**Evaluation of Hypovirus Introduction in an American Chestnut Stand in Wisconsin**

Mark L. Double, Andrew M. Jarosz, Dennis W. Fulbright, William L. MacDonald, and Anita L. Davelos Baines

First and fourth authors: Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV 26506.

Second author: Department of Plant Biology and Ecology, Evolutionary Biology and Behavior Program, Michigan State University, East Lansing, Michigan 48824.

Third author: Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, Michigan 48824.

Fifth author: Department of Biology, University of Wisconsin-La Crosse, La Crosse, WI 54601.

Corresponding author: A. Davelos Baines; E-mail address: abaines@uwlax.edu

**ABSTRACT**

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Hypovirus-infected *Cryphonectria parasitica* strains were introduced in a large stand of American chestnut (> 4000 individuals) in western Wisconsin (USA) to demonstrate whether they could affect biological control. They were deployed from 1992 to 1997 and again from 2004 to 2014. After sixteen years of hypovirus introductions within an area of the stand with the longest history of disease, isolation of hypovirus-infected strains increased from 55% in 1994 to 83% in 2014 for treated trees. Over this same period, prevalence increased from 29% to 72% on non-treated cankers on treated trees and from 15% to 84% for cankers on non-treated trees. Introduction of hypovirus has resulted in the regrowth of the crowns of many large-diameter trees. As of 2014, tree survivorship for hypovirus-treated trees was 51%, compared to 31% for non-treated trees. Putative recovery of American chestnut in this stand provides evidence that prolonged hypovirus treatment can act as a biological control when limited numbers of vegetative compatibility types of *C. parasitica* exist.

*Additional keywords:* *Cryphonectria parasitica*, biological control, dsRNA, epidemiology, mycovirus, chestnut blight, hypovirus

Mycoviruses, viruses that infect fungi, are found in all major fungal phyla (Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota), and there is increasing evidence of their potential to be effective biological control agents for a wide range of plant diseases (Pearson et al. 2008; Xie and Jiang 2014). The presence of mycoviruses has been shown to reduce the virulence (hypovirulence) of several plant pathogens, including *Botrytis cinerea* (Castro et al. 2003), *Sclerotinia sclerotiorum* (Yu et al. 2010), and *Ophiostoma novo-ulmi* (Doherty et al. 2006).

The most well-known example of hypovirulence, in which intracellular mycoviruses (hypoviruses) partially attenuate virulence, is for the chestnut blight fungus, *Cryphonectria parasitica* (Murr.) Barr. Hypovirulence in *C. parasitica* was described on European chestnut (*Castanea sativa* [Mill.]) by French mycologist Jean Grente (Grente 1981; Grente and Bertheley-Sauret 1978). Field trials by Grente and Bertheley-Sauret using hypovirulent (mycovirus-infected) strains were so successful arresting canker expansion on European chestnut that the French Ministry of Agriculture set up a biological control program to assist chestnut growers with blight control (Heiniger and Rigling 1994). Success in many European field trials produced interest in North America where chestnut blight devastated 200 million acres of American chestnut (*Castanea dentata* [Marsh.] Borkh*.*) during the first half of the 20th century (Kuhlman 1978; Van Alfen et al. 1975), relegating it to understory sprouts from extant root systems (Clark et al. 2016). Field applications of hypovirus-containing *C. parasitica* isolates in North America have met with limited success, possibly owing to the high diversity in vegetative compatibility (vc) types in *C, parasitica*, which has been suggested to slow the spread of hypovirus (Anagostakis et al. 1986, Cortesi and Milgroom 1998; Krstin et al. 2008); the higher level of susceptibility of American chestnuts when compared to their European relatives (Viéitez and Merkle 2005); and the inability of infected chestnuts to compete successfully with other tree species in eastern forests (Griffin 2000; Heiniger and Rigling 1994; MacDonald and Fulbright 1991). Here we report on the long-term application of hypoviruses in a stand of American chestnut near West Salem, Wisconsin.

