*Article*

# Differing Responses to *Cryphonectria parasitica* at Two Indiana Locations

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**Abstract:** Chestnut blight, a disease that has spread rampantly among American (*Castanea dentata* (Marsh.) Borkh.) and European chestnut (*C. sativa* Mill.) trees, results from infection by the fungal pathogen *Cryphonectria parasitica* (Murrill) M.E. Barr (*C. parasitica*). This fungus was introduced in the early 1900s and has almost functionally eliminated chestnut trees from the North American landscape. In 2017, we collected chestnut blight samples from two sites (Site B, (Fulton Co., IN) and Site C (Marshall Co., IN)). At the Fulton County planting, Site B, cankers had formed, healed over, and the trees were healthy. However, at the second site in Marshall County, (Site C), cankers continued to propagate until all of the chestnut trees had died back to the ground. Research evidence worldwide has indicated that these visual clues likely result from the presence of a hypovirus. Upon closer inspection and the subsequent isolation and reproduction of spores, no hypovirus has been identified from either site. Here, we present a curious coincidence where one site has completely succumbed to the disease, while the other has been able to spring back to health.

**Keywords:** American chestnut; *Castanea dentata*; chestnut blight; *Cryphonectria parasitica*; Indiana

## 1. Introduction

Chestnut blight is a plant disease caused by the fungus *Cryphonectria parasitica* (*C. parasitica*; formerly known as *Endothia parasitica*). Originally from Asia, the disease has decimated American (*Castanea dentata* (Marsh.) Borkh.) and European chestnut (*C. sativa* Mill.) tree populations [1]. The loss of billions of American chestnuts from native landscapes has severely impacted wildlife species dependent upon the mast crops to survive, detrimentally affected the nut and lumber industry economy, and completely reconfigured forests throughout its native range. In Europe, chestnut has fared similarly. Native to Eastern Asia, the fungus has evolved alongside Chinese (*C. mollissima*) and Japanese (*C. crenata*) species. Thus, due to genetic evolution, high levels of resistance have been found in Asian chestnut species [2–4]. *C. parasitica* infection is characterized by the formation of necrotic lesions (cankers) on the bark and branches of susceptible trees [5,6]. These cankers are also a significant source of oxalic acid, a chemical toxic to chestnut tissues [7]. Studies in European chestnut indicated that the greatest concentration of cankers was most often found on larger (>30 cm DBH), more mature trees [8,9].

An extremely persistent fungal pathogen found primarily on chestnut trees, *C. parasitica* will also colonize other tree species such as sessile oak (*Quercus petraea*) [10], pedunculate oak (*Q. robur*), and common beech (*Fagus sylvatica*) to some degree [11]. Susceptible American chestnut trees typically exhibit 100% mortality rates after infection, although the longevity of the tree afterwards is variable [12–14].

A hallmark of a virulent *C. parasitica* infection is the formation of sunken cankers caused by the accelerated deterioration and death of tissue beneath the bark [15]. An enlarged,

primarily superficial canker often forms in trees infected with the hypovirus [2,16–18]. While these morphologies are often seen with the respective infection types, researchers have not indicated conclusively that these generalizations are reliable [19].

Several other *Cryphonectria* spp. can be found on chestnut (i.e., *Cryphonectria japonica*, *Cryphonectria radicalis*, *Cryphonectria naterciae*); however, these strains fail to cause cankers [20]. The majority of efforts to curtail the spread of *C. parasitica* such as burning, herbicide application, felling, and quarantine [21–24] have failed. Much greater success for the control of chestnut blight has come from the use of mycoviruses, viruses capable of replication within fungal cells, as biological control agents [25–27].

