FUNGAL COMMUNITY SUCCESSION AND INTERACTIONS IN CHESTNUT BLIGHT CANKERS IN MICHIGAN AND WISCONSIN

By

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ABSTRACT

FUNGAL COMMUNITY SUCCESSION AND INTERACTIONS IN CHESTNUT BLIGHT CANKERS IN MICHIGAN AND WISCONSIN

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This dissertation research contributes to understanding the role of fungal community succession in chestnut blight cankers on American chestnut trees and interactions between the chestnut blight pathogen, *Cryphonectria parasitica*, virulence-altering hypoviruses, and secondary fungal invaders. Hypoviruses infect the pathogen mycelium directly and are known to decrease pathogen virulence (*i.e.* hypovirulent). These viral infections can slow pathogen growth, decrease the rate of canker expansion, and ultimately lower the probability of girdling. Secondary fungi also invade the expanding canker and may antagonize *C. parasitica* and reduce pathogen growth. The main objectives of this research are: (i) describe the spatial and temporal dynamics of the fungal community within cankers from six American chestnut populations and correlate that community with the severity of cankers and the likelihood of girdling; (ii) assess the ability of different fungal taxa, isolated from low severity cankers, to inhibit the growth of virulent and hypovirulent forms of *C. parasitica* in dual culture tests; and (iii) evaluate the combined effect of fungal antagonism and a hypovirus on canker expansion rates with treatment of experimental cankers.

The spatial distribution of virulent and hypovirulent *C. parasitica* and non-*C. parasitica* fungi within a canker differed from the spatial structuring we predicted and resembled a mosaic. Fungal communities within cankers were unstable. The fungal community structure in one year was classified differently the next year. There was a net shift of the community toward

abundant non-*C. parasitica* fungi in cankers on surviving stems. Cankers on surviving trees containing hypovirulent *C. parasitica* consistently were invaded by non-*C. parasitica* fungi and were associated with declining survivorship over time. Fungal invasion into the canker community may facilitate canker expansion via greater inhibition of hypovirulent *C. parasitica* relative to the virulent form of the pathogen. Commonly occurring, non- *C. parasitica* fungi in chestnut blight cankers, including *Trichoderma*, may inhibit hypovirulent *C. parasitica* more than virulent *C. parasitica* based on dual culture testing. This may allow virulent *C. parasitica* to escape hypovirus infection and resume rapid canker expansion. Inoculations at the margin of experimental cankers on American chestnut stems using hypovirulent *C. parasitica* were effective at slowing canker expansion. However, inoculations of potentially antagonistic fungi such as *Trichoderma* did not reduce the rate of canker expansion. Applying antagonistic fungi to the inner area of a canker may offset the influence of hypovirulent *C. parasitica*.

This work investigates the fungal community within chestnut blight cankers and the role it plays in canker expansion and stem girdling. Although *Trichoderma* is noted as a biological control agent in other plant-pathogen systems, it may be too inhibitory of hypovirulent *C. parasitica*, which is known to slow canker expansion and delay tree girdling. The presence of invading fungi into the canker does not seem to slow canker expansion and may prevent dissemination of hypovirulent *C. parasitica* in a canker and a forest.

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CHAPTER 1

INTRODUCTION

Chestnut blight cankers

American chestnut (*Castanea dentata* [Marsh.] Borkh.) was once a dominant canopy tree in the hardwood forests of eastern North America (Griffin *et al.* 1986). At the beginning of the 20th century, the species began to decline dramatically after the accidental introduction of the chestnut blight fungus, *Cryphonectria parasitica*, (Murrill) Barr (previously *Endothia parasitica*) from Asia. This fungal pathogen caused the decimation of billions of mature American chestnut trees just decades after the introduction of the pathogen near New York City (Merkel 1905). All attempts to prevent chestnut blight from spreading were largely unsuccessful (reviewed in Anagnostakis 1987), and three to four billion mature American chestnut trees were either killed by blight or reduced to understory ramets (Keever 1953).

Chestnut blight infections begin as ascospores, conidia, or mycelia of *C. parasitica* enter through wounds in the outer bark of the tree (Hebard *et al.* 1984), perhaps at weak branch points and natural branch scars (Garrod *et al.* 1985). Mycelial fans proliferate within the living bark of the tree and extract resources from host cells causing a lesion that enlarges in size to form a canker. Over time, a canker may expand completely around the circumference of the stem or branch (*i.e.* girdled). Once the vascular cambium is destroyed and no new conductive xylem can be produced, all plant tissues distal to the infection die (Ewers *et al.* 1989; McManus *et al.* 1989). Cankers on the main stem can kill the trunk of a large canopy tree and reduce it to a number of small sprouts produced at the root collar (Paillet 1982).

The chestnut blight canker and the expanding margin is the interface of host-pathogen contact, yet the time from initiation of the canker to it girdling a chestnut stem is not straightforward: cankers neither expand at a uniform rate (citations?), nor do cankers always girdle an infected stem (citations?). Several factors of the pathogen, the host tree, and the environment all contribute to and influence disease (Stevens 1960). Thus, the rate of canker expansion and the probability of stem girdling are likely to depend on a combination of factors (reviewed in Fulbright 1999; Milgroom and Cortesi 2004; Rigling and Propsero 2018).

Hypovirulence and host response

A similar blight epidemic of European chestnut (*Castanea sativa* [Mill]) seemed destined to follow the fate of American chestnut (reviewed in Heiniger and Rigling 1994). However, plant pathologist Antonio Biraghi noticed recovering trees in Italy and reported that these trees did not have girdling cankers, but instead the pathogen was restricted to the outer bark and failed to destroy the cambium (Biraghi 1953). Samples from these trees were sent to French mycologist Jean Grente and yielded strains of *C. parasitica* that grew abnormally in culture and had reduced virulence (Grente 1965). Grente called these strains "hypovirulent" and demonstrated with J. Bertheley-Sauret that these hypovirulent strains of *C. parasitica* could be inoculated into the margin of imminently lethal cankers to convert it to a non-girdling canker phenotype (Grente and Bertheley-Sauret 1978). Researchers in North America found that these hypovirulent strains also could limit canker expansion on American chestnut in a similar manner (Anagnostakis and Jaynes 1973; Van Alfen *et al.* 1975; Day *et al.* 1977; Jaynes and Elliston 1980). Day *et al.* (1977) showed that hypovirulent

strains were correlated with the presence of double-stranded ribonucleic acid (dsRNA) in the pathogen mycelium. Choi and Nuss (1992) would establish that dsRNA viruses caused the reduction of virulence of the pathogen.

The effects of hypoviruses on reducing pathogen virulence (*i.e.* hypovirulence) have been studied extensively (reviewed in Macdonald and Fulbright 1991; Dawe and Nuss 2001; Hillman and Suzuki 2004). A diversity of dsRNA viruses infecting *C. parasitica* within cankers includes species within the family *Hypoviridae* based on their genome organization (Dawe and Nuss 2001; Hillman and Suzuki 2004). CHV1 (*Cryphonectria Hypovirus 1*) is a group of hypoviruses from hypovirulent strains found throughout Europe and China and is known to debilitate *C. parasitica* by reducing sporulation and virulence (Shapira *et al.* 1991; Peever *et al.* 1997; Gobbin *et al.* 2003). CHV2 was discovered in New Jersey and also reduces the development and fecundity of *C. parasitica* (Hillman *et al.*1992; 1994). GH2, the type species of CHV3, was found naturally in Michigan chestnut populations (Fulbright *et al.* 1983). Strains of *C. parasitica* containing CHV3 dsRNA viruses have also been found in other parts of Michigan and North America (Paul and Fulbright 1988; Peever *et al*. 1997; Melzer and Boland 1999). CHV4 types have been found in Michigan and eastern North America but seem to have no effect on virulence (Enebak et al. 1994).

The discovery of hypoviruses in Europe and North America seemed encouraging for blight management and restoration of chestnut, especially as a way to better understand the variability of canker expansion that allowed trees to stave off girdling. Indeed, hypovirulent *C. parasitica* has become established over time within chestnut populations across the

European continent, either naturally or through experimental disseminations (reviewed in Heiniger and Rigling 1994; Milgroom and Cortesi 2004; Bryner *et al.* 2012). Hypovirulence is now ubiquitous in recovering European chestnut populations in Spain, Italy, France, and southern Switzerland (Turchetti *et al.* 2008; Robin *et al.* 2010; Prospero and Rigling 2012; Zamora *et al.* 2014). Yet in North America, the establishment of hypoviruses in *C. parasitica* populations infecting American chestnut has largely failed, despite experimental introductions within and outside the natural range (reviewed in MacDonald and Fulbright 1991; Milgroom and Cortesi 2004).

A leading hypothesis for the successful establishment of hypovirulence in parts of Europe and not in North America is the low genetic diversity of *C. parasitica* in European chestnut populations (Milgroom and Cortesi 2004). Through the work of Day *et al.* (1977), hypovirulence was found to be transmissible as hypovirus passed between strains of *C. parasitica* via hyphal anastomosis (*i.e.* cytoplasmic fusion of vegetative hyphae). However, transmission of hypovirus from an infected *C. parasitica* strain to a hypovirus-free strain is limited through the formation of barrage zones when hyphal anastomosis fails due to vegetative incompatibility genes (*vic* genes; Anagnostakis 1977; Anagnostakis and Day 1979; Liu and Milgroom 1996). Liu and Milgroom (1996) demonstrated that hypovirus transfer is negatively correlated with the number of *vic* genes that differ between neighboring strains. In addition, Huber (1996) reported that individual alleles at *vic* gene loci have varying effects on transmission.

Indeed, in the few instances of recovery in North American chestnut populations, cankers with hypovirulent *C. parasitica* are frequent and correlated with low *vic* diversity in the pathogen population (Springer *et al.* 2013). The authors discuss, however, that low *vic* diversity may actually be a result of hypovirus infection negating sexual recombination in *C. parasitica*, lowering the formation of new *vic* genotypes, and selection favoring the most fit hypovirus-pathogen genotype combination (Springer *et al.* 2013).

Certain *Castanea* spp. are better able to prevent virulent or hypovirulent *C. parasitica* from advancing around the stem or branch by compartmentalizing *C. parasitica* through the formation of wound periderm (Hebard *et al.* 1984; McManus *et al.* 1989). Chinese chestnut (*C. mollissima* Blume) and Japanese chestnut (*C. creneta* Siebold and Zucc.) have higher resistance to blight than European chestnut, which is likely more resistant to blight than American chestnut (Griffin *et al.* 1983; Hebard *et al.* 1984; Viéitez and Merkle 2005). Although the rate and extent of wound periderm formation in *Castanea* spp. is similar (Hebard *et al.* 1984), mycelial fans of the pathogen are restricted to the outer bark by resistant *C. mollissima*, forming superficial cankers. Hebard *et al.* (1984) also demonstrated that wound periderm formation in susceptible American chestnut trees was delayed and subsequently halted following penetration of periderm by *C. parasitica* mycelial fans. Not all individuals of American chestnut responded in this way, however, especially in their response to a hypovirulent strain of the pathogen. A "slightly-to-moderately resistant" American chestnut tree (Hebard *et al.* 1984) was able to restrict the slow-growing mycelia of these hypovirulent strains to the outer bark, forming superficial cankers. Other trees throughout the natural range of American chestnut are reported to have some level of

resistance to the pathogen, and are often associated with hypovirulent strains of the pathogen (Griffin *et al.* 1983). However, penetration and destruction of wound periderm by hypovirulent *C. parasitica* often is only delayed compared to virulent *C. parasitica*, and stems of American chestnut still become girdled. Thus, chestnut populations may vary in response to the pathogen with and without hypovirus.

In Europe, Bryner *et al.* (2013) sampled nearly 700 cankers across *C. sativa* populations with blight to determine if the morphology of cankers could be used to predict the presence or absence of hypovirulent *C. parasitica*. Bryner *et al.* (2013) found that different features of cankers (*e.g.* stem encircling, canker length, canker depth) did not reliable predict the presence or absence of hypovirulent *C. parasitica* in a canker using logistic regression. The difficulty in predicting hypovirus presence based on canker morphology and tree response to infection could be due to the pathogen becoming infected with hypovirus at different times after canker establishment (Bryner *et al.* 2013). In addition, the mycelia of *C. parasitica* may become only partially infected with hypovirus in a canker, or that cankers merge together on a stem. Thus, direct isolation of the *C. parasitica* from cankers and laboratory assessment for hypovirus infection was considered critical for predicting the time to girdling (Bryner *et al.* 2013).

There are, however, additional factors that influence canker expansion rates and the probability of stem girdling. These include external factors in the environment that can influence canker development on a chestnut stem or branch. Cankers are vulnerable to invasion by other fungal taxa (*e.g.* Akilli *et al.* 2009; Double *et al.* 2013; Ćurković-Perica*et al.*

2017). Long-term monitoring within a stand of American chestnuts near West Salem, Wisconsin where hypoviruses were being disseminated found that for non-girdling cankers on surviving trees, the prevalence of non-*C. parasitica* fungi (Non-CP) increased over time while the prevalence of *C. parasitica* decreased (Double *et al.* 2013). Additionally, the prevalence of *C. parasitica* isolates containing hypovirus remained relatively constant within these cankers at a rate of approximately one-third of isolates from a canker in a given year. The influence of invading fungi and bacteria on canker expansion is largely unknown, although other fungi within healthy (*e.g.* Russin and Shain 1984; Bissegger and Seiber 1994; Tattar *et al.* 1996; Wilhelm *et al.* 1998; Groome *et al.* 2001) and diseased chestnut stem tissue (*e.g.* Arisan *et al.* 1995; Akilli *et al.* 2009) have been implicated as potential biological control options to supplement hypovirulence in a canker.

Secondary microorganisms in cankers

Previous research of microorganisms other than *C. parasitica* and hypoviruses that influence canker expansion includes bacteria and fungi isolated from healthy and diseased bark tissue, as well as microbes in the soil. Weidlich (1978), knowing that *C. parasitica* does not infect the root system of chestnut applied compresses of soil from the area surrounding the base of an infected chestnut tree and found that after several months with the compress, cankers appeared swollen with callus tissue and non-girdling. When autoclaved soil compresses failed to initiate the same non-girdling response in the tree, Weidlich (1978) concluded that the microorganisms within the soil were responsible. Among the microbes isolated were the fungus *Trichoderma* and the bacterium *Bacillus* (Weidlich 1978), which also have been

associated with healthy chestnut stems and cankers (Arisan-Atac *et al.* 1995; Tattar *et al.* 1996; Wilhelm *et al.* 1998; Groome *et al.* 2001; Akilli *et al.* 2009; Ćurković-Perica *et al.* 2017).

The presence of *Trichoderma* in naturally-occurring chestnut blight cankers is intriguing for canker management, as this fungal genus is known for utilizing different antagonistic mechanisms (*e.g.* mycoparasitism, competition) against plant pathogenic fungi (reviewed in Harmen 2006; Lorito *et al.* 2011). Tattar *et al.* (1996) isolated *Trichoderma* strains from the bark of healthy American chestnut trees and demonstrated their ability to outgrow *C. parasitica* in dual culture. Tattar *et al.* (1996) found that the *Trichoderma* strains could prevent *C. parasitica* from colonizing excised chestnut bark using a spore solution of the "antagonistic fungus". Akilli *et al.* (2011) demonstrated that *Trichoderma* strains could limit canker expansion as effectively as hypovirulence when applied to induced cankers on young sapling *C. sativa* trees in a greenhouse. The *Trichoderma* strains used by Akilli *et al.* (2011) were isolated from cankers on European chestnut trees in Turkey.

Other fungal genera (*e.g. Pestalotiopsis, Phomopsis, Botryosphaeria, Gnomoniopsis*) found at low levels in chestnut blight cankers could be "canker disease agents" and exhibit some level of pathogenicity toward chestnut (Akilli *et al.* 2009). Previous research has suggested that even before *C. parasitica* infects and causes cankering, the bark of chestnut is likely composed of endophytic fungi that may interact with the host as weak pathogens (*e.g. Amphiporthe*) or others (*e.g. Pezicula*) that may interact with the pathogen to prevent infection (Baird 1991; Bissegger and Seiber 1994). A study by Russin and Shain (1984) evaluated the succession of *Ceratocystis* and *C. parasitica* on both living and excised chestnut stems, showing that

Ceratocystis was more likely to colonize and persist in a canker compared to healthy chestnut bark. Thus, the fungal community in chestnut and cankers can change over time and suggests other fungi beside *C. parasitica* may contribute to canker expansion.

Given the breadth of recent research of the plant microbiome as modifiers of disease (discussed below), fungi inhabiting chestnut blight cankers other than *C. parasitica* may be important to explain variability in canker expansion, whether affecting the pathogen, the tree, or both. A brief review of recent literature on the plant microbiome and disease affecting properties is provided in the next section, followed by a set of testable questions that will be addressed in Chapters 2, 3, and 4 of this dissertation to better understand how the fungal community that becomes established in chestnut blight cankers may potentially influence disease severity, that is canker expansion and the likelihood of stem girdling.

Plant microbiomes and disease

Plant microbiomes (*i.e.* communities of plant-associated microorganisms) can have profound impacts on plant growth and survival (*e.g.* Rodriguez *et al.* 2009; Partida-Martinez and Heil 2011; Hardoim *et al.* 2015; Busby *et al.* 2017). Fungi of the plant microbiome include pathogens, which destroy and consume tissues and negatively affect plant fitness (Thrall *et al.* 2007; Brown and Tellier 2011). In contrast, mycorrhizae, which form relationships with plant roots and exchange nutrients from the soil for carbon, represent a critical plant mutualism (Hoeksema *et al.* 2010). Beyond pathogens and mycorrhizae, an enormous diversity of other plant-associated fungi exists without well-defined functional

roles and interactions with respect to their host plants (reviewed in Arnold *et al.* 2007; Peay *et al.* 2008; Rodriguez *et al.* 2009).

Recent research suggests that other fungi that occupy healthy plant tissue, and the fungi that invade diseased plant tissue may affect disease dynamics (*e.g.* Carroll 1988; Woodward and Boddy 2008; Mejia *et al.* 2008; Parfitt *et al.* 2010; Ridout and Newcombe 2015; Busby *et al.* 2016a). In certain cases, endophytic fungi that symbiotically inhabit healthy plant tissues may protect plants and lessen disease severity (*e.g.* Arnold *et al.* 2003; Busby *et al.* 2016b), while other fungi in the environment may facilitate disease (*e.g.* Ragazzi *et al.* 2003; Giordano *et al.* 2009; Delaye *et al.* 2013; Busby *et al.* 2016b). Thus, this symbiosis likely depends on the environmental context and factors including the host plant, surrounding plant community, and other fungi within the plant tissue (*e.g.* Carroll 1988; Schulz and Boyle 2005). For example, the leaves of black cottonwood, *Populus trichocarpa* (Torr. and A.Gray ex Hook.) are attacked by a leaf rust pathogen (*Melampsora*). Fungal endophytes were reported to alter disease severity experimentally, and the abundance of certain endophytes, including *Trichoderma* and *Cladosporium*, was correlated with low disease levels in the wild (Busby *et al.* 2016b). These fungi were described as antagonists to the pathogen that lessened disease severity. Yet other endophytes in the community of diseased leaves were considered "facilitators of disease" and associated with higher disease levels (Busby *et al.* 2016b). Ragazzi *et al.* (2003) found that the branches of declining oak trees in Italy were more likely to be colonized by a greater diversity of fungal endophytes compared to lower diversity and colonization in healthy trees. Some endophytes associated with declining trees were considered weakly pathogenic to oak (Ragazzi *et al.* 2003). Thus, the diversity of

fungi that inhabit healthy and diseased plant tissues may be important to consider when assessing disease in natural plant populations and communities (Seiber 2007; Saikkonen 2007; Partida-Martinez and Heil 2011; Clay 2014; Desprez-Loustau *et al.* 2016).

The diversity of fungi within the tissues of trees is extensive, especially in tropical species (*e.g.* Arnold *et al.* 2000; Arnold and Lutzoni 2007); however, the microbial community ecology of the bark and wood of trees and disease-affecting properties of these communities is unclear (see Fisher and Petrini 1990; Carroll 1995; Ragazzi *et al.* 2003; Giordano *et al.* 2009; Kolarik *et al.* 2011; Hacquard and Schadt 2015). In some cases, recent disease epidemics of forests are caused by pathogens not previously described until after the invasion (citations?). Determining whether a new disease is caused by a non-native pathogen, or whether existing microbial populations have evolved virulence in a now favorable environment is hard due to incomplete data on fungal biogeography (Linzer *et al.* 2009; Shaw and Osborne 2011; Garcia-Guzman and Heil 2013; Desprez-Loustau *et al.* 2016).

Unintentional movement of insect pests and pathogens around the globe also has led to a series of emergent tree diseases that are plaguing natural and agricultural systems in North America (*e.g.* Sturrock *et al.* 2011; Ploetz *et al.* 2013). Sudden oak death (Rizzo *et al.* 2002), thousand cankers disease of walnut (Kolarik *et al.* 2011), laurel wilt (Hulcr and Dunn 2011), and citrus greening (Bove 2006) are only a few of the devastating diseases that have appeared in recent years. Thus, a community ecology approach to stem diseases of trees may be necessary to elucidate a more complete set of biotic factors that contribute to disease

severity and progress. The chestnut blight system has an extensive history of research investigating microbial communities in bark tissue and is a classic example of an introduced bark pathogen decimating a native host tree. Thus, it creates an excellent opportunity to investigate aspects of the fungal community in diseased bark to better understand disease severity and ultimately the fate of diseased trees.

Model of fungal community dynamics in cankers

My dissertation research will combine approaches from what is known about hypovirulent strains of *C. parasitica* and potentially antagonistic microbes, mainly fungi isolated from diseased chestnut bark to characterize the microbial community composition within a canker, and describe how changes in this community are associated with disease and girdling. Previous work with collaborators from Michigan State University, West Virginia University, and the University of Wisconsin at La Crosse were involved in developing a model to explain canker expansion and girdling as a response to the temporal succession and spatial arrangement of hypoviruses and fungi within chestnut blight cankers.

My qualitative model includes non-girdling and girdling cankers with fungal communities that differ both spatially and temporally (Figure 1). For non-girdling cankers, hypoviruses become established along the canker margin early in the development of the canker (Bell 2004). Hypovirus infection (HCP) slows canker expansion allowing the response of the tree to wall-off a less virulent pathogen, or at least delay destruction of callus tissue by the pathogen (Hebard *et al.* 1984). As the slowly expanding canker ages, the inner area becomes

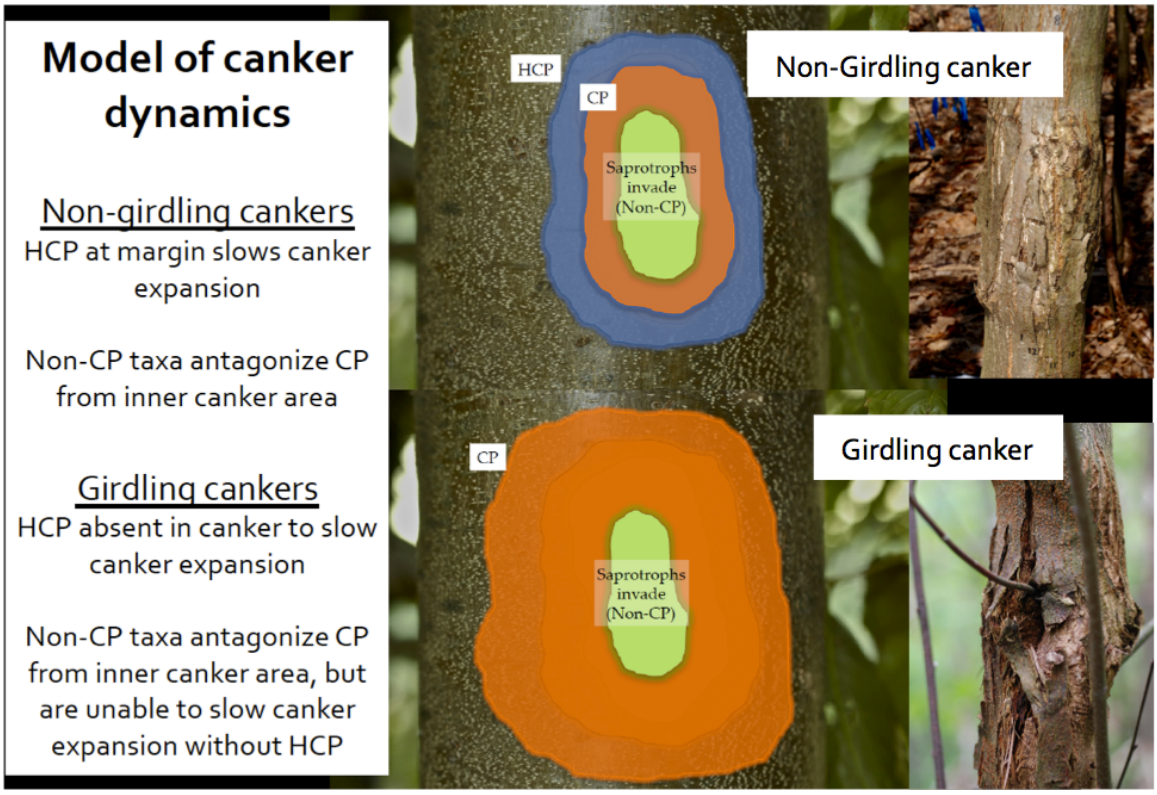

Figure 1-1: Model of fungal communities within non-girdling and girdling cankers. Non-girdling cankers: Hypoviruses become established along the margins of cankers (HCP), which reduces the rate of cankers expansion. Eventually the inner area of these cankers senesce and become vulnerable to invasion by variety of other fungal taxa (Non-CP). The Non-CP fungi compete with *C. parasitica* (CP) for resources within the inner region of the canker, further reducing the rate of canker expansion. Girdling cankers: CP promotes a very high rate of canker expansion in the absence of HCP. While Non-CP may invade the inner portion of the canker, their influence is too weak to prevent girdling of the infected stem.

vulnerable to invasion by saprophytic, weakly pathogenic, or antagonistic fungi (*i.e.* Non-CP) that compete with CP for plant resources (Prospero *et al.* 2006). The combined effects of HCP infection of CP and Non-CP antagonism act to slow canker expansion and increase the probability that tree defenses prevent girdling of the infected stem (Figure 1). The fungal community within these non-girdling cankers increases in diversity over time, with more Non-CP overall within the inner areas of the canker, and the importance of CP decreasing as HCP and Non-CP both limit CP growth and breakout from host defenses.

Girdling cankers, however, are expected to quickly kill an infected American chestnut stem. I expect a virulent pathogen can be isolated from these cankers while HCP is absent from these cankers (Figure 1). Without hypovirus infection to limit CP from destroying host tissue at the margin of cankers, expansion will occur rapidly in susceptible trees. While Non-CP may invade the center of the canker, any antagonism of *C. parasitica* will have minimal effect on expansion rate due to spatial separation in the canker. The fungal community in these cankers will continue to be dominated by CP until the stem is girdled.

Structure of dissertation

In chapter 2, I investigated how spatial and temporal dynamics of the fungal community influence canker severity and the likelihood of girdling by sampling and monitoring cankers at six chestnut populations for up to five years. The chestnut populations differed with respect to the degree of recovery of infected trees. Cankers in recovering populations generally have a less severe morphology and often contain HCP; in epidemic populations, cankers are severe and typically contain little HCP (Springer *et al.* 2013). At the West Salem,

Wisconsin site, cankers were treated with hypoviruses with the goal of establishing HCP mycelium at the margins of the canker for over 20 years beginning in 1992 (Double *et al.* 2018).

The abundance of CP, HCP, and Non-CP within cankers at recovering populations is hypothesized to differ from those at epidemic populations. The ratio of HCP to CP is predicted to be higher at recovering populations compared to epidemic populations. Non-CP taxa also may vary in their interaction with chestnut and *C. parasitica*. Previous research of *Trichoderma* inhabiting chestnut bark and the soil has demonstrated its potential to influence disease severity when introduced to the canker community (Akilli et al. 2011; Arisan-Atac et al. 1995; Tattar et al. 1996; Weidlich 1978). Many of the Non-CP commonly isolated from non-girdling cankers at the West Salem, WI were *Trichoderma* (Double *et al.* 2013). Other Non-CP within cankers may be weak pathogens of chestnut and exacerbate disease (Bissegger and Sieber 1994), or depend on colonization of healthy chestnut by CP in order to complete their life cycle before decay fungi invade (Russin and Shain 1984). Thus, the presence of HCP and Non-CP taxa antagonistic toward CP is predicted to be abundant in cankers at recovering populations and less frequent in epidemic populations.

In chapter 3, I quantified the ability of different Non-CP taxa commonly recovered from non-girdling cankers to inhibit the growth of CP and HCP. We chose ten Non-CP isolates collected in 2012 from cankers on American chestnut trees at West Salem, WI and at two recovering American chestnut populations in Michigan. Hypovirulence and the interactions

between CP and HCP within cankers and in the laboratory has been critical in chestnut blight research, yet little is known about how CP and HCP might interact differently with species of Non-CP. The pathogen must kill host cells to extract resources to continue to grow. It would follow that HCP obtains resources at a slower rate. Therefore, with fewer resources, HCP may be limited in its ability to defend territory within the canker. The intent of this work is to provide a baseline of Non-CP antagonism for my model of canker dynamics and disease management schemes using antagonistic fungi and hypovirulence to slow canker expansion and prevent girdling.

In chapter 4, three experiments were carried out over a four-year period (2013-2017) to determine the effect of spatial and temporal aspects of the model described above on canker expansion rates on American chestnut. The first experiment examined the effectiveness of HCP in different areas of a canker; along with a separate set of treatments evaluating whether *Trichoderma* applied to the inner canker areas would further reduce the rate of canker expansion beyond hypovirulence treatments alone. In order to most effectively slow canker expansion experimentally, a combination of the conversion capability of HCP to lower the virulence of CP at the margin and antagonism by Non-CP taxa toward CP in the canker interior should be utilized. One should consider spatially restricting Non-CP to the inner area of cankers to compete with CP only, preventing antagonism of HCP at the margin where conversion of CP to HCP occurs. This research can be useful to microbial ecologists, as well as plant pathologists and will increase understanding of ecology and evolution of cryptic fungal communities in a forest can play a role in preventing or increasing virulence in a disease system. In addition, this work will be useful in agricultural

science to address challenges in orchard management practices, especially as chestnut becomes are more widely planted tree fruit crop.