The West Salem chestnut stand is an example of relocation of a tree species outside its natural range. This introduction occurred in the 1880s when 8 to 10 American chestnuts from the native range were planted at a site near West Salem, WI (Cummings Carlson et al. 1998; McGrath 1992). These trees served as the founders for a population of chestnuts with > 4,000 individuals and some trees as large as 64 cm in diameter that became the dominant species on a portion of this 36-hectare mixed hardwood stand (Paillet and Rutter 1989). The stand was free of chestnut blight until 1987 when cankers were detected on four trees. From 1987 through 1991 attempts were made to eradicate the disease by felling and burying infected trees. Despite these efforts, the number of infected trees rose steadily, prompting the landowners to support the introduction of hypoviruses in 1992.

Several characteristics of the stand were thought to increase the probability that hypovirus introductions would be successful. First, a single vc-type (WS-1, described below) was identified from strains isolated from cankers at the site (McGuire et al. 2005) between 1990 and 1991. Since the *C. parasitica* population was essentially clonal, the West Salem chestnut stand offered a unique opportunity to initiate biological control using hypoviruses because the barriers to hypovirus infection, imposed by vc, were lacking. Second, the site shared several characteristics (e.g., isolated population and vigorous chestnut growth) with sites in Michigan where American chestnuts were undergoing naturally occurring hypovirus-associated recovery (Day et al. 1977; Fulbright et al. 1983), leading to the decision to use the site as a natural laboratory for the deployment of hypovirus. Cankers were treated with hypoviruses from 1992 to 1997 and again from 2004 to 2014. Here, we detail the results of deploying hypoviruses in a portion of the West Salem stand where *C. parasitica* infections originated (hereafter referred to as the ‘Disease Center’; Fig. 1), and concentrate on the acquisition of hypovirus within the thallus of *C. parastica* in cankers and the growth and survivorship of trees. The major question following 23 years of annual treatment/assessment is are chestnut trees in the area of the disease focus recovering from blight, and if so, to what extent?

# Materials and Methods

***C. parasitica* hypovirulent strains.** Several hypoviruses were available as potential biological control agents, each imparting different effects on *C. parasitica* (Hillman et al*.* 2000). The decision was made to use a hypovirus, *Cryphonectria* hypovirus 3 (CHV 3), from Manistee County, Michigan (designated COLI) (Fulbright et al. 1983; Peever et al. 1997) for two reasons. First, COLI was associated with recovery of American chestnut populations in Michigan, and second, there were fewer restrictions associated with obtaining state and federal approval for the release of a hypovirus from North American. As discussed below, COLI did not spread well within the West Salem stand, and a second hypovirus designated EURO7 was deployed starting in 1995. The fungal strains and hypoviruses used in this study are described in Supplementary Table S1.

**Hypovirus transmission into West Salem *C. parasitica* genetic backgrounds.** The hypovirus COLI was successfully transmitted to vegetative compatibility (vc) type WS-1 when a COLI-containing strain and a hypovirus-free WS-1 strain were co-inoculated side-by-side on excised stems of *C. dentata*. Presumptive WS-1 (COLI) converted strains were isolated from the WS-1 side of the pairings and sub-cultured on potato dextrose agar (PDA, Difco, Detroit, MI). When slow-growing orange colonies were chosen as putatively infected, transmission of the COLI hypovirus into the WS-1 background was routinely demonstrated on PDA by changes in colony morphology (described below). The second deployed hypovirus, designated EURO7, *Cryphonectria* hypovirus 1 (CHV1), was transmitted readily into WS-1 and two additional common vc types, WS-2 and WS-3 (described below), when paired on PDA. Infection of virulent *C. parasitica* isolates by the COLI and EURO7 hypoviruses resulted in unique morphologies on PDA (Fig. 2). Slow-growing orange strains were categorized as CHV3 (COLI)-infected; fast-growing white strains as CHV1 (EURO7)-infected; and fast-growing orange strains as virulent, hypovirus-free (Fig. 2). The two hypoviruses also had differing banding patterns upon dsRNA extraction and gel electrophoresis (Morris and Dodds 1979). Morphology and dsRNA patterns together were used to distinguish hypovirus-infected isolates in the laboratory from each other and wild-type isolates collected from field samples.