Hypovirulence is achieved when a cytoplasmic determinant within a mycovirus capable of infecting *C. parasitica* and leading to its attenuation is transmitted to a virulent strain. Mycoviruses use hyphae fusion (i.e., anastomosis) to transfer the virus from an infected strain to the virulent *C. parasitica* strain aided by the CHV-1 hypovirus (*Cryphonectria hypovirus* 1) [28]. The single stranded RNA (ssRNA) CHV-1 hypovirus can be identified by a trademark reduction in *C. parasitica* pigmentation, limited sporulation, and a negation of female fungus fecundity [29]. Three additional hypovirulent mycoviruses have been identified from *Cryphonectria* spp., but all have varied effects on the persistence of lethality in *C. parasitica* [30]. CHV-2 severely limits *C. parasitica* fertility, proper growth and development, and virulence [31], while CHV-3 primarily contributes to the diminished virulence of the fungus [32]. The least obvious of the CHV hypoviruses is CHV-4 as it has little effect on *C. parasitica* overall.

The *C. parasitica* fungus shows little genetic diversity, specifically within vegetative compatibility (vc) types, in studies from Azerbaijan [33], Spain [34], and Romania [35,36], but studies in Croatia and Slovenia report considerable diversity [37]. Increased diversity within vc types is often considered a negative outcome of sexual reproduction; however, these data may be misleading when natural populations rather than laboratory populations are examined [38–44].

The incorporation of hypovirulence with the CHV-1 hypovirus was most effective in efforts to save European chestnut when populations steeply declined throughout Europe [45,46]. Efforts to contain the spread of *C. parasitica* infection in urban areas where individual trees were infected have been extremely successful [22,23], while large-scale eradication efforts at the stand level have had limited success [47].

In other European chestnut studies, it was noted that cankers healed on a number of trees because of naturally occurring hypovirulence. However, the conversion from lethal to healing cankers appeared to be variable in studies of inoculation by Diamandis et al. [48], though percentages varied considerably. Celiker et al. [47] indicated a prevalence of healed cankers on shaded western slopes, and also proposed that ants and snails may have contributed to the observed spread of hypovirulence found in areas where no hypovirulent strains were previously inoculated. Observed over time, assisted inoculation proved less effective than natural hypovirulence as many trees eventually succumbed to blight, despite the presence of healed or healing cankers [47]. Systematic inoculation to propagate hypovirulence may be an appropriate standard practice to slow chestnut blight infection [48]; however, a lengthy time period, often between 20 and 50 years, exists between initial chestnut blight observation and the subsequent appearance of healed cankers [49]. Thus, regions with little evidence of a natural hypovirus may be coming to the end of the lag period and could soon reveal presence of a natural hypovirus. Perpetuation of *C. parasitica* and hypovirus spread has also shown to be influenced by the presence of other fungal species [50] as those groups may effectively hinder hypovirus spread. Therefore, *C. parasitica* spread and rapid canker expansion may be aided by the prevalence of competitive fungi inhibitors of the hypovirus.

Ten years ago, it was thought that the treatment of chestnut blight cankers with an inoculation of a transgenic hypovirus would initiate the spread of the treatment to other nearby trees, as transgenic hypovirulent strains of *C. parasitica* can transmit hypoviruses with 100% efficiency to ascospore progeny through asexual spores [51]. Unlike natural strains,

transgenic versions possess full-length infectious cDNA copies of virulence-attenuating hypoviruses that have been chromosomally integrated [51]. Double et al. [51] used inoculum reservoirs and direct application of the hypovirus at different plots over the course of a decade to measure the natural and assisted spread of chestnut blight treatment. The Double et al. [51] data also indicated that punch-initiated infections led to the greatest mortality rates; however, the overall health of the trees before the study may have led to those results. Outcrossing with naturally occurring strains can perpetuate the hypovirus, although dissemination rates varied depending upon fungal isolate type [52], location [53], treatment method [51], and vegetative compatibility [53–57]. Regardless of the undulating success of researchers, work by Zlatanov et al. [24] indicated spread of chestnut blight has slowed in Europe as our understanding and implementation of hypovirulence studies has increased. Hypovirulence has only marginally controlled chestnut blight in several U.S. locations (Connecticut, CHV1 and CHV3; Michigan, CHV3; Virginia, CHV1; and West Virginia, CHV3) but has failed in others (Wisconsin, CHV1, CHV3) [3]. Hypovirus application in the U.S. thus far is a concentrated effort to keep individual American chestnut trees alive for use in breeding but is not recommended for blanket protection because of poor natural dissemination [3]. Recent work by Ko et al. [58] on *C. parasitica* DNA methylation and by Demené et al. [59] on genomic sequencing and chromosomal rearrangement within the fungus may provide clues to slowing the infection and spread of the fungus within the United States.

