compatibility (vc) types in stands and regions varies greatly depending on the number of infections, if sexual reproduction is occurring, and the time since infection. The West Salem stand in Wisconsin contains 15 vc types (Mark Double, personal communication). In Michigan, 30 vc types have been identified (Springer *et al.* 2013). A study throughout Italy detected 20 vc types (Cortesi *et al.* 1996),while in Macedonia and Greece only 5 vc types were detected (Sotirovski *et al.* 2004). In forest and orchard sites in southern France there were 30 known vc types and 10 isolates with unknown vc types that did not match any previously identified strains

(Robin *et al.* 2000). In Japan and China there is high vegetative compatibility diversity. When 79 *C. parasitica* isolates in Japan were analyzed 71 of the 79 had unique vc types, and when *C. parasitica* isolates were tested in China, 54 of 64 isolates had unique vc types (Liu and Milgroom 2007).

Many sites in the United States and Europe have dominant vc types even when greater vc diversity is present. In Wisconsin at West Salem, WS-1 is dominant, comprising 85% of the population (McGuire *et al.* 2005). In Michigan many stands are dominated by one vc type or a combination of a few vc types (Springer *et al.* 2013). Stands in Italy are dominated by I-1, 1-2, and 1-5 in the North, while I-10 and 1-12 are most prevalent in the southern populations (Cortesi *et al.* 1996). Similarly, in the study looking at the vc diversity of Greece and Macedonia 96% of the isolates were vc type EU-12 (Sotirovski *et al.* 2004). Of the more than 30 vc types detected in France, four were most frequent, with EU-33, EU-2, EU-72, and EU-66 constituting approximately 70% of the population (Robin *et al.* 2000).

Effects and Limitations of Hypovirus Treatment

Across the United States hypovirus has had very mixed results. In Michigan, the naturally occurring CHV-3 hypovirus is associated with recovery in several local populations, where trees are diseased but able to coexist with the fungal pathogen (MacDonald and Fulbright 1991). In Wisconsin, CHV-3 and CHV-1 have been used to treat trees, with moderate levels of success. Many of the treated trees had calloused cankers and 82% of the trees treated with hypovirus contained hypovirus five years after treatment (Milgroom and Cortesi 2004). Other areas of the United States (e.g., West Virginia, Connecticut, and Virginia) have also used hypovirus treatments, but have had

lesser degrees of success. In all three states, use of the hypovirus had some success treating individual cankers on single trees, but where the treatment fell short, and continues to disappoint, is in the lack of horizontal transmission (Liu *et al.* 2002, Milgroom and Cortesi 2004).

Outside of the United States hypovirus tends to spread more readily, and in cases where treatments have been used, the results have been much more positive. In southeastern France, 31 to 90% of cankers contain CHV-1 hypovirus (Robin *et al.* 2010). Naturally occurring hypovirus CHV-1 is also present in Slovenia where it was found in six out of seven populations tested with frequencies from 11.1 to 72.2% (Krstin *et al.* 2011). In this example vc type diversity was high with 15 types present, but the stand was dominated by only a few vc types. This result somewhat contradicts findings that the dispersal of hypovirus should be higher in populations with low diversity, as transmission was as low as 11.1% (Liu and Milgroom 1996). This low transmission level can be partially explained by high levels of sexual reproduction of *C. parasitica* like what has been documented in the adjacent country of Croatia, and also by the substantial levels of naturally occurring hypovirus, resulting in these variable findings. In Croatia, biocontrol could be successful because of the naturally occurring hypovirus, but the sexual reproduction of the fungus should increase the vc diversity resulting in less horizontal transmission (Krstin *et al.* 2008).

The geography of the population can also play a large role in the dispersal of hypovirus. In Northwest Spain for example only 15 of 539 *C parasitica* isolates collected were hypovirulent despite that only six vc types were present. In this study, only the León province contained hypovirus, but it is separated from the other regions of

study by a mountain resulting in only approximately 3% of the isolates containing hypovirus (Montenegro *et al.* 2008).

From these studies it is clear that the success of hypovirus in an area is dependent on several important factors. The first is applying the sub-type of the hypovirus with the highest invasiveness or greatest ability to spread in an area, the second is working in an area of low vc diversity with the lowest amount of sexual reproduction by the fungus to minimize an increase in vc types, and the third is that successful hypovirus spread can only occur in areas that lack physical barriers that would otherwise impede its spread. In the United States, despite this knowledge and strategies, it has not been enough to stop the spread of the disease even in ideal treatment conditions. Originally, the West Salem, WI stand was seen as an ideal site for hypovirus treatments, since it had a relatively low number of vc types and was dominated by type WS-1 (McGuire *et al.* 2005). However, counter to what was first thought, the pathogen was spreading faster than the hypovirus (Jarosz *et al.* 2002).

Use of *Streptomyces* to Combat Fungal Pathogens

Treating with hypovirus has been the focus of study in the field for decades and as populations of American chestnut trees continue to disappear it is important to look at less utilized avenues that might yield more success. One of these avenues is the use of mudpacks, which has been shown to inhibit fungal growth (Larry Severeid, personal communication). These mudpacks are essentially soil, rich with *Streptomyces*, which are members of the Streptomycetaceae family and are aerobic, gram-positive bacteria (Dworkin et al 2006). The life cycle of *Streptomyces* consists of a DNA replication phase followed by the filamentous growth forms, primary filaments, secondary filaments, and

finally division. Specifically, spores germinate and complete vegetative growth to form the vegetative filaments and then undergo aerial filaments to form aerial filaments. These aerial filaments then septate and produce chains of spores that can be pinched off and triggered to germinate, giving rise to the next generation (McGregor 1954, Tillotson *et al.* 1998).