**Canker sampling, hypovirus assessment, and vegetative compatibility type determination**. In order to assess vc type and hypovirus acquisition in the *C. parasitica* cankers in the West Salem stand, 12 2-mm-diameter bark plugs were removed from every canker annually with a bone marrow instrument (Lee-Lok, 11-guage, 4-inch) and processed according to McGuire et al. (2005). Cultures from these samples were used to ascertain the hypovirus infection status of the *C. parasitica* strains using the morphological criteria described above. Further, any non-*C. parasitica* isolates that were recovered were designated as non-C.p. These data were compiled to provide a longitudinal characterization of hypovirus acquisition by each sampled canker.

Vegetative compatibility was assayed on PDA containing bromocresol green (PDA-bgt; Powell, 1995), amended with 600 mg/liter tannic acid and 0.5 ml/liter Tween-20, added before autoclaving. Isolates were paired on PDA-bgt in total darkness for 7 to 10 days at 22°C. Plates were scored on the presence/absence of a barrage (Anagnostakis 1977). Prior to 1995, one representative virulent isolate from each canker was paired on PDA-bgt with isolate 25-1, representing vc group WS-1. After additional vc types were found in the stand in 1995 (WS-2) and 1997 (WS-3), a virulent isolate from each canker was tested against WS-1, WS-2 (isolate 108-1), and WS-3 (isolate 133-1). All isolates identified as incompatible with these three vc groups were retested. Isolates incompatible with WS-1, WS-2, and WS-3 a second time were then paired with tester isolates representing WS-4 through WS-15 (McGuire et al*.* 2005).

**Hypovirus treatment**. Trees were assessed annually (late May/early June) from 1992 to 2014. Each tree was assigned a unique number when first infected and cankers were numbered sequentially on individual trees as they developed. Between 1992 and 1994, an agar slurry of WS-1 (COLI) was used to treat newly discovered cankers (from the base to 3.7 m off the ground) using the margin-punch method of inoculation (Bell 2004; Grente and Bethelay-Sauret 1978) The WS-1 (COLI) slurry was made by combining 20 PDA plates of 7 to 10 day-old cultures of WS-1 (COLI), 1.5 liter of sterile water, and 1 liter of solidified sterile water agar (2.5%) per batch; eight batches were made annually to treat cankers. Ingredients were blended for 2 to 3 min in a 4 liter stainless steel Waring® commercial blender. Similar slurries were made for WS-1 (EURO7), WS-2 (EURO7), and WS-3 (EURO7) inoculum that were used for treatments from 1995 to 1997 and from 2004 to 2014. Newly discovered cankers (from 2004 to 2014) were treated initially with WS-1 (EURO7). In subsequent years, treatments were changed to WS-2 (EURO7) or WS-3 (EURO7) when vc testing of strains from bark samples revealed that the canker was incited by strains representing WS-2 or WS-3. In an attempt to discern if hypoviruses were able to disseminate without continual application, there was no hypovirus treatment from 1998 to 2003; however, bark plugs were obtained for analysis from all existing cankers. Professional tree climbers were hired to sample and treat cankers between 3.7 to 24 m in 1996 and 1997 and in 2004 and 2005. When cankers merged with one another, they typically were treated as a single merged unit rather than as individual cankers. Trees were designated ‘dead’ when there were no living epicormic sprouts on the main stem or root sprouts from the base of the tree.

**Canker rating and tree health assessment**. All cankers were rated annually from 1998 to 2014 using a 1-4 scale, where 1 = heavy callus with no sporulation; 2 = heavy callus with some sporulation; 3 = slight-to-moderate callus with moderate-to-heavy sporulation; and 4 = no callus and heavy sporulation. The main stem of each tree was assessed annually from 2001 to 2015 and used to characterize the long-term recovery of trees treated with hypovirus compared to untreated control trees. Trees were rated on a 1-5 scale, where 1 = no active cankers but tree has died back due to blight; 2 = tree infected with only virulent cankers; 3 = tree infected with a combination of callousing and virulent cankers; 4 = tree infected with only callousing cankers; and 5 = tree has never been infected (healthy).