Determination of a particular treatment for chestnut blight relies primarily on the downstream uses for the trees. Owners interested in nut production are assured a valuable harvest, while those dependent upon market prices for chestnut products have less incentive to continue chestnut plantation treatment and management [60]. The influx of chestnut blight has limited market demand for chestnut products. Thus, abandonment, or substitution for exotic species rather than implementation of expensive chestnut blight prevention measures, may become commonplace for timber plantations unless the chestnut wood products market can be revived [60]. Olivia et al. [60] suggested that the lack of economical disease and pest treatments for other low-value timber species may prevent landowners from attempting to save those species on the landscape as well. The main objectives of this study were to observe the spread of a natural *C. parasitica* infection within two size- and age-matched *C. dentata* stands, isolate the *C. parasitica* fungus and the potential hypovirus from Site B, attempt to convert virulent strains into hypovirulent ones by hyphal fusion, and identify a cause for the differing results observed between Site B and Site C.

## 2. Materials and Methods

### 2.1. Site Information

Two privately owned size- and age-matched orchards (each ≤50 ha) were used in this work. Orchard one (Site B) was located in Fulton Co., IN, USA, while Orchard two (Site C) was located 32 km away in Marshall Co., IN, USA (Figure 1). Both orchards were planted in 2000 using the same batch of pure American chestnut seedlings (*Castanea dentata*) from the American Chestnut Cooperative (https://www.accf-online.org/; accessed on 14 April 2021) and occupy similar well-drained silty loam soils. Seedlings were identified as 'McDaniels' half-sibs from an open pollinated grafted orchard.
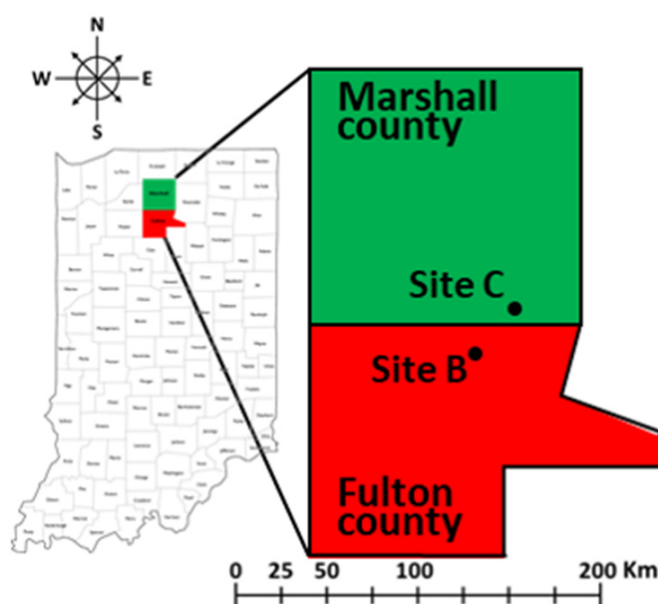
**Figure 1.** Site locations. Illustration of the relative distance between the Site B and Site C study sites.

## 2.2. Growth Measurements and Blight Evaluation

Tree growth was determined using Haglöf Mantax Blue calipers (Haglof Inc., Madison, MS, USA) specifically designed for the measurement of DBH in 2011 and 2017. Blight evaluations are subjective and were conducted by the same person to limit variability. Ratings ranged from one to three where 1 = low (few cankers present with no large cankers), 2 = moderate (a few or several large cankers present in a general area), or 3 = severe (obvious cracks and numerous large cankers present throughout the tree) disease infection, based on canker number and size.