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CHAPTER 2

SPATIAL AND TEMPORAL DYNAMICS OF THE FUNGAL MICROBIOME OF CHESTNUT BLIGHT CANKERS ON AMERICAN CHESTNUT (*CASTANEA DENTATA*) IN MICHIGAN AND WISCONSIN

ABSTRACT

Chestnut blight cankers caused by the fungal pathogen *Cryphonectria parasitica* on infected American chestnut trees are susceptible to invasion by non-*C. parasitica* fungi, although the role of these fungi in disease is unclear. Hypoviruses also invade cankers by infecting *C. parasitica* mycelia and lowering pathogen virulence (*i.e.* hypovirulence), thus decreasing the rate of canker expansion and the probability of stem girdling. Fungi that invade cankers may be capable of altering *C. parasitica* growth and further reducing the probability of girdling. However, the combined effects of fungal antagonism and hypovirulence in chestnut blight cankers have not been evaluated, especially with respect to how these fungi and hypoviruses are distributed within cankers and their persistence over time. To investigate how spatial and temporal dynamics of the fungal community within cankers correlate with the severity of cankers and the likelihood of girdling, blight cankers from six American chestnut populations were monitored from 2012-2016. The spatial distribution of virulent and hypovirulent *C. parasitica*, along with non-*C. parasitica* fungi within a canker resembled a mosaic that differed from the spatial structuring we predicted. Fungal communities within cankers also were unstable, to the extent that the community structure in one year was more often than not classified differently the next year. In cankers on surviving stems, there was a net shift of the community type toward abundant non-*C. parasitica* fungi; However, these cankers were not associated with higher stem survivorship over time compared to cankers with abundant hypovirulence. In addition, cankers on

surviving trees containing hypovirulence consistently were invaded by non-*C. parasitica* fungi, which we show is associated with declining survivorship over time. These results suggest that there is no stable canker community, and the volatility of the fungal community indicates that cankers can change quickly from less severe to more severe cankers through the loss of hypovirulence. In addition, fungal invasion may facilitate canker expansion through greater inhibition of hypovirulent *C. parasitica* relative to the virulent form of the pathogen, allowing virulent *C. parasitica* to escape hypovirus infection and resume rapid canker expansion. This research can improve our understanding and management of forest diseases and the role of other species of fungi in the community in preventing or increasing disease.

INTRODUCTION

Plant microbiomes and disease

Plant microbiomes (*i.e.* communities of plant-associated microorganisms) can have profound impacts on plant growth and survival (*e.g.* Rodriguez *et al.* 2009; Partida-Martinez and Heil 2011; Hardoim *et al.* 2015; Busby *et al.* 2017). Fungi of the plant microbiome include pathogens, which destroy and consume tissues and negatively affect plant fitness (Thrall *et al.* 2007; Brown and Tellier 2011). In contrast, mycorrhizae, which form relationships with plant roots through exchange of nutrients in the soil, represent a critical plant mutualism in stressful environments (Hoeksema *et al.* 2010). Beyond pathogens and mycorrhizae, an enormous diversity of other plant-associated fungi exists without well-defined functional roles and interactions with respect to their host plants (reviewed in Schulz and Boyle 2005; Peay *et al.* 2008; Rodriguez *et al.* 2009).

Recent findings suggest that fungi that occupy healthy plant tissue and others that invade diseased plant tissue may affect disease dynamics (*e.g.* Arnold *et al.* 2003; Woodward and Boddy 2008; Ridout and Newcombe 2015; Busby *et al.* 2016a). In certain instances, fungi that symbiotically inhabit healthy plant tissues (*i.e.* endophytes) may protect plants and mitigate disease severity (*e.g.* Arnold *et al.* 2003; Busby *et al.* 2016b). Other fungi in the environment – endophytic or not, may facilitate and intensify disease (*e.g.* Ragazzi *et al.* 2003; Giordano *et al.* 2009; Busby *et al.* 2016b). Thus, endophytic fungi might be viewed as a misnomer, as this latent symbiosis likely depends on multiple factors including the host plant, surrounding plant community, and other microorganisms in the community. For example, the leaves of black cottonwood, *Populus trichocarpa*, are attacked by a leaf rust

pathogen (*Melampsora*). Fungal endophytes were reported to alter disease severity experimentally, and the abundance of certain endophytes, including *Trichoderma* and *Cladosporium*, was correlated with low disease levels in the wild (Busby *et al.* 2016b). These fungi were described as antagonists to the pathogen that lessened disease severity. Yet other endophytes in the community of diseased leaves were considered "facilitators of disease" and associated with higher disease levels (Busby *et al.* 2016b). Ragazzi *et al.* (2003) found that the branches of declining oak trees in Italy were colonized by a greater diversity of fungal endophytes compared to a lower diversity of fungi in healthy trees. Some endophytes associated with declining trees were considered weakly pathogenic to oak (Ragazzi *et al.* 2003). Understanding the diversity of fungi that inhabit healthy and diseased plant tissues may be important factors to consider when assessing disease in natural plant populations and communities (Partida-Martinez and Heil 2011; Clay 2014; Desprez-Loustau *et al.* 2016).

The diversity of fungi within the tissues of trees can also be extensive (*e.g.* Arnold *et al.* 2000; Arnold and Lutzoni 2007). Microbial communities within the bark and wood of trees are often unknown and unclear with respect to the ecology and disease-affecting properties of these communities (*e.g.* Ragazzi *et al.* 2003; Giordano *et al.* 2009; Kolarik *et al.* 2011; Hacquard and Schadt 2015). In some instances, forest epidemics are caused by pathogens not previously described until after the invasion. Due to incomplete data on fungal biogeography, determining whether a new disease is caused by a non-native pathogen, or whether existing microbial populations have evolved virulence in a now favorable environment is difficult to determine (Linzer *et al.* 2009; Shaw and Osborne 2011; Desprez-

Loustau *et al.* 2016). In addition, the unintentional movement of insect pests and pathogens around the globe has created a series of emergent tree diseases that are plaguing natural communities in North America (*e.g.* Sturrock *et al.* 2011; Ploetz *et al.* 2013). Sudden oak death (Rizzo *et al.* 2002), thousand cankers disease of walnut (Kolarik *et al.* 2001), laurel wilt (Hulcr and Dunn 2011), and dogwood anthracnose (Daughtry *et al.* 1996) are a few of the devastating diseases that have appeared in recent decades.

A community ecology approach to tree diseases may enable researchers to explain more completely the biotic factors that contribute to disease severity and mortality. Chestnut blight in North America and Europe has an extensive history of research investigating microbial communities in bark tissue and is a classic example of an introduced bark pathogen decimating a naïve host tree (reviewed in Anagnostakis 1987; Heiniger and Rigling 1994; Milgroom and Cortesi 2004). Thus, it serves as an excellent opportunity to investigate fungal community dynamics in diseased bark to better understand disease severity and ultimately the fate of diseased trees.

Chestnut blight

The chestnut blight pathogen (*Cryphonectria parasitica*) is a fungus that was unintentionally imported into the United States in the early 1900s (Merkel 1905) In the ensuing decades, it decimated American chestnut (*Castanea dentata*) and an estimated three-to-four billion mature trees in eastern North America were either killed or reduced to understory ramets (Keever 1953; Paillet 1982). Infections are initiated when *C. parasitica* spores enter the host through wounds in the outer bark (Hebard *et al.* 1984). Mycelial fans proliferate and extract

resources from the bark causing a localized, sunken canker on the infected stem. Over time, the canker can expand around the stem or branch and girdle it – destroying tissues down to the vascular cambium and killing the stem distal to the infection. Resistant and slightly resistant chestnut trees can slow or prevent canker expansion by compartmentalizing the pathogen through the formation of wound periderm (Hebard *et al.* 1984; McManus *et al.* 1989).

Besides forming wound periderm, another key factor in determining the probability that an infected stem will be girdled is the virulence of *C. parasitica*, which can be reduced by mycovirus infection of the fungal mycelium. Strains of *C. parasitica* infected with these double-stranded RNA mycoviruses (*i.e.* hypoviruses, Family Hypoviridae) (Choi and Nuss 1992; Day *et al.* 1977), initially were found in chestnut blight cankers in the 1950s on European chestnut (*Castanea sativa* Mill.) in Italy (Biraghi 1953) and France (Grente 1965). The hypovirulent strains were isolated from non-girdling cankers that did not completely destroy the vascular tissue, as host-produced callus tissue from the tree appeared to wall off the hypovirulent pathogen (Grente and Sauret 1978). In North America, hypovirulent *C. parasitica* strains also were found in cankers on American chestnut. These strains were shown to be less virulent than their virus-free counterparts (Fulbright *et al.* 1983; Hillman *et al.* 1992), although Hebard *et al.* (1984) demonstrated that infected American chestnuts are still susceptible and stems can be girdled even when the canker is composed of hypovirulent *C. parasitica.* Despite the variability, multiple studies support that American chestnut trees containing hypovirulent *C. parasitica* within cankers generally survive at higher rates

compared to trees without hypovirus infection within cankers (*e.g.* Griffin *et al.* 1983; Double *et al.* 2018).

The origin of hypoviruses is unknown, and in a canker, infection of virulent *C. parasitica* by a hypovirus is not straightforward (reviewed in Dawe and Nuss 2001; Milgroom and Cortesi 2004). Hyphal anastomosis (*i.e.* cytoplasmic fusion of vegetative hyphae) is required to successfully transfer hypoviruses, but transmission of the hypovirus is limited via vegetative incompatibility (*vic*) genes (Anagnostakis 1977; Anagnostakis and Day 1979; Huber 1996; Liu and Milgroom 1996). In the few instances of recovery in North American chestnut populations, cankers with hypovirulent *C. parasitica* are frequent in the chestnut populations and correlated with low *vic* diversity in the pathogen population (Springer *et al.* 2013). In addition, a persistent source of hypovirulent *C. parasitica* spores (*i.e.* spores infected with hypovirus) must be present in the environment to spread to hypovirus-free cankers, thus promoting long-term recovery of chestnut (MacDonald and Fulbright 1991). Bryner *et al.* (2013) discuss more complexities in canker dynamics on European chestnut; (1) hypovirus infection of the pathogen may occur at different times after canker establishment on a tree; (2) mycelia within a canker become only partially infected with hypoviruses; and (3) virulent spores can form new infections on the tree and cankers may merge. These variables, led to the conclusion that direct isolation of the pathogen from cankers and laboratory assessment for hypovirus infection is important for predicting the time to girdling (Bryner *et al.* 2013).

Cankers are known to succumb to invasion by other fungal taxa (*e.g.* Akilli *et al.* 2009; Double *et al.* 2013; Ćurković-Perica *et al.* 2017), but the influence of these invading fungi on canker expansion rates is largely unknown. Within a stand of American chestnut near West Salem, Wisconsin U.S.A where hypoviruses were being disseminated, annual monitoring revealed that non-girdling cankers accumulated non-*C. parasitica* fungi (hereafter: Non-CP) (Figure 1). Over time, these non-girdling cankers became diverse fungal communities with unexpected temporal patterns: the percentage of *C. parasitica* isolates containing hypovirus (hereafter: HCP) remained relatively constant within the canker; the percentage of virulent isolates of *C. parasitica* without hypovirus (hereafter: CP) decreased over time; and the percentage of Non-CP increased over the same period. After several years of hypovirus treatment of cankers, surviving trees exhibited fungal communities that were diverse, often containing more Non-CP overall than either virulent or hypovirulent *C. parasitica* (Double *et al.* 2013; Kolp *et al.* 2017). This pattern suggests that Non-CP may influence canker expansion rates and the probability that a canker will girdle an infected stem.

Model of fungal community dynamics in cankers

Non-girdling and girdling cankers harbor fungal communities that differ spatially (Figure 2). For non-girdling cankers, hypoviruses become established along the canker margin early in canker development (Figure 1; Bell 2004). Hypovirus infection of *C. parasitica* (HCP) slows canker expansion allowing the defense response of the tree to more effectively wall-off a less virulent pathogen, or at least delay the destruction of callus tissue by the pathogen (*e.g.* Hebard *et al.* 1984). As the slowly expanding canker ages, the inner area where the infection

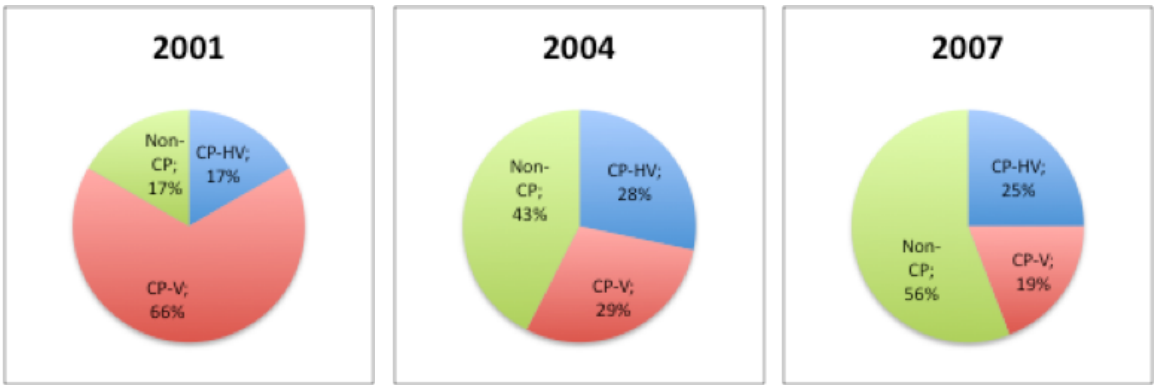

Figure 2-1: Temporal pattern of isolate frequencies (CP-V = *C. parasitica* without suspected hypovirus infection; CP-HV = *C. parasitica* with suspected hypovirus infection; Non-CP = fungi or bacteria other than *C. parasitica*) for 263 cankers. (MacDonald *et al.,* unpublished data).

began yields to invasion by saprophytic and weakly pathogenic fungi (*i.e.* Non-CP), which compete with CP for resources (Prospero *et al.* 2006). The combined effects of HCP and Non-CP act to decrease the presence of CP in the canker and the canker expansion rate, which increases the probability that the tree defenses will wall-off the canker and prevent girdling of the infected stem (Figure 2). This qualitative model also postulates that the fungal community within non-girdling cankers increases in diversity over time, with greater Non-CP overall within the inner areas of the canker The abundance of CP is predicted to decrease due competition with Non-CP taxa and hypovirus infection by HCP.

Girdling cankers are at the other extreme and quickly kill an infected stem. HCP is expected to be rare in these cankers (Figure 2), and without hypovirus to limit CP destroying host tissue at the margin, expansion will occur rapidly. While Non-CP may invade the center of the rapidly expanding cankers, their effect on canker expansion via competition will be minimal. The fungal community in these cankers will continue to be dominated by CP until the stem is girdled.

Objectives

To test this model and investigate how spatial and temporal dynamics of the fungal community within cankers influence the severity of cankers and the likelihood of girdling, chestnut blight cankers from six chestnut populations were monitored for up to five years. The composition, distribution, and persistence of CP, HCP, and Non-CP within a canker is predicted to influence the severity rating of a canker and the probability that a canker will girdle an infected stem. The chestnut populations differed with respect to the degree of

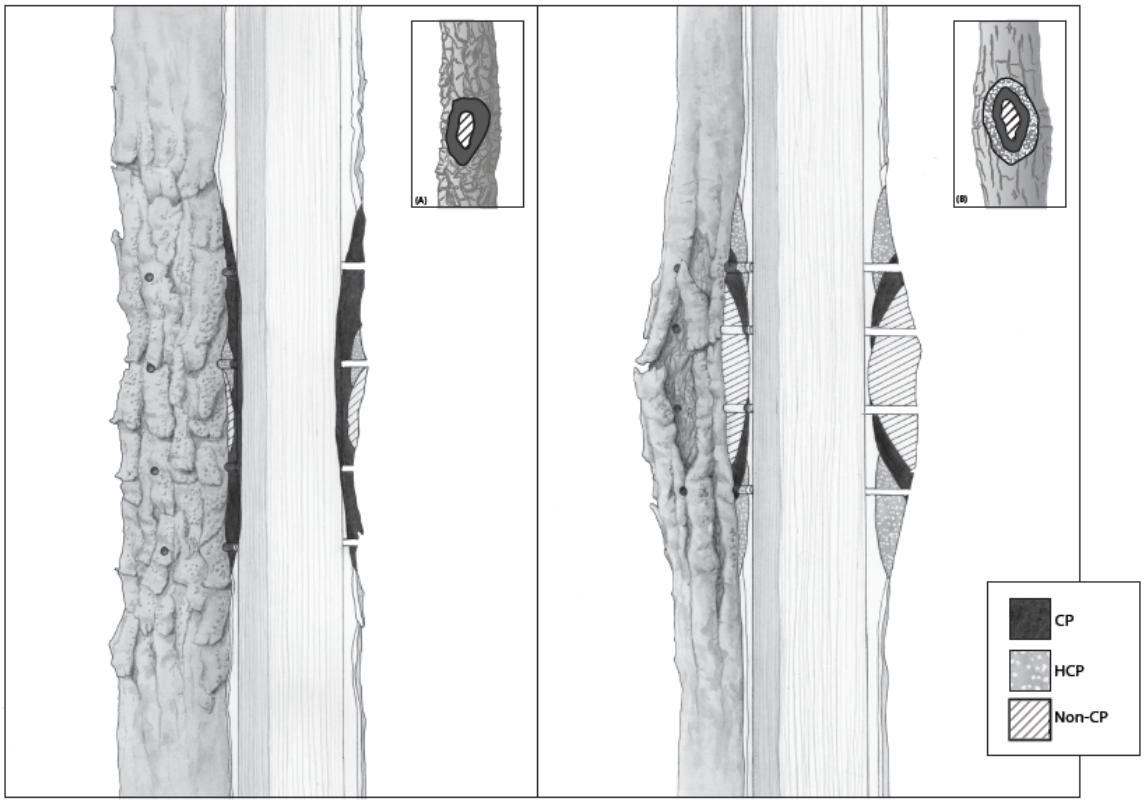

Figure 2-2: Model of fungal communities within girdling (left) and non-girdling (right) cankers on infected chestnut (cross-section). Left Panel (Girdling canker): Virulent *C. parasitica* (CP) promotes a very high rate of canker expansion in the absence of hypovirus-infected *C. parasitica* (HCP) at the canker margin. While a variety of other fungal taxa (Non-CP) may invade the inner portion of the canker, their influence on CP at the margin is negligible to prevent girdling of the infected stem. Right Panel (Non-girdling canker): Hypoviruses become established along the margins of cankers (HCP), which reduces the rate of cankers expansion by CP. Eventually the inner area of these cankers senesce and become vulnerable to invasion by Non-CP. The Non-CP fungi compete with CP for resources within the inner region of the canker, further reducing the rate of canker expansion.

recovery of infected trees. Cankers in recovering populations generally have a less severe morphology (described below) and often contain HCP. In contrast, cankers in epidemic populations have a more severe morphology and typically contain little HCP (Springer *et al.* 2013). At the West Salem site in Wisconsin, cankers were *treated* with hypoviruses with the goal of establishing HCP mycelium at the margins of the canker (Double *et al.* 2018).

The Non-CP community within cankers in recovering populations is hypothesized to differ from those at epidemic populations, especially if Non-CP taxa vary in their interaction with chestnut and *C. parasitica*. Previous research suggests that *Trichoderma* spp. isolated from chestnut bark and the soil have the potential to influence disease severity when introduced to the canker (Akilli *et al.* 2011; Arisan-Atac *et al.* 1995; Tattar *et al.* 1996; Weidlich 1978). In addition, *Trichoderma* is among the Non-CP commonly isolated from non-girdling cankers at the West Salem, WI chestnut population (Double *et al.* 2013). Other Non-CP within cankers may be weak pathogens of chestnut and exacerbate disease (Bissegger and Sieber 1994), or depend on colonization of healthy chestnut by virulent *C. parasitica* in order to complete their life cycle before decay fungi invade (Russin and Shain 1984). Thus, those Non-CP taxa antagonistic toward *C. parasitica* are predicted to be abundant in canker communities associated with recovering chestnut populations and less frequent in epidemic populations.

MATERIALS AND METHODS

Sampling sites

The six chestnut populations used in this study originated from different American chestnut trees planted on early homesteads in Michigan and Wisconsin – outside the native chestnut range where the major blight epidemic began in the early 20th century (Paillet and Rutter 1989; Brewer 1995). Five of the six populations are located in the northern Lower Peninsula of Michigan (Davelos and Jarosz 2004; Springer *et al.* 2013) and the sixth near West Salem, WI in the southwestern part of the state (Paillet and Rutter 1989). The five Michigan populations were monitored intermittently between the 1970s and mid-1990s (Fulbright *et al.* 1983; Brewer 1995). A demographic survey began in 1996 for four of the five sites (Davelos 1999; Davelos and Jarosz 2004; Springer *et al.* 2013). Surveying of chestnut demography began at the sixth Michigan population (Roscommon) in 2007 (Springer *et al.* 2013). A survey of the West Salem, WI site began in 2002 using methodology similar to that used in the Michigan populations (Jarosz and Davelos Baines, unpublished data).

Epidemic chestnut populations – Leelanau [LE] and Missaukee [MS]

The Leelanau (LE) and Missaukee (MS) chestnut populations have experienced severe epidemics since blight was first found in 1997 (Springer *et al.* 2013). The prevalence of hypoviruses is less than 10% at each site (Davelos 1999; Springer *et al.* 2013), and the majority of cankers at these sites are girdling, although some non-girdling cankers exist (AM Jarosz, unpublished data).

Two stands of chestnut in Missaukee County were treated as separate populations in the past because blight was present at one site and absent at the other (see Davelos and Jarosz 2004; Springer *et al.* 2013). However, blight migrated to the previously disease-free site (called 'Missaukee Healthy' [MH]) and a severe epidemic has developed similar to the diseased site ('Missaukee Diseased' [MD]). Therefore, the two sites are combined as one population (MS) since they are separated by only ~0.25 km. Some trees at both sites have been treated with hypovirus in the past (Springer 2013) and some of those cankers have been included in this study.

Recovering populations – County Line [CL], Roscommon [RC], and Frankfort [FR]

The County Line (CL), Roscommon (RC), and Frankfort (FR) chestnut populations have naturally occurring hypovirus infection of *C. parasitica* (Fulbright *et al.* 1983; Peever *et al.* 1997; Davelos 1999; Springer *et al.* 2013). At CL, greater than 90% of chestnut blight cankers sampled during the 1990s and 2000s contained hypovirus (Davelos 1999; Springer *et al.* 2013). At RC, greater than 90% of cankers sampled in the 2000s contained hypoviruses (Springer *et al.* 2013). Although girdling cankers are found at CL and RC, they are less frequent compared to the epidemic LE and MS sites. CL and RC have many large, reproductively mature trees despite cankers forming on stems and branches (Davelos and Jarosz 2004; AM Jarosz, unpublished data).

Hypovirus prevalence at FR was >90% in the 1990s (Davelos 1999). However, hypovirus prevalence had fallen to 76% by 2009 (Springer *et al.* 2013), which may be contributing to a larger proportion of girdling cankers relative to the CL and RC populations (AM Jarosz,

unpublished data). Davelos and Jarosz (2004) reported that FR is intermediate in recovery from the blight epidemic, as recruitment of seedlings is lower compared to CL and RC despite many large, reproducing trees.

Hypovirus treated population – West Salem [WS]

The WS chestnut population is located in southwestern Wisconsin (43°57'09"N, 91°03'19"W). Over 4,000 chestnut trees are interspersed within this mixed hardwood stand (Paillet and Rutter 1989). The present stand is derived from progeny of ten American chestnuts planted at the site in the 1880s. WS was blight-free until 1987 (Double *et al.* 2013). Beginning in 1992, a large, experimental dissemination of two hypoviruses (CoLi and EURO7; Double *et al.* 2013) began by inoculating newly discovered cankers with hypovirus-infected *C. parasitica* at the canker margin (Double *et al.* 2018). Annual treatments ceased in 1998, but began again in 2004 on a subset of trees. Treatments ceased again in 2016.

Canker sampling

In 2012, sampling began at the CL, RC, and WS sites, and in 2013 at LE, FR, and MS. Cankers were sampled annually through 2016 so the presence and persistence of CP, HCP, and Non-CP taxa could be evaluated temporally. In 2012 and 2013, 12 bark samples spaced along the margin of each canker were obtained using a bone marrow instrument (Lee-Lok, 11-guage, 4-inch, Lee Medical, Skillman, NJ), collecting bark layers in a plug down to the cambium. From 2014-2016, 24 bark samples were obtained per canker: 12 samples from the inner canker area in addition to 12 around the canker margin. Cankers were sampled differently depending on how much of the canker area had expanded around the

circumference of the stem or branch: The 'Clock' sampling method was used when the canker had not expanded around the infected stem. Samples were taken around the margin as in a clock face. Inner area bark samples were collected within the clock face of the canker. The 'Ring' sampling method was used when the canker had expanded completely around the stem or nearly so (Figure 2). Six samples were taken at the top and bottom of the canker margin around the stem or branch, in addition to the two rings of six samples collected within the canker area. Bark samples were collected and stored per Double *et al.* (2013) until processed (see below).

Cankers were rated annually using an ordinal system similar to Double *et al.* (2013). Cankers that displayed healing symptoms (*e.g.* plant callus tissue forming at or near margin of canker) with very little signs of the disease (*e.g.* stroma breaking through outer bark) were assigned a rating of '1'. Cankers rated as '4' had a sunken appearance due to dead or dying plant cells and displayed abundant pathogen stroma erupting through the outer bark. Cankers rated '2' had some callus tissue but also small areas of collapsed, sunken bark tissue and slight-to-moderate amounts of stroma erupting through the bark. Cankers rated '3' had some callus tissue but large areas of the canker had a sunken appearance with moderate-to-high amounts of stroma.

Trees were monitored annually to determine whether they were alive or dead (*i.e.* girdled) above the sampled canker. If the tree was alive, the diameter of the tree at breast height (DBH) in cm was measured. While all dead trees were removed from the study, approximately half of the cankers causing tree death were sampled the year the tree died.

Canker height from the ground also was recorded; however, it was not found to be an important predictor of either disease rating nor correlated with the composition of the canker fungal community. Thus, canker height was not included in the analyses.

Isolation and identification of fungi

All bark samples collected from diseased chestnut were initially surface-sterilized following the protocols of Double *et al.* (2013). Bark pieces were then transferred aseptically to petri plates containing ~25mL of solidified potato dextrose agar (39g of PDA in 1 L water; Difco, Becton, Dickinson, and Company, Sparks, MD) and incubated at room temperature. Emerging fungal colonies were transferred to new PDA plates via hyphal tipping. Some bark samples yielded two unique fungal colonies. In these cases, both isolates were recorded as occupying that bark sample.

Cryphonectria parasitica isolates were easily distinguished from Non-CP isolates and were characterized further as either virulent (CP) or hypovirulent (HCP). In culture, dark, orange-pigmented mycelia with abundant pycnidia were indicative of virulent *C. parasitica* (CP); slow vegetative growth with crenulated edges was typical of hypovirulent *C. parasitica* (HCP) from CL, RC, FT, LE, and MS; and white-pigmented mycelia with few pycnidia was characteristic of hypovirus infection at WS. In the past, *C. parasitica* isolates from the WS site have been tested for hypovirus using protocols from Morris and Dodds (Method 1, 1979) and culture morphology (Double *et al.* 2018). Michigan isolates have been evaluated historically based on culture morphology (*e.g.* Fulbright *et al.* 1983; Fulbright 1984; Peever *et al.* 1997; Smart *et al.* 1999; Springer *et al.* 2013). Several isolates also were tested in 2013 for

the presence of dsRNA using the procedure in Morris and Dodds (1979; data not shown). To confirm our categorization of virulence, a subset of CP and HCP isolates were tested for their pathogenicity on Golden Delicious apples using the protocols of Fulbright (1984); data not shown).

Isolates of Non-CP were grouped into distinct operational taxonomic units (OTU) based on colony and spore morphologies (Dugan 2006; St-Germain and Summerbell 2011). Representative isolates of 15 common OTUs were selected for DNA sequencing using internal transcribed spacer (ITS) region (White *et al.* 1991). Mycelia from fresh cultures of each representative OTU were scraped into a cold mortar and ground using a cold pestle and liquid nitrogen. Genomic DNA from ground mycelia was extracted using a QIAGEN® DNeasy Plant Kit following the manufacturer's instructions. The ITS (ITS1, 5.8S gene, ITS2) region was amplified using the primer pair of ITS1 and ITS4. Amplification via polymerase chain reaction (PCR) of DNA was executed in a 25µL or 50µL reaction volume containing GoTaq® Green Master Mix. PCR was carried out following the thermal cycling program: 1min initial denaturation at 94ºC, followed by 30 cycles of 1 min denaturing at 94ºC, 1 min of primer annealing at 50ºC, 90 seconds of extension at 72ºC, and a final extension at 72ºC for 7 min. PCR products (6µL per well) were examined by electrophoresis at 83 V for 70 min in a 1.5% agarose gel that was pre-stained with ethidium bromide in 1X TAE buffer and viewed under ultraviolet light. PCR products were purified using a mix of 0.125µL FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific), 0.15µL Exonuclease I (Thermo Scientific), and 5µL DNase-free water for each 6µL of PCR product. Forward and reverse sequences were

assembled using Geneious 9.0.2 (http://www.geneious.com, Kearse *et al.* 2012). Contigs were queried against GenBank® (Clark *et al.* 2016) using BLAST (Altschul *et al.* 1990).

Long-term storage of a subset of CP and HCP isolates (at least one of each per canker, if present) involved growing isolates on PDA plates with pieces (2~3 cm^2) of cellulose filter paper on the surface of the medium (Whatman®; Millipore Sigma – Darmstadt; Germany.). After two weeks, CP or HCP isolates grew across the filter paper, and with tweezers, the mycelium-containing filter paper was placed into autoclaved coin envelopes and stored at -20ºC in a laboratory freezer.