Streptomyces has a long history in the development of antibiotics such as early work with streptomycin and neomycin in the 1940s (Waksman and Lechevalier 1949). Antibiotics are produced in these bacteria after the soluble nutrients in their surrounding environment have been exhausted. In *Streptomyces,* each compartment secretes species-specific antibiotics along with enzymes to degrade insoluble nutrients. This dual secretion protects this food source from other micro-organisms that might try to utilize these now readily available nutrients, and additionally these now dead potential competitor bacteria become nutrients for *Streptomyces* (Chater 2006).

In addition to their ability to kill bacteria, several *Streptomyces* species have been identified as containing chitinolytic enzymes. These enzymes could make *Streptomyces* a very effective biocontrol of fungi like *C. parasitica* since they are capable of degrading the chitin in the fungal cell walls (Narayanna and Vijayalakshmi 2009). The chitinase activity was first documented in 1996 where researchers isolated a protein that contained the activity from *Streptomyces* N174 and reported its x-ray crystal structure (Marcotte *et al.* 1996). Some *Streptomyces* strains also produce proteases that have antifungal effects by inhibiting spore germination and the extension of hyphae (Singh and Chhatpar 2011). Using these approaches the *Streptomyces* have a unique advantage where they can remove potentially competitive fungi and maximize their access to resources.

Various *Streptomyces* isolates have shown promising results in the field as effective agents of biocontrol against a variety of pathogens. *Streptomyces globisporus* was found to inhibit mycelial growth on many pathogenic fungi especially *Magnaporthe oryzae* the causal agent of rice blast disease as well as against *C.parasitica* and *Bipolaris maydis* on solid media (Li *et al.* 2011). Specifically, the *S. globisporus* JK-1 had an inhibition zone of 15.0 ± 0.9mm against *M. oryzae*, 13.4 ± 1.3mm against *B. maydis*, and 11.3 ± 1.0mm against *C. parasitica*. In a study with the fungal infection bird's eye spot disease in tea leaves, *Streptomyces* acted as an effective barrier to the entry of new pathogens and was the best biocontrol tested at reducing infection in trail plots (Balasubramanian *et al.* 2012). *Streptomyces* sp MJM5763 was found to be an effective biocontrol against yam anthracnose, which is caused by the fungus *Colletotrichum gloeosporiodes*. The treatment reduced the severity of the disease by 86% and the frequency by 75% in field settings (Palaniyandi *et al.* 2011).

RESEARCH OBJECTIVES

Current hypovirus treatments have proven ineffective in the majority of tested stands in the United States. If the chestnut trees that are still in existence are to survive, additional treatment options should be explored. The first goal of this study was to characterize the local vc diversity, in eforts to effectively introduce the selected hypovirus CHV-1. The second goal was to identify a *Streptomyces* isolate or group of isolates that could be used to effectively inhibit growth of all known Wisconsin strains of *Cryphonectria parasitica* in the lab setting. The third goal was to compare hypovirus and *Streptomyces* treatments on American chestnut trees at two Wisconsin stands. At each location trees infected with *C. parasitica* were selected and then divided evenly into three groups including hypovirus treatment, *Streptomyces* treatment, and no treatment which served as a control. Over the course of September-May the cankers were measured to determine the expansion rates of the *C. parasitica* cankers.

MATERIALS AND METHODS

Study Sites

The stand at Galesville is located around 10 miles south of the city center of Galesville, WI and contains several hundred American chestnut trees all which can be traced back to approximately 12 large parent trees that were planted in 1865 from seed obtained from Pennsylvania. Chestnut blight was first detected at the Galesville stand between 2009 and 2010 and spread quickly throughout the property infecting nearly all the American chestnut trees (Phil Lunde, personal communication). The stand in Rockland, Wisconsin contains hundreds of American chestnut trees that were grown from seed obtained from the Galesville, WI stand and from a stand near Trempealeau, WI between 1995 and 1996. Chestnut blight was detected within the last decade at Rockland, WI (Jacobs and Severeid 2004).

Cryphonectria parasitica Isolation and Vegetative Compatibility Testing

Bark samples were obtained by cutting a section of the outer bark from an active canker using a flame sterilized scalpel. Fungal isolates were cultured by placing approximately 300mm^2 sections of bark in a 0.3% bleach solution for one minute to surface sterilize the sample. Four to six bark pieces, approximately 25 mm^2, were placed on a PDA (potato dextrose agar) (Difco) plate and grown for 2-4 days. The mycelium that grew from the edges of the bark was subcultured on a new PDA plate. Once pure cultures of these isolates were obtained, single spore isolates were generated for each

sample. An 5 mm diameter plug was taken from the edge of an actively growing culture on PDA and used for serial dilutions in sterile distilled water; 100 µL of the 10^{-3} and 10^{-4} dilutions of the conidia suspension were spread plated on water agar plates. The plates were checked daily under the dissecting microscope for the presence of germinating spores. An individual spore was selected from each sample and placed on a PDA plate. This single-spore isolate was then used in the vegetative compatibility (vc) testing.