## 2.3. Cryphonectria parasitica Isolates

Three confirmed pure isolates of *C. parasitica* ('SG' strain) [61] were provided by The American Chestnut Foundation (TACF, www.acf.org; accessed on 14 April 2021).

## 2.4. Isolate Growth and Attempted Conversion

Bark samples collected from branch crotches and putatively healed cankers on the trunk and scaffold limbs 2 ft to 12 ft from the base of the tree in 2017 were cultured in half-strength potato dextrose media before being plated on PDA (Figure 2). Isolates were grown on half-strength PDA plates in alternating 12 h dark/light cycles at 25 °C for 7 d according to protocols by [62]. Resultant culture mycelium were orange, yellow, or white and were consistent with *C. parasitica* isolates. Orange and yellow cultures grew quickly and displayed abundant pycnidia [9,63]. White mycelium grew slower, showed no pigmentation, and displayed few or no pycnidia or evidence of sporulation as reported for hypovirulent strains [9,63]. Attempted transmission, where compatible virulent strains were converted to hypovirulence, involved plating potentially virulent isolates (orange) with those potentially expressing hypovirus (white) together on a single plate in the dark at 25 °C for 14 d. Samples of both the orange and white isolates were then submitted for genomic sequencing analysis to the Purdue University Plant and Pest Diagnostic Lab (PPDL; https://ag.purdue.edu/btny/ppdl/Pages/default.aspx; accessed on 14 April 2021). The PPDL sequenced each submitted sample using internal transcribed spacer 1 (ITS1) and 4 (ITS4). Sequences were then subjected to BLAST (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 14 April 2021), where a match was proclaimed 98–100% identity to *C. parasitica*.
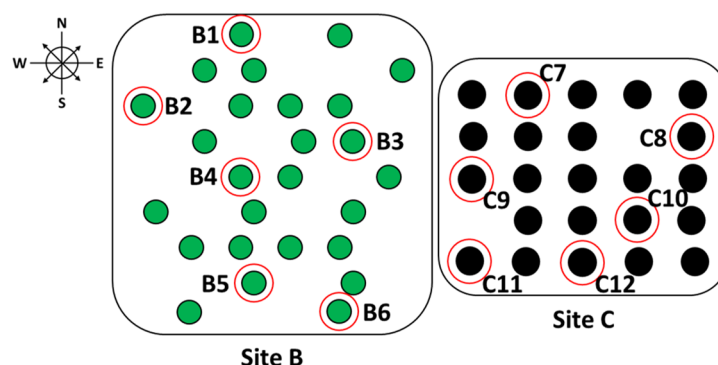
**Figure 2.** Sampling. A total of twelve samples were taken from Site B and Site C in 2017 after it was noted that Site B was healthy again and Site C had not recovered.