**Permanent plots**. Between 1992 and 2000, all trees in the 36-hecatare stand were monitored. Following the 2000 disease survey, it was evident that disease progress exceeded our ability to sample all infected trees. This situation resulted in the establishment of twelve permanent plots in 2001 representing differing levels of disease incidence in the stand (Fig. 1). Plots 2, 3, 4, and 6 were in the area of the stand where the disease was first discovered in 1987, subsequently referred to as the ‘Disease Center’. Approximately 80% of the trees infected by 2000 were in the 12 permanent plots. Two-thirds of the trees in each plot were treated with hypovirus-containing isolates. The remaining one-third were untreated to assess tree-to-tree hypovirus dissemination. When a main stem died, resulting sprouts were treated similarly to the main stem. The results discussed in this paper represent data collected from the four Disease-Center plots (2, 3, 4, and 6) (Fig. 1).

**Analyses.** All statistical analyses were conducted using SAS 9.3 (SAS Institute, Inc., Cary, NC). In the analyses described below, hypovirus incidence is defined as the proportion of cankers that had at least one out of 12 isolates per canker from bark plugs showing infection by hypovirus. Hypovirus frequency represents the proportion out of the 12 isolates per canker that showed infection by hypovirus. Correlations between ranked canker ratings and frequency of hypovirulent isolates within a canker were evaluated using Spearman’s rank test (PROC CORR). Differences among mean rating categories were compared by analysis of variance (PROC GLM). Significant differences among means were determined with least significant differences. The frequency of hypovirulent isolates found in a canker (out of 12 samples per canker) was compared with a binomial model (PROC GENMOD) (Agresti 2002; Schabenberger and Pierce 2002). Significant differences were determined with the likelihood ratio statistic.

**Results**

**Disease incidence and canker dynamics.** Disease incidence (i.e., proportion of trees infected), in the four Disease Center plots, increased steadily from 1992 so that by 2014, 120 of the 121 trees in the Disease Center plots were infected. The cumulative number of cankers detected over time displayed a nearly logistic pattern (Fig. 3, black bars). As the epidemic progressed in the early 1990s, the proportion of cankers treated with hypovirus decreased steadily after 1997, especially with the cessation of hypovirus treatment from 1998-2003 (Fig. 3, white bars). When treatments resumed in 2004, the number of cankers treated in the Disease Center remained low because limb dieback in the larger trees prevented efforts of tree climbers to treat cankers safely in the crowns of trees. In addition, a decrease in the number of sampled cankers over time was not necessarily solely the result of tree death. As hypovirulent cankers slow the growth of the infecting fungus, tree survivorship is lengthened and cankers often merge with each other so that they are no longer assessed as individual infections, but as merged units. As of 2011, 27% of the cankers in the four Disease Center plots had merged (M. L. Double and W. L. MacDonald, *unpublished data*).

**Vegetative compatibility group diversity over time.** WS-1, the initial vc type to infect chestnuts at West Salem, was the only vc type detected in the Disease Center prior to 1999 (Table 1). Across all years of monitoring (from 1998 to 2014), 95% of the samples were WS-1. WS-2 was first detected in a portion of the stand distant (~1000 m) from the Disease Center in 1995, but was not found in the Disease Center until 1999. While WS-2 was detected every year thereafter, its frequency never rose above 6% (Table 1). WS-3, the third vc group, was found in 1997, but not in the Disease Center until 2005. WS-3 was never detected above 2% in the Disease Center (Table 1). The mating type of WS-1 and WS-3 is MAT-2 while WS-2 is MAT-1 (McGuire et al. 2004). Matings between isolates representing the two mating types may have resulted in recombination leading to additional vc types (WS-4 through WS-15) detected in the stand (Short et al. 2015). Despite the discovery of 15 different vegetative compatibility groups during the study, WS-1 remained the most commonly isolated vegetative compatibility group in the Disease Center. Its frequency exceeded 80% each year (Table 1) and its aggregate frequency across all years was 95%.