The vc testing was conducted on bromocresol green media: 2.4% (w/v) PDA pre-mix (Difco), 1.5% Agar, 0.7% malt extract (Difco), 0.2% yeast extract, 0.06% tannic acid, 0.005% bromocresol green (Mark Double, personal communication). In Wisconsin, 15 standard vc types are present (Mark Double, personal communication). At the Rockland site 25 isolates were tested to determine the most common vc types. The field-collected single spore isolates were paired once against all the known WI vc types. The ability of the isolates with different vc types to anastomose is limited by their vegetative incompatibility (*vic*) loci. If the two samples did not match at these *vic* alleles they formed a zone of inhibition due to hyphal death (see Figure 5). The vc type of an unknown isolate is presumed to be the same as a known isolate with which it successfully forms anastomoses. In this initial study the vc type WS-2 was found to be dominant, making up 60% of the population, and was selected for use in this study. The actual number of vc types might be underestimated due to homoallelism, or alternate forms of the gene.

Streptomyces Inoculum

To select a *Streptomyces* isolate to test as a biological control in the field 16 isolates were chosen that were previously obtained from agricultural soils in Minnesota or Texas (Davelos *et al.* 2004; Martinez Blanco *et al.* 2007). These isolates were initially screened against a randomly selected Wisconsin isolate of *C. parasitica* (WS-6) to determine their potential biological control effects towards the pathogen *in vitro*. 5µL of each *Streptomyces* spore suspension (10^6 to 10^8 spores/µl in 20% glycerol) was pipetted onto a water agar plate and grown at room temperature for three days. A 5 mm diameter plug of *C. parasitica* (isolate WS-6) was placed 3 cm away from the *Streptomyces* isolate. Isolates were then grown for 10-14 days. The zone of inhibition was measured at four positions (Figure 3). The zone of inhibition for each *Streptomyces* isolate was calculated as the mean of these four measurements. In the laboratory setting, 14 of the 16 isolates could inhibit the growth of *C. parasitica* (Table 1).

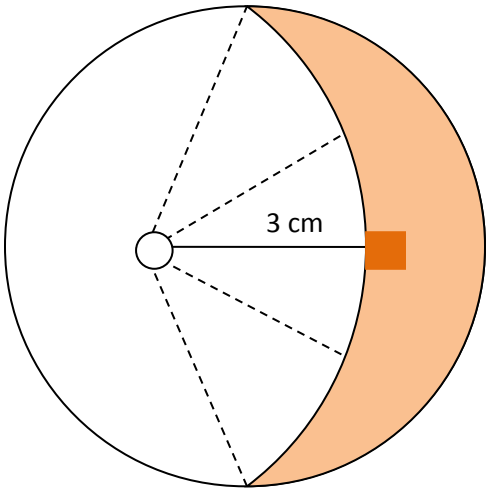

Figure 3. Measuring Zones of Inhibition (dashed line) between *Streptomyces* (circle) and *Cryphonectria* (square).

Table 1. Mean length of inhibition zone (mm) (SD) of *Streptomyces* isolates inhibiting *Cryphonectria parasitica* isolate WS-6.

Streptomyces Isolate	Mean Inhibition Zone Size (mm) (SD)
LK4-20	20.25 (5.90)
LK4-2	16.25 (6.80)
WI2E5	15.88 (5.25)
DL93	15.50 (6.99)
LK4-24	14.50 (9.25)
WI1B5	11.88 (3.00)
LK2-12	11.88 (3.09)
DL87	10.13 (3.52)
WI2E3	10.12 (4.55)
LK4-21	8.00 (7.33)
WI1B2	4.13 (4.61)
WI2E4	3.75 (4.55)
WI1B4	2.38 (2.56)
LK4-16	1.50 (0.76)
WI242	0.00
WI243	0.00

The isolates LK4-2, LK4-20, and WI2E5 were determined to have the largest mean zone of inhibition against the randomly selected WS-6 vc type. LK4-2, LK4-20, and WI2E5 were then used in vc testing against all 15 Wisconsin *C. parasitica* vc types. From this single round of testing the objective was to choose one individual isolate that inhibited growth of all known Wisconsin vc types, or if necessary a group of isolates that together inhibited all Wisconsin *C. parasitica* vc types. The goal was to obtain the biocontrol that would most limit canker expansion in stands with any number of vc types. In the laboratory setting both isolates LK4-2 and LK4-20 were capable of inhibiting the growth of all known local *C. parasitica* strains. Isolate WI2E5 failed to inhibit growth of 3 of the 15 WI vc types. Isolate LK4-2 had the largest average zone size and was selected for use in the biocontrol field study (Table 2).

Table 2. Mean size of inhibition zones (mm) (± SD) of *Streptomyces* isolates with the highest level of inhibition paired with all known WS *Cryphonectria parasitica* isolates.

	LK4-20	LK4-2	WI2E5
Mean Inhibition (mm) (± SD)	21.1 (3.0)	15.2 (2.2)	11.5 (6.1)

Inoculum Preparation

The hypovirus inoculum was prepared by growing a strain of *C. parasitica*, WS-2 containing hypovirus CHV-1 Euro7 for 10-14 days on PDA. To prepare the virus/fungi inoculum, the entire contents of 20 plates was mixed with 1 L of fragmented 2.5% water agar and 1.5 L of sterile water in a non-sterilized blender and blended for 3 minutes. The prepared mixture was refrigerated until used to inoculate the trees (personal communication, Mark Double).