## 3. Results

Blight evaluations conducted in 2011 noted that 26 of 50 seedlings survived in Site B, while 23 of 25 seedlings survived in Site C (Figure 3). The dramatic loss in Site B was attributed to flooding rather than disease. We observed numerous buck rubs and significant browse damage at both sites consistent with an overwhelming ungulate presence. Thus, numerous entry points were available for *Cp* infection.
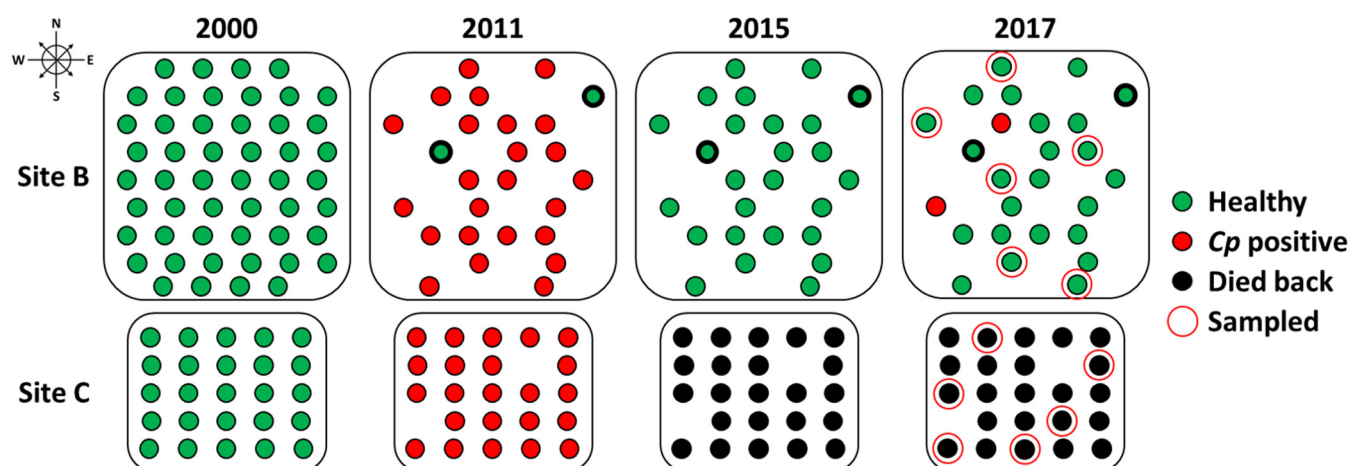


**Figure 3.** Disease progression. Representative depiction of disease presence at the Site B and Site C sites across several years. Tree mortality from causes unrelated to blight are excluded from 2011 onward.

At the conclusion of the 11th year (2011), 92% of trees at Site B and 100% of trees at Site C were diseased (Table 1). Canker ratings on the severity of disease for each of the trees within Site B (1.6) and Site C (1.5) were similar, with few trees displaying canker ratings above moderately affected (rating 2). Further, after repeated visits, these data showed the disease was initiated about 8 years prior and had progressed to a mild to moderate state for all trees. Measurements taken in 2011 at each site indicated growth was initially more rapid at Site C, with an average annual growth rate of 0.47 in/yr and DBH averaging 5.1 in. Trees at Site B demonstrated average annual growth rates of 0.29 in/yr and DBH values of 3.2 in (Table 1). Diseased trees at Site B began to recover in 2012 and showed no new, active cankers in 2013. Site B exhibited no new canker development from 2014 to 2017, and those trees that died back as a result of disease all resprouted with numerous 4 to 7 year old healthy stems. Disease progression was markedly more pronounced at Site C, with a steady increase in disease severity until all 23 trees and prospective resprouting stems died by the end of 2015 (Figure 3). A visit to the site in Fall 2020 showed several trees at Site C had resprouted.

**Table 1.** Visual characteristics. Measurements and canker ratings from 2011.

| Orchard | Avg. Height (ft) | Avg. DBH (in) | Avg. Canker Rating (1–3) | Avg. Disease Progress (%) |
|---|---|---|---|---|
| Site B | 17.5 | 3.2 | 1.6 | 92 |
| Site C | 22.1 | 5.1 | 1.5 | 100 |

Nine potential *Cp* isolates were recovered from Site C but none from Site B. The 21 initial fungal samples recovered from Site B were *Pestalotia* spp. and other saprophytic fungi (Table 2). Attempts to assay biocontrol with microbes derived from Site B were ineffective and isolates from Site B and Site C were inconclusive for hypovirulence. A total of 310 isolates were obtained from 19 individual trees (13 Site B, 6 Site C) (Tables 2 and 3). Upon plating, it was observed that isolates from Site B often appeared to be fluffy and white, while those from Site C were most often of an orange hue (Figure 4). Further investigation indicated nine isolates from Site C and two from Site B appeared positive for *Cp* (Tables 2 and 3).