**Hypovirus incidence in cankers over time.** When hypovirus incidence in treated cankers was first assessed in 1994, the CHV3-COLI hypovirus was found in 55% of the cankers to which hypovirulent inoculum had been introduced in 1992 and 1993. However, the CHV3-COLI hypovirus was detected in only 29% of non-treated cankers on treated trees (between canker spread) and 15% of new cankers on non-treated trees (between tree spread) in 1993 (Table 2). We attributed the limited canker-to-canker and tree-to-tree spread of CHV3-COLI to low transmission of hypovirus to fungal conidia since CHV3-COLI was detected in only 3% of conidia produced by this strain in laboratory tests (M. L. Double and W. L. MacDonald, *unpublished data*). Additionally, the CHV3-COLI hypovirus was so debilitating that few conidia were produced under field conditions (M. L. Double and W. L. MacDonald, *personal observation*). These characteristics were presumed to explain the low spread of this hypovirus in the stand, prompting the decision to introduce a CHV1 hypovirus (EURO7) in 1995. EURO7 was less debilitating to the West Salem strains of *C. parasitica*. CHV1-EURO7-infected strains also colonized more bark and more readily produced conidia *in situ* (M. L. Double and W. L. MacDonald, *personal observation*). Additionally, 93% of conidia from WS-1 (EURO7) strains were shown to contain the EURO7 hypovirus *in vitro* (M. L. Double and W. L. MacDonald, *unpublished data*). Replacement of CHV3-COLI with CHV1-EURO7 resulted in hypovirus detections in 78% of treated cankers and from 55% of non-treated cankers on treated trees by 1997 (Table 2). While spread within treated trees increased with CHV1-EURO7 from 1995 to 1997, tree-to-tree spread was only 10% in 1997 (Table 2). In the absence of hypovirus introductions from 1998 to 2003, tree-to-tree spread dropped to 2% in 2002. However, over the course of 2005 to 2014, there was an increase in hypovirus incidence on non-treated trees from 53% in 2005 to 84% in 2014 (Table 2). Although CHV3-COLI hypovirus has not been introduced as treatment inoculum since 1994, it was detected in 1% of the bark samples in 2014 (M. L. Double and W. L. MacDonald, *unpublished data*).

**Hypovirus frequency within cankers.** The twelve samples obtained from a canker in any given year revealed that most cankers were a complex mixture of *C. parasitica* without hypovirus (virulent, V), isolates with hypovirus (HV), and fungi that were not *C. parasitica* (NCP; Figure 4). There were significant differences among the years in the proportion of isolates that were classified as V, HV, or NCP (Χ2 = 1106.78, df = 8, P < 0.0001) with V isolates decreasing and HV isolates increasing over time. There was a significantly greater proportion of HV and NCP isolates found in cankers on treated trees than on non-treated trees (Χ2 = 1039.80, df = 2, P < 0.0001); the proportion of V isolates from cankers on non-treated trees was more than triple the proportion found from cankers on treated trees (0.55 and 0.15, respectively) (Table 3).

Fungal components of individual cankers changed over time for new cankers found in 2001 (Table 4). The 271 cankers initially detected in 2001 were dominated by *C. parasitica* isolates without hypovirus (78%) (Table 4). Over the period of 2001-2014, the fungal components of these cankers changed: the number of virulent isolates decreased (from 78% to 28%); the hypovirus-containing isolates increased (6%-36%) and the number of samples with secondary fungal colonizers increased (16%-36%). For cankers previously treated (1992-2000), components of cankers were similar over the course of 2001-2014; each component (virulent and hypovirulent *C. parasitica* and Non-cp) was recovered in nearly equal amounts (30% each) (Table 4).