Streptomyces inoculum was prepared by placing the most effective *Streptomyces* isolate (LK4-20) in 1L of nutrient broth (Difco) and allowing the isolate to grow for seven days on a shaking platform at room temperature. The liter of the nutrient broth containing the *Streptomyces* was blended with a non-sterilized blender for 3 minutes with 1L of fragmented 2.5% water agar to obtain a consistency similar to the hypovirus inoculum and stored in the refrigerator until used.

Tree Selection

At the Galesville and Rockland, WI locations, trees were selected to receive future treatment based on the following criteria:

1. Live trees
2. On each tree, a killing canker is present, denoted by active stroma and a sunken appearance relative to the surrounding tissues (Anagnostakis 1987, McManus *et al.* 1989, and Milgroom and Cortesi 2004).
3. The tree has not received any previous treatments

Treatment Application and Evaluation

In September 2012, at each of the two sites, 30 trees were selected and one canker per tree was marked to receive the treatments. Prior to treatment, bark samples of the active cankers were taken and vc testing was conducted on the isolates obtained as described above. After tree selection and sampling the inocula were prepared as described above.

For each canker, the longest and widest active portion of the canker was measured using a measuring tape. After 3 weeks, the cankers were measured again to establish baseline growth rates of the cankers in the absence of treatment. Baseline growth rates varied significantly. Using a two way ANOVA the average change in the baseline growth of length (9/10/2012 – 10/1-2012) was found to vary significantly between the two sites and the trees assigned to each treatment groups at the sites and treatment trees. There was not a significant interaction between site and trees selected for treatments in the baseline length data (Table 3). The average change in baseline growth of width did not vary significantly between sites but did vary significantly between trees at the sites. There was not a significant interaction between site and trees selected for treatments in baseline width data (Table 4). Because baseline data varied significantly, tree-specific adjustments were used to standardize post-treatment canker expansion values.

Table 3. Two way analysis of variance of baseline *Cryphonectria parasitica* canker length expansion.

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Site	0.664	1	0.664	5.321	0.025
Treatment	1.039	2	0.52	4.164	0.021
Site * Treatment	0.314	2	0.157	1.257	0.293
Error	6.738	54	0.125		

Table 4. Two way analysis of variance of baseline *Cryphonectria parasitica* canker width expansion.

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Site	0.081	1	0.081	0.883	0.352
Treatment	0.792	2	0.396	4.343	0.018
Site * Treatment	0.410	2	0.205	2.249	0.116
Error	4.652	51	0.091		

Treatments were applied randomly. The stands were separated into blocks of 10 trees. Treatment or control was used based on random selection of the blocks. At each site, ten of the cankers received hypovirus treatment. Hypovirus treatment was applied by drilling a series of holes approximately every 3 cm with a power drill using a 1/4 inch drill bit on the periphery of the canker and then injecting the hypovirus treatment into these openings using a squirt bottle. On another 10 trees the *Streptomyces* treatment was applied using the same method. At the final 10 trees, the same series of holes was drilled around the periphery of the canker but no treatment was applied. Canker length and width was measured every 3 weeks throughout the fall and once again in the spring for a total of 3 additional measurements of canker expansion. Growth of cankers for each

measurement date (other than the initial date) was calculated by subtracting the previous measurement from the current measurement for both length and width.

The canker expansion measurements were adjusted by finding baseline expansion and dividing by the average change for each stand. The post treatment changes in expansion were divided by the baseline adjustment. This ratio allowed for the data to be standardized relative to the changes in canker expansion of the average tree. This treatment removed biases in the data due to pre-experimental differences between the trees at the different sites.

RESULTS

The Galesville and Rockland Sites have Minimal vc Diversity

At the Galesville stand using vc testing on the cankers of the 30 sampled trees only two known vc types were detected based on pairwise crosses to the 15 identified vc types at the West Salem, WI stand (Figure 4). These types were WS-1 and WS-2. The most common vc type at Galesville, WI was WS-2 (Figure 5). Additionally, at the Galesville site was the occurrence of two isolates with unknown vc types.

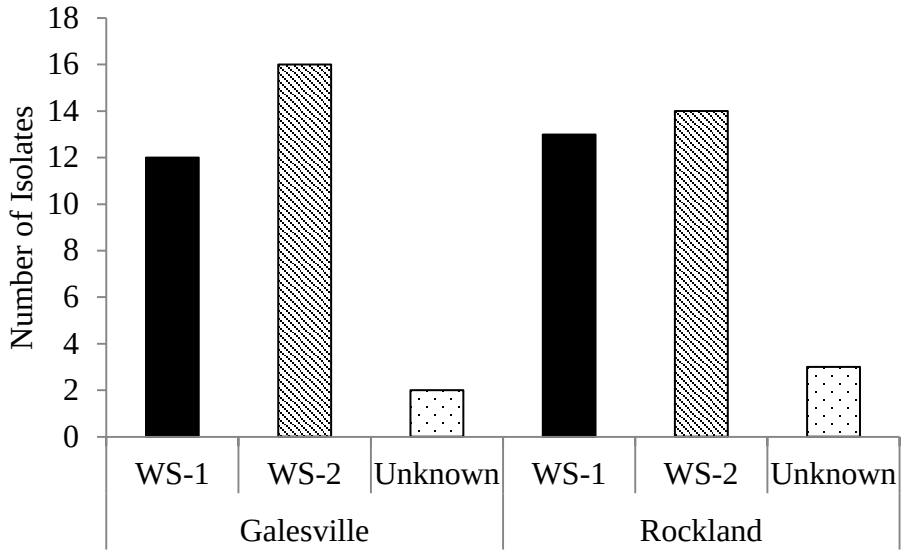

Figure 4. Vegetative compatibility types of *Cryphonectria parasitica* identified at the Galesville and Rockland WI stands.