**Table 2.** Initial samples and isolates from 2017 after 2 d incubation on PDA media.

| Orchard | Tree | No. Bark Samples (6 mm) | No. Fungal Isolates | Isolates per Sample (%) | Confirmed *Cp* Isolates |
|---|---|---|---|---|---|
| Site B | B1 | 9 | 6 | 67 | 0 |
| Site B | B2 | 10 | 2 | 20 | 0 |
| Site B | B3 | 9 | 1 | 11 | 0 |
| Site B | B4 | 12 | 7 | 58 | 0 |
| Site B | B5 | 14 | 1 | 7 | 0 |
| Site B | B6 | 12 | 4 | 33 | 0 |
| Site C | C7 | 8 | 3 | 38 | 2 |
| Site C | C8 | 5 | 2 | 40 | 2 |
| Site C | C9 | 7 | 4 | 57 | 0 |
| Site C | C10 | 6 | 3 | 50 | 1 |
| Site C | C11 | 7 | 4 | 57 | 3 |
| Site C | C12 | 8 | 2 | 25 | 1 |
| Totals | | 107 | 39 | 39 * | 9 |

* Average percentage of isolates per sample.

**Table 3.** Secondary samples and isolates from 2017. Two days after plating on PDA.

| Orchard | Tree | No. Bark Samples (6 mm) | No. Fungal Isolates | Isolates per Sample (%) | No. Putative *Cp*HV Isolates |
|---|---|---|---|---|---|
| Site B | B2 | 14 | 6 | 43 | 0 |
| Site B | B5 | 12 | 3 | 25 | 0 |
| Site B | B6 | 8 | 2 | 25 | 0 |
| Site B | B7-y ‡ | 10 | 4 | 40 | 0 |
| Site B | B7-o † | 18 | 4 | 22 | 0 |
| Site B | B8-1 | 8 | 2 | 25 | 0 |
| Site B | B8-2 | 29 | 11 | 38 | 0 |
| Site B | B9-1 | 13 | 2 | 15 | 0 |
| Site B | B9-2 | 5 | 1 | 20 | 0 |
| Site B | B10 | 11 | 3 | 27 | 0 |
| Site B | B11 | 9 | 1 | 11 | 0 |
| Site B | B12 | 13 | 5 | 38 | 0 |
| Site B | B12-*Cp* | 19 | 6 | 32 | 1 |
| Site B | B13 | 13 | 5 | 38 | 0 |
| Site B | B13-*Cp* | 21 | 5 | 24 | 1 |
| Totals | | 203 | 60 | 28 * | 2 |

* Average, ‡, (yellow color), †, (orange color).
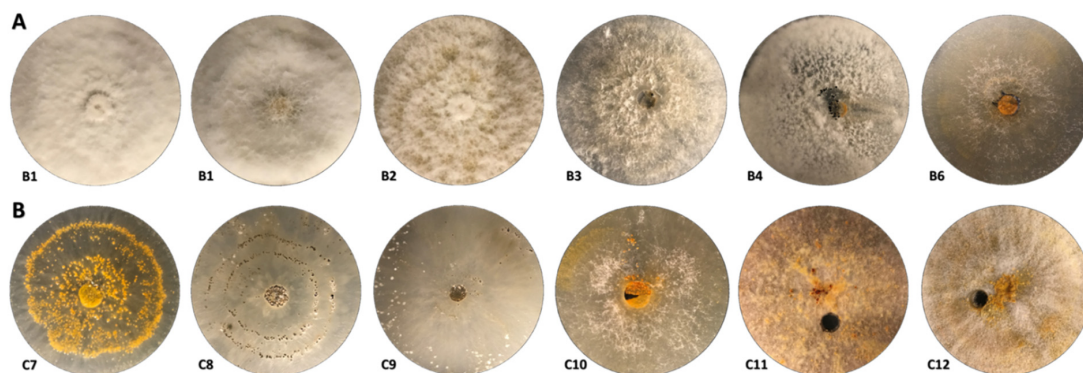
**Figure 4.** Isolate growth. Of the different isolates from Site B (**A**), the primary color observed was white, a color usually attributed to hypovirulent colonies. The six isolates from Site C (**B**) were primarily orange, a color typically indicative of virulent isolates. The punch holes observed were used to initiate a new plate of growth and were made before the previous plates became confluent.