Long-term storage of a subset of Non-CP isolates (at least one isolate for each Non-CP OTU) involved removing two-to-three plugs of mycelia using a 3-millimeter cork borer and placing them into 15 x 45 mm 1 dram glass vials (Kimble Glass, Inc.), filled with approximately 2 mL of a 30% glycerol solution and then sealing with parafilm. Two duplicate vials were generated for each stored Non-CP isolate: One set was placed at -80ºC freezer, the other on a shelf at room temperature in the laboratory.

Statistical analyses

All statistical analyses were performed in R (V3.2.2). Shannon-Weiner index (*H'*) was used to describe diversity within factors: population, canker rating, canker area, sampling year, and tree health using the package 'vegan'. Diversity within cankers of varying tree sizes (DBH) was assessed using a generalized linear model (Poisson distribution) and an offset to account for differences in sampling effort (12 samples/canker in 2012 and 2013; 24

samples/canker in 2014-2016). Following the recommendations of Paliy and Shankar (2016), the microbial community of each canker sample was characterized using a k-means clustering analysis to classify observations into four clusters (canker community clusters A-D) based on species composition and abundances within each canker. Principal component analysis (PCA) was used (R package 'ggfortify') to characterize the major axes of variation, reduce the dimensionality of the dataset, and visualize trends among clusters. A cumulative link mixed model (R packages 'lme4' and 'ordinal') was used to test for differences among canker ratings and community clusters, tree size, and tree health. The sampling year, population, and trees within each population were treated as random effects.

RESULTS

Overall composition

The 201 chestnut blight cankers that were sampled yielded 12,322 fungal isolates (Table 1). Two-thirds of all isolates that accounted for the canker community were *C. parasitica* – 33% HCP and 33.2% CP (Table 2). The other third (33.8%) of the canker community was composed of at least 59 other Non-CP taxa (Table 2). The four most common Non-CP taxa were isolates of *Penicillium* (851 isolates of 12,322 or 6.9%), followed by *Trichoderma* (3.9%), *Pezicula* (3.7%), and *Nectria* (2.9%). The fifth most common Non-CP, *Umbelopsis isabellina* made up only 1.7% of all fungal isolates. To assess our sampling effort for less frequently occurring Non-CP taxa, we plotted our accumulation of new taxa with each new canker and sampling year (Figure 3).

Table 2-1: Summary of sampling of chestnut blight cankers during the summers in 2012-2016. Shannon-Weiner diversity (*H'*) was calculated for each chestnut population, canker disease rating, designated areas within cankers, year of sampling, and whether the tree was alive or dead above the canker.

Canker Rating	Cankers sampled	Bark samples	Fungal Isolates	*H'*
1	109	2,207	2,067	*1.31*
2	225	4,632	4,516	*1.27*
3	187	3,720	3,525	*1.12*
4	112	2,232	2,214	*0.90*
Canker Area*				
Margin	633	7,884	7,579	*1.08*
Inner Area	550	4,907	4,743	*1.14*
Population				
County Line	130	2,532	2,446	*1.31*
Roscommon	132	2,604	2,547	*1.13*
Frankfort	115	2,423	2,266	*1.37*
Leelanau	59	1,272	1,233	*1.28*
Missaukee	48	984	973	*0.90*
West Salem	149	2,976	2,857	*0.97*
Sampling year**				
1st year	201	2,846	2,706	*0.84*
2nd year	168	3,419	3,251	*1.11*
3rd year	126	3,096	2,959	*1.43*
4th year	100	2,470	2,450	*1.45*
5th year	38	936	956	*1.54*
Tree Health				
Alive	593	11,687	11,276	*1.17*
Dead***	40	1,104	1,046	*1.18*
Totals	**633**	**12,791**	**12,322**	

***** = Beginning in 2014, the area within the canker margin (*i.e.* inner area) was sampled in addition to the margin

****** = Canker observations are separated by sampling year, meaning a canker sampled in 2012 is counted again in 2013 and each year until death of the tree.

*** = Tree dead at or above canker

Table 2-2: List of non-C. parasitica, fungal OTUs (Non-CP) in order of prevalence (1 = most prevalent) and the total number of Non-CP isolates obtained from chestnut blight canker sampling in 2012-2016.

OTU Name/ID	Isolate ID for PCR	Accession #[a]	Closest taxa match	Similarity Index (%)[b]	Accession match[c]	Total isolates
1. Penicillium	MKBG10_2013	MH384912	*Penicillium spinulosum*	99.6	KF646101	851
	MKBG15_2013	MH384915	*Penicillium glabrum*	100.0	MG659664	
	MKBG51_2014	MH384920	*Penicillium citreonigrum*	99.9	KJ780798	
2. Trichoderma	MKBG11_2013	MH384913	*Trichoderma harzianum*	99.8	MF567525	481
	MKBG13_2014	MH384914	*Trichoderma citrinoviride*	99.2	MG878433	
	MKBG5_2013	MH384911	*Trichoderma atroviride*	100.0	KY225624	
3. Pezicula	MKJP2014 81_2012	MH384928	*Pezicula cinnamomea*	100.0	KR859235	457
	MKJP2012_3 53_2012	MH384930	*Pezicula ericae*	98.0	KR859173	
4. Nectria	MKBG45_2014	MH384919	*Nectria cinnabarina*	99.3	KP305907	358
5. Umbelopsis	MKJP2012_3 31_2012	MH384929	*Umbelopsis isabellina*	98.9	LC100011	210
6. Valsaceae	MKBG25_2013	MH384917	*Valsaceae* sp.	98.1	KC963923	179
7. Strasseria	n/a	n/a	n/a	n/a	n/a	178
8. Unknown 7	n/a	n/a	n/a	n/a	n/a	151
9. Gnomoniopsis	MKBG20_2013	MH384916	*Gnomoniopsis smithogilvyi*	99.8	MG495621	93
	MKJP2_2012 20b_2012	MH384925	*Gnomoniopsis castanea*	100.0	KU886073	
10. Sarocladium	MKBG52_2014	MH384921	*Sarocladium implicatum*	100.0	GU189520	92
11. Unknown 30	n/a	n/a	n/a	n/a	n/a	86
12. Diplodia	MKJP2_2012 25a_2012	MH384927	*Diplodia corticola*	100.0	KF766156	64
	MKJP2_2012 3dota_2012	MH384924	*Diplodia seriata*	100.0	KY608885	
13. Alternaria	MKJP2014 5b_2014	MH384922	*Alternaria alternata*	98.7	MF141010	59
	MKJP2014 5a_2014	MH384923	*Alternaria brassicae*	98.3	JF439433	
14. Unknown 4	n/a	n/a	n/a	n/a	n/a	57
15. Mucor	MKBG42_2014	MH384918	*Mucor circinelloides*	99.2	KT207740	53
	MKJP2_2012 22b_2012	MH384926	*Mucor fragilis*	98.4	GU566275	
16. Unknown 24	n/a	n/a	n/a	n/a	n/a	52
17. Unknown 6	n/a	n/a	n/a	n/a	n/a	51
All 42 others[d]	n/a	n/a	n/a	n/a	n/a	689
Total	n/a	n/a	n/a	n/a	n/a	4161

a = Deposited isolate sequence accession number
b = Level of similarity for pairwise alignment with the closest match
c = Closest match accession number
d = Each OTU <0.4% of all fungal isolates collected

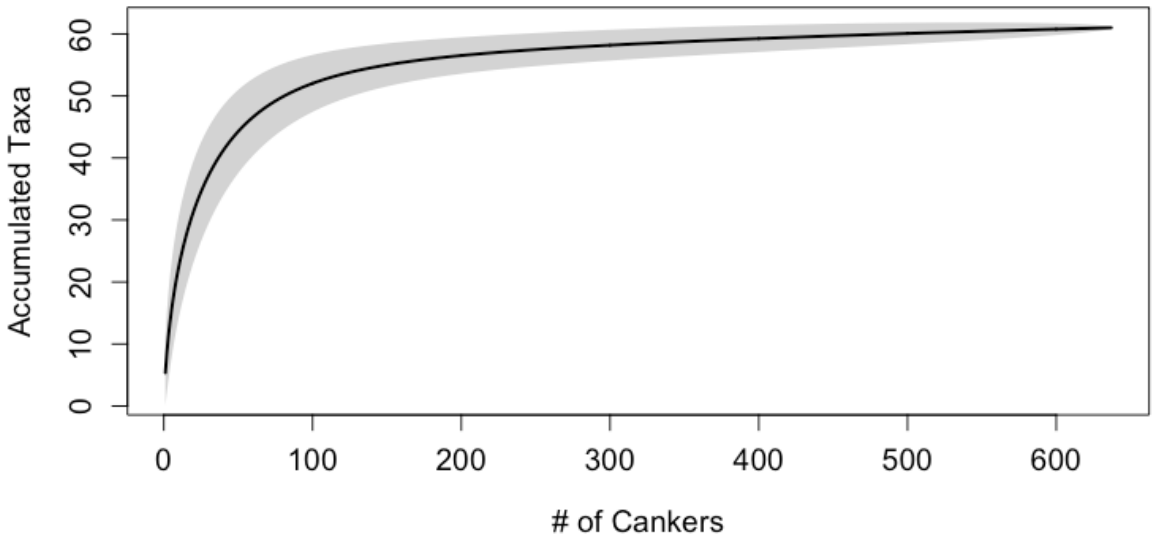

Figure 2-3: Species accumulation curve for sampling of fungal community within chestnut blight cankers. Sixty-one OTUs were assigned to 11,900 fungal isolates collected from 633 chestnut blight cankers during 2012-2016.

Disease severity and spatial distribution of fungi

The frequency of CP, HCP, and Non-CP varied depending on whether a canker was rated 1 or 2 (*i.e.* low severity cankers) compared to cankers rated 3 or 4 ($\chi^2 = 343.2$, df = 1, $p < 0.0001$). Cankers rated '4' (*i.e.* the most severe rating) also were the least diverse overall as measured by Shannon-Weiner index compared to cankers rated 1 and 2 (Table 1). More HCP (36.6%) and Non-CP (39.1%) were recovered from cankers rated 1 or 2 compared to cankers rated 3 or 4 (28.3% HCP and 28% Non-CP). Only 24.3% of isolates from cankers rated 1 or 2 were CP, compared to 43.7% CP isolates from cankers rated 3 or 4.

The occurrence of CP, HCP, and Non-CP in the inner area and margin of cankers differed significantly ($\chi^2 = 23.5$, df = 1, $p < 0.0001$). Approximately one-quarter of all isolates at the margin of cankers rated 1 or 2 were CP (24.8%), compared to 45.4% CP at the margin of cankers rated 3 or 4 (Table 3). HCP at the margin of cankers rated 3 or 4 occurred less often (26.5%) compared to HCP within the inner area (33%). There was no difference in the spatial distribution of HCP within cankers rated 1 or 2 (Table 3). Like HCP, Non-CP taxa also were isolated more frequently from cankers rated 1 or 2 compared to cankers rated 3 or 4; however, there was no difference between the frequencies of total Non-CP at the margin compared to the inner area among canker ratings (Table 3).

Overall, the margin of chestnut blight cankers was less diverse than the inner canker area based on Shannon-Weiner index (Table 1). Some Non-CP taxa were more commonly isolated from the inner area of cankers (*e.g. Penicillium, Trichoderma*) compared to the margin (Table 3). In contrast, *Pezicula* and *Nectria* displayed the opposite pattern. Several Non-CP

taxa found at low frequency in cankers were more likely to be isolated from the margin or inner area of cankers (Table 3). For example, an unknown fungus identified only to the family Valsaceae was consistently found at a higher frequency in the margin than the inner area, regardless of canker rating (Table 3); *Gnomoniopsis* and *Strasseria* also followed this pattern. An unknown fungus (unknown7) and *Umbelopsis isabellina* were more likely to be found in the inner area of cankers.

Population differences

There was no general trend between population status (recovery vs. epidemic) and canker community diversity as measured by Shannon-Weiner index (Table 1). Cankers at FR and CL, two of the three recovering chestnut sites, were among the most diverse.

The third population considered in recovery, RC, had only the fourth highest diversity measure among the six sites (Table 1). Epidemic site MS had the lowest diversity within cankers, although the other epidemic site (LE) had the third highest diversity. HCP was more prevalent in cankers from the three recovering populations (CL, RC, and FT) compared to the two epidemic sites (MS and LE; Table 4). Nearly half (45.9%) of isolates from cankers at CL were categorized as HCP, compared to only 14.8% and 21.5% HCP at MS and LE, respectively. In contrast, over half of isolates at MS were CP (57.8%), followed by the other epidemic site LE at 41.9%. The range of total Non-CP within cankers among the six populations varied from a high of 38% at CL, to a low of 27.1% at WS (Table 4). Some Non-CP taxa were more likely to be isolated from cankers at particular populations.

Table 2-3: Frequency of CP, HCP, and Non-CP within the margin and inner area of cankers rated 1 or 2 and cankers rated 3 or 4. Values are percentages of isolates within taxon across all years and populations.

Taxon	Overall	All Cankers		Canker Rating 1 or 2		Canker Rating 3 or 4	
		Margin	Inner Area	Margin	Inner Area	Margin	Inner Area
CP	33.2%	34.8	30.7	24.8	23.4	45.4	38.7
HCP	33.0	31.4	35.4	36.0	37.6	26.5	33.0
Non-CP	33.8	33.8	33.9	39.2	39.0	28.1	28.3
	↓	↓	↓	↓	↓	↓	↓
Penicillium	6.9	4.5	7.1	4.8	7.9	4.2	6.1
Trichoderma	3.9	3.8	4.4	5.6	6.1	1.7	2.4
Pezicula	3.7	4.5	2.1	5.1	2.7	4.5	1.8
Nectria	2.9	3.4	1.8	2.6	1.8	3.7	1.9
Umbelopsis	1.7	0.8	1.7	1.0	2.0	0.7	1.6
Valsaceae	1.5	2.0	0.4	2.4	0.3	2.0	0.5
Strasseria	1.4	1.6	1.1	2.2	1.6	1.2	0.5
unknown7	1.2	0.6	2.2	0.7	2.3	0.6	2.3
Gnomoniopsis	0.8	0.8	0.4	0.9	0.3	0.7	0.4
Acremonium	0.8	0.6	1.0	0.7	0.8	0.6	1.0
unknown30	0.7	0.7	0.5	1.0	0.7	0.7	0.4
Botryosphaeria	0.5	0.5	0.4	0.6	0.2	0.4	0.4
Alternaria	0.5	0.4	0.4	0.6	0.2	0.4	0.4
unknown4	0.5	0.5	0.3	0.5	0.2	0.6	0.2
Mucor	0.4	0.5	0.3	0.5	0.4	0.4	0.2
unknown24	0.4	0.3	0.4	0.4	0.2	0.4	0.4
unknown6	0.4	0.2	0.7	0.3	0.9	0.2	0.8
all 42 others	5.7	8.1	9.0	9.3	10.3	5.2	7.0
% = percentage of isolates across all years and populations							

For example, *Trichoderma* made up 11% of all isolates from WS (Table 5). No other site had higher than 4% *Trichoderma.* The frequency of *Penicillium* in cankers also varied across chestnut populations, making up 9.6% of isolates at RC but only 1.5% of isolates at WS (Table 5). *Pezicula* was relatively common at CL (7.6% of isolates) and RC (5.9% of isolates), two of the three recovering populations; however, *Pezicula* was also found at a high frequency at MS, an epidemic site (6.5% of isolates; Table 5).

There was no relationship between species richness in the canker community and tree size (Figure 4). Survivorship and change in tree size were monitored from 2013 and followed until 2016 at all six populations (Table 4). All three recovering populations exhibited higher survivorship (>74%) compared to the two epidemic sites (<27%) and WS (46.4% survivorship; Table 4). Average tree size increased by 28% and 27% at CL and RC, respectively (Table 4). In contrast, tree size did not change at FR despite high survivorship.

Canker communities as "clusters"

The k-means clustering analysis grouped canker communities into four clusters (A, B, C, or D) with respect to the presence, absence, and abundance of 61 different fungal taxa isolated from cankers during this study (Table 6). Principal components analysis revealed an association between the response variable (cluster) and two primary axes of the multivariate community dataset, which explained 81% of all the variation among cankers (Figure 5). The primary axis (PC1) explained 66% of community-level variation among cankers, predominantly between canker communities with either abundant HCP or CP. The second

Table 2-4: Average tree size (measured as stem diameter at breast height in cm), cohort size (n), percent survivorship, and average percent CP, HCP, and total Non-CP for cankers of living trees sampled annually beginning in 2013 through 2016 at six chestnut populations.

Population	Avg. Treesize (cm) 2013	n	Avg. Treesize (cm) 2016	n	% Survivorship	Avg. % CP	Avg. % HCP	Avg. % Non-CP
CL	9.9	29	12.7	26	89.7	16.1	45.9	38.1
RC	9.5	31	12.0	23	74.2	28.3	35.7	36.0
FR	9.6	31	9.3	25	80.6	29.9	32.6	37.5
LE	11.8	15	13.0	4	26.7	41.9	21.5	36.6
MS	7.0	18	7.2	2	11.1	57.8	14.8	27.3
WS	22.0	28	21.4	13	46.4	43.1	29.9	27.0

Table 2-5: Frequency of fungi sampled from cankers in each chestnut population. Species richness = total unique Non-CP taxa. Shaded cells indicate taxon not found at that population.

Taxon	Overall	Recovering populations			Epidemic populations		Treated
		County Line	Roscommon	Frankfort	Leelanau	Missuakee	West Salem
CP	33.2%	16.1	28.3	29.8	41.9	57.8	43.1
HCP	33.0	45.9	35.7	32.6	21.5	14.8	29.8
NonCP	33.8	38.0	36.0	37.6	36.6	27.4	27.1
Richness	59 ↓	53 ↓	46 ↓	49 ↓	42 ↓	34 ↓	52 ↓
Penicillium	6.9%	5.2	9.6	5.9	5.4	6.6	1.5
Trichoderma	3.9	1.6	2.3	1.7	0.9	3.3	11.0
Pezicula	3.7	7.6	5.9	1.1	0.4	6.5	0.2
Nectria	2.9	1.2	0.9	6.0	11.7	0.1	0.3
Umbelopsis	1.7	1.1	1.7	1.0	1.2	1.2	0.7
Valsaceae	1.5	2.4	0.2	1.7	3.3	0.7	0.9
Strasseria	1.4	2.4	1.0	1.6	1.3	0.1	1.1
unknown7	1.2	0.9	0.2	4.5	1.2	0.2	0.1
Gnomoniopsis	0.8	1.1	0.0	0.7	0.0	0.2	1.1
Acremonium	0.8	0.7	1.8	0.2	1.0	0.0	0.7
unknown30	0.7	0.8	0.3	0.3	0.3	0.0	1.1
Botryosphaeria	0.5	0.2	0.2	0.3	0.3	0.3	1.7
Alternaria	0.5	0.2	1.1	0.1	0.6	0.0	0.3
unknown4	0.5	0.6	0.0	1.1	0.6	0.4	0.1
Mucor	0.4	1.0	0.2	0.0	0.1	0.1	0.5
unknown24	0.4	0.3	0.5	0.2	0.1	0.2	0.1
unknown6	0.4	0.8	0.8	0.3	0.1	0.2	0.1
all 42 others	5.7	9.9	9.3	10.9	8.1	7.3	5.6
% = percentage of isolates across all years and populations							

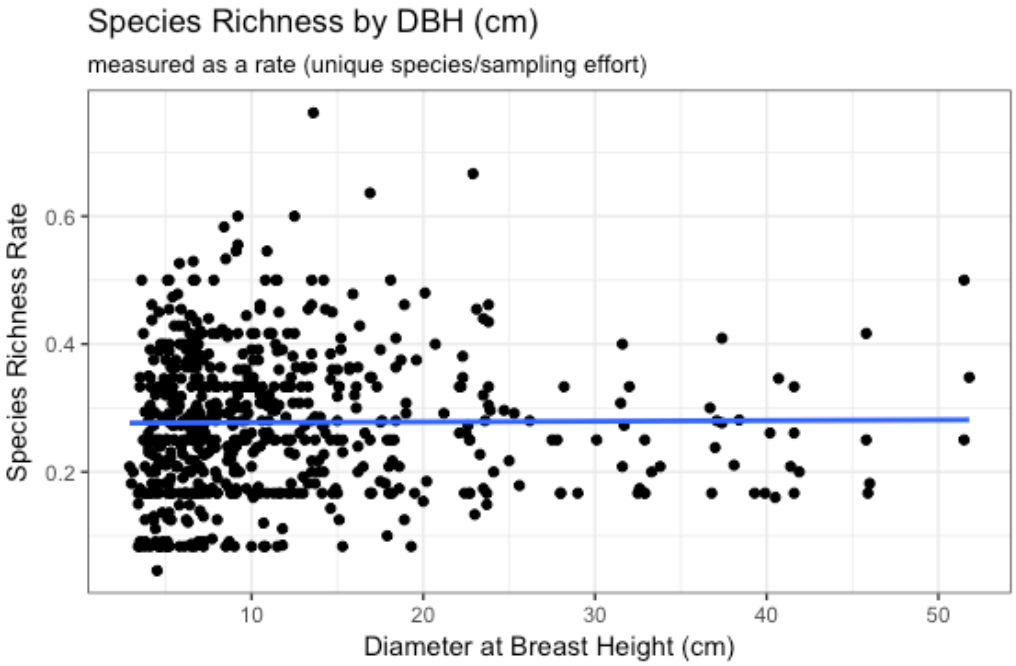

Figure 2-4: Tree size (Diameter at Breast Height [DBH] in cm) and species richness rate (species richness / sampling effort or number of isolates) within cankers sampled from 2012-2016. Twelve samples were taken per canker in 2012-2013; 24 samples per canker in 2014-2016.

Table 2-6: Number of canker-year observations (n) and proportions of common fungal taxa for each canker community cluster (A-D) as calculated via K-means clustering analysis for all cankers sampled from 2012-2016. From Cluster A to D the frequency of HCP (hypovirulent *C. parasitica*) decreases, whereas the frequency of CP (hypovirulent *C. parasitica*) increases. The Total Non-CP column represents total Non-CP for that community cluster. Infrequent Non-CP taxa are pooled as a percentage of the remaining abundance within each community cluster type.

CLUSTER (n)	CP	HCP	Total Non-CP	*Penicillium*	*Trichoderma*	*Pezicula*	*Nectria*	*Umbelopsis*	All 54 others
A (82)	11.8	68.9	19.3	2.6	2	2.2	1.2	1.3	10
B (177)	15.9	30.9	53.2	11.3	7.2	5.9	3.7	2.4	22.7
C (184)	49.8	20.4	29.8	6.2	3.2	2.6	3.8	1.2	12.8
D (190)	83.5	5.7	10.8	1.5	1	1.3	1	0.1	5.9

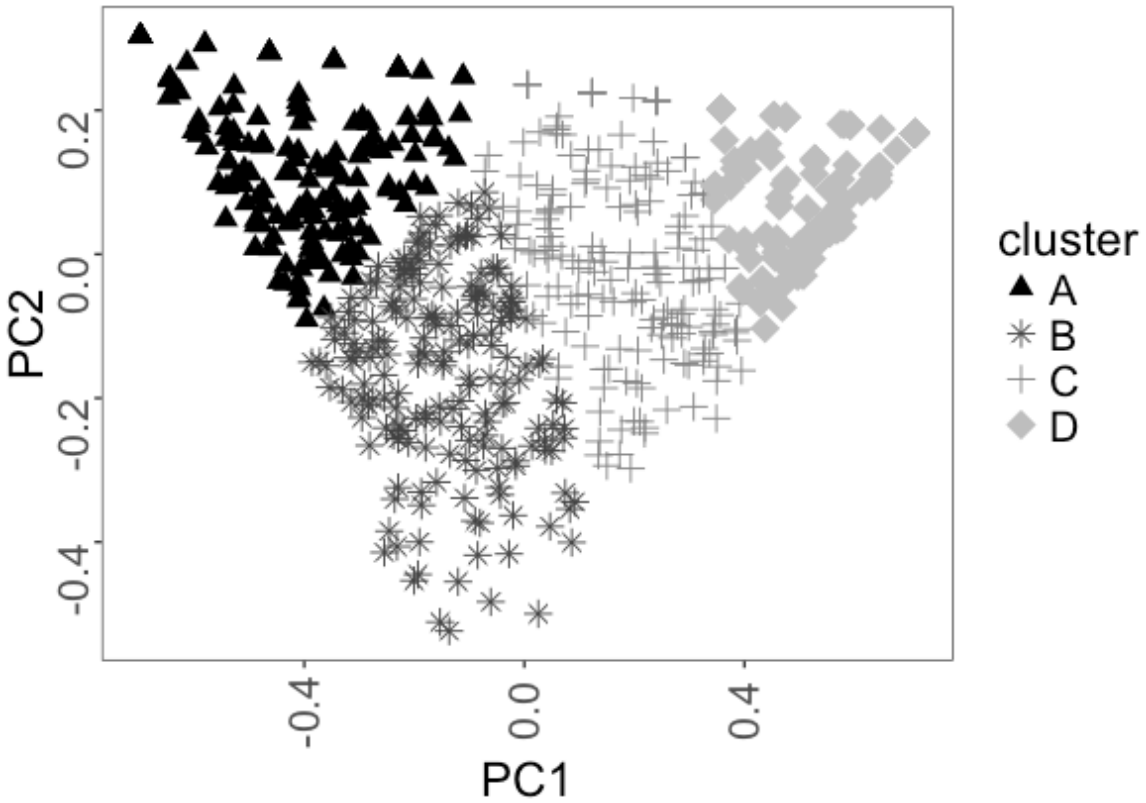

Figure 2-5: Principal Components Analysis (PCA) plot of four community clusters from canker-year observations (n = 633) of 61 fungal taxa sampled. PC1 separates observations based on the ratio of CP to HCP, where greater values represent greater amounts of CP relative to HCP in a canker. PC2 separates observations based on the amount of Non-CP, where smaller (negative values) represent more Non-CP in a canker relative to CP and HCP.

principal component (PC2) explained 15% of the remaining variation. Abundant *Penicillium* and *Trichoderma*, as well as total Non-CP in the canker community, were strongly associated with this axis. Each canker-year observation was assigned to a cluster. Generally, the abundance of CP increased from cluster A to D, while HCP decreased (Table 6). Total Non-CP was relatively low in clusters A and D, which were dominated by HCP and CP, respectively. However, total Non-CP peaked in cluster B – representing more than 50% of the canker community. *Trichoderma* and *Penicillium* averaged 7.4% and 11.3% within cluster B cankers, respectively.

Cluster associations with disease severity and tree survivorship

Clusters were associated with canker rating (Figure 6). More than 80% of cluster D cankers, which had a very high frequency of CP, were rated either 3 or 4, the most severe ratings. In contrast, clusters A and B were associated with low severity cankers (*i.e.*, ratings 1 and 2). The fourth cluster, C, was associated with intermediate ratings, 2 or 3.

Trends in survivorship were evaluated based on the first year of sampling, which was considered year one for the canker. Cankers first sampled in 2012 were monitored for up to five years, while those cankers first sampled in 2013 were monitored for only four years, etc. Tree survivorship among populations was correlated with cluster type assigned in the first year of sampling (Figure 7). Cankers assigned initially to cluster A survived better than cankers assigned clusters B or C, which survived better than cankers assigned to cluster D. The pattern of survivorship followed the trend in

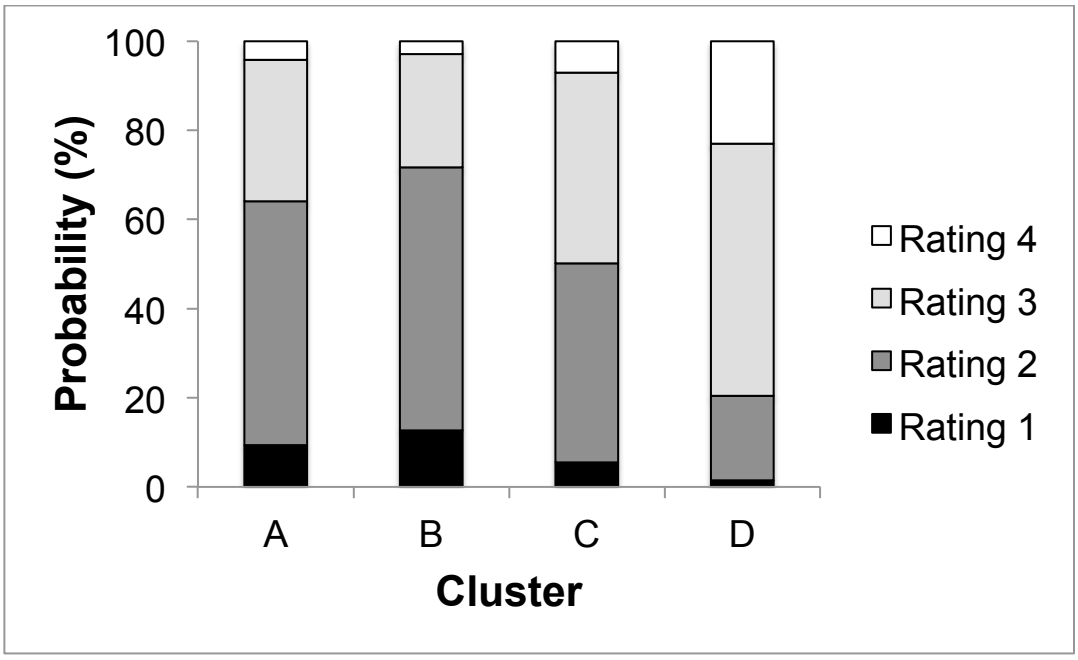

Figure 2-6: Community clusters for each canker-year observation (n = 633) and the probability that a cluster being associated with each canker rating: Rating '1' is the least severe canker rating; Rating '4' is the most severe. Data based on all years (2012-2016) and across all populations.