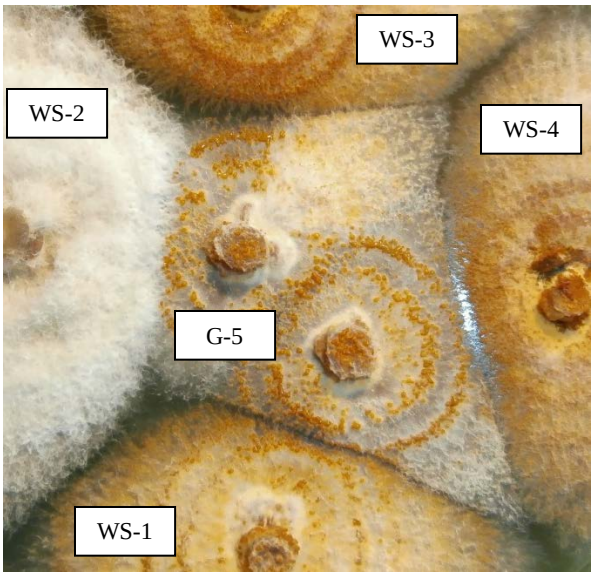

Figure 5. Vegetative compatibility test on the *Cryphonectria parasitica* isolate Galesville-5. Barrage lines shown between isolates WS-1, WS-3, WS-4 and Galesville-5. WS-2 and Galesville-5 have not formed a barrage line and are in the same vegetative compatibility group.

The vc types detected at Rockland, Wisconsin followed a similar pattern. At Rockland from the 30 trees sampled only WS-1 and WS-2 were detected from the 15 known local vc types. At the stand, WS-2 was the most frequently detected isolate at 53%. In addition to the detection of the two known vc types, three isolates were from unknown vc types (Figure 4). Between the sites the vc types did not significantly vary and were found equally in a 1:1 ratio (χ^2=0.64, df=1, p=0.05). The unknown types were not tested against the unknowns from the other sites or isolates not found in WI.

Streptomyces **Treatment Resulted in the Greatest Fungal Inhibition**

The growth in the width and length of cankers containing *C. parasitica* was most limited in the cankers that were treated with the *Streptomyces* based treatment. At the Galesville stand the cankers continued to expand in length and width, but the cankers

inoculated with the *Streptomyces* treatment appeared to experience a decrease after treatment application at day 21 (Figures 6,7).

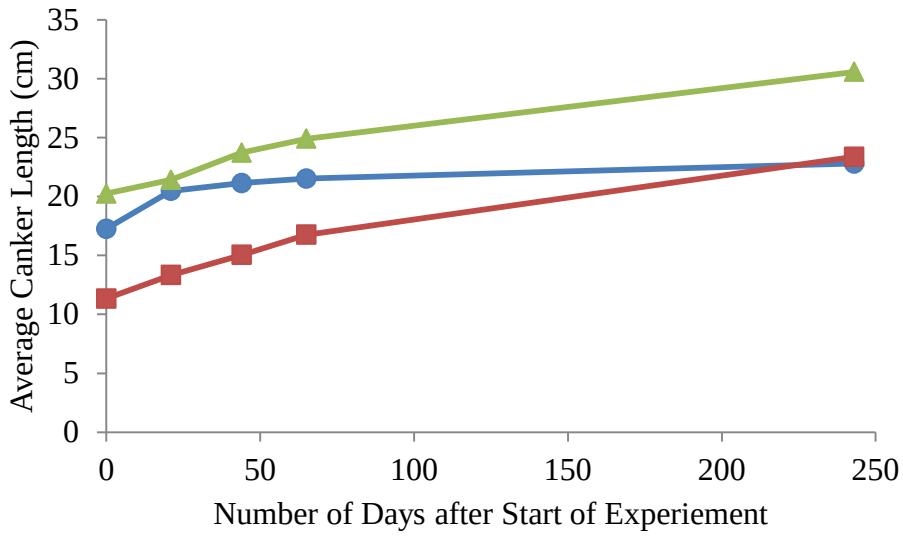

Figure 6. Average *Cryphonectria parasitica* canker length at Galesville, Wisconsin with *Streptomyces* based treatment (circle), hypovirus treatment (triangle), and control (square), (n=30, standard error bars shown).