Colonies from both Site B and Site C were plated against pure *Cp* strains in an attempt to induce conversions (Figure 5). Each plated pair resulted in incompatible plate reactions; thus, we were unable to confirm conversion from our plated cultures. The potential positive isolates from Site B (B12, B13) had an orange color that later became white before sequencing confirmed they were not positive for *Cp* (Figure 6). The Purdue University Plant and Pest Diagnostic Lab (PPDL; https://ag.purdue.edu/btny/ppdl/Pages/default.aspx; accessed on 14 April 2021) confirmed the orange Site C isolates were *Cp* and the Site B isolates were other fungal species (Supplemental Table S1. Sequencing results using ITS primers for evaluation of potential *Cryphonectria parasitica* isolates.).
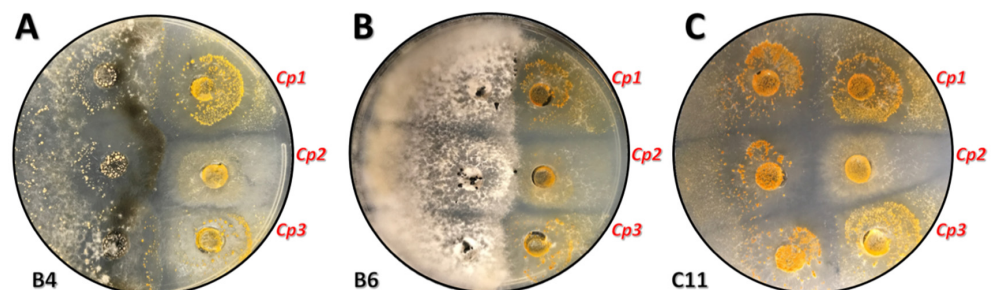


**Figure 5.** Conversion attempts. Isolates from Site B (**A**,**B**) and Site C (**C**) were plated with pure strains of *Cp1*, *2*, and *3* to induce conversion. Clear barrage zones appeared between each colony, an indication of both vegetative incompatibility and a lack of transmission.



**Figure 6.** Putative *Cp*HV from secondary sampling. Two Site B isolates (B12*Cp*HV?, B13*Cp* *) began with an orange hue before transitioning to white and were considered putatively positive *Cp*HV isolates. * B13*Cp* transition not captured.

## 4. Discussion

Past work has shown that there are a number of interacting variables involved in the chestnut response to *C. parasitica* and, while populations in Europe seem to be doing slightly better in response to hypoviruses, stresses such as drought and insect pests (i.e., the Asian chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu) [64]) represent another barrier to the restoration of this majestic species. In addition, continuous observation of the diversity of vc types and hypovirus ranges within existing chestnut populations is necessary to track progress. Advances in research regarding hypovirulence have slowed the spread of chestnut blight in Europe [24]. Natural hypovirulence may be essential for the effective biological control of the chestnut blight disease [41]. Unfortunately, recombination events could lead to increased vc type diversity, thus potentially interfering with natural controls [23]. In addition, research data also noted that success is more apt to occur in intensely managed stands rather than abandoned ones irrespective of vc diversity [65].

In the United States, evaluations of American chestnut for potential restoration have shown its distribution is significantly influenced by soil parameters and site topography, with pH and slope angle serving as primary predictors [66]. Additional parameters such as soil composition and precipitation during the growing season [66] and relatedness of tree species [54] also play significant roles. It is thought that chestnut distribution on the landscape varies with genotype as studies have noted pure American chestnut and hybrid varieties require differing environments for optimal growth [66,67].