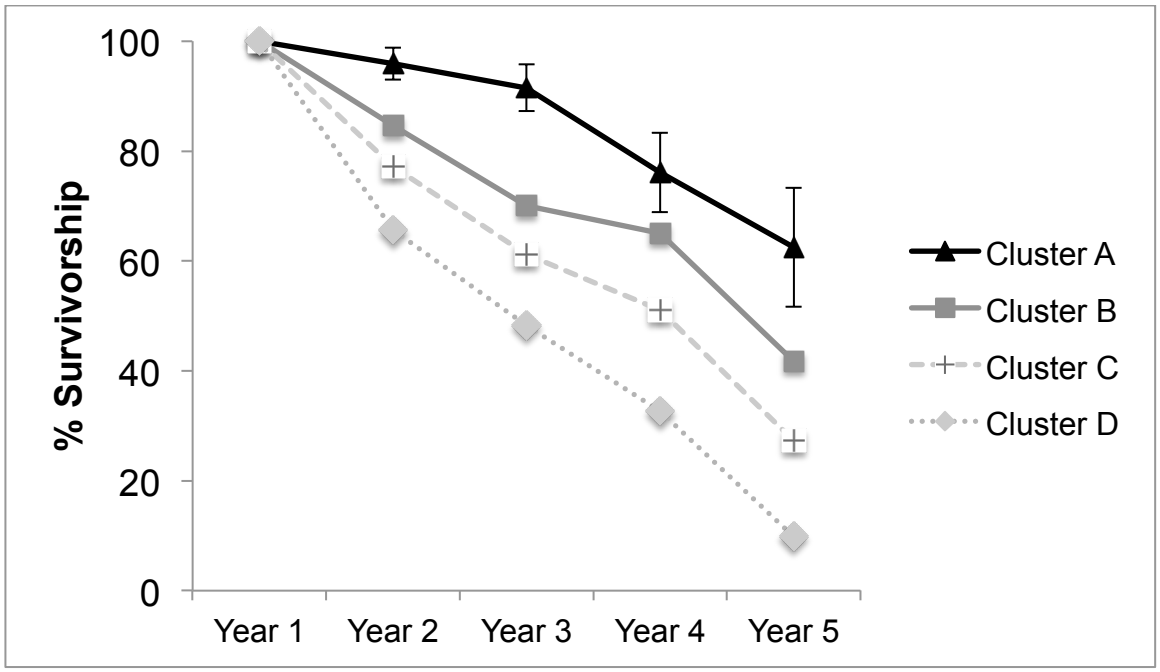

Figure 2-7: Survivorship of infected trees across all populationsby cluster type (A-D) assigned based on fungal taxa presence, absence, and abundance in cankers in first sampling (Year 1).

the frequency of CP and HCP within a canker community (see Table 6): higher prevalence of HCP (*i.e.* cluster A) in the canker community correlated with high survivorship, while high prevalence of CP (*i.e.* Cluster D) was associated with low tree survivorship. This pattern of survivorship held across years. After five years, 62.5% of the cankers in cluster A survived, while only 9.8 % of the cankers in cluster D survived (Figure 7).

Within the recovering populations, stems with cankers assigned cluster A survived at the highest rate (78.5%) over four years (Figure 8). At West Salem, survivorship was low among all cluster community types (Figure 9). Only 37.5 % and 14.3% of stems with cankers assigned cluster A surviving more than three and four years, respectively. There were not enough cankers assigned cluster A or B to compare survivorship curves between clusters at epidemic populations.

Cluster transitions over time

Although cankers assigned community cluster A at initial sampling survived at the highest rate, fungal communities changed over time (Table 7; top panel). Among all populations, less than a third of cankers assigned clusters A or C remained as A or C in the next year (Table 7), while nearly 50% of cankers assigned cluster B remained as cluster B from one year to the next. Movement of the canker community toward cluster B, which comprised only 25% of the cankers in the second sampling of cankers, increased to 50% and 67% of all cankers on trees that survived to year 4 and 5, respectively. Frequencies of other community types on surviving trees decreased between the first and fifth year. In the case of cankers assigned cluster D, tree death was more likely to occur than transition to a new

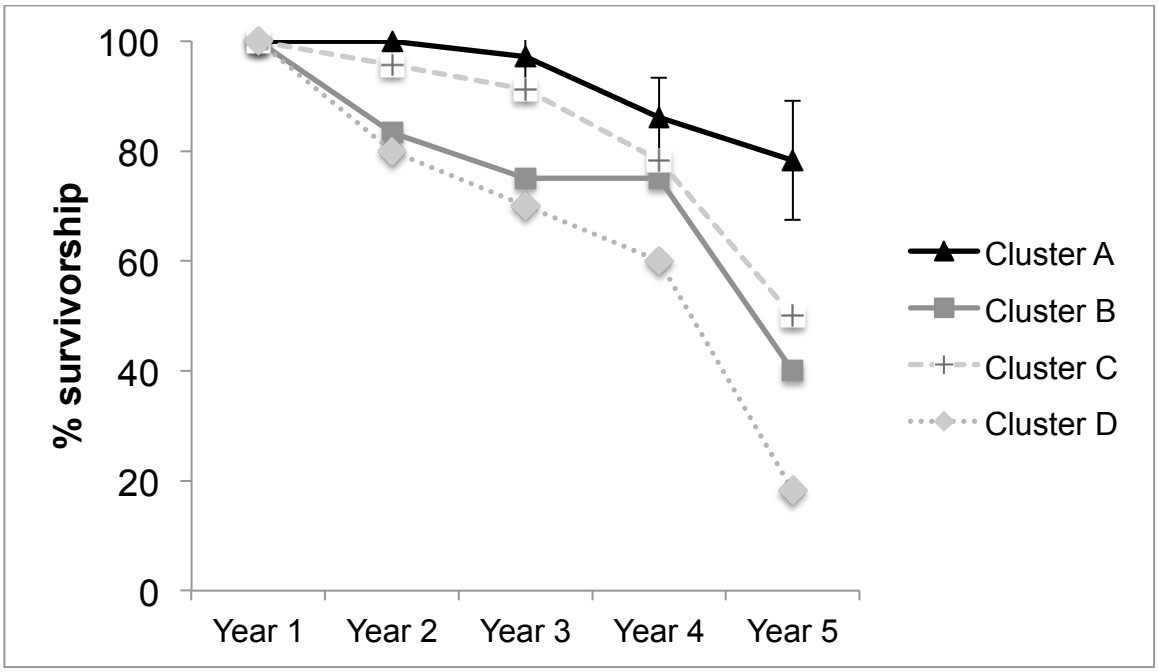

Figure 2-8: Survivorship of infected trees at recovering chestnut populations (County Line [CL], Roscommon [RC], and Frankfort [FR]) by cluster type (A-D) assigned based on fungal taxa presence, absence, and abundance in cankers in first sampling (Year 1).

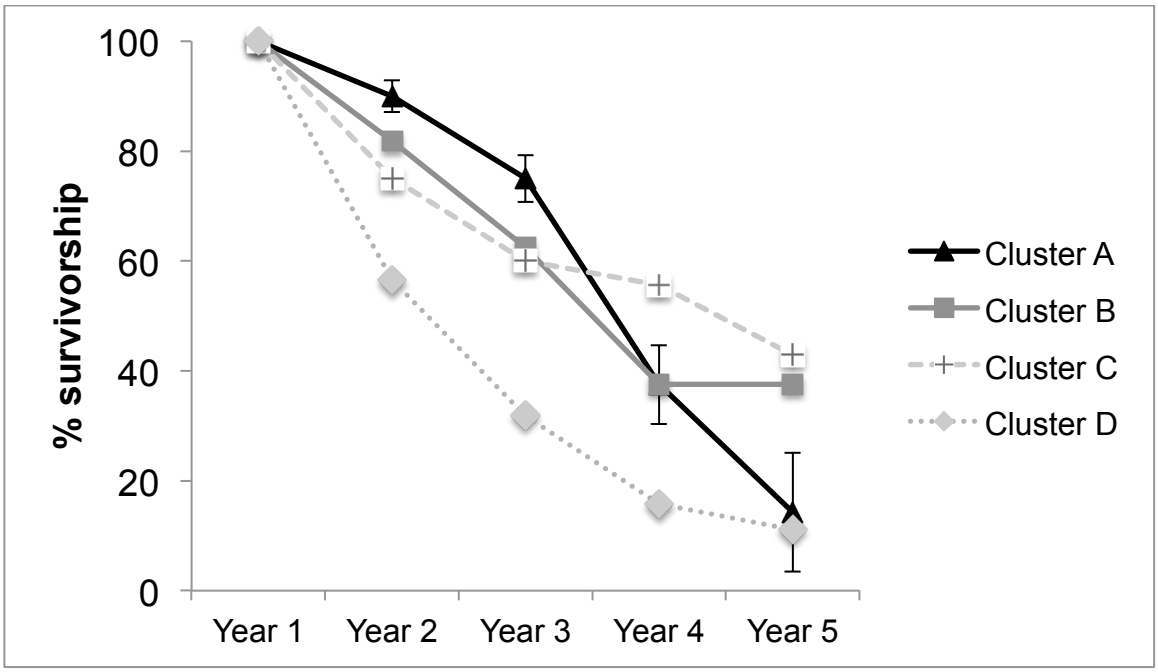

Figure 2-9: Survivorship of infected trees at West Salem population by cluster type (A-D) assigned based on fungal taxa presence, absence, and abundance in cankers in first sampling (Year 1).

Table 2-7: (Top panel): Frequencies of transition between community clusters across all populations in successive sampling years, as well as the frequency that a community cluster is associated with tree death the following year. EOS* = end of study; count for cankers on surviving trees still alive at the end of study (2016). (Bottom four panels): Counts of transitions for all canker-year observations and transitions. Cankers were assigned a cluster based on the fungal community in first sampling year (t = 1), and a cluster assignment based on the fungal community in the following sampling years (t = 2,3,4 and 5). Row % in each column is the percentage of cankers that transition to each cluster type for that sampling year.

	CLUSTER (t + 1)					
CLUSTER (t)	*A*	*B*	*C*	*D*	*Dead*	*EOS**
A	32.3	31.5	18.1	7.1	11.0	17
B	23.1	49.6	12.0	5.1	10.2	37
C	13.7	33.6	29.0	6.1	17.6	18
D	11.1	12.2	28.9	7.8	40.0	6
	CLUSTER (t = 2)					
CLUSTER (t = 1)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	21	8	12	6	2	5
B	5	9	4	3	4	0
C	6	15	16	2	13	0
D	5	4	20	6	22	0
%	26.1	25.4	36.6	12.0		
	CLUSTER (t = 3)					
CLUSTER (t = 2)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	9	12	10	1	4	1
B	6	15	3	2	1	9
C	9	15	12	3	7	5
D	5	6	3	1	12	1
%	25.9	42.9	25.0	6.3		
	CLUSTER (t = 4)					
CLUSTER (t = 3)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	8	12	1	1	8	1
B	13	22	7	0	6	1
C	2	9	8	3	3	3
D	0	1	2	0	2	2
%	25.8	49.4	20.2	4.5		
	CLUSTER (t = 5)					
CLUSTER (t = 4)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	3	8	0	1	0	10
B	3	12	0	1	1	27
C	1	5	2	0	0	10
D	0	0	1	0	0	3
%	18.9	67.6	8.1	5.4		

cluster type or remaining cluster D (Table 7; top panel). The lack of cankers assigned cluster A or B at epidemic populations made inferences regarding cluster transitions at these sites difficult to substantiate (Table 8).

At recovering populations, 37.5% cankers assigned cluster A remained in cluster A (Table 9), similar to A-to-A transitions across all sampling years and populations (32.3%; Table 7). In addition, transition to cluster B was the most likely transition for cankers on surviving stems at the recovering sites, regardless of the previous year's community cluster (Table 9). Transition to cluster B from cluster D occurred 28.1% of the time at recovering populations, which is higher than cluster transitions from D to B among all populations (12.2%; Table 7). Interestingly, transition from cluster D to A was lower in recovering sites (6.3%) than the rate across all populations (11.1%; Table 7).

Transitions between canker clusters at West Salem were unique among the other chestnut populations (Table 10), likely due to canker treatments with HCP inoculum in the past and during the study period. Cluster B cankers were three times more likely to transition to community cluster A than to remain in cluster B (Table 10). Only 10% of cluster A cankers remained cluster A in the following year, compared to over 40% likelihood of transition to cluster C.

DISCUSSION

The fungal community within cankers of diseased American chestnut trees in recovering and epidemic populations was monitored to follow the distribution and succession of fungi

Table 2-8: (Top panel): Frequencies of transition between community clusters across epidemic populations (Leelanau [LE] and Missaukee [MS]) in successive sampling years, as well as the frequency that a community cluster is associated with tree death the following year. EOS* = end of study; count for cankers on surviving trees still alive at the end of study (2016). (Bottom four panels): Counts of transitions for all canker-year observations and transitions. Cankers were assigned a cluster based on the fungal community in first sampling year (t = 1), and a cluster assignment based on the fungal community in the following sampling years (t = 2,3,4 and 5). Row % in each column is the percentage of cankers that transition to each cluster type for that sampling year.

	CLUSTER (t + 1)					
CLUSTER (t)	*A*	*B*	*C*	*D*	*Dead*	*EOS**
A	37.5	25.0	0.0	0.0	37.5	0
B	0.0	42.9	14.3	0.0	42.9	7
C	9.1	3.0	36.4	9.1	42.4	8
D	3.6	7.1	25.0	17.9	46.4	1
	CLUSTER (t = 2)					
CLUSTER (t = 1)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	1	0	0	0	0	0
B	0	3	1	0	2	0
C	2	1	7	1	10	0
D	1	2	6	3	9	0
%	14.3	21.4	50.0	14.3		
	CLUSTER (t = 3)					
CLUSTER (t = 2)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	2	0	0	0	2	0
B	0	0	0	0	1	5
C	1	0	4	1	3	5
D	0	0	0	2	2	0
%	30.0	0.0	40.0	30.0		
	CLUSTER (t = 4)					
CLUSTER (t = 3)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	0	2	0	0	1	0
B	0	0	0	0	0	0
C	0	0	1	1	1	1
D	0	0	1	0	2	0
%	0.0	40.0	40.0	20.0		
	CLUSTER (t = 5)					
CLUSTER (t = 4)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	0	0	0	0	0	0
B	0	0	0	0	0	2
C	0	0	0	0	0	2
D	0	0	0	0	0	1
%	0.0	0.0	0.0	0.0		

Table 2-9: (Top panel): Frequencies of transition between community clusters across recovering populations (County Line [CL], Roscommon [RC], and Frankfort [FR]) in successive sampling years, as well as the frequency that a community cluster is associated with tree death the following year. EOS* = end of study; count for cankers on surviving trees still alive at the end of study (2016). (Bottom four panels): Counts of transitions for all canker-year observations and transitions. Cankers were assigned a cluster based on the fungal community in first sampling year (t = 1), and a cluster assignment based on the fungal community in the following sampling years (t = 2,3,4 and 5). Row % in each column is the percentage of cankers that transition to each cluster type for that sampling year.

	CLUSTER (t + 1)					
CLUSTER (t)	*A*	*B*	*C*	*D*	*Dead*	*EOS**
A	38.3	38.3	12.8	5.3	5.3	12
B	27.4	48.4	18.9	1.1	4.2	21
C	18.2	51.5	21.2	4.5	4.5	8
D	6.3	28.1	31.3	9.4	25.0	2
	CLUSTER (t = 2)					
CLUSTER (t = 1)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	16	7	10	4	0	0
B	2	4	4	1	1	0
C	3	11	5	2	0	0
D	0	5	9	3	5	0
%	24.4	31.4	32.6	11.6		
	CLUSTER (t = 3)					
CLUSTER (t = 2)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	7	12	2	0	0	0
B	8	14	4	0	1	0
C	7	13	6	0	1	1
D	2	4	1	0	3	0
%	30.0	53.8	16.3	0.0		
	CLUSTER (t = 4)					
CLUSTER (t = 3)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	9	10	0	1	4	0
B	13	18	10	0	2	0
C	2	6	2	1	2	0
D	0	0	0	0	0	0
%	33.3	47.2	16.7	2.8		
	CLUSTER (t = 5)					
CLUSTER (t = 4)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	4	7	0	0	1	12
B	3	10	0	0	0	21
C	0	4	1	0	0	7
D	0	0	0	0	0	2
%	24.1	72.4	3.4	0.0		

Table 2-10: (Top panel): Frequencies of transition between community clusters at West Salem population in successive sampling years, as well as the frequency that a community cluster is associated with tree death the following year. EOS* = end of study; count for cankers on surviving trees still alive at the end of study (2016). (Bottom four panels): Counts of transitions for all canker-year observations and transitions. Cankers were assigned a cluster based on the fungal community in first sampling year (t = 1), and a cluster assignment based on the fungal community in the following sampling years (t = 2,3,4 and 5). Row % in each column is the percentage of cankers that transition to each cluster type for that sampling year.

	CLUSTER (t + 1)					
CLUSTER (t)	*A*	*B*	*C*	*D*	*Dead*	*EOS**
A	9.1	22.7	40.9	13.6	13.6	7
B	33.3	11.1	11.1	18.5	25.9	5
C	12.1	24.2	27.3	18.2	18.2	6
D	7.9	18.4	18.4	15.8	39.5	2
	CLUSTER (t = 2)					
CLUSTER (t = 1)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	2	2	2	2	1	5
B	5	2	1	2	3	0
C	2	1	3	4	2	0
D	0	4	3	5	10	0
%	22.5	22.5	22.5	32.5		
	CLUSTER (t = 3)					
CLUSTER (t = 2)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	0	1	7	0	0	1
B	1	0	2	2	1	3
C	1	2	1	1	2	2
D	2	2	2	1	5	1
%	16.0	20.0	48.0	16.0		
	CLUSTER (t = 4)					
CLUSTER (t = 3)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	0	1	0	0	2	1
B	1	1	0	0	2	1
C	0	2	5	1	2	2
D	1	1	1	0	0	1
%	14.3	35.7	42.9	7.1		
	CLUSTER (t = 5)					
CLUSTER (t = 4)	*A*	*B*	*C*	*D*	*Dead*	*EOS*
A	0	1	0	1	0	0
B	2	0	0	1	1	1
C	1	3	0	0	0	2
D	0	0	1	0	0	0
%	30.0	40.0	10.0	20.0		

as a canker ages. The spatial distribution of CP, HCP and Non-CP within a canker differed from the predicted structure, and instead resembled a mosaic (Figure 10). Fungal communities within cankers also were dynamic, to the extent that more than half of cankers with a particular community composition type (*i.e.* cluster A-D) in a given year were classified differently in the next (Table 7). This constant shifting of the fungal community and mosaic spatial distribution indicates that cankers may not follow distinct trajectories leading to girdling or non-girdling. Rather, CP, HCP and Non-CP are interacting in complicated ways throughout the diseased bark to influence canker expansion and ultimately the fate of the tree. A high prevalence of CP within a canker (cluster D) had a 40% probability of girdling the infected stem within one year, yet these cluster D cankers also had an 11% chance of becoming cluster A fungal communities (Table 7; top panel). While fungal community clusters were labile across years (Figure 5), there was a succession toward the cluster B fungal community in cankers on surviving stems (Table 7), which contained >50% total Non-CP.

The ecology of Non-CP in the chestnut blight system is largely unknown, but one role could include colonizing dead plant tissue after *C. parasitica* has caused necrosis via canker expansion (*e.g.* Russin and Shain 1984; Prospero *et al.* 2006). However, Non-CP may also be interacting with CP and HCP within the expanding canker and competing with the pathogen for host tissue. In addition, antagonism by Non-CP may influence conversion of CP by HCP within a canker, a critical process that can slow canker expansion (*e.g.* Jaynes and Elliston 1980; Bell 2004). Of common Non-CP taxa described here, *Trichoderma* spp. are noted for an assortment of antagonistic mechanisms (*e.g.* mycoparasitism, competition),

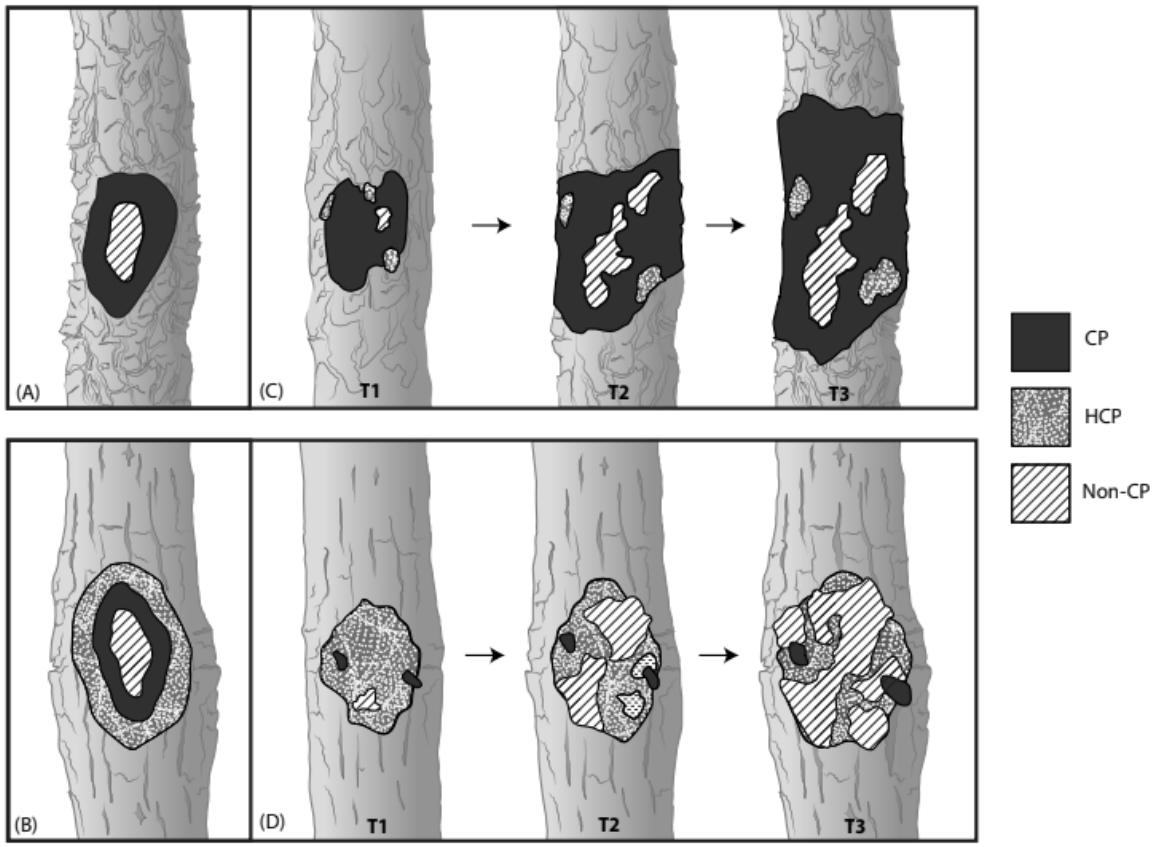

Figure 2-10: Outdated spatial model of fungal community within girdling cankers (A) and non-girdling (B) cankers on infected chestnut (Also see Figure 2). (C) Updated time series model of mosaic fungal community in girdling cankers, which increase in virulent *C. parasitica* (CP) and total non-*C. parasitica* fungi (Non-CP) abundance relative to hypovirus-infected *C. parasitica* (HCP) and are likely to girdle in T2 or T3. (D) Time series of mosaic fungal community in non-girdling cankers, which increase in total Non-CP relative to CP; HCP decreases in abundance over time. Survivorship is expected to be higher in T2 and T3 compared to girdling cankers; however, survivorship over time is expected to decrease with the decline of HCP abundance in non-girdling cankers.

which have been exploited for biological control in other plant-pathogen systems (reviewed in Harman 2006; Lorito *et al.* 2010). *Trichoderma* have been isolated from blight cankers in other studies as well (*e.g.* Tattar *et al.* 1996; Akilli *et al.* 2009).

The ability of various Non-CP taxa to inhibit growth of CP and HCP under laboratory conditions suggests that common Non-CP like *Trichoderma* may have a greater negative influence on HCP relative to CP (Kolp thesis, Chapter 3). This suggests that Non-CP may cause a net reduction in the prevalence of HCP over time and allow CP to continue rapid canker expansion. In the present study, cankers on surviving trees tended to shift from a community of mostly HCP (cluster A) to communities with a higher prevalence of Non-CP (Clusters B and C). This has been noted in cankers monitored in other chestnut populations as well (*e.g.* Ćurković-Perica *et al.* 2017). In contrast, CP-dominated cankers (Cluster D) were just as likely to girdle an infected stem as to survive and succumb to Non-CP invasion (Table 7).

If *Trichoderma* and other Non-CP taxa inhibit HCP in cankers, this also may reduce the amount of hypovirulent inoculum in a forest and decrease the likelihood of conversion of CP in new cankers by HCP. Spore production by HCP *in planta* is lower compared to CP because hypoviruses are not transmitted vertically via the sexual ascospores of *C. parasitica* (Carbone *et al.* 2004; Prospero *et al.* 2006). In addition, the rate of hypovirus transmission into asexual, conidiospores can vary depending on the hypovirus and *C. parasitica* genotypes (Peever *et al.* 2000; Bryner and Rigling 2012), as well as whether the host tree is alive or dead (Prospero *et al.* 2006). Reduced levels of hypovirulent inoculum in the environment

could have long-term consequences for chestnut,, pathogen, and hypovirus populations (*e.g.* Davelos and Jarosz 2004; Springer *et al.* 2013; reviewed in Rigling and Prospero 2018). For example, Springer *et al.* (2013) reported that the frequency of HCP at the FR recovering population had declined from 90% of isolates sampled in 1996 (Davelos 1999) to 76% in 2009. Increasing diversity among *vic* genes in the pathogen population at FR could limit hypovirus transfer (Springer *et al.* 2013), but an alternative could be that the reduction of HCP is driven by Non-CP antagonism of HCP within cankers.

While Non-CP may be antagonizing *C. parasitica,* some Non-CP taxa may actually be weak pathogens of chestnut. The ability of a pathogen to infect and cause disease in a host is hypothesized to increase with evolutionary relatedness of known host plants (Gilbert and Webb 2007; Vienne *et al.* 2009; Parker *et al.* 2015), although some *Phytophthora* spp. infect a broad range of plant species (Grunwald *et al.* 2008; Lamour *et al.* 2011). In addition, the relatedness and distribution of tree species in a forest may increase the potential risk for pathogen spillover and to infect closely related hosts (Saikkonen 2007; Desprez-Loutau *et al.* 2016; Gilbert *et al.* 2012).

Among common Non-CP taxa found in cankers, *Nectria* and *Pezicula* are fungal genera containing pathogenic species of trees within the Fagaceae. Native and introduced species of *Nectria* contribute to beech bark disease of American beech (*Fagus grandifolia*; Houston 1994), while *Pezicula cinnamomea* is a known bark endophyte and weak pathogen of many hardwood species of the Fagaceae, including red oak (*Quercus rubra*; Kehr 1992). Bissenger and Seiber (1994) noted that *Pezicula cinnamomea* is isolated commonly from apparently

healthy sapwood of European chestnut and discussed the potential of the endophyte to prevent *C. parasitica* infection in coppice shoots. Although the healthy bark tissue or sapwood was not examined in this study, Kolp *et al.* (2018) reported that a *Pezicula* strain isolated from a canker on an American chestnut was among the poorest inhibitors of *C. parasitica* growth in dual culture. *Pezicula* and *Nectria* were twice as prevalent at the margin of blight cankers compared to the inner area (Table 3) and may reflect their ability to invade cankers at the expanding edge, or their identity as endophytes that colonize chestnut bark prior to *C. parasitica.*

Gnomoniopsis is another Non-CP taxa that may be weakly pathogenic against chestnut. This Non-CP was recovered from cankers at all sites and is known to interact with *Castanea* spp. in dramatically different ways. For example, the *Gnomoniopsis* species complex (*G. smithogilvyi* in Shuttleworth *et al.* 2015, *G. castanea* in Visentin *et al.* 2012; Tamietti 2016) is known in Europe and Australia as the causal agent of brown rot of chestnut kernels from European chestnut (*C. sativa*), Japanese chestnut (*C. creneta*), and hybrid orchard trees. Yet the same fungus may act as an endophyte capable of biocontrol against the chestnut gall wasp, a pest of chestnut shoots and leaves (Vannini *et al.* 2017). Further, *Gnomoniopsis* has been confirmed as a stem cankering pathogen on twigs and scions of European chestnut (Pasche *et al.* 2016). The taxonomic uncertainty surrounding *Gnomoniopsis* remains important to resolve given the ecological and pathogenic variability, as well as possible threat to nascent chestnut fruit production industries in Michigan (Fulbright DW, pers. comm.).

The role of these Non-CP taxa warrants further investigation, especially given that an abundance of Non-CP within canker communities (cluster B) on surviving trees was associated with higher survivorship over time relative to canker communities dominated by CP (cluster D). Compared to cluster C cankers with more CP and less Non-CP relative to cluster B (Table 6), survivorship was only slightly higher. Whether this difference in survivorship over time is due to less HCP or more CP in the canker remains unclear. Cluster B cankers, which contained the second highest prevalence of HCP (~30%), survived at the second highest rate; Cluster A cankers, which contained ~70% HCP, survived at the highest rate over the course of this study. Over time, as cankers on surviving trees are more likely to be overrun by Non-CP invasion (Table 7), the negative influence of Non-CP on HCP and declining survivorship may be inevitable. Thus, the probability of girdling may depend on the rate at which HCP infection spreads within the canker relative to the rate at which Non-CP invades and inhibits HCP. Within a chestnut forest, higher levels of HCP within cankers (*e.g.* Recovering sites CL, RC, and FR in Table 4) may lead to higher HCP dispersal between cankers that offsets Non-CP invasion into cankers. Canker-to-canker dispersal of HCP may be impeded by Non-CP and warrants further investigation.

Canker communities are far from stable, and that even for cankers composed of predominantly HCP there exists some probability of stem girdling. The dynamic succession of the fungal community indicates that cankers can change quickly from less severe to more severe cankers through the loss of HCP, perhaps due to Non-CP invasion. In addition, Non-CP may facilitate disease and allow CP to escape conversion by HCP and resume fast canker expansion.