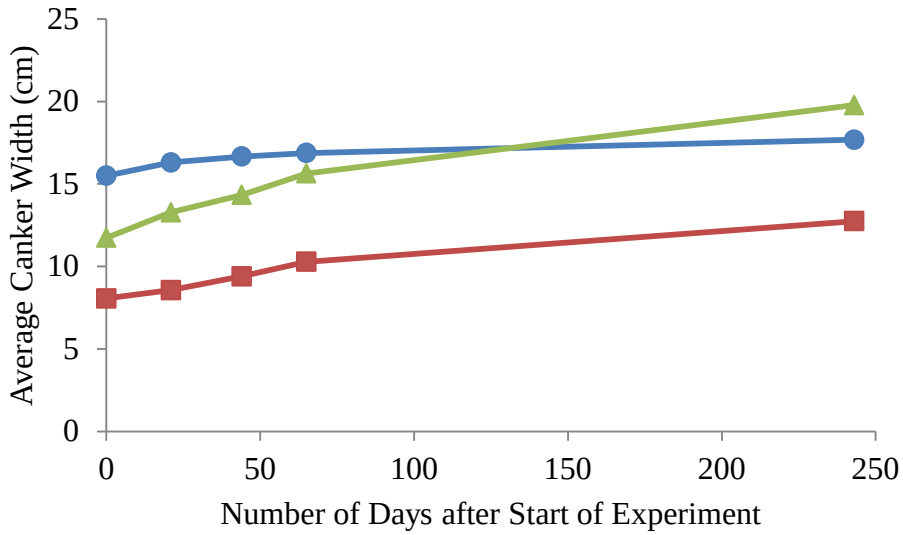

Figure 7. Average *Cryphonectria parasitica* canker width at Galesville, Wisconsin with *Streptomyces* based treatment (circle), hypovirus treatment (triangle), and control (square), (n=30, standard error bars shown).

The lengths and widths of the cankers were observed in this study using a two-way ANOVA that was performed using log transformed data to normalize the distribution after the data was adjusted to account for baseline variation (see above). The control treatment showed the largest changes between the baseline canker expansion and the post treatment measurements when comparing these adjusted values (Table 5).

Table 5. Changes in adjusted *Cryphonectria parasitica* canker length and width prior to and post *Streptomyces,* hypovirus, and control treatment at Galesville, WI.

	Change in Canker Length (cm) (SD)		Change in Canker Width (cm) (SD)	
	Baseline 9/10/12 – 10/1/12	Post Treatment 10/24/12 – 5/11/13	Baseline 9/10/12 – 10/1/12	Post Treatment 10/24/12 – 5/11/13
Streptomyces	1.53 (0.88)	3.00 (3.93)	0.85 (0.83)	2.54 (3.59)
Hypovirus	0.53 (0.48)	26.13 (23.44)	1.63 (1.45)	7.51 (5.87)
Control	1.04 (0.83)	19.24 (25.37)	0.53 (0.43)	10.30 (9.12)

At the Rockland stand the cankers had continuous expansion in length and width, but the cankers inoculated with the *Streptomyces* and hypovirus treatment appeared to experience a decrease after treatment application at day 21 while the control continued to have high canker expansion (Figures 8,9).

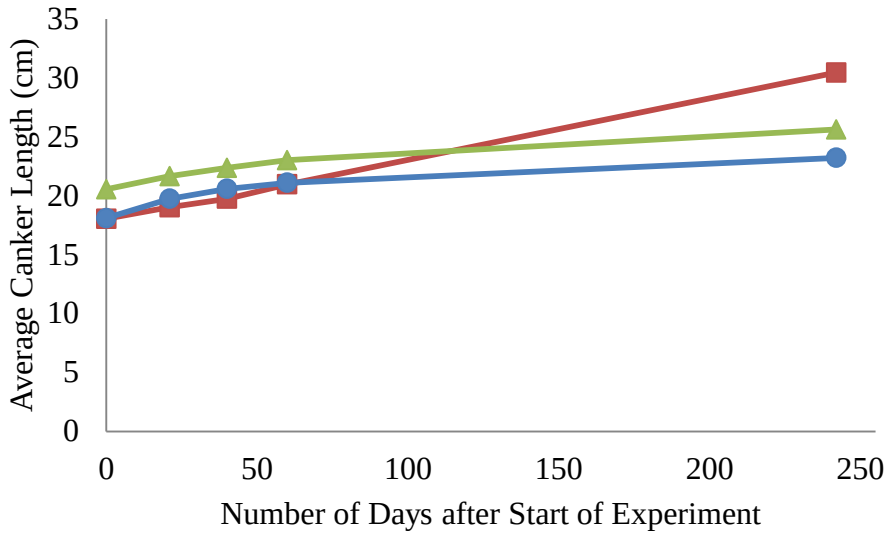

Figure 8. Average *Cryphonectria parasitica* canker length at Rockland, Wisconsin with *Streptomyces* based treatment (circle), hypovirus treatment (triangle), and control (square) (n=30, standard error bars shown).

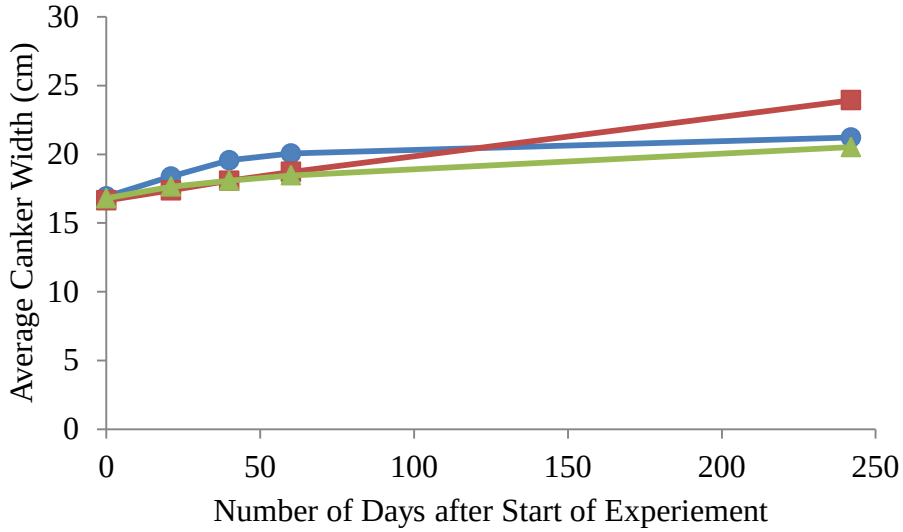

Figure 9. Average *Cryphonectria parasitica* canker width at Rockland, Wisconsin with *Streptomyces* based treatment (circle), hypovirus treatment (triangle), and control (square) (n=30, standard error bars shown).