Because CHV-1 subtype can vary, correct identification of the strain you have isolated is paramount. Hoegger et al. [68] reported that CHV-1 subtype-I can be established in native *C. parasitica* populations but is less effective than CHV-1 subtypes F1 and F2. CHV-1 subtypes F1 and F2 dramatically decrease the virility of *C. parasitica* when used on isolated urban trees or individual cankers; however, dissemination efforts have been primarily ineffectual [69]. Studies of natural chestnut populations stated CHV-1 subtype-I slowed canker development by greater than 80% and, on this basis, is being considered for use as a biological control agent [49]. Ježić et al. [68] noted that CHV-1-infected wild chestnut trees were able to recover after exposure; however, grafted chestnut failed to form healed cankers and later succumbed to *C. parasitica* infection.

Future efforts to improve biological control success require in-depth details regarding the relationship between *C. parasitica* and various hypovirus strains capable of attenuation. At this point, more research is needed to illuminate this complex relationship. On the other hand, sexual reproduction and the increase of vc type diversity by recombination could obstruct natural biological control. Since sustainable biological control of chestnut blight requires a comprehensive knowledge of the dynamics of the Cryphonectria–hypovirus interaction, additional population studies of the fungus and the hypovirus will be necessary. Alternative means used to investigate resistance levels have ranged from backcrossing [70] and interspecific crosses [43] to the generation of transgenics [71], genotyping [60] and genome editing [72]. New research published by Meyer et al. [73] noted *C. parasitica* and the *Cp*HV can survive on the bark of fresh dead chestnut for over two years. This suggests fresh dead wood could be used as a vector for transmission of both agents in the forest, recommendations for the removal of fresh dead chestnut from the forest may be detrimental if *Cp*HV is present [73], and that time is a critical factor [74].

A clear explanation as to the sudden recovery of Site B when a similar site, Site C, was completely decimated remains a topic for further discussion. Our hypotheses led us to believe that *Cp*HV was present at Site B. Despite our best efforts to isolate and verify this, our samples did not confirm the presence of *Cp*HV after several rounds of sample collection and subsequent sequencing. If we can obtain a confirmed *Cp* isolate from Site B, we aim to culture the *Cp*HV strain and offer it as an alternative biocontrol method in Indiana. Other theories to explain the phenomena observed at Site B involve an unintended benefit from the flooding event or from the presence of an unknown environmental factor in the area encompassing Site B. We have continued to focus on genotyping the nine *Cp* isolates recovered from Site C for later reports on the genetic diversity within native *Cp* populations.

Additional efforts will pursue the identification and isolation of *Cp* isolates at Site B from younger branches at a range of positions within the canopy. Scaling up field sterilization and removal of outer bark will be carried out to limit saprophyte contamination. Intensive sampling from the two trees in Site B unaffected at any point with chestnut blight will be performed in conjunction with the continued monitoring and measurement of the Site B study site.

Previous research by Bissegger et al. [75] noted strain conversion was successful if previously orange strains underwent a color change and exhibited characteristics similar to those of white cultures. This observation was further confirmed by later researchers [45,69,76,77]. However, we recognized, as did Celiker et al. [47], the need to verify the presence of hypovirulence in white isolates, as color alone cannot confirm the presence of the hypovirus. Cultures were thought to be virus-infected if their morphology was akin to that of confirmed CHV-1 hypoviruses. In our study, several orange cultures appeared to develop white characteristics, but further evaluation by experts failed to confirm presence of a hypovirus. In addition, incompatible plate reactions were observed between ascospore colonies plated together during conversion testing similar to those of Double et al. [20] and Rigling and Prospero [51].

## 5. Conclusions

Our goal was to recover *Cp* isolates at both orchards and then to identify hypovirulent isolates of *Cp* (*Cp*HV). Without confirmation via an isolate collected at Site B, we must refrain from concluding that *Cp*HV is responsible for the dramatic difference in survival at these two sites. Ultimately, we aimed to identify and culture a native Indiana *Cp*HV strain as a biocontrol for chestnut blight in Indiana. This would allow for progression of American chestnut restoration efforts in its native range and throughout the region.

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