Across each of our study sites, survivorship of infected trees was positively correlated with the frequency of HCP sampled from cankers (Table 4). The amount of HCP within cankers at WS was lower (~30%) than expected given that hypovirulent treatment of cankers has occurred at WS for the better part of the last two decades (Double *et al.* 2018). Double *et al.* (2018) found that in the area of WS where chestnut blight was first discovered, the amount of HCP in cankers has increased over time (55% of isolates in 1994; 86% in 2014). In this study, canker sampling at WS from 2012-2016 (149 canker-year observations) included areas beyond the disease center and suggests a high variability of HCP spread within WS. The abundance of *Trichoderma* at WS is intriguing (11% of canker community; Table 5). The higher frequency of this antagonistic fungus within cankers at WS could explain why HCP may not be prolific within parts of the stand.

Non-CP taxa were more prevalent at some sites, which may reflect environmental and plant community factors specific to each site. For example, the evolutionary relatedness of the neighboring plant community among chestnut trees could influence the diversity of Non-CP taxa that are present to invade cankers at a particular site (Saikkonen 2007; Gilbert *et al.* 2012; Parker *et al.* 2015). At CL and RC, the chestnut populations are set within predominantly pine forest or plantations. In contrast, the MS site is interspersed within beech and maple hardwoods that potentially serve as primary or opportunistic hosts to weakly pathogenic Non-CP. Future work should describe plant community and environmental factors that may act to influence the presence or abundance of antagonistic

or weakly pathogenic Non-CP taxa in certain chestnut populations. These factors may be useful in predicting and managing Non-CP invasion of hypovirulence-containing cankers.

These results provide another example in which fungal communities influence the outcome of plant-pathogen interactions. Plant microbiomes can have profound impacts on plant responses to the environment, including interactions with fungal pathogens (*e.g.* Arnold *et al.* 2003). Furthermore, the ecological and evolutionary overlap among fungal endophytes, pathogens, and saprotrophs can make it difficult to define cryptic communities within and across plant species (Arnold 2007; Peay *et al.* 2008; Partida-Martinez and Heil 2011; Delaye *et al.* 2013; Garcia-Guzman and Heil 2013). Diverse fungal communities and an abundance of Non-CP in chestnut blight cankers may negatively influence the persistence of hypoviruses that can lower virulence of the chestnut blight pathogen. These hypoviruses have known benefits of increasing survival, growth, and reproduction of the infected tree when present in cankers (*e.g.* Davelos and Jarosz 2004; Springer *et al.* 2013). However, Non-CP are predicted to antagonize HCP and inhibit the spread of hypoviruses in a canker. Over time, the amount of hypovirulent inoculum may decrease and fail to spread throughout a forest. In addition, chestnut blight and canker expansion may be facilitated by Non-CP taxa that are weak pathogens of chestnut.

The fungal community of chestnut blight cankers is dynamic, and a management strategy to slow canker expansion and delay or prevent girdling of an infected tree should consider the separate effects of HCP and Non-CP on the virulent pathogen (CP). Management of canker expansion using HCP has been research extensively, while using Non-CP antagonism of CP

to slow expansion is unclear. Slow canker expansion may not occur when HCP and Non-CP are used in concert.

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CHAPTER 3

INHIBITION OF VIRULENT AND HYPOVIRULENT *CRYPHONECTRIA PARASITICA* GROWTH IN DUAL CULTURE BY FUNGI COMMONLY ISOLATED FROM CHESTNUT BLIGHT CANKERS

ABSTRACT

Chestnut blight cankers, caused by the fungus *Cryphonectria parasitica*, are prone to invasion by other microorganisms as the canker ages. This microbial community has the potential to alter canker expansion, which may influence the probability that the canker girdles the infected stem. Hypoviruses infect the pathogen mycelium directly and are known to decrease pathogen virulence (*i.e.* hypovirulent). These viral infections can slow pathogen growth, decreasing the rate of canker expansion and lowering the probability of girdling. Saprophytic fungi also invade the expanding canker and may antagonize *C. parasitica* leading to reduced pathogen growth. The combined effects of fungal antagonism and a hypovirulent pathogen could work in combination to reduce the probability of girdling the infected stem. We assessed the ability of different fungal taxa, isolated from low severity cankers, to inhibit the growth of virulent and hypovirulent forms of *C. parasitica* in dual culture tests on two cultural media. Percent growth inhibition of virulent *C. parasitica* by potentially antagonistic fungi ranged from 2% to 34%, while inhibition of hypovirulent *C. parasitica* ranged from 18% to 54%. Only one isolate, identified as *Umbelopsis isabellina* (*Umbelopsis*WS) inhibited the virulent form of the pathogen more than the hypovirulent form. All three *Trichoderma* isolates caused the greatest growth inhibition of virulent *C. parasitica*, but they, like all other fungal isolates tested, inhibited the hypovirulent form of the pathogen more than the virulent form. These results suggest that commonly occurring fungi in chestnut blight cankers, including *Trichoderma*, may inhibit the hypovirulent *C. parasitica*

more than virulent *C. parasitica*. Thus, the presence of other fungi in cankers may not enhance the effect of hypovirulent *C. parasitica* to delay cankers from girdling a stem but instead intensify canker development.

INTRODUCTION

Cryphonectria parasitica [Murr.] Barr) causes chestnut blight and, after being introduced accidentally to both North America (Merkel 1905) and Europe (Biraghi 1953), instigated severe epidemics on American (*Castanea dentata* [Marsh.] Borkh.) and European (*Castanea sativa* Mill) chestnut (reviewed in Griffin *et al.* 1986). The pathogen infects through wounds in the outer bark of chestnut trees (Hebard *et al.* 1984). Mycelial fans grow out from the infection court resulting in a sunken canker that expands outward from the point of infection. If canker expansion is unchecked, the canker edges will eventually encompass the full circumference of the infected stem (*i.e.*, girdling) resulting in the death of all plant tissue distal to the canker. However, not all cankers girdle infected stems, and canker expansion is influenced by the combined effects of tree resistance (Hebard *et al.* 1984), pathogen virulence (Griffin *et al.* 1983; Enebak *et al.* 1994), and other abiotic and biotic factors.

While American and European chestnuts are susceptible to blight, Chinese chestnut (*C. mollissima* Blume) is resistant. Trees of this species produce wound periderm tissue that can halt mycelia growth and prevent girdling of an infected stem (Hebard *et al.* 1984). It has been postulated that the relative rates of mycelial fan growth and wound periderm formation determine whether the canker is contained or girdles a stem (Hebard *et al.* 1984; Griffin *et al.* 1986).

Cryphonectria parasitica strains can differ in virulence (*e.g.* Grente and Berthelay-Suaret 1978; Fulbright *et al.* 1983; Hillman *et al.* 1992; Enebak *et al.* 1994; Zhang and Nuss 2016) but most, if not all, *C. parasitica* strains in North America possess enough virulence to girdle stems

when infecting American chestnut. However, biologically important changes in pathogen virulence can be attained by hypovirus infection of *C. parasitica*. Double-stranded RNA hypoviruses reside in the fungal cytoplasm and reduce pathogen virulence by inhibiting sexual reproduction, and reducing both mycelial growth and conidia production (*e.g.* Carbone *et al.* 2004; Prospero *et al.* 2006; Zhang and Nuss 2016).

The diversity of dsRNA viruses infecting *C. parasitica* includes at least four species (family *Hypoviridae*) separated taxonomically based on their biogeography, genome organization, and ability to alter fungal virulence (reviewed in Dawe and Nuss 2001; Hillman and Suzuki 2004). Hypoviruses have spread extensively throughout Europe leading to recovery of many European chestnut populations since trees can produce enough wound periderm to contain cankers produced by hypovirulent strains of *C. parasitica* (reviewed in Milgroom and Cortesi 2004). Hypoviruses have not spread extensively in North America, but isolated *C. dentata* populations have recovered from *C. parasitica* epidemics and recovery is usually associated with the prevalence of hypovirus infection of the pathogen (Fulbright *et al.* 1983; Davelos and Jarosz 2004; Springer *et al.* 2013).

Hypovirus infection of the pathogen represents one biotic influence on the fate of a canker. Monitoring of the fungal community within chestnut blight cankers revealed that non-*Cryphonectria parasitica* (Hereafter, Non-CP) fungi increase in prevalence as a canker ages (Double *et al.* 2013; Kolp, unpublished). In some cases, these species may influence the fate of a canker. *Trichodermia* spp., which have been isolated from blight cankers, have been investigated as potential biological control agents for the management of chestnut blight

(Akilli *et al.* 2011; Arisan-Atac *et al.* 1995; Tattar *et al.* 1996; Weidlich 1978). Weidlich (1978) demonstrated that soil compresses containing *Trichoderma* could prevent canker expansion and girdling. Tattar *et al.* (1996) found that *Trichoderma* strains could prevent *C. parasitica* from colonizing excised chestnut bark using a *Trichoderma* spore solution. Akilli *et al.* (2011) also demonstrated that *Trichoderma* strains from cankers on European chestnut could limit canker expansion when applied to induced cankers on young sapling *C. sativa*.

The main objective of this study was to compare the ability of different Non-CP isolates (recovered from non-girdling chestnut blight cankers) to inhibit the growth of virulent and hypovirulent *C. parasitica*. We chose ten Non-CP isolates collected in 2012 from cankers on American chestnut trees in West Salem, Wisconsin (Double *et al.* 2013; Paillet and Rutter 1989) and in two recovering American chestnut populations in Michigan with a high frequency of hypovirus in the pathogen population (Davelos 1999; Springer *et al.* 2013). Growth inhibition was quantified by measuring colony growth of *C. parasitica* in dual culture. This work will provide information that will improve our understanding of how hypoviruses and Non-CP interact to influence canker expansion and the probability that a canker will girdle an infected stem.

MATERIALS AND METHODS

Fungal isolates

Non-CP were recovered in the summer of 2012 during a survey of the fungal community inhabiting cankers from three American chestnut populations: one near West Salem, Wisconsin (Double *et al.* 2013; Paillet and Rutter 1989) and two in northern Michigan

(County Line: see Davelos and Jarosz 2004; and Roscommon: see Springer *et al.* 2013). Non-CP taxa chosen for this study were commonly isolated from non-girdling cankers at each site in 2012. However, some Non-CP taxa used in this study have been found in girdling cankers as well (Kolp *et al.* 2017). Non-CP taxa included three strains of *Trichoderma* (one strain from each chestnut population), two strains each of *Epicoccum nigrum* and *Umbelopsis isabellina*, and one each of *Penicillium*, *Gnomoniopsis*, and *Pezicula* (Table 1).

Three virulent (without hypovirus infection) isolates of *C. parasitica* were selected to measure their growth response to Non-CP (Non-pathogenic, potentially antagonistic fungi) in dual culture testing. Isolate EP155 from Connecticut is a standard virulent isolate commonly used in chestnut blight research (Anagnostakis and Day 1979). Isolate LE221 represents a *vic* genotype (MI-5) common to naturalized America chestnut populations in the northern Lower Peninsula of Michigan (Springer *et al.* 2013). Isolate RBO15, another representative of a common *vic* genotype (MI-3 in Springer *et al.* 2013), was isolated from a canker on a European (*C. sativa*) x Japanese (*C. crenata*) hybrid cv. 'Colossal' tree in an orchard near New Era, Michigan.

Using the protocols of Huber (1996), infection of virulent isolates LE221 and RBO15 with hypoviruses was accomplished by pairing mycelial plugs of each virulent isolate with a "donor" hypovirulent isolate (GH2 infected with CHV3-GH2 hypovirus) 5 mm apart at the edge of a petri plate containing potato dextrose agar (PDA; Difco, Becton, Dickinson and Company, Sparks, MD). Hypovirus infection was considered successful when morphology

Table 3-1: Summary of dual culture tests to assess growth inhibition of *C. parasitica* in the presence of potential fungal antagonist (Non-CP). Non-CP were isolated from chestnut blight cankers on American chestnut trees at two populations in Michigan (County Line and Roscommon) and Wisconsin (West Salem).

	***Cryphonectria parasitica* isolates**				
Non-CP isolates	EP 155 (V[a])	LE221 (V)	LE221 (HV)	RBO15 (V)	RBO15 (HV)
*Trichoderma*WS[c]	22[b]	20	8	8	8
*Trichoderma*RC[d]	22	19	8	8	8
*Trichoderma*CL[e]	22	20	8	8	8
*Umbelopsis*WS	22	19	8	8	8
*Umbelopsis*RC	22	16	-	-	-
*Epicoccum*WS	22	15	-	-	-
*Epicoccum*CL	22	17	8	8	8
*Peziculа*RC	-	4	8	7	8
*Penicillium*RC	19	8	-	-	-
*Gnomoniopsis*CL	22	16	-	-	-
Agar Plug (control)	22	20	8	8	8

[a] = Virulence level (V = virulent; HV = hypovirulent; *i.e.* infected with CHV3 hypovirus)
[b] = Number of replicate plates
[c] = collected from West Salem; [d] = Roscommon; [e] = County Line

of the virulent isolate changed to mimic the donor hypovirulent isolate – reduced or abnormal vegetative growth due to the replication of the hypovirus within its new fungal mycelium (*i.e.* conversion from "virulent" to "hypovirulent"; Anagnostakis and Day 1979). The GH2 isolate was originally obtained from a now extirpated American chestnut grove near Grand Haven, Michigan (Fulbright *et al.* 1983). In laboratory tests, CHV3 hypovirus infection of *C. parasitica* can result in a less virulent pathogen on excised chestnut branches (Fulbright *et al.* 1983; Melzer *et al.* 1997). CHV3 is endemic to Michigan and is distantly related to hypoviruses found in Europe and Asia (Peever *et al.* 1998; Smart *et al.* 1999). The closest relative of CHV3 is CHV4, another hypovirus found in North America (Linder-Basso *et al.* 2005) that does not significantly reduce the virulence of *C. parasitica* on American chestnut (Enebak *et al.* 1994).

Dual culture tests of antagonism

To generate each dual culture test (n = 492), a petri plate (100 x 15 mm) containing either 20 mL of sterile PDA or endothia complete media (ECM; Atlas 2010) was first inoculated with 0.5 cm^2 agar plug obtained from the hyphal edge of a one-week-old isolate of *C. parasitica*. Two 2 days later, the petri plate was inoculated with a 0.5 cm^2 agar plug obtained from the hyphal edge of a one-week-old Non-CP isolate. *C. parasitica* and Non-CP were separated by 4 cm (Figure 1). Dual culture test combinations were replicated at least four times in one of four experimental runs. Control plates contained an agar plug of *C. parasitica* and a sterile plug of PDA or ECM in place of the Non-CP and were also replicated four times in each experimental run. Dual culture tests were incubated at 25°C in the dark for ten days.

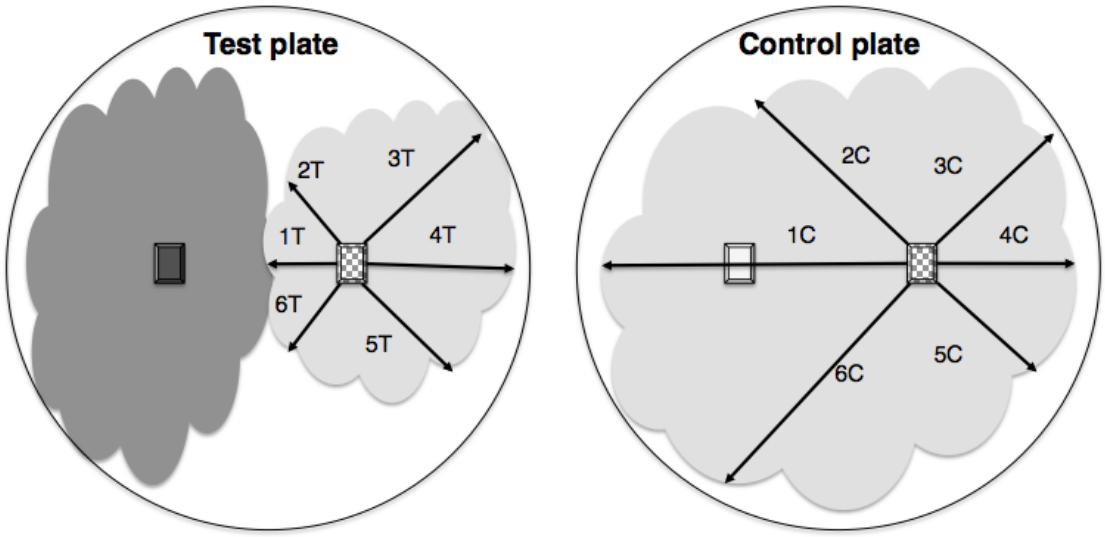
Percentage inhibition was measured as mycelial growth of *C. parasitica* when paired with individual Non-CP relative to control plates. Mycelial growth was quantified by measuring colony growth (mm) along six vectors (Figure 1). Measurements occurred every second day for ten days, at which point *C. parasitica* mycelia on control plates had reached the edge of the plate. Measures of antagonism across all time points were similar in pattern (data not shown), so only antagonism at ten days is reported here.

Growth reductions due to hypovirus infection

Hypovirus infection of *C. parasitica* can be characterized *in vitro* as abnormal and reduced growth on culture media (*e.g.* Anagnostakis and Day 1979; Dawe and Nuss 2001). Thus, the reduction of growth associated with the CHV3 hypovirus infection was assessed in the absence of Non-CP antagonism in order to understand how Non-CP affects mycelial growth of virulent and hypovirulent *C. parasitica* differently. Two of the three *C. parasitica* isolates (LE221 and RBO15) were used to compare growth of the virulent *C. parasitica* on control plates relative to the corresponding hypovirulent strain growing on control plates using our formula (Figure 1).

Statistical analyses

Linear regression was used to analyze the relationship between antagonism at ten days and the effects of four different factors: Non-CP isolate, *C. parasitica* isolate, virulence, and media type, as well as all possible two-way interactions. Differences among group means were determined using an analysis of variance (ANOVA). Multiple comparisons within factor levels were made post-hoc using Tukey's HSD. All analyses were conducted in R



$$\% \text{ Inhibition} = 100 \times \left(1 - \left(\frac{(1T/1C) + (2T/2C) + (3T/3C) + (4T/4C) + (5T/5C) + (6T/6C)}{6}\right)\right)$$

Figure 3-1: Schematic and formula to assess growth inhibition of *C. parasitica* in dual culture. Light gray and checkered plug indicates position of *C. parasitica*, with light gray growth over time. Dark gray plug indicates position of potential fungal antagonist isolate with dark gray growth over time. White plug indicates position of agar plug on control plates.

(version 3.2.2). Despite the unbalanced and incomplete experimental design, assumptions of a linear model were not violated.

RESULTS

ANOVA analyses determined that all four main effects: two media (PDA vs. ECM), three *C. parasitica* isolates, two virulence levels (virulent [V] vs. hypovirulent [HV]), and ten Non-CP isolates had significant influence on the level of antagonism (Table 2). Virulence also produced significant two-way interactions with each of the other three main effects indicating that V and HV responses of LE221 and RBO15 were influenced differently by the presence of the CHV3 hypovirus.

Influence of hypovirus on C. parasitica *growth in the absence of Non-CP*

Growth inhibition associated with hypovirus infection was measured at ten days and differed between the two *C. parasitica* isolates, LE221 and RBO15. Growth of control plates was reduced by 16.5% when RBO15 was infected with the CHV3 hypovirus, while hypovirus infection only reduced growth by 6.3% for LE221. This represents the amount growth inhibition associated with hypovirus infection in the absence of any antagonism by Non-CP.

Influence of Non-CP on the growth of virulent and hypovirulent C. parasitica

The three virulent *C. parasitica* isolates differed in overall growth inhibition when grown in dual culture with Non-CP (Table 2). EP155 (Average growth inhibition due to antagonism by Non-CP = 17.4%) was least inhibited by the presence Non-CP, while inhibition of LE221

Table 3-2: ANOVA source table for percentage growth inhibition of different *C. parasitica* isolates, of different virulence levels, subjected to dual culture plating with potentially antagonistic fungi (Non-CP), on different culture media.

Factor	**df**	**Sum Sq.**	**Mean Sq.**	**F-value**	**Pr(>F)**
Virulence (virulent or hypovirulent)	1	13912	13912	96.24	**<0.0001**
C. parasitica (CP) Isolate	2	1882	941	6.51	**0.0016**
Non-CP Isolate	9	40386	4487	31.04	**<0.0001**
Media	1	1513	1513	10.47	**0.0013**
CP Isolate x Virulence	1	1006	1006	6.96	**0.0086**
Non-CP Isolate x Virulence	5	3000	600	4.15	**0.0011**
Virulence x Media	1	1546	1546	10.7	**0.0012**
Non-CP Isolate x CP Isolate	13	1214	93.4	0.65	0.815
CP Isolate x Media	1	609	305	2.11	0.123
Non-CP Isolate x Media	9	679	76	0.52	0.859
(Residuals)	447	64619	145	-	-

and RBO15 was 21.3 and 21.2%, respectively (Table 3). Infection by the CHV3 hypovirus was associated with a larger degree of growth inhibition in the presence of Non-CP of both LE221 (29.9%) and RBO15 (38.8 %).

There was a significant interaction, however, between the level of virulence (V or HV) for *C. parasitica* and the growth inhibition response to a particular Non-CP isolate (Figure 2; Table 3). The three *Trichoderma* isolates caused the greatest growth inhibition in both virulent and hypovirulent *C. parasitica*, followed by *Penicillium*RC (Figure 2; Table 3). *Umbelopsis*WS displayed a distinct pattern: growth inhibition did not increase much when LE221 or RBO15 was infected with the CHV3 hypovirus relative to the virulent form (Table 3). For all other Non-CP, hypovirulent *C. parasitica* displayed a significantly greater degree of growth inhibition. For example, *Pezicula*RC inhibited virulent *C. parasitica* on average by only 3.3%, yet both hypovirulent LE221 and RBO15 were inhibited on average by over 20% (Table 3).

Virulent LE221 and RBO15 reacted nearly identically overall with 21.3% and 21.2% growth inhibition in the presence of Non-CP, respectively (Table 3). In contrast, hypovirulent LE221 growth was inhibited by 29.9% overall in the presence of the Non-CP compared to 38.8% growth inhibition of hypovirulent RBO15 (Table 3). Hypovirulent LE221 and RBO15 also varied in their responses to the various Non-CP isolates. For LE221, the difference in the amount of growth inhibition between virulent and hypovirulent forms of the pathogen (**Δ**) averaged 8.6%, ranging from 3.1% against *Trichoderma*RC to 16.6% against

Table 3-3: Growth inhibition, measured as the percentage change relative to control plates, of virulent (V) and hypovirulent (HV) forms of *C. parasitica* isolates when interacting with other fungi (Non-CP) in dual culture. For each *C. parasitica* and Non-CP isolate combination, Δ represents the difference in growth inhibition of the hypovirulent and virulent form (HV – V = Δ).

Non-CP isolates	EP155 (V)	LE221 (V)	LE221 (HV)	LE221 (Δ)	RBO15 (V)	RBO15 (HV)	RBO15 (Δ)
*Trichoderma*RC	28.5^{a}	32.5^{a}	35.6^{abc}	+3.1	29.0^{a}	46.5^{ab}	+17.5
*Trichoderma*CL	25.5^{ab}	34.0^{a}	39.2^{ab}	+5.2	30.0^{a}	54.1^{a}	+24.1
*Trichoderma*WS	26.5^{ab}	27.4^{ab}	41.2^{a}	+13.8	33.0^{a}	53.4^{a}	+20.4
*Penicillium*RC	19.2^{bc}	24.9^{abc}	-	-	-	-	-
*Umbelopsis*WS	11.7^{cd}	22.2^{abc}	17.8^{c}	-4.4	21.1^{ab}	18.4^{c}	-2.7
*Umbelopsis*RC	12.7^{cd}	13.4^{bc}	-	-	-	-	-
*Epicoccum*WS	12.4^{cd}	16.9^{bc}	-	-	-	-	-
*Epicoccum*CL	12.5^{cd}	8.8^{c}	25.4^{abc}	+16.6	10.1^{ab}	35.9^{abc}	+25.8
*Gnomoniopsis*CL	7.7^{d}	12.7^{bc}	-	-	-	-	-
*Peziculа*RC	-	5.9^{c}	20.1^{bc}	+14.2	1.8^{b}	24.3^{bc}	+22.5
Overall	17.4	21.3	29.9	+8.6	21.2	38.8	+17.6

Note: Values followed by the same letter are not statistically different ($P < 0.05$) based on Tukey's HSD. Contrasts are made within *C. parasitica* isolate (EP155, LE221, RBO15) and virulence (V or HV).

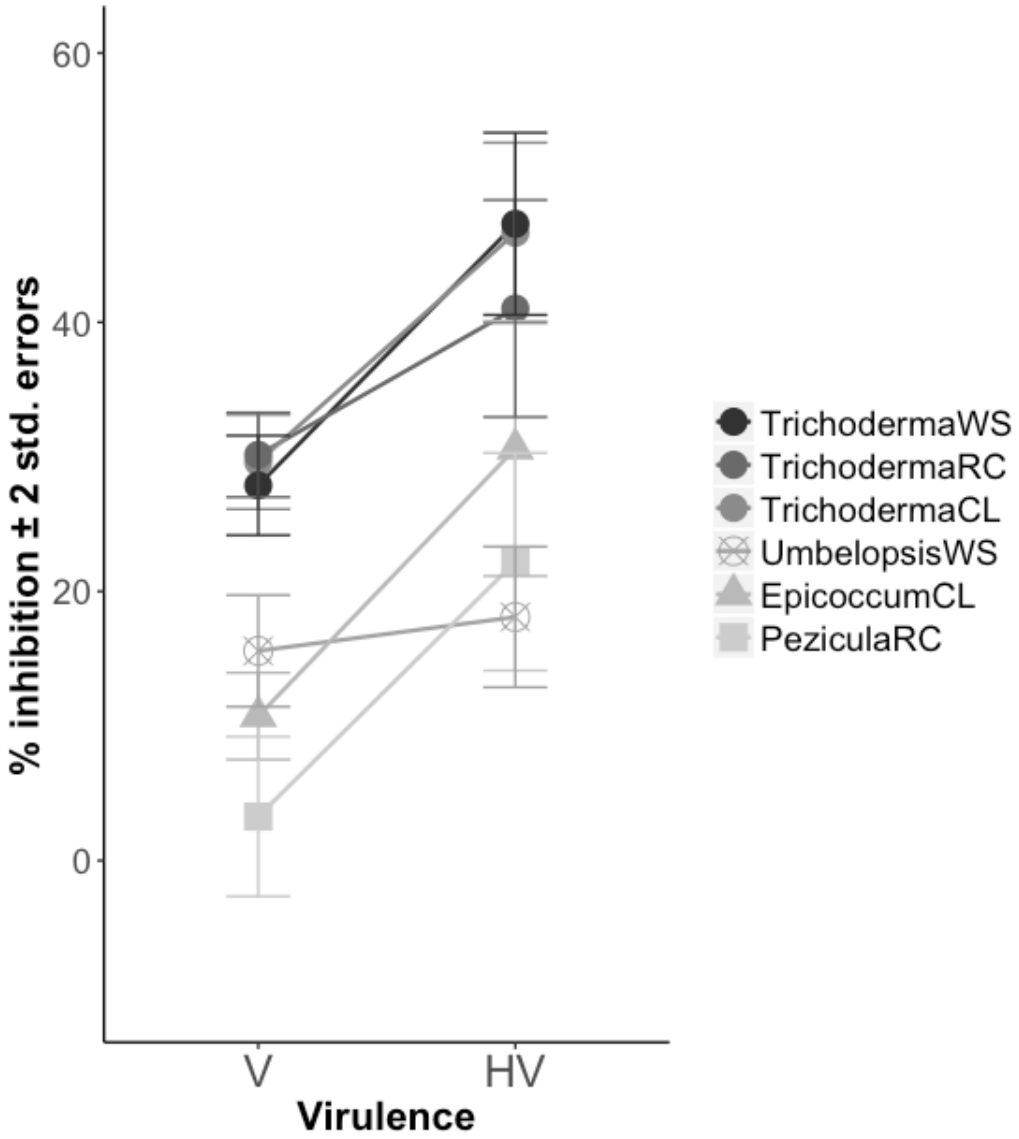


Figure 3-2: Overall percent inhibition of virulent and hypovirulent *C. parasitica* isolates in dual culture tests with six different Non-CP isolates. Colors represent different Non-CP isolates; shapes represent different fungal genera. Not all pairwise tests performed – see Table 1 for a summary of all tests.

*Epicoccum*CL (Table 3). The difference in inhibition between virulent and hypovirulent RBO15 (Δ = 17.6%) was double that displayed by LE221. It also was more variable – ranging between -2.7% against *Umbelopsis*WS (*i.e.* the virulent form was inhibited more) to 25.8% against *Epicoccum*CL. *Trichoderma*RC and *Trichoderma*CL displayed large growth inhibitions against virulent *C. parasitica*, yet virulent and hypovirulent forms of LE221 and RBO15 reacted differently to these *Trichoderma* strains. The difference in growth inhibition of virulent and hypovirulent RBO15 was 17.5% against *Trichoderma*RC and 24.1% against *Trichoderma*CL. The differences for virulent and hypovirulent LE221 in the presence of *Trichoderma*RC and *Trichoderma*CL was only 3.1% and 5.2%, respectively.

Influence of media on the growth of C. parasitica

The media on which *C. parasitica* and Non-CP interacted also influenced growth inhibition; average inhibition was 24% on PDA compared to only 20.5% on ECM. Further, the interaction between *C. parasitica* virulence and culture media was also highly significant (Table 2). While hypovirulent isolates almost always displayed greater growth inhibition compared to virulent isolates regardless of the media, inhibition was significantly lower for virulent *C. parasitica* on ECM media (Figure 3). Growth inhibition was similar, however, for hypovirulent *C. parasitica* on ECM and PDA.