The lengths and widths of canker expansion at Rockland were also observed in this study using a two-way ANOVA that was performed using log transformed data to

normalize the distribution after the data was adjusted to account for baseline variation (see above). The control treatment here had the smallest baseline expansion, but at the end of the study had the largest expansion values (Table 6).

Table 6. Changes in adjusted *Cryphonectria parasitica* canker length and width prior to and post *Streptomyces*, hypovirus, and control treatment at Rockland, WI.

	Change in Canker Length (cm) (SD)		Change in Canker Width (cm) (SD)	
	Baseline 9/11/12 – 10/2/12	Post Treatment 10/21/12 – 5/11/13	Baseline 9/11/12 – 10/2/12	Post Treatment 10/21/12 – 5/11/y13
Streptomyces	1.32 (1.11)	5.52 (6.45)	1.44 (0.71)	3.69 (7.75)
Hypovirus	0.90 (0.82)	10.39 (8.08)	0.83 (1.16)	7.75 (13.44)
Control	0.79 (0.47)	19.37 (12.05)	0.73 (0.37)	10.62 (6.97)

After treatment (10/24/12- 5/11/2013) there was a significant interaction where the cankers inoculated with *Streptomyces* had a greater increase in canker length at the Rockland site than the Galesville site. Cankers inoculated with hypovirus had greater increases in canker length at Galesville than Rockland. There was significant variation in canker length expansion between the treatments at the sites, where hypovirus and the control were similar in length and the *Streptomyces* had a significantly reduced canker length expansion rate (Table 7). *Streptomyces* treatments resulted in the greatest reduction in the canker expansion rates in width and significantly varied from the control

treated trees. The sites did not vary significantly in the width of canker expansion and there was no interaction between the sites and the treatments (Table 8).

Table 7. Two way analysis of variance of the final adjusted *Cryphonectria parasitica* canker length expansion using log transformation.

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Site	0.459	1	0.459	0.412	0.524
Treatment	32.056	2	16.028	14.393	0.000
Site * Treatment	10.694	2	5.347	4.801	0.012
Error	59.021	53	1.114		

Table 8. Two way analysis of variance of the final adjusted *Cryphonectria parasitica* canker width expansion using log transformation.

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Site	0.023	1	0.023	0.026	0.872
Treatment	10.566	2	5.283	5.952	0.005
Site * Treatment	2.256	2	1.128	1.271	0.29
Error	42.602	48	0.888		

In addition to growth rates of the cankers, the *Streptomyces* based treatment also resulted in lowest mortality at the stands although not statistically significant. Of the 60 trees in the study nine did not survive. The majority of the dead trees were found within the control group, where five trees did not survive the winter. In addition to these trees, an additional three trees from the hypovirus groups and only one tree from the trees treated with *Streptomyces* did not persist throughout the study period.

DISCUSSION

vc Diversity is Limited

The limited vc diversity at Galesville and Rockland WI likely stems from the limited amount of vc diversity in the area. This conclusion is supported by the fact that there are only 15 vc types at West Salem. (Mark Double, personal communication). Of the types WS-1 makes up approximately 85% of the population and the remaining infections are mostly caused by types WS-2, WS-3, and occasionally WS-4 (McGuire *et al.* 2005). At West Salem hypovirus Euro7 has been frequently infected in WS-1 and applied in the stand, which led to a limitation of the production of spores and dispersal potential (Smart et. al 1999). Many trees were not exposed to hypovirus and still had the ability to produce normal levels of spores that could have directly infected the sites of Galesville and Rockland WI through airborne spores or indirectly through carriers ranging from insects to researchers. The vc type WS-2 is found in approximately 10% of the trees at West Salem, WI (McGuire *et al.* 2005) and at this percent, the infection at the nearby stands is expected. Once these isolates traveled to the stands they quickly infected and spread throughout, infecting the majority of the trees. WS-1 and WS-2 vc types spread evenly at a 1:1 ratio at Galesville and Rockland WI (see Figure 4), suggesting that neither type had a competitive advantage at the stands over the other.

It is very important to consider the role that founder effect has played in the vc diversity of these populations. Firstly, the recent infection of both of the sites within the past decade has limited the vegetative diversity of the stands. Founder effect has been seen in areas with recent infection by the pathogen resulting in low diversity of stands in the Swiss Alps and Northern Switzerland where a maximum of one or two vc types were identified (Bissegger and Heiniger 1991, Hoegger *et al.* 2000). An older stand approximately 100km away had nine vc types detected (Hoegger *et al.* 2000). Second, the lack of gene flow to the new population can greatly limit diversity. Founder effect has been observed in areas that have restricted gene flow from larger populations like the edges of a valley (Cortesi *et al.* 1998). Low dispersal of spores and low gene flow has also been seen in France where sites are isolated by distance, resulting in limited diversity (Breuillin *et al.* 2006). These fungi are not constantly in contact with the source population in West Salem and had a limited number of infections. Rockland is approximately 13 km east of the West Salem stand, and the Galesville stand is approximately 30 km north west of the West Salem stand. As a result they should contain decreased genetic diversity compared to the source population due to founder effect and restricted gene flow.