DISCUSSION

Chestnut blight cankers that expand quickly are more likely to girdle infected trees compared to slowly expanding cankers. Hebard *et al.* (1984) reported that the rate of canker expansion is largely determined by mycelial fan formation of the pathogen and whether the

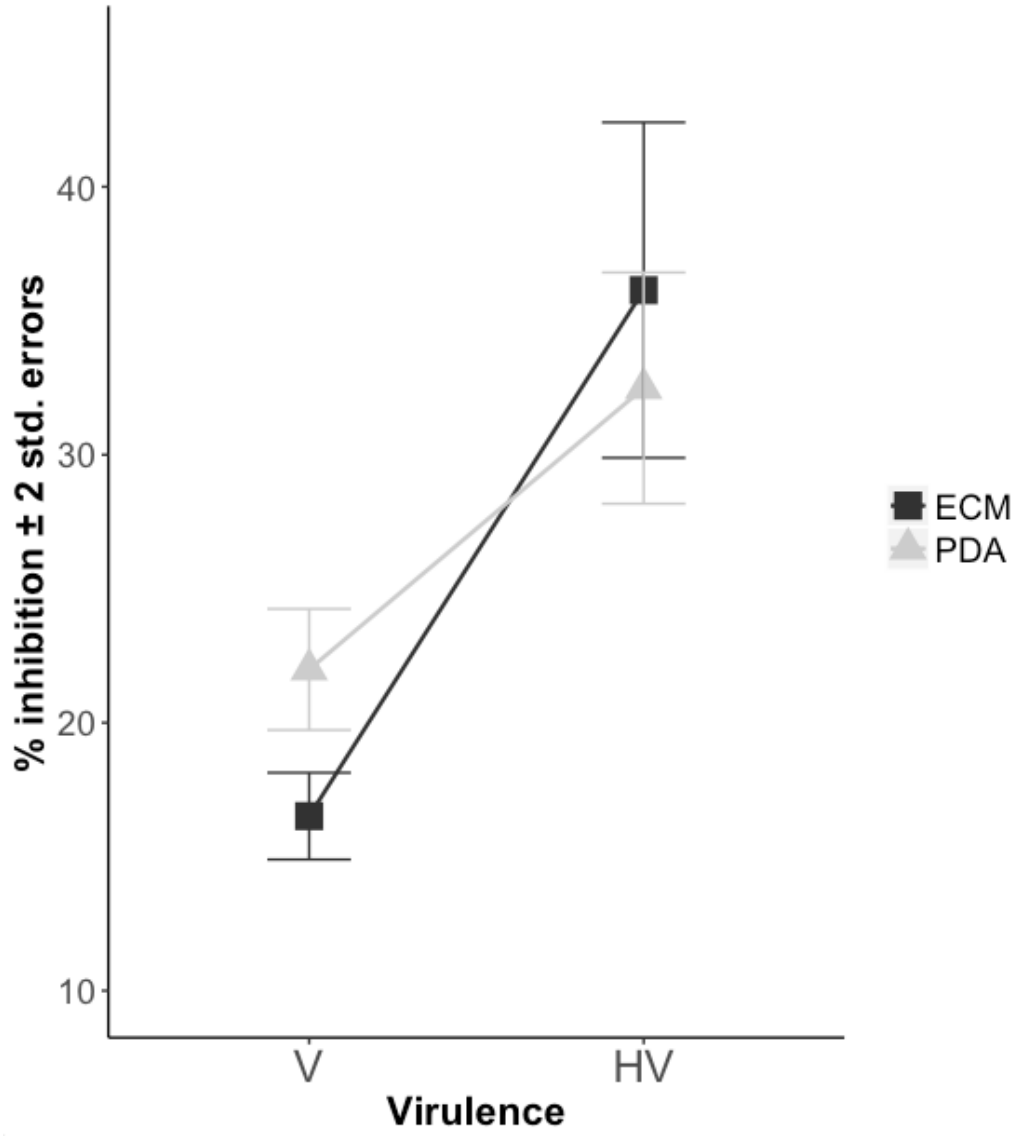


Figure 3-3: Overall % inhibition of virulent (V) and hypovirulent (HV) *C. parasitica* isolates in the presence of potential fungal antagonist isolates on different media types: Endothia complete media (ECM) and potato dextrose agar (PDA).

host is able to slow infection and restrict the pathogen to the outer bark (*i.e.* level of resistance). Virulent *C. parasitica* can destroy and bypass wound periderm created by chestnut – this happens particularly quickly in susceptible American chestnut trees (Hebard *et al.* 1984). Resistant trees can delay destruction of wound periderm by *C. parasitica* or prevent it altogether. However, when *C. parasitica* is infected with a hypovirus, host defense responses, even in some susceptible individuals, are more effective against the hypovirulent pathogen (Hebard *et al.* 1984). Thus, hypovirus infection, either inoculated on to cankers (*e.g.* Anagnostakis and Waggoner 1981; Bell 2004; Jaynes and Elliston 1980) or in a forest (*e.g.* Springer *et al.* 2013; Double *et al.* 2018), can act as a biological control by effectively slowing canker expansion and decreasing the probability of girdling.

The presence of Non-CP in cankers has been noted in field situations before (Akilli *et al.* 2009; Double *et al.* 2013; Ćurković-Perica *et al.* 2017), but the potential of Non-CP to influence the interaction between the infected chestnut tree and virulent or hypovirulent *C. parasitica* is unknown. If Non-CP are antagonistic toward *C. parasitica*, then they could further decrease pathogen growth. Over time, certain Non-CP may outcompete *C. parasitica* and become the dominant fungi in cankers on surviving chestnut trees (*e.g.* Double *et al.* 2013; Kolp 2018). This could benefit the host tree by complementing the known influence of hypovirus infection of *C. parasitica* slowing canker expansion. However, all but one Non-CP isolate tested in this study antagonized hypovirulent strains to a greater degree than corresponding virulent strains of the same *C. parasitica* isolate (Figure 2). In addition, sampling of blight cankers in natural chestnut populations in Michigan (Kolp 2018), determined that virulent and hypovirulent *C. parasitica* were often intermixed with Non-CP

taxa within a canker. Thus, the potential exists for Non-CP to antagonize both virulent and hypovirulent strains of the pathogen in a canker. If hypovirulent *C. parasitica* is already less effective at infecting and colonizing the host tree (*e.g.* Hebard *et al.* 1984), it may also be a poor competitor with Non-CP for resources within a canker.

Our results suggest that Non-CP commonly occurring in chestnut blight cankers may antagonize hypovirulent *C. parasitica* to a greater degree than the virulent form of the pathogen. The ability of Non-CP recovered from chestnut blight cankers to inhibit growth of *C. parasitica* in dual culture depended on the Non-CP strain, the *C. parasitica* strain, the cultural medium, and most importantly, whether *C. parasitica* was virulent or hypovirulent. The effect of virulence complicated the response of *C. parasitica* to Non-CP *in vitro*, as virulence interacted with each of the other three main effects (Table 2).

*Umbelopsis*WS was the only isolate to antagonize virulent *C. parasitica* more than the hypovirulent form (Δ = -2.7% with RBO15; Table 3); however, it was consistently among the worst antagonists against virulent *C. parasitica*: EP155 (5th out of 9 Non-CP isolates tested), LE221 (7th of 10), and RBO15 (4th of 6). Maximum growth inhibition of virulent *C. parasitica* was observed when paired with *Trichoderma*. Although *Trichoderma*RC and *Trichoderma*CL displayed relatively low Δs when paired with hypovirulent LE221 (3.1 and 5.2%, respectively; Table 3), they were particularly aggressive against hypovirulent RBO15. Interestingly, LE221 and RBO15 differed in their response to the same antagonistic fungi despite being infected with the same hypovirus.

Results presented here potentially explain why hypovirulent treatments sometimes fail to prevent girdling, or why hypovirulent *C. parasitica* rarely becomes the dominant fungus within chestnut blight cankers (*e.g.* Ćurković-Perica *et al.* 2017; Double *et al.* 2013; Kolp 2018). Hypovirulent forms of *C. parasitica* appear to be outcompeted at a faster rate than virulent *C. parasitica.* Given the spatial juxtaposition of Non-CP with hypovirulent and virulent forms of *C. parasitica* within a canker (Kolp *et al.* 2017), the dynamics of canker expansion will be determined, in part, by the relative rates of Non-CP competing with virulent and hypovirulent *C. parasitica*. Our results suggest that Non-CP taxa will generally outcompete hypovirulent *C. parasitica* more efficiently than virulent *C. parasitica*. Thus, the fate of a canker might be determined by the relative rate of hypovirus spread in the *C. parasitica* mycelium within a canker compared with the rate at which Non-CP eliminates hypovirulent mycelia. Spatial and temporal monitoring of cankers on American chestnut trees in Michigan and Wisconsin suggest that Non-CP taxa interact with both CP and HCP throughout the canker area, and that the potential for Non-CP taxa to reduce the abundance of HCP in a canker over time exists (Kolp 2018). The fungal community within a canker often stabilizes at a composition of with approximately 31% hypovirulent *C. parasitica*, 12% virulent *C. parasitica* and 57% Non-CP (Kolp *et al.* 2017). Thus, tree resistance will largely determine whether this community results in a slowly expanding canker that does not girdle the infected tree.

Despite being the superior fungal antagonist in this experiment and against other plant pathogens (review in Harman *et al.* 2004), *Trichoderma* may not supplement the beneficial effects of hypovirulence in the chestnut blight system. Each dual culture test between the

three *Trichoderma* isolates and hypovirulent LE221 and RBO15 exhibited more growth inhibition compared to virulent *C. parasitica*. The presence of *Trichoderma* in a canker with both virulent and hypovirulent *C. parasitica* may result in the hypovirulent form of the pathogen being excluded from the canker over time. Since both virulent and hypovirulent forms of the pathogen are often isolated from non-girdling cankers (*e.g.* Bryner *et al.* 2013; Ćurković-Perica *et al.* 2017; Double *et al* 2013; Kolp *et al.* 2017), the virulent form may persist and continue killing host cells.

Previous work with *Trichoderma* provided results similar to those presented here. Tattar *et al.* (1996) observed that *Trichoderma* "arrested growth of *C. parasitica*" in dual culture, and isolates "completely overgrew *C. parasitica* within 14 days" to the point that the pathogen was no longer able to be isolated from the test plate. Many of the dual culture tests involving *Trichoderma* resulted in a similar exclusion of *C. parasitica* from the petri plate (Figure 4A and 4B); however, *Trichoderma* were more likely to overgrow hypovirulent *C. parasitica* than the virulent form. No other Non-CP tested inhibited the growth of virulent or hypovirulent *C. parasitica* in a similar way as *Trichoderma* (Figure 5A and 5B).

Researchers of other disease systems have experimented with *Trichoderma* to find biological control solutions via fungal antagonism of other plant pathogens as well (*e.g.* Campanile *et al* 2007; Card *et al.* 2009; Mejia *et al.* 2008). Campanile *et al.* (2007) noted that although some species of oak were declining near the Mediterranean Sea due to the fungus *Diplodia corticola*, the diseased bark of trees contained a variety of other fungi, including species of *Trichoderma.* Results from their dual culture experiments identified an isolate of *Trichoderma viride* as

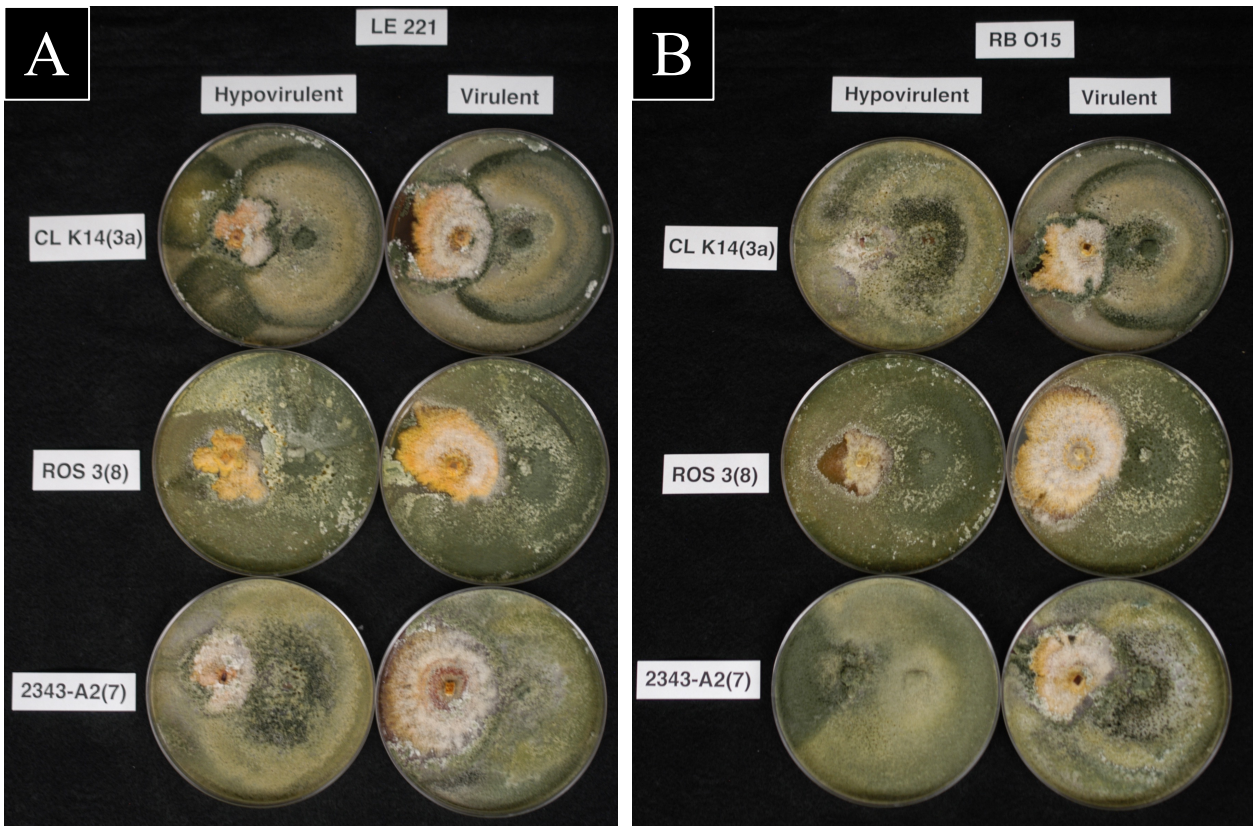


Figure 3-4: (A): Competitive interactions of virulent and hypovirulent *C. parasitica* isolate RBO15 with *Trichoderma*CL (CL K14(3a)),*Trichoderma*RC (ROS 3(8)), and *Trichoderma*WS (2343-A2(7)) on PDA after ten days. (B): Competitive interactions of virulent and hypovirulent *C. parasitica* isolate LE221 with *Trichoderma*CL (CL K14(3a)),*Trichoderma*RC (ROS 3(8)), and *Trichoderma*WS (2343-A2(7)) on PDA after ten days.

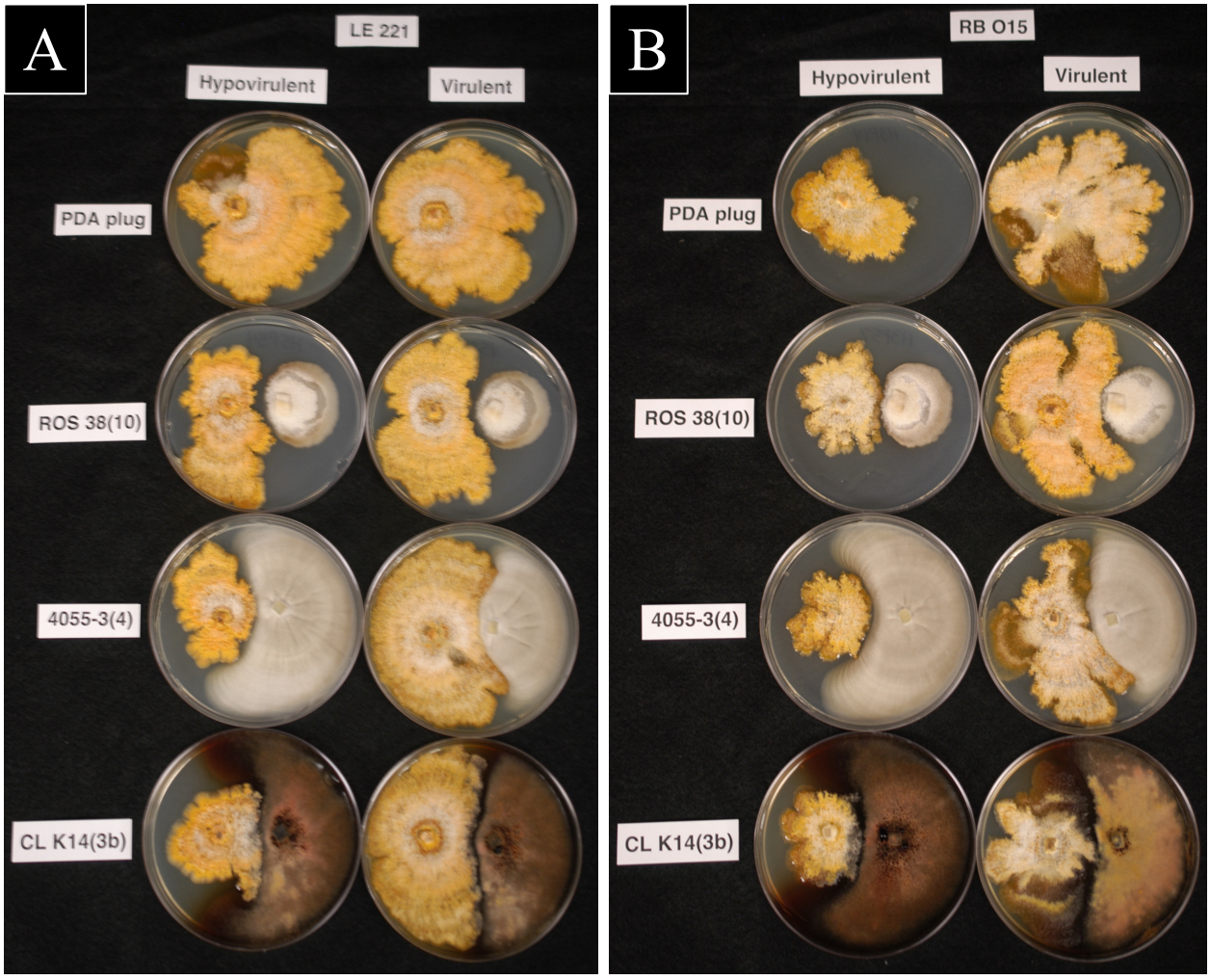


Figure 3-5: (A): Competitive interactions of virulent and hypovirulent *C. parasitica* isolate RBO15 with *Pezicula*RC (ROS 38(10)), *Umbelopsis*WS (4055-3(4)), and *Epicoccum*CL ((CL K14(3b)), on PDA after ten days. (B): Competitive interactions of virulent and hypovirulent *C. parasitica* isolate LE221 with *Pezicula*RC (ROS 38(10)), *Umbelopsis*WS (4055-3(4)), and *Epicoccum*CL ((CL K14(3b)), on PDA after ten days.

having a maximum inhibitory effect on the oak decline pathogen. Among the other fungi used by Campanile *et al.* (2007) was *Epicoccum nigrum*, a species isolated from the bark of oak. *Epicoccum*CL, isolated from chestnut and used in this study, was among the worst antagonists of virulent *C. parasitica*. Furthermore, it displayed three to four times greater growth inhibition of hypovirulent *C. parasitica.* Other, seemingly weak fungal antagonists may also inhibit hypovirulent *C. parasitica* to a greater degree in chestnut blight cankers than virulent *C. parasitica.* Although this study did not investigate interactions between Non-CP taxa, future work with Non-CP should investigate how antagonism among Non-CP taxa might impact *C. parasitica* with and without hypovirus infection.

The interaction between cultural media and virulence also indicated that virulent and hypovirulent *C. parasitica* may react differently to Non-CP depending on environmental conditions, such as the type of resources available for growth. The ability of *C. parasitica* to grow is related to its ability to obtain nutrients and resist inhibition by the host tree, both of which are variable within and among species of chestnut (Hebard *et al.* 1984). Therefore, interactions between the pathogen and potentially antagonistic fungi are likely complicated by the environment and host chemistry. Other studies that have screened for antagonistic microorganisms of other plant pathogens *in vitro* and *in planta* echo this (*e.g.* Arnold *et al.* 2003). Arnold *et al.* (2003) found that younger leaves of *Theobroma cacao* (chocolate) were less susceptible to pathogen damage compared to mature leaves, but that both leaf types incurred less damage when co-inoculated with foliar fungal endophytes and an important leaf pathogen (*Phytophthora* sp.).

For the purposes of managing chestnut blight via slowing canker expansion and delaying the time to girdling, we sought bark fungi that displayed higher levels of antagonism toward virulent *C. parasitica* than toward hypovirulent *C. parasitica.* Based on distribution and diversity of Non-CP and virulent and hypovirulent *C. parasitica* within cankers, these antagonistic interactions between fungi become more important to consider. Our results demonstrate that Non-CP may antagonize hypovirulent *C. parasitica* to a larger degree than the virulent form of the pathogen. This may lead hypovirulent *C. parasitica* never becoming the dominant fungus of a chestnut blight canker and exclusion from the canker over time. The fate of different Non-CP taxa and hypovirulent *C. parasitica* in chestnut blight cankers warrants further study to investigate how the fungal community changes over time to slow canker expansion and prevent girdling.

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CHAPTER 4

EXPERIMENTAL TREATMENT OF CHESTNUT BLIGHT CANKERS WITH HYPOVIRULENT *CRYPHONECTRIA PARASITICA* AND POTENTIALLY ANTAGONISTIC FUNGI TO SLOW CANKER EXPANSION

ABSTRACT

Chestnut blight cankers, caused by *Cryphonectria parasitica*, are susceptible to invasion by hypoviruses and other fungi in the environment. Hypovirus infections can slow pathogen growth, decreasing the rate of canker expansion and lowering the probability of girdling the infected stem. Fungi that invade the expanding canker also may antagonize *C. parasitica* leading to further reductions in pathogen growth. Experimental cankers were initiated on living stems and treated with hypovirus and/or fungi recovered from cankers that had not girdled the stem at recovering chestnut populations in Michigan. The study was designed to evaluate the combined effect of fungal antagonism and a hypovirus on canker expansion rates. Treatments were designed to determine spatial and temporal aspects of the fungal community on canker expansion rates on infected American chestnut. Additionally, two European X Japanese hybrid chestnut cultivars ('Colossal' and 'Nevada') with naturally occurring cankers were treated with either *Trichoderma* or hypovirulent *C. parasitica* to prevent canker expansion on orchard trees. Hypovirus inoculations at the margin of cankers on American chestnut stems were effective at slowing canker expansion, while inoculations to the inner area of the canker did not affect canker expansion rates. Inoculations of potentially antagonistic fungi such as *Trichoderma* to either the inner area of the canker or the margin did not reduce the rate of canker expansion. Applying antagonistic fungi to the inner area of a canker actually offset the influence of hypovirulence added to the canker margin. The delayed addition of potentially antagonistic fungi to the inner canker

areas failed to slow canker expansion beyond the no treatment control. Cankers on hybrid orchard trees treated with *Trichoderma* controlled canker expansion 50% of the time, while 72% of hypovirus-treated cankers were successful and did not require retreatment within three years. Across experiments, there were several uncontrolled factors that may have led to high variation that reduced the statistical power to detect differences among treatments.

INTRODUCTION

Chestnut blight, caused by the stem-cankering fungus *Cryphonectria parasitica* (Murr.) Barr., is a destructive disease of American chestnut (*Castanea dentata* [Marsh.] Borkh.), European chestnut (*C. sativa* Mill.), and certain hybrid *Castanea* spp. grown in orchard settings (Fulbright, pers. comm.; Rigling and Prospero 2018). Infection begins as *C. parasitica* spores enter through wounds in the outer bark (Griffin and Elkins 1986). The pathogen's mycelial fans eventually break through wound periderm created by the tree and destroy tissues down to the cambium (Hebard *et al.* 1984). Death of the cambium leads to death of the xylem ray parenchyma, resulting in loss of sapwood function (Ewers *et al.* 1989; McManus *et al.* 1989). Canker expansion around the circumference of an infected stem can lead to girdling. Cankers on the main stem can eventually girdle the trunk of a large canopy tree and reduce it to understory sprouts produced at the root collar (Paillet 1984).

The canker is the interface of host-pathogen contact, yet the time from initial infection to girdling is not straightforward: cankers do not always expand at the same rate, nor do cankers always girdle an infected stem. Several factors of the pathogen, the host tree, and the environment all contribute to and influence disease (Stevens 1960). Thus, the canker expansion rate and the probability of stem girdling are likely to depend on a combination of factors (reviewed in Fulbright 1999; Milgroom and Cortesi 2004; Rigling and Propsero 2018).

The interaction between pathogen virulence and host resistance have been investigated in the past (*e.g.* Griffin *et al.* 1983; Hebard *et al.* 1984). In Europe and North America,

"hypoviruses" infecting the pathogen were discovered (Paul and Fulbright 1988; Hillman *et al.*1992; Peever et al. 1997), some with virulence-attenuating effects (Grente and Bertheley-Sauret 1978; Fulbright *et al.* 1983; Hillman *et al.*1992) and others without effect (Enebak *et al.* 1994). When inoculated to the margin of quickly expanding cankers on European chestnut, hypoviruses could slow canker expansion, resulting in a non-girdling response (Grente and Bertheley-Sauret 1978). Researchers in North America found that hypovirulent strains (*C. parasitica* infected with hypoviruses) could be used in the same way to limit canker expansion on American chestnut (Van Alfen *et al.* 1975; Anagnostakis and Waggoner 1981).

Certain species of *Castanea* spp. are better able to prevent the virulent or hypovirulent pathogen from advancing around the stem or branch by compartmentalizing *C. parasitica* through the formation of wound periderm (Hebard *et al.* 1984; McManus *et al.* 1989). Chinese chestnut (*C. mollissima* Blume) and Japanese chestnut (*C. creneta* Siebold and Zucc.) have higher resistance to blight than European chestnut, a species that may be more resistant to blight than American chestnut (Griffin *et al.* 1983; Hebard *et al.* 1984; Viéitez and Merkle 2005). Further, chestnut populations may vary in response to the pathogen with and without hypovirus, although this claim has not been widely substantiated. Hebard *et al.* (1984) demonstrated that individuals of American chestnut differed in their response to virulent and hypovirulent forms of the pathogen, but that even when the pathogen is hypovirulent, susceptible American chestnut stems can eventually become girdled.

Direct isolation of the *C. parasitica* from cankers and laboratory assessment for hypovirus infection may be critical for predicting the time to girdling (Bryner *et al.* 2013). Bryner *et al.*

(2013) found that for European chestnut (*C. sativa* Mill.), different features of cankers (*e.g.* stem encircling, canker length and depth) were not reliable predictors of the presence or absence of hypovirus in the canker. Bryner *et al.* (2013) discussed the difficulty in predicting hypovirus presence based on canker morphology could be due to the pathogen become infected with hypovirus at different times after canker establishment. In addition, the mycelia of *C. parasitica* may only become partially infected with hypovirus in a canker, or that cankers merge together on a stem.

The environment in which host-pathogen interactions occur is also important to consider, especially considering that chestnut blight cankers are vulnerable to invasion by other fungal taxa (*e.g.* Akilli *et al.* 2009; Double *et al.* 2013; Ćurković-Perica *et al.* 2017; Kolp thesis, Chapter 2). Monitoring within a stand of American chestnuts near West Salem, Wisconsin where hypoviruses were being disseminated revealed that non-girdling cankers on surviving trees accumulated non-*C. parasitica* fungi (Non-CP) over time (Double *et al.* 2013). These non-girdling cankers became diverse fungal communities: the percentage of *C. parasitica* isolates containing hypovirus (HCP) remains relatively constant within the canker. In contrast, the percentage of virulent isolates of *C. parasitica* without hypovirus (CP) decreases over time, while the percentage of Non-CP increases over the same period.

Certain Non-CP taxa that invade cankers may antagonize *C. parasitica* by competing with the pathogen for dead plant tissue (Prospero *et al.* 2006). Kolp *et al.* (Kolp thesis, Chapter 2) found that the majority of cankers on surviving American chestnut trees in northern Michigan also transitioned to communities dominated by Non-CP taxa. However, stems

with cankers with abundant Non-CP within the canker community had a lower rate or survival (~42% survivorship over five years) compared to stems with HCP-dominated cankers (~63%; Kolp thesis, Chapter 2, Figure 4). In addition, dual culture experiments with CP and HCP showed that *Trichoderma* and other common canker fungi actually inhibit HCP to a greater degree than CP (Kolp thesis, Chapter 3). Previous research found that *Trichoderma* spp. were capable of antagonizing *C. parasitica in vitro* (Tattar *et al.* 1996) and *in planta* (*e.g.* Weidlich 1978; Tattar *et al.* 1996; Akilli *et al.* 2011), but the community and fate of a canker over time may depend on the rate at which HCP infection spreads to CP within the canker and the rate at which Non-CP inhibits HCP.

CP, HCP, and Non-CP were distributed as a mosaic in natural cankers monitored across six sites over five years, suggesting that CP, HCP, and Non-CP may all interacts within a canker (Kolp thesis, Chapter 2). In order to most effectively slow canker expansion experimentally, one should consider spatially restricting Non-CP to the inner area of cankers to compete with CP only. This will prevent antagonism of HCP at the margin where conversion of CP to HCP also occurs. This approach is hypothesized to combine the conversion capability of HCP to lower the virulence of CP, as well as the antagonistic nature of certain Non-CP taxa toward *C. parasitica*.

Model of fungal communities within cankers

Non-girdling and girdling cankers develop fungal communities that differ both spatially and temporally (Figure 1). In non-girdling cankers, hypoviruses become established along the margin of cankers early in canker development (Bell 2004). Hypovirus infection (HCP)

slows expansion rates, which allow the defense response of the tree to more effectively wall-off or at least delay destruction of callus tissue by a less virulent pathogen (Hebard *et al.* 1984). As a non-girdling and slowly expanding canker ages, the inner area where the infection began becomes prone to invasion by saprophytic, potentially antagonistic fungi (*i.e.* Non-CP), which compete with *C. parasitica* for resources (Prospero *et al.* 2006). The combination of HCP and Non-CP acting to decrease the canker expansion rate with hypovirulence at the margin and fungal antagonism within the inner areas of the canker will further increase the likelihood that tree defenses delay or prevent girdling of the infected stem (Figure 1). Within these non-girdling cankers, the fungal community increases in diversity over time, with more Non-CP overall within the inner areas of the canker, and the threat of CP decreases as HCP and Non-CP both limit CP growth.

Girdling cankers are generally quick to girdle an infected stem. As a result, it is expected that HCP is largely absent from these cankers (Figure 1). Without hypovirus to limit CP destroying host tissue at the margin, cankers will expand rapidly. While Non-CP may invade the center of the canker, their effect on canker expansion will be minimal because they are spatially separated from CP at the expanding margin. The fungal community in these cankers will continue to be dominated by CP until the stem is girdled. Over a four-year period (2013-2017), the experiments described herein were executed and designed to determine the effect of spatial and temporal aspects of the model described above regarding canker expansion rates on American chestnut. In the first experiment ("East Farm"), one series of treatments examined the effectiveness of inoculating

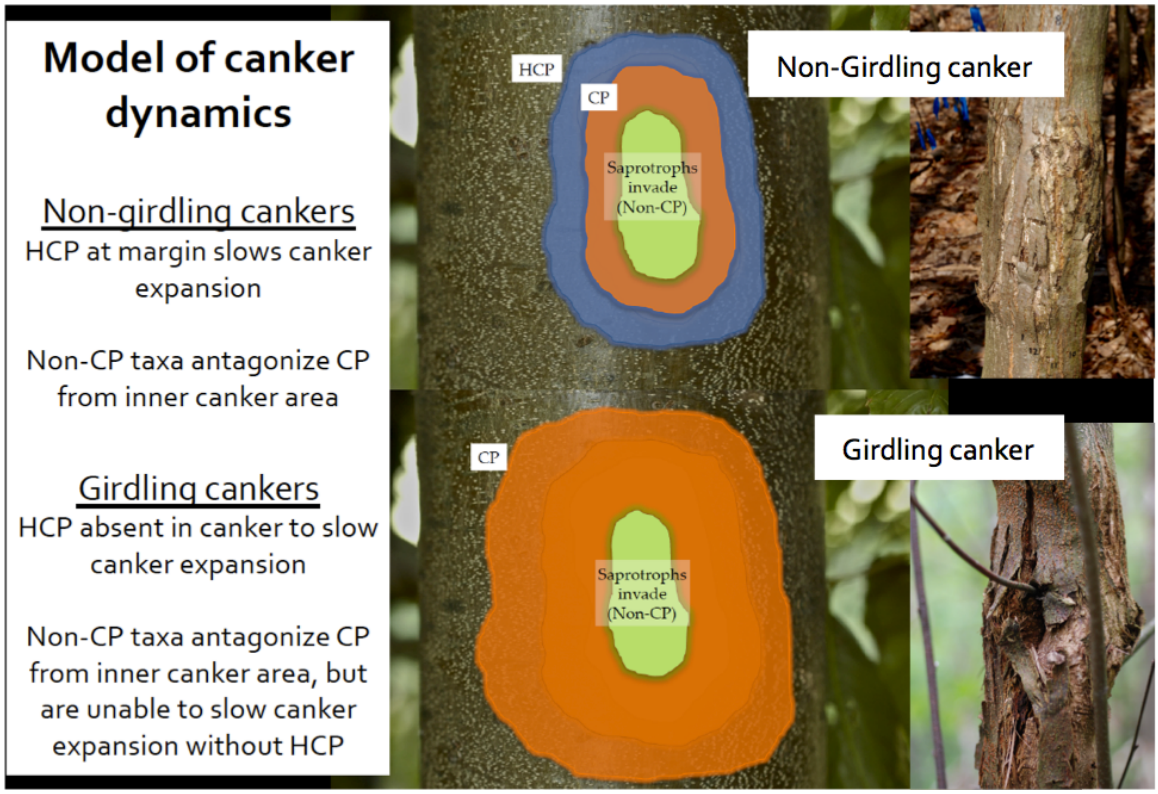

Figure 4-1: Model of fungal communities within non-girdling and girdling cankers. Non-girdling cankers: Hypoviruses become established along the margins of cankers (HCP), which reduces the rate of cankers expansion. Eventually the inner area of these cankers senesce and become vulnerable to invasion by variety of other fungal taxa (Non-CP). The Non-CP fungi compete with *C. parasitica* (CP) for resources within the inner region of the canker, further reducing the rate of canker expansion. Girdling cankers: CP promotes a very high rate of canker expansion in the absence of HCP. While Non-CP may invade the inner portion of the canker, their influence is too weak to prevent girdling of the infected stem.

hypovirulent *C. parasitica* to the margin or inner area of a canker. In parallel, a separate set of treatments evaluated whether *Trichoderma* inoculations applied to the inner areas of a canker further reduced the rate of canker expansion beyond hypovirulence treatments alone. It is hypothesized that canker expansion will be delayed beyond the effect of HCP treatment alone by adding Non-CP to the inner area of cankers already treated with HCP inoculum at the margin. In the second experiment ("County Line"), treatments evaluated whether the timing of Non-CP addition to the inner canker area would influence canker expansion rates compared to only hypovirulent treatment at the margin. The timing of Non-CP addition to a canker may be important in preventing unwanted Non-CP antagonism of HCP. Thus, cankers were treated either three months after HCP treatment or over a year after HCP treatment.

A third experiment examined whether *Trichoderma* isolates secured and covered to the entire canker area (*i.e.* "soil compress" in Weidlich 1978) would effectively slow canker expansion on natural cankers occurring on European X Japanese hybrid chestnut cultivars ('Colossal' and 'Nevada') compared to hypovirulent treatment at the canker margin. To test whether treatment of Non-CP and HCP inoculum survived within cankers across these experiments, subsets of experimental cankers were sampled.

MATERIALS AND METHODS

East Farm inoculation experiment (2013-2014)

To test the spatial effects of HCP and Non-CP on canker development, 59 stems from 39 American chestnut trees were inoculated. Trees were non-clonal, seedling derived-trees

planted on the botany farm on the south campus of Michigan State University in 1997. Stems were between 4 cm and 23 cm in diameter at breast height at the time of inoculation. Two inoculations on each stem were made using the hypovirus-free, virulent strain LE221 of *C. parasitica* on July 30, 2013. LE221 represents a *vic* genotype (MI-5) common to naturalized America chestnut populations in Michigan (Springer *et al.* 2013). Stems were disease-free for at least 10 cm below and above the point of inoculation. Inoculations were made on the stem near the base of the tree and up to seven feet up the stem to maximize the number of canker treatments on an individual stem. Inoculations were performed by removing the outer bark with a leather punch instrument (5 mm diameter) down to the cambium and inserting a PDA agar plug containing LE221 mycelia side facing outward. The inoculation point was covered with masking tape for one week for reduce desiccation of the inoculum plug.

Fourteen treatments were used in the experiment to differentiate the individual effects of HCP and Non-CP, as well as their combined influence on canker expansion with respect to their spatial orientation within a canker (Table 1). With the exception of the virulent controls (Treatment 14), the margin and/or inner area of all cankers were treated on September 16, 2013 – 48 days after inoculation. A hypovirulent form of LE221 was used for HCP treatments (Treatments 4-11; Table 1) because the hypovirus was the same genotype as LE221, the canker-inciting strain, and could transfer readily to extant cankers. Hypovirulent LE221 was created by infecting the hypovirus-free virulent LE221 with a CHV-3/GH2 hypovirus in the laboratory by pairing it with a "donor" hypovirulent strain (GH2 infected with CHV-3/GH2 hypovirus) 5 mm apart at the edge of a 10-cm-diameter

Table 4-1: Treatment groups for East Farm 2013 experiment. Cankers were initiated on day 0, and all treatments were applied to cankers 45 days post inoculation. All treated cankers, with the exception of the no treatment control [#14], were subjected to wounding prior to adding treatment inoculum. All *Trichoderma* treatments were applied to the inner canker areas. If HCP and *Trichoderma* were both added as a treatment (treatments #6-11), HCP was added first. "n" indicates the number of replicate cankers within each treatment.

Treatment ID	*Trichoderma* isolate	HCP added	n
1	2343-A2(7)	none	9
2	CL-K14(3a)	none	9
3	ROS-3(8)	none	9
4	none	margin	9
5	none	inner	9
6	2343-A2(7)	margin	9
7	CL-K14(3a)	margin	9
8	ROS-3(8)	margin	8
9	2343-A2(7)	inner	8
10	CL-K14(3a)	inner	8
11	ROS-3(8)	inner	8
12	PDA control (inner)	none	8
13	PDA control (margin)	none	8
14	No Treatment	none	8

petri dish containing Potato Dextrose Agar (PDA; Difco, Becton, Dickinson and Company, Sparks, MD) as in Huber (1996). Conversion was successful when morphology of LE221 changed to mimic the donor hypovirulent strain – reduced or abnormal vegetative growth due to the replication of the hypovirus within its new host fungus (Anagnostakis and Day 1979). From the converted LE221 strain, several dozen hypovirulent LE221 isolates were then created via hyphal tipping. To create hypovirulent treatment inoculum, ten plates of the hypovirulent LE221 strain were mixed with sterile PDA plates (10) and water (~300 mL) in a blender to achieve a viscous solution similar to the methods of Double *et al.* (2018).

Three *Trichoderma* strains were utilized for the Non-CP treatments (Treatments 1-3 and 6-11; Table 1). The strains were collected from cankers in different chestnut populations: County Line: CL-K14(3a), Roscommon: ROS 3(8), or West Salem: 2343-A2(7). For population details, see Davelos and Jarosz (2004), Springer *et al.* (2013) and Double *et al.* (2018), respectively. These three strains of *Trichoderma* also were chosen as Non-CP with known antagonistic effects toward *C. parasitica* in dual culture experiments (Tattar *et al.* 1996; Kolp, thesis Chapter 3), as well as effective canker treatments when applied to chestnut blight cankers (Akilli *et al.* 2011). Each strain was hyphal tipped and then sub-cultured on PDA agar for use as inoculum. The inoculum slurry was prepared as described above for the hypovirulent inoculum. Slurry of sterile PDA plates (20) and water (~300 mL) was used as the no-*Trichoderma* control. A box-cutter knife was used to wound healthy bark just beyond the canker area (*i.e.* margin) or throughout the diseased bark of the canker area (*i.e.* inner area). Treatment inoculum was then applied by painting wounds. No cover

or wrap was used to secure inoculum to each canker to simulate natural invasion of cankers by fungal spores.

The canker area was measured as lesions expanded away from inoculation point beginning on August 23 2013, 24 days after inoculation. The outline of a canker was traced onto an acetate transparency that was placed over the canker. To approximate canker area (cm^2), the equation for an ellipse was utilized using the product of π, half of the longest length of the canker, and half the longest width. Weekly measurements continued until the end of the experiment on November 25, 119 days after inoculation.

Two cankers from each treatment were sampled in the summer of 2014 to assess the colonization success of treatment inoculum. Six bark samples were taken from each canker: four from the canker margin and two near the inoculation point of each canker. Samples were collected using a bone marrow instrument (Lee-Lok, 11-guage, 4-inch, Lee Medical, Skillman, NJ) down to the cambium of the stem. Samples were stored frozen, then thawed and surface sterilized before plating and isolation as described in Double *et al.* (2013).

County Line inoculation experiment (2015-2017)

To test the temporal effects of HCP and Non-CP on canker development, 44 American chestnut trees (*Castanea dentata*) located in a stand in the northern Lower Peninsula of Michigan (Manistee Co., 44°31'00" and 86°06'25") were used for this study. These trees are natural seedlings representing the progeny of mature American chestnut planted at this site by early settlers (Brewer 1995) and trees at the site have been monitored since 1995

(Davelos and Jarosz 2004; Jarosz unpublished data). Some of the trees had multiple stems and were considered the same tree but as different stems (n = 55). Stems were between 4 cm and 21 cm in diameter at breast height at the time of inoculation. Stem inoculations were made on May 22, 2015 using the same virus-free, virulent LE221 strain of *C. parasitica* used in the East Farm experiment and covered for one week. Inoculations that resulted in cankers (n = 120) ranged from one-to-four per stem and were separated by at least 10 cm above and below; making sure stem area was disease-free. Inoculations were made on the stem near the base of the tree and up to seven feet up the stem. Since the vigor of the tree may influence canker expansion (*e.g.* Herms and Mattson 1992), the growth rate of each stem was estimated by coring the stem at breast height in the fall of 2016 and then dividing the diameter of each stem by the number of annual growth rings.

Nine treatments were used to test if delaying Non-CP treatment would have a different effect on canker expansion than through application of Non-CP and HCP on the same day, as in the East Farm inoculation experiment (Table 2). With the exception of the LE221 virulent control (Treatment 9), all cankers were treated with hypovirulent LE221 (see East Farm experiment above) around the margin of cankers on July 29, 2015. Stem tissue just beyond the margin of cankers was wounded using a box-cutter knife or a Dremel® power tool with a metal cutting wheel. Hypovirulent slurry was then applied using a paintbrush (see above for details). No cover or wrap was used to secure inoculum to each canker to simulate natural invasion of cankers by fungal spores.

Table 4-2: County Line cankers resulted from inoculating chestnut stems with virulent LE221 isolate of *C. parasitica* on May 22, 2015 and schedule of treatments thereafter. "Hypovirus" – hypovirulent strain of *Cryphonectria parasitica* (LE221); "*Trichoderma*" – ROS 3(8); "*Pezicula* 1" – CL A52(10), and "*Pezicula* 2" – ROS 38(10). NT = No treatment. "n" indicates the number of replicate cankers within each treatment. Cankers on dead stems were discovered as early as 337 days post inoculation and as late as 832 days. These cankers were removed from statistical analyses.

Treatment ID	HCP treatment July 29, 2015 68 dpi	2015 Non-CP treatment Oct. 11, 2015 139 dpi	2016 Non-CP treatment Aug. 14, 2016 425 dpi	n	cankers on dead stems
1	Hypovirus	NT	NT	12	2
2	Hypovirus	PDA control	NT	12	1
3	Hypovirus	*Trichoderma*	NT	12	2
4	Hypovirus	*Pezicula* 1	NT	12	2
5	Hypovirus	*Pezicula* 2	NT	12	3
6	Hypovirus	NT	*Trichoderma*	16	0
7	Hypovirus	NT	*Pezicula* 1	17	0
8	Hypovirus	NT	*Pezicula* 2	16	2
9	NT	NT	NT	11	3

Non-CP treatments were applied first to a subset of hypovirulent-treated cankers on October 11, 2015, 71 days post-hypovirulent treatment (Table 2). Non-CP inoculum was prepared as described above. The ROS 3(8) strain of *Trichoderma* from the East Farm experiment, which slowed canker expansion more effectively than the other two *Trichoderma* strains was tested, along with two strains of *Pezicula*: CL A52(10) and ROS 38(10) collected from cankers in the County Line and Roscommon chestnut populations, respectively. *Pezicula* is a common Non-CP collected from cankers in these two recovering chestnut populations in Michigan (Kolp thesis, Chapter 2). In addition, Bisseger and Seiber (1994) noted that *Pezicula* warranted further study as an effective chestnut blight biocontrol on European chestnut. In dual culture tests, however, Kolp *et al.* (Chapter 3, Kolp thesis) demonstrated that a strain of *Pezicula* [ROS 38(10)] was among the poorest inhibitors of CP among several Non-CP taxa tested. Unfortunately, these results (Kolp thesis, Chapter 3) were obtained after this experiment was set up and thus, may not have warranted further testing of antagonism *in planta*.

A box-cutter knife was used to wound throughout the inner canker area after which Non-CP slurry could be applied as described above. Slurry of sterile PDA plates and water (recipe above) also was used as a control for treatment of the inner area of cankers, first by wounding and then painting on the slurry (PDA control; Table 2). The second Non-CP treatments were applied to the remaining subset of hypovirulent-treated cankers on August 14, 2016, 54 weeks after the hypovirulent treatments (Table 2). The same three Non-CP strains were used to create slurry (described above) in these Non-CP treatments. Strains were maintained by sub-culturing to new PDA plates every two to three months, wrapping

plates with Parafilm® (Bemis Company Inc., Oshkosh WI), and stored on a laboratory bench.

Canker area was initially measured on June 11, 2015 by overlaying the canker with acetate transparency and tracing around the canker margin using a permanent marker. This procedure was repeated eight times during the first growing season, approximately once per month, until December 10, 2015. In 2016, six more canker measurements were taken – beginning on May 2, 2016 until October 23, 2016. Canker measurements began again in 2017 on May 17 and continued until the final measurement (t = 20) on November 8, 873 days post inoculation of stems with virulent *C. parasitica*.

Cankers from each of the nine treatments were sampled on June 13, 2016 and again on March 27, 2017 to assess the colonization success of HCP and Non-CP. Four cankers from each treatment were sampled (two cankers in 2016; two more cankers in 2017) by taking four bark samples at the margin of each canker, as well as two samples near the inoculation point of each canker (inner area). Samples were stored frozen, then thawed and surface sterilized before plating and isolation as per Double *et al.* (2013).

Blackwell experiment (2014-2017)

To test the efficacy of HCP and Non-CP treatment on development of naturally occurring chestnut blight cankers, 53 European X Japanese hybrid chestnut trees (cv. 'Colossal' [n=41] or 'Nevada' [n=12]) located in an orchard near New Era, MI were selected during an orchard survey for blight infections on June 3, 2014. These trees were planted in the

early 2000s, each approximately 15-years old and reproductively mature at the time of this study. Each tree contained a newly discovered blight canker on either the main stem or a reproducing branch and was assumed to be a canker less than one-year old.

One of two treatments were applied to cankers during the summer of 2014: hypovirulence treatment (n=43; 33 'Colossal' and 10 'Nevada' trees) or *Trichoderma* treatment (n=10; 8 'Colossal' and 2 'Nevada' trees). Hypovirulence treatment involved creating hypovirulent inoculum as previously described using the GH2 donor strain paired with a representative strain of *C. parasitica* isolated from the canker, or a mix of hypovirulent *C. parasitica* representing all five *vic* genotypes found in the orchard. Cankers were wounded first using a chainsaw or, in the case of difficult to reach cankers, scratchers (Double *et al.* 2013) around the margin of the canker. The slurry of hypovirulent inoculum was applied to the wounds using a paintbrush. Treatments were covered using plastic laboratory bench paper and masking tape for at least two weeks.

Trichoderma treatments involved growing strains of *Trichoderma* used in the East Farm experiment (CL K14(3a), ROS 3(8), and 2343-A2(7)) individually on Petri plates containing PDA for one week. A mix of plates containing each *Trichoderma* strain was applied to each canker mycelia facing inward until entire canker area was covered at least 5 cm beyond the canker margin. Treatments were covered using plastic lab bench paper and masking tape for nine months similar to Weidlich (1978).

Canker treatments were evaluated annually during orchard surveys in the summers of 2015-2017. Treatments were considered failures if 2014 treatment was inadequate to control canker expansion and subsequent treatment was necessary. In some cases, severe failure of treatments resulted in tree death and removal from orchard. Each of the ten *Trichoderma* treatment cankers was sampled to assess the colonization success of treatment inoculum. Three bark samples were taken at both the margin and inner area of each canker using a 1 cm leather punch. Samples were stored frozen, then thawed and surface sterilized before plating and isolation as per Double *et al.* (2013).

RESULTS

East Farm experiment

ANOVA analyses based on the final canker measurements determined that there were no significant differences between the fourteen treatments due to high variance among replicates within each treatment group, although the overall p value was marginally significant ($p < 0.07$; Table 3; Figure 2). When pooled together, however, treatments involving HCP inoculum applied at the margin of cankers (Treatments 4 and 6-8) resulted in cankers that were significantly smaller in size (cm^2) compared to cankers treated with HCP throughout the inner canker area or no HCP treatment (Table 3). Also, there was a significant wounding effect, with wounding of the inner canker area resulting in significantly larger cankers compared to no wounding or wounding at the margin treatments (Table 3).

There was a tendency for *Trichoderma* strains to accelerate canker expansion when HCP treatment was added to the margin, although this trend was not statistically significant.

Table 4-3: ANOVA table for East Farm 2013 experiment for three individual models, all with final canker area size (cm^2) as the response variable. Tukey's honestly significant differences (HSD) with the same letter are not statistically different ($P < 0.05$).

MODEL Canker Area ~ Treatment

Factor	df	Sum Sq.	Mean Sq.	F-value	Pr(>F)
Treatment	13	9550	734.6	1.708	0.0696
(Residuals)	105	45153	430.6	-	-

Treatment ID	Mean Canker Area	Tukey's HSD	Treatment ID	Mean Canker Area	Tukey's HSD
1	45.2	a	8	22.7	a
2	47.1	a	9	42.9	a
3	30.7	a	10	33.7	a
4	15.0	a	11	31.7	a
5	38.4	a	12	33.8	a
6	27.1	a	13	28.7	a
7	26.7	a	14	22.9	a

MODEL Canker Area ~ HCP

Factor	df	Sum Sq.	Mean Sq.	F-value	Pr(>F)
HCP	2	4189	2094.7	4.81	0.0098
(Residuals)	116	50514	435.5	-	-

HCP added to	Treatment ID	Mean Canker Area	Tukey's HSD
Margin	4, 6, 7, 8	22.8	b
Inner	5, 9, 10, 11	36.8	a
None	1, 2, 3, 12, 13, 14	35.1	a

MODEL Canker Area ~ Wounding

Factor	df	Sum Sq.	Mean Sq.	F-value	Pr(>F)
Wounding	2	6006	3003	7.153	0.0012
(Residuals)	116	48698	419.8	-	-

Wounding canker at	Treatment ID	Mean Canker Area	Tukey's HSD
Margin	4, 6, 7, 8, 13	23.9	b
Inner	1, 2, 3, 5, 9, 10, 11, 12	38.1	a
None	14	22.9	b

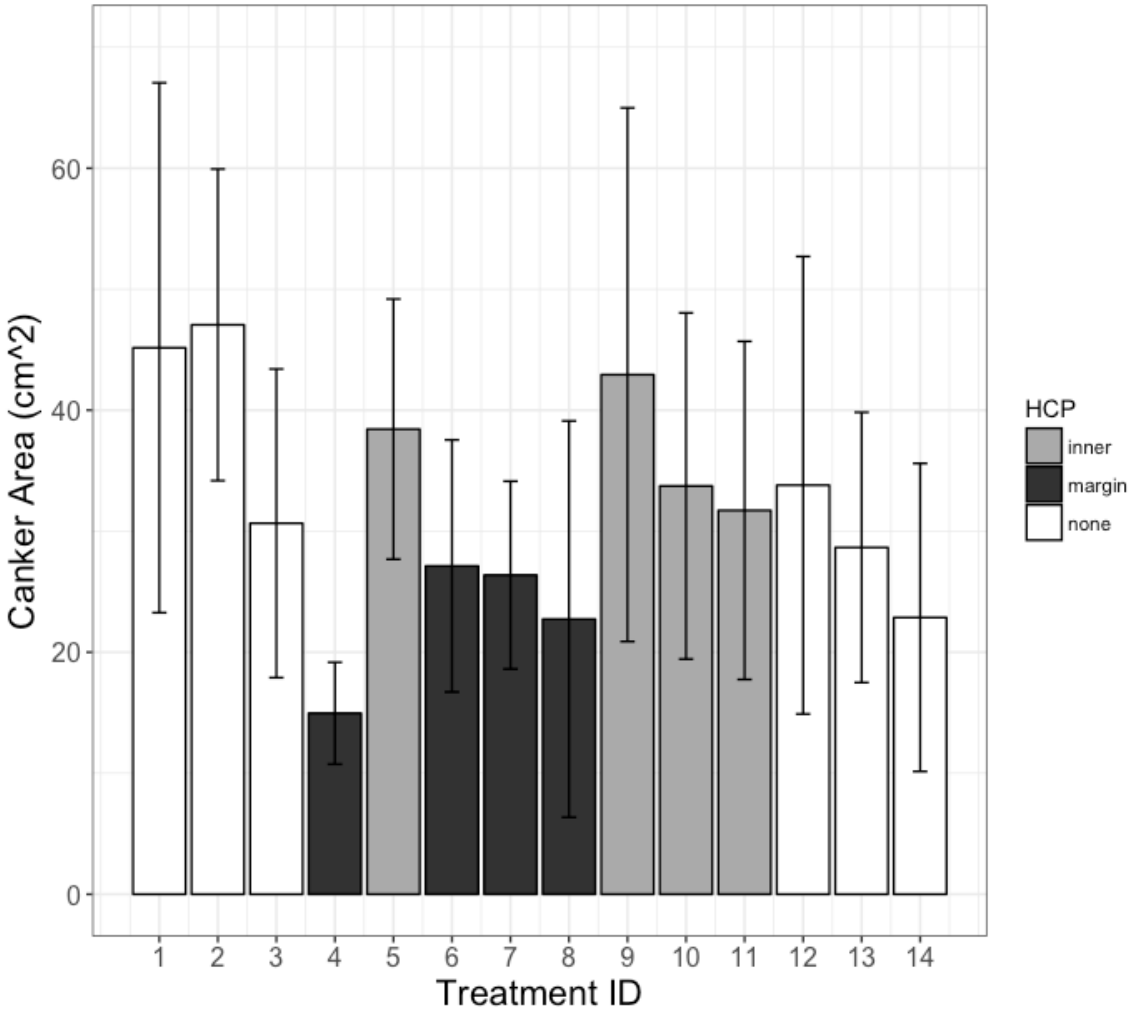

Figure 4-2: Mean canker size (cm^2) for East Farm stems 117 days post inoculation with +/- 2 standard errors. Columns represent treatment groups; column fill indicates HCP treatment location in cankers. Stems were inoculated on August 2, 2013. All treatments were added to expanding cankers 48 days post inoculation. *Trichoderma* treatments involved adding a slurry of *Trichoderma* to the inner canker area: Strain 2343-A2(7) for treatments #1, 6, and 9; CL-K14(3a) for treatments #2, 7, and 10; and ROS-3(8) for treatments #3, 8, and 11. For treatments #12 and #13, wounding and addition of non-*Trichoderma,* PDA control slurry was added to the inner area or margin of cankers, respectively. Treatment #14 served as a no treatment, no wounding control.

This pattern can be observed by comparing treatment 4 (HCP at margin; no *Trichoderma* added) to treatments 6-8 (HCP at margin; *Trichoderma* at inner canker area; Table 3; Figure 2). In addition, treatments that involved adding both HCP and *Trichoderma* to the inner canker area were among the worst at preventing canker expansion (treatments 9-11; Figure 2). Among the three *Trichoderma* strains tested, ROS 3(8) appeared to be most effective at limiting canker expansion compared to other *Trichoderma* strains, as well as when used with HCP, although this result was not statistically significant (Table 3; Figure 2).

County Line experiment

Over the course of this three-year experiment, 7 of the 55 stems used in this study died (Table 2). The majority of stems used in this study had one or more cankers in addition to the experimentally-initiated cankers, so it is not possible to state that the death of a stem was due to the canker we created and treated. The first stem died 337 days post inoculation, the last at 832 days. Canker observations on those stems (n=15) were removed from the data set.

Among the nine treatments (Table 2), significant differences were found between the virulent control (Treatment 9) and treatments 1, 4, 7 and 8 but only at time point 7 (Table 4; Figures 3 and 4). Early in canker development, however, HCP treatment at the margin of cankers was effective at limiting canker expansion relative to the no treatment control (Treatment 9) just before 2015 Non-CP treatments occurred (Table 4; Figure 4, left panel). However, an effect of HCP treatment at the margin to slow canker expansion diminished by the midpoint of the experiment (Table 4; Figure 4, middle panel).

Table 4-4: ANOVA table for County Line experiment for three individual models, all with final canker area size (cm^2) as the response variable. Tukey's honestly significant differences (HSD) with the same letter are not statistically different ($P < 0.05$).