It is important to consider the potential consequences of the presence of both WS-1 and WS-2 at the stands in this study. WS-1 exclusively consists of mating type 2 while WS-2 is nearly entirely composed of mating type 1 (approximately 95%) (McGuire *et al.* 2005). As unknown vc types were detected at both stands there are two potential explanations for this result. The first possibility is that an error was made during the vc testing. This outcome is unlikely due to the controls that were performed. The more

likely explanation is that the presence of both mating types allowed for the fungus to reproduce sexually and generate new combination of *vic* alleles resulting in new vc types.

Sexual reproduction of *C. parasitica* has large ramifications for *C. dentata*. Sexual reproduction is often rare, as many stands have a dominant mating type, limiting the fungus to asexual reproduction (Hoegger *et al.* 2000, Springer *et al.* 2013). It has been shown that sites with a more even ratio of mating types have higher levels of sexual reproduction resulting in more vc types; and this may also be associated with stands with longer infections (Hoegger *et al.* 2000). This pattern was also seen in Michigan where a nearly even ratio of mating types was detected at sites with long term infections (Springer *et al.* 2013). With sexual reproduction present, there will be an increased number of vc types which would then result in the decreased success of hypovirus and the increased likelihood that the stands will not survive (Krstin *et al.* 2011, Liu and Milgroom 1996). Over time, more vc types may be formed through sexual reproduction of the fungus or by subsequent infections from nearby stands.

Hypovirus and *Streptomyces* based treatments were applied in areas with low vc diversity yet hypovirus treatments were still relatively ineffective. Hypovirus treatments did have average canker growth rates that were frequently lower than the controls, but cankers were still growing at increased rates in comparison to the *Streptomyces* inoculum. Overall, the infected trees treated with hypovirus did not respond well to hypovirus treatment. This finding is not unique in North America, and several studies have used hypovirus without reductions in canker expansion and continued death of the trees. A study in West Virginia followed the effects of different combinations of hypovirus inoculations. At the stand the majority of the trees died within the first five

years. When the stand was tested for the presence of hypovirus 12 years later the majority of the remaining trees were hypovirus free (Liu *et al.* 2002). In a Connecticut study some trees persisted following the application of a mixture of hypoviruses but overall the hypoviruses did not protect the trees from developing lethal cankers (Milgroom and Cortesi 2004).

Streptomyces Treatment Resulted in the Overall Greatest Fungal Inhibition

The inoculum containing the *Streptomyces* decreased the growth rate of the cankers in length and width. It was significantly found to be the best treatment with the lowest canker expansion at Galesville, WI and significantly outperformed the control trees at this stand. At Rockland, WI the *Streptomyces* based treatment resulted in the smallest increase in canker expansion over the course of the study, although the canker expansion rates in width were not significantly different between the *Streptomyces* and hypovirus treatments. In addition to limiting the canker expansion rates the trees treated with *Streptomyces* also had the lowest mortality levels, when compared to the hypovirus and control group trees. While the exact mechanism was not studied here, it is likely the ability of many *Streptomyces* to produce chitinolytic enzymes and protease enzymes plays a role in the effectiveness of the biocontrol, by degrading the fungal cell walls, inhibiting spore germination and limiting the extension of hyphae (Narayanna and Vijayalakshmi 2009, Singh and Chhatpar 2011). This study looks at the effect of the *Streptomyces* in vivo, on the actual trees, showing the true effects of *Streptomyces* activity. Agar based assays are a useful starting point, but do not have accurate conditions and may not indicate what is actually occurring in the field..

One complication seen in this study was the high occurrence of girdled trees. 18/60 trees or 30% were fully or nearly girdled. As cankers cannot be removed using these treatments, the occurrence of statistically significant decreases in the mean width of canker expansion were noteworthy. In the future this study and similar studies could be improved by working in stands with relatively young infections, or initiating cankers on larger stems, and by working on trees that are not girdled so the full effects of each treatment could be better observed.

Streptomyces Inoculum should be used in Areas with Limited Success of Hypovirus

Many chestnut stands such as in Michigan or outside of North America respond well to hypovirus treatments or have naturally dispersing hypovirus. In areas where this has not been typical including the majority of American chestnut stands in the United States, the application of *Streptomyces* based inoculum should be considered as an alternative. *Streptomyces* inoculum proved to be easy to culture and implement as one inoculum may be used for a multitude of sites without first performing vegetative compatibility testing, since *Streptomyces* do no need to overcome the barrier of vegetative compatibility.

Where effective, hypovirus based treatments should continue to be used as they offer the potential for a systemic treatment not offered here. Researchers should continue to develop new biocontrols using antagonistic microorganisms like *Trichoderma*, *Penicillium*, and *Bacillus* which have all been previously shown to inhibit the growth of *Cryphonectria parasitica* (Akilli *et al.* 2011). *Streptomyces* based treatment has proved to be a valid alternative to hypovirus treatment and could potentially be used be used to save the remaining American chestnut trees.

In the future, additional isolates should be tested to determine if there are *Streptomyces* species that provide even greater inhibition against *C. parasitica*. This could be enhanced by determining the molecular mechanisms behind the inhibition of the isolate 4-20 tested here, first looking for possible protease or chitinase activity. Once this mechanism (or mechanisms) is elucidated potential treatments could be screened much more efficiently.

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