MODEL Canker Area @ timepoint 7 ~ Treatment

Factor	df	Sum Sq.	Mean Sq.	F-value	Pr(>F)
Treatment	8	4652	581.5	2.886	0.00634
(Residuals)	96	19342	201.5	-	-

Treatment ID	Mean Canker Area	Tukey's HSD	Treatment ID	Mean Canker Area	Tukey's HSD
1	23.1	b	6	29.5	ab
2	34.9	ab	7	22.3	b
3	26.7	ab	8	25.5	b
4	25.9	b	9	47.8	a
5	29.4	ab			

MODEL Canker Area @ timepoint 13 ~ Treatment

Factor	df	Sum Sq.	Mean Sq.	F-value	Pr(>F)
Treatment	8	39485	4936	1.204	0.305
(Residuals)	96	393379	4098	-	-

Treatment ID	Mean Canker Area	Tukey's HSD	Treatment ID	Mean Canker Area	Tukey's HSD
1	74.5	a	6	88.3	a
2	111.5	a	7	60.5	a
3	120.0	a	8	81.7	a
4	81.4	a	9	97.3	a
5	115.9	a			

MODEL Canker Area @ FINAL timepoint ~ Treatment

Factor	df	Sum Sq.	Mean Sq.	F-value	Pr(>F)
Treatment	8	81580	10197	0.529	0.832
(Residuals)	96	1851366	19285	-	-

Treatment ID	Mean Canker Area	Tukey's HSD	Treatment ID	Mean Canker Area	Tukey's HSD
1	215.8	a	6	257.1	a
2	293.0	a	7	232.2	a
3	272.7	a	8	239.8	a
4	235.9	a	9	260.8	a
5	313.8	a			

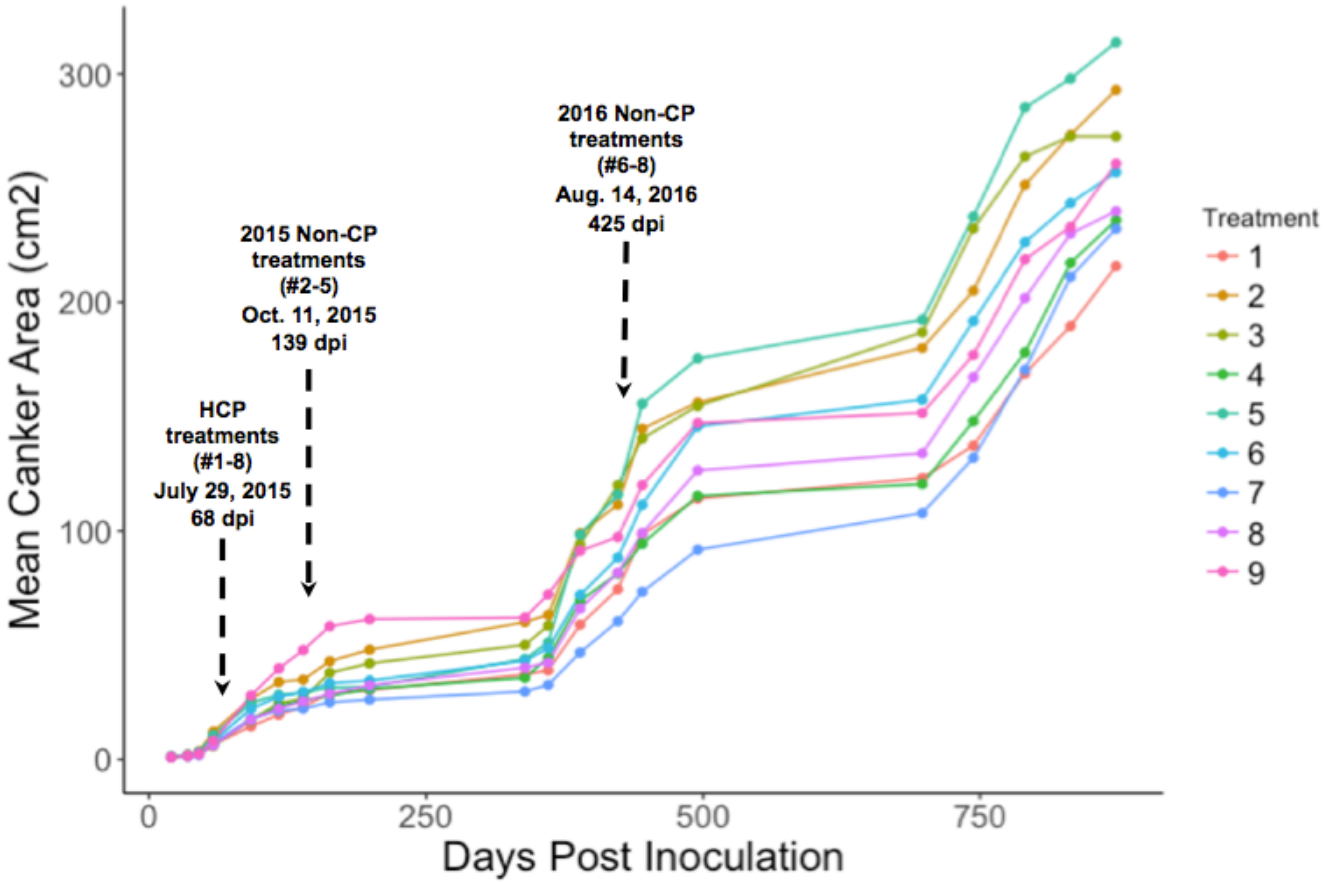

Figure 4-3: Mean canker area (cm2) at County Line for each treatment plotted across twenty time points over 873 days; canker measurement at greatest length and greatest width, then approximating using the formula to calculate the area of an ellipse: area (cm2) = (longest length [cm] / 2) x (longest width [cm] / 2) x π. Treatment ID and schedule of treatments in days post-inoculation (dpi) are included in Table 2.

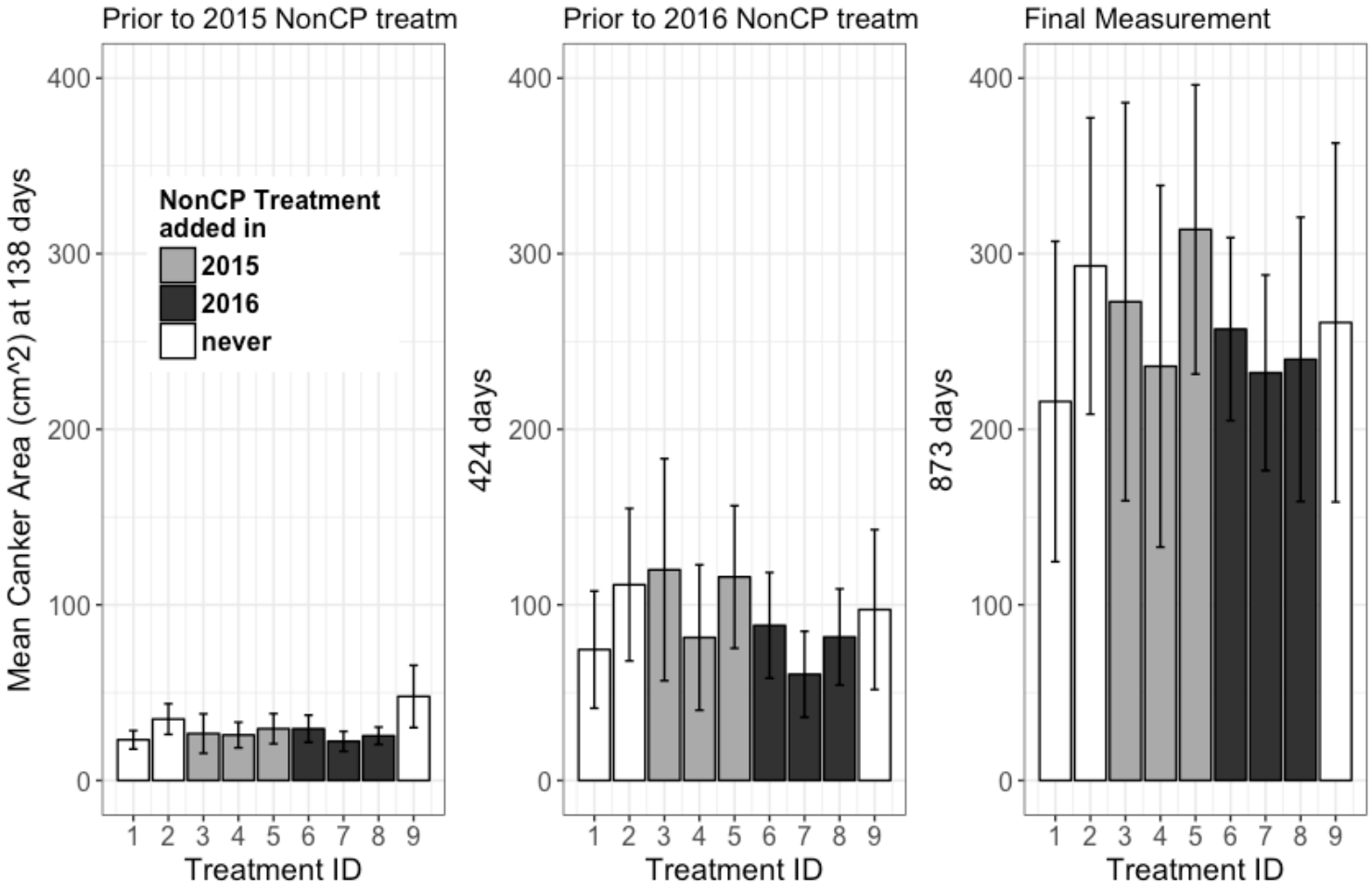

Figure 4-4: Mean canker area (cm2) for each treatment at County Line plotted across twenty time points over 873 days; canker measurement at longest length and longest width, then approximating using the formula to calculate the area of an ellipse: area (cm2) = (longest length [cm] / 2) x (longest width [cm] / 2) x π. Treatment ID and schedule of treatment post-inoculation is included in Table 2.

Growth rate among stems varied between 0.267 and 0.927 rings per cm. Tree size (stem diameter just below canker in cm) or growth rate based on tree ring data was not significant in predicting canker size at any point during the experiment.

Blackwell experiment

Hypovirulent treatment to natural cankers on 'Colossal' and 'Nevada' orchard trees was more successful (*i.e.* did not require further treatment) compared to *Trichoderma* treatments ($\chi^2 = 6.81$, $p < 0.01$; Table 5). Five of the ten cankers treated using *Trichoderma* were successful and did not need further treatment with HCP in order to prevent further canker expansion. In contrast, 72% of cankers treated using hypovirulent isolates were successful and did not need further treatment as recently as summer 2017, or three years post-treatment.

Sampling cankers for inoculum survivability

Across the three experiments, at least four bark samples per treatment were taken, and the ability to successfully isolate HCP and Non-CP from cankers post-treatment was variable (Table 6). HCP isolates were recovered from 11 of the 12 treatments involving HCP application to the margin of cankers. Only two of four treatments of HCP to the inner canker area yielded HCP isolates from either the inner area or canker margin in the East Farm experiment. The presence of *Trichoderma* in the inner canker area was confirmed in over half (7 of 12) of treatments across the three experiments (Table 6). In addition, 33% of the isolates recovered from treated cankers in the Blackwell experiment were *Trichoderma*.

Table 4-5: At an orchard near Ludington, MI, 53 newly discovered natural chestnut blight cankers were treated in the summer of 2014 with either HCP or Trichoderma inoculum on European X Japanese hybrid 'Colossal' and 'Nevada' chestnut. Treatment successes were those cankers on trees that did not result in girdling or require further treatment to prevent canker expansion in 2015-2017 (** = $p < 0.01$).

	Hypovirulent treatment	*Trichoderma* treatment
New Cankers in 2014	43	10
Treatment Success	31	5
% Success	72.1%**	50%

Table 4-6: Sampling of treated cankers for each experiment. Gray rows indicate unsuccessful colonization by treatment of canker. * = Both HCP and *Trichoderma* were added to inner canker area.

		Canker Margin			Inner Canker Area		
Experiment	Treatment ID	Treatment	Bark samples	Isolates	Treatment	Bark samples	Isolates
East Farm	1	NT	8	-	*Trichoderma*	4	0
	2	NT	8	-	*Trichoderma*	4	1
	3	NT	8	-	*Trichoderma*	4	0
	4	HCP	8	2	NT	4	-
	5	NT	8	-	HCP	4	0
	6	HCP	8	1	*Trichoderma*	4	0
	7	HCP	8	3	*Trichoderma*	4	0
	8	HCP	8	0	*Trichoderma*	4	1
	9*	HCP (inner)	4	2	*Trichoderma*	4	2
	10*	HCP (inner)	4	0	*Trichoderma*	4	2
	11*	HCP (inner)	4	2	*Trichoderma*	4	3
	12	NT	8	-	PDA control	4	-
	13	PDA control	8	-	NT	4	-
	14	NT	8	-	NT	4	-
County Line	1	HCP	16	3	NT	8	-
	2	HCP	16	3	PDA control	8	-
	3	HCP	16	2	*Trichoderma*	8	0
	4	HCP	16	7	*Pezicula*	8	0
	5	HCP	16	2	*Pezicula*	8	0
	6	HCP	16	7	*Trichoderma*	4	2
	7	HCP	16	5	*Pezicula*	4	0
	8	HCP	16	8	*Pezicula*	4	0
	9	NT	16	-	NT	8	-
Blackwell	*Trichoderma*	*Trichoderma*	30	10	*Trichoderma*	30	5

We were unable to recover *Pezicula* from cankers treated with this fungus at County Line, although *Pezicula* isolates were recovered from the non-treated areas of other experimental cankers (Figure 5). Isolates of *C. parasitica* were most likely sampled from canker areas that were not treated, or treated with the control agar slurry (Figure 5). Virulent *C. parasitica* (CP) isolates accounted for over three quarters of no treatment isolates at East Farm, yet only 17% of isolates from County Line. Hypovirulent *C. parasitica* (HCP) isolates made up nearly 40% of no treatment isolates at County Line, followed by various non-*C. parasitica* fungi that were not added to cankers experimentally ("unknowns" in Figure 5).

DISCUSSION

Experimentally induced chestnut blight cankers on American chestnut stems, as well as natural cankers on European X Japanese hybrid chestnut trees were treated with combinations of hypovirulent *C. parasitica* (HCP) and other, potentially antagonistic fungi (Non-CP) to determine the spatial and temporal effects of HCP and Non-CP on canker expansion. HCP treatments at the margin of cankers on American chestnut stems at East Farm were effective at slowing canker expansion; however, addition of *Trichoderma* to the inner canker area did not further reduce the rate of canker expansion. Indeed, *Trichoderma* treatments seemed to offset the influence of HCP applied to the canker margins compared to no treatment and PDA controls (Figure 2). At County Line, neither HCP treatments at the margin nor the delayed Non-CP addition to the inner canker areas appeared to slow canker

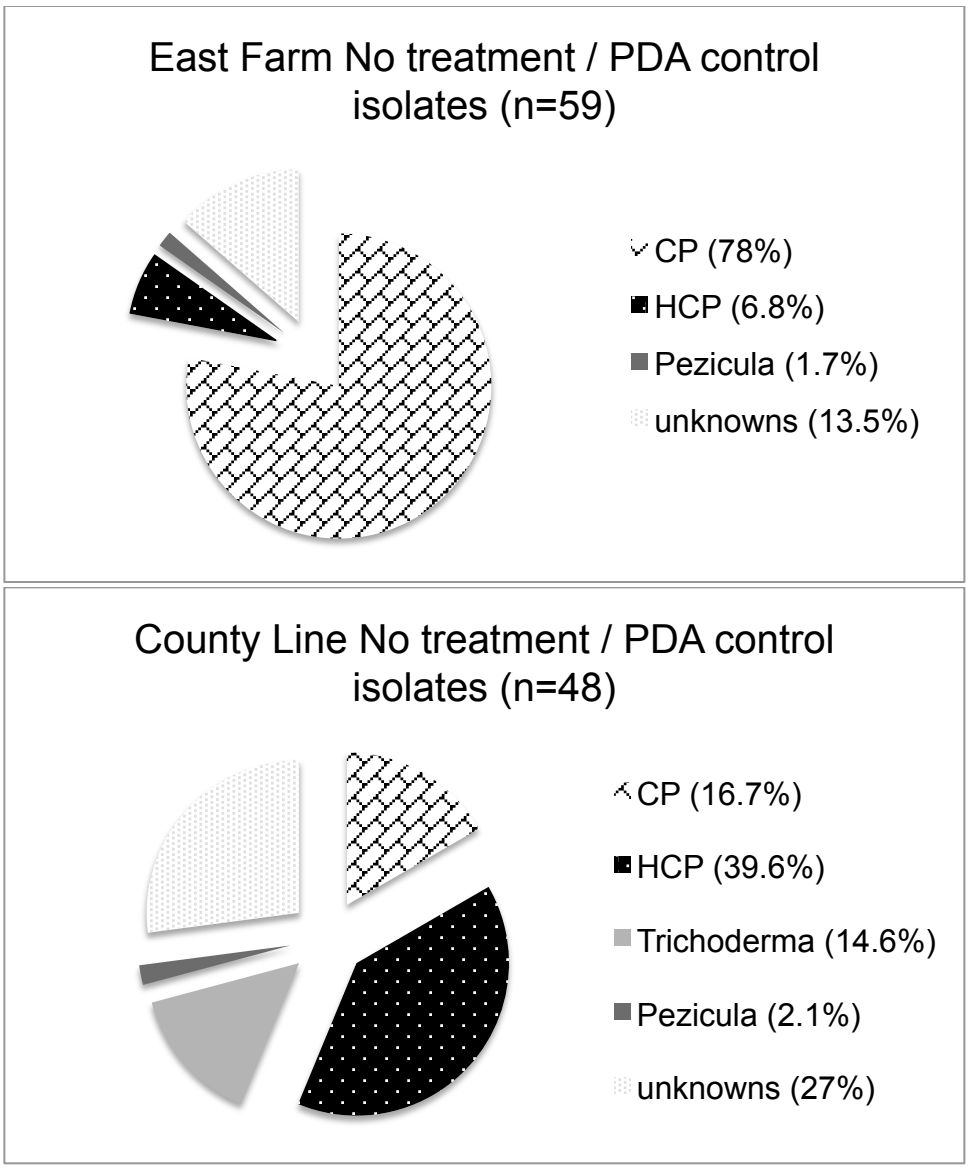

Figure 4-5: (Top panel): Frequency of isolates sampled from areas of experimental cankers in which "No Treatment" or PDA control were used at East Farm. Cankers were sampled at least eight months after treatment (Bottom panel): Frequency of isolates sampled from areas of experimental cankers in which "No Treatment" or PDA control were used at County Line. Cankers were sampled between at least eight months after treatment.

expansion on those American chestnut stems compared to the no treatment control. Finally, *Trichoderma*-treated cankers on European X Japanese hybrid orchard trees were considered treatment failures (*i.e.* required further treatment to prevent girdling) more often than HCP-treated cankers three years after initial treatment.

Fungal communities within diseased plant tissues were predicted to influence interactions between host and pathogen (*e.g.* Arnold *et al.* 2003; Campanile *et al.* 2007; Busby *et al.* 2016b). In chestnut blight cankers, multiple factors were known to contribute to disease severity and the fate of a tree, including pathogen virulence, host resistance, and a variable biotic environment where potentially antagonistic fungi invade cankers. Based on these results we suspect that several uncontrolled factors may have led to high variation within treatments across the East Farm and County Line experiments. Those factors are discussed below.

Our sampling of cankers post treatment suggests that HCP was successfully isolated from treatment areas in most treatment groups at East Farm and all HCP treatments at County Line (Table 5). Research into the treatment of chestnut blight cankers using HCP has been shown that HCP inoculum around the margin of cankers to be more effective at slowing canker expansion compared to inoculum placed within the inner area of cankers (Bell 2004). HCP inoculum at East Farm or County Line was not secured to treated trees with coverings in an attempt to simulate natural dissemination and colonization of cankers by HCP. For this reason, it is possible that the amount of HCP inoculum that managed to survive within

cankers desiccated or diminished due to rain before HCP could transfer virus to slow the virulent pathogen from continuing its destruction of host tissue.

Another explanation for the apparent lack of canker expansion control by HCP inoculum may be due to how stems were wounded prior to treatment. Bell (2004) provided evidence that not wounding cankers prior to HCP treatment significantly lowers the transmission of hypoviruses to virulent *C. parasitica.* Grente and Berthelay-Sauret (1978) established a protocol for canker treatment using a leather punch to create holes around the margin of cankers, and while other studies using this method have shown it to be effective (*e.g.* Double *et al.* 2018), it is time consuming. We used a box-cutter knife to wound trees before treatment similar to the method of Bell (2004), as well as power tools to wound trees at County Line and the Blackwell orchard. Although labor intensive, the margin-punch method (Grente 1978) may allow HCP to colonize deeper bark layers and lead to better conversion of virulent *C. parasitica*, especially if the virulent pathogen is penetrating deeper into the bark during canker development. Further, if HCP does not penetrate into the bark sufficiently, it may be more vulnerable to antagonism by invading Non-CP in the outer bark layers.

Attempts were made to establish cankers on as many genetically identical ramets as possible at East Farm and County Line; however, there were not enough multi-stemmed chestnut trees at either site to control for individual tree variability (*e.g.* randomized block design). Differences in responses among trees at both East Farm and County Line may have contributed to the large variability in canker expansion and blurred any fungal effect, in part

because these stems are the result of seedlings planted previously with unknown genetic makeup. After the East Farm experiment, it was proposed to add more replications within treatment groups at County Line. Over time, however, variability among replicates within each treatment at County Line only increased (Figures 2 and 3). This suggests that certain stems are equipped to effectively defend against the pathogen. Further, the timing of host defense breakdown in susceptible stems likely occurred early in canker development, perhaps after HCP inoculum was added and host defense in some trees could not even impede hypovirulent *C. parasitica*. Previous research suggests within population variation for host resistance in American chestnut, and that a "tree effect" will accentuate any differences among individual responses to blight (Griffin *et al.* 1983; Hebard *et al.* 1984). Rather than inoculate more seedling chestnut stems, using clonal sapling trees planted out into a forest or in a greenhouse would be a plausible solution to minimize tree effects and detect more subtle differences between HCP and Non-CP treatments.

Trees at Blackwell orchard were one of two germplasm (*i.e.* 'Colossal' or 'Nevada' European X Japanese hybrids). Despite similar genetic makeup, 30% of hypovirulent treatments failed to prevent canker expansion suggests other factors may contribute to variable host responses. For example, these cankers were naturally forming and detected for the first time in summer 2014, yet they may have initiated at different times prior to detection. In addition, the health and development of each orchard tree prior to infection, as well as the severity of each canker prior to treatment may preclude any host response. Half of cankers treated with coverings of *Trichoderma* failed to prevent canker expansion, which is in contrast to Akilli *et al.* (2011) who demonstrated that *Trichoderma* and

hypovirulent treatments were equally effective at controlling canker expansion. However, this work was conducted in a greenhouse using three-year-old European chestnut saplings (cultivar 'Osmanoglu') and with experimentally initiated cankers. Further, canker expansion was only measured for 58 days (Akilli *et al.* 2011).

The apparent lack of influence of HCP and various Non-CP strains on canker expansion rates in American chestnut may mean that fungal communities in diseased tree bark are difficult to manipulate. These experiments were designed to test the spatial structure and temporal dynamics of a model of girdling and non-girdling cankers (Figure 1). Fungi and hypoviruses were found throughout the canker (Kolp thesis, Chapter 2), and the temporal trends revealed an increasing prevalence of Non-CP and decreasing prevalence of HCP (Double *et al.* 2013; Kolp thesis, Chapter 2). While it may be true that both HCP conversion of CP, and Non-CP antagonism of HCP contribute to canker expansion, these two interactions seem to act in opposition with respect to benefitting the tree in preventing girdling. HCP conversion of CP enables host resistance and slows canker expansion, while Non-CP may antagonize HCP to a greater degree than CP (Kolp thesis, Chapter 3) and disrupt conversion of CP and allow it to resume rapid growth.

The *Trichoderma* strains used in these experiments do not support the hypothesis that this fungus can slow canker expansion rates via fungal antagonism (*e.g.* Tattar *et al.* 1996; Akilli *et al.* 2011) on American chestnut (East Farm and County Line), or at least beyond the effect that hypovirulence may provide (Blackwell experiment). Natural cankers on hybrid orchard trees treated with *Trichoderma* needed retreatment more often than the conventional

treatment method of adding HCP inoculum to wounds at the canker margin (Bell 2004). Treatment of HCP at the margin of chestnut blight cankers is partially supported as a method of slowing canker expansion rates, and that Non-CP addition to a canker may exacerbate disease progress. Future research directions should include attempts to limit the high variability among replicates within treatments in these experiments.

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CHAPTER 5

CONCLUSIONS

My dissertation research investigated chestnut blight cankers on American chestnut with the goal of assessing how the fungal community and interactions therein influenced survival of infected chestnuts. The presence of secondary fungal invaders (Non-CP) has been implicated as a disease-altering factor in canker expansion and girdling (*e.g.* Tattar *et al.* 1996; Akilli *et al.* 2011). This work represents the first spatial and temporal survey of the canker community for fungi other than *Cryphonectria parasitica* (CP) or hypovirulent *C. parasitica* (HCP) in cankers. A community ecology approach allowed me to describe the composition, distribution, and persistence of pathogenic and non-pathogenic fungi in cankers and how changes in the community correlated and likely influenced the health of the tree over time (Chapter 2).

My survey (Chapter 2) falsified our initial hypothesis that non-girdling cankers are spatially structured with HCP on the canker margins enclosing a middle area of CP, and that a central core of Non-CP develops as the canker ages (Chapter 1, Figure 1). Although approximately one third of the total canker community diversity consisted of Non-CP taxa (59 total operational taxonomic units), I found that cankers are a spatial mosaic, and that both CP and HCP interact with Non-CP that invade as a canker ages (Figure 2-10).

My survey also suggests that cankers where hypoviruses fail to establish (community cluster D) have a high probability of quickly girdling infected branches (Figure 2-7; Table 2-7). Host defense is likely insufficient at preventing canker expansion when the canker

community consists of mostly CP (Hebard *et al.* 1984). If hypoviruses infect CP and HCP becomes abundant in a canker, then the probability of girdling a stem is diminished, as host defense are able to delay expansion when the pathogen is hypovirulent (Hebard *et al.* 1984). Non-CP are thought to antagonize and inhibit growth of *C. parasitica* within the canker, as they are known to inhibit *C. parasitica* in dual culture tests (Tattar *et al.* 1996; Chapter 3). However, the fungal community can range from a high prevalence of HCP (community cluster A) to a high prevalence of Non-CP (community cluster B), and in fewer cases, can revert back to a community dominated by CP (community cluster D). Thus, the fungal community within cankers on surviving chestnut trees was unstable – more often than not, the fungal community of a canker in one year was classified differently in the next. Of cankers on trees that survived at least four years, approximately half transitioned to a final community dominated by Non-CP taxa (cluster B), yet chestnut stems with a canker community >50% Non-CP had a lower survivorship compared to cankers dominated by HCP. Although there is a slight probability of girdling even when the canker consists of mostly HCP, the volatile transitions of the fungal community over time suggests that cankers can change from less severe to girdling quickly. Through the loss of HCP in a canker, which is likely to occur over time via Non-CP invasion, CP may escape hypovirus infection and resume rapid canker expansion. Some Non-CP taxa also may be weak pathogens of chestnut and facilitate disease progress. Thus, canker expansion and the likelihood of girdling may be determined by the relative rates of HCP converting CP, and Non-CP preventing HCP from remaining prevalent in the canker community.

Non-CP invasion of chestnut blight cankers was shown to have a strong influence in the girdling process of chestnut blight and may be detrimental to the establishment of HCP in a canker and possibly a forest. Chapter 2 represents yet another example in which cryptic fungal communities of the plant microbiome are capable of affecting the outcome of plant-pathogen interactions. Future investigation of Non-CP should include the bacterial and fungal endophyte communities in healthy bark tissues beyond the margin of the canker and their effect on canker expansion and girdling. Understanding the combined effect of HCP and undetected Non-CP in diseased and healthy tissue on canker expansion and the probability of girdling will also guide future biological control strategies for chestnut blight. Additionally, the source of these Non-CP taxa in the environment (*e.g.* soil, other host trees), their ability to invade cankers, and their pathogenicity should be investigated further.

I suspect that the interactions between Non-CP, HCP, and CP govern the observed dynamics of the fungal community within a canker. If certain Non-CP taxa are antagonistic toward *C. parasitica*, Non-CP may slow canker expansion by inhibiting *C. parasitica* growth within cankers. While the presence of double-stranded RNA viruses infecting *C. parasitica* (HCP) within cankers is known to slow canker expansion and reduce the probability of girdling, HCP in cankers does not always prevent girdling. Dual culture tests (Chapter 3) of the competitive interactions between commonly isolated Non-CP from cankers and virulent and hypovirulent *C. parasitica* demonstrated hypovirus infection of *C. parasitica* results in a less competitive strain of the pathogen against all but one Non-CP taxon (*Umbelopsis isabellina*). Overall, *Trichoderma* strains inhibited the growth of CP and HCP the most. The greater degree of inhibition of HCP by most Non-CP *in vitro* supports the hypothesis that

HCP is a poor competitor in chestnut blight cankers, and may explain the tendency for Non-CP to invade and replace HCP as the dominant fungi within cankers of surviving American chestnut trees.

Although competition was the only form of antagonism tested here, future investigation of Non-CP taxa should examine other forms of antagonism (*e.g.* mycoparasitism, antibiosis) by these fungi or other microorganisms in cankers. In screening for microorganisms as biological controls of plant pathogens, *in vitro* testing should be complimented by *in planta* experiments, as results do not always translate. Studies on live chestnut stems are not always feasible, and testing on excised chestnut stems could serve as an initial proxy because they involve testing chestnut tissues directly, as well as controlling for environmental variability that may not be possible in the field.

The original model of canker dynamics (Figure 1-1; Figure 4-1) predicted that slowly expanding cankers with the lowest probability of stem girdling would have the combined effect of HCP converting CP at the margin of cankers, and antagonism of CP by Non-CP within the inner canker areas. Manipulating the spatial distribution and temporal progression of HCP and Non-CP in the canker community in experimentally created cankers (Chapter 4) did not affect canker expansion rates in the predicted way. Instead, *Trichoderma* treatments had a general tendency to offset any influence of HCP had on slowing canker expansion. When applied without HCP treatment at the margin, treatments of *Trichoderma* failed to slow canker expansion beyond no treatment controls. In addition, delaying the timing of Non-CP treatment to the inner canker nearly two years after

treatment with HCP at the margin did not result in a significant effect on slowing canker expansion compared to no treatment controls. In an attempt to simulate the natural invasion of cankers by HCP and Non-CP spores, coverings that are often used to secure treatment inoculum to experimental cankers were not used. Thus, inoculum washing away in rain or drying out before establishing in cankers may have contributed to lack of differences among treatments, although the majority of cankers sampled after treatments did contain at least some treatment inoculum.

Wounding of the canker margin and inner area was done using a small knife instead of a more labor-intensive, punch method that is thought to allow better colonization of HCP within the bark (*e.g.* Grente and Berthelay-Sauret 1978; Bell 2004). This also may have influenced the lack of differences among treatments. The host response of the tree was discussed as a possible source of the considerable variation among replicates within treatment groups, especially since seedling American chestnut trees were used for these two experiments. A third canker manipulation experiment was conducted by treating natural cankers found on European X Japanese hybrid 'Colossal' and 'Nevada' chestnut trees in an orchard with either *Trichoderma* throughout the margin and inner area or HCP treatment at the margin. Despite ~30% failure of HCP treatments, half of *Trichoderma* treatments failed to slow canker expansion and needed to be treated again with HCP to prevent girdling.

Future efforts to manipulate components of the fungal community with chestnut blight cankers to understand canker expansion could be modified to include the overall health of the stem prior to initiating cankers, as the stems used in these experiments often contained

other, natural cankers. Although *Trichoderma* is noted as a biological control agent in other plant-pathogen systems, it may be too inhibitory of HCP, and the presence of this fungus and perhaps other fungi in chestnut blight cankers does not seem to slow canker expansion.

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