**Canker ratings, hypovirus incidence and frequency, and hypovirus treatment.** In 2000, 37% of cankers in the Disease Center were rated as 1 (Table 5). Cankers of this type expand slowly or not at all and rarely girdle infected stems. Cankers rated as 4 (abundant stroma and no host callus tissue at the canker margins) had a frequency of 22%. The distribution of ratings changed over time with 11% more cankers being rated as 1 in 2014 than in 2000 and 14% fewer cankers rated as 4 (Χ2 = 20.83, df = 6, P < 0.002; Table 5). The relationship between canker ratings and hypovirus incidence (at least one isolate out of 12 per canker contained hypovirus based on the morphology of *C. parasitica* isolated from bark samples) of all cankers sampled in the Disease Center in 2000, 2009, and 2014 was compared (Table 5). Hypovirus incidence did not differ among years (Χ2 = 1.85, df = 2, N.S.) with 52-76% of cankers containing at least one isolate out of twelve infected with hypovirus across the years (Table 5). There was no difference in hypovirus incidence between ratings overall (Χ2 = 5.61, df = 3, N.S.) but there was a trend for a greater incidence of hypovirus in cankers rated 1 and lower incidence in cankers rated 4. Within years, hypovirus incidence was associated with canker rating in 2014 with only 29% of cankers rated as 4 containing hypovirus (Χ2 = 8.36, df = 3, P < 0.04; Table 5) but not in 2000 or 2009 (Χ2 = 2.32, df = 3, N.S. and Χ2 = 3.97, df = 3, N.S., respectively; Table 5). The canker rating system (1-4) was correlated with the frequency of hypovirus detected (percent of isolates out of 12) over all years (rs = -0.16, P < 0.002) indicating that cankers rated 1 (abundant callus and no stroma) have more hypovirus than cankers rated 4 (no callus and abundant stroma) (Table 5).

The rating of two-year old cankers was influenced by three factors: (1) rating of the canker in year 1, (2) whether the canker received hypovirus treated with hypovirus in the first year and (3) the hypovirus treatment history of the tree (Table 7). Generally, canker ratings in year 2 were lowest for cankers that had a one rating in the first year. The lowest canker ratings were correlated with two factors: (1) trees with a history of hypovirus treatment; and (2) if the canker itself received hypovirus treatment in their first year.

**Tree Health.** The response of whole trees to hypovirus treatments is an important measure of biocontrol success. The impact of hypovirus on tree health was assessed by comparing cohorts of trees with and without hypovirus treatment. The first cohort (59 trees) included all trees infected between 1992 and 1997 that were treated with hypovirus in either the first or second year after disease was discovered. The second cohort (61 trees) included trees initially infected between 1998 and 2003 that were not treated with hypovirus until at least 2004 (Figure 3). During the annual census, main stem dieback and whole tree mortality were assessed. (Note: Trees often survive dieback of the main stem and begin producing root collar sprouts. Some trees rebuilt their crowns). Ninety-three percent of the main stems from trees in the 1998-2002 cohort died back by 2009, while only 47% of trees in the 1992-1997 cohort experienced main stem dieback (data not shown). Whole tree assessment showed that proportion of trees alive in 2014 for the treated 1992-97 cohort was 51%, compared to 31% for the non-treated 1998-2003 cohort. The lower main stem dieback and tree mortality in the 1992-1997 cohort is even more striking given that trees in the 1992-1997 cohort have been infected for a longer period of time (6-11 years).

**Discussion**

There is a general perception that hypovirus-mediated biological control of chestnut blight is widely successful in many areas of Europe, since chestnut trees are recovering and that recovery is associated with the presence of hypoviruses (Heiniger and Rigling 1994; Robin and Heiniger, 2001). In North America, despite some short-term success, there has been little or no biological control of blighted populations of American chestnut in the natural range of American chestnut (Griffin 1999). Several factors have been proposed to explain the inability of hypoviruses to control blight in North America. *Cryphonectria parasitica* populations are more diverse for vegetative compatibility in North America (Anagostakis et al. 1986; Cortesi and Milgroom 1998; Deutch et al. 2012), which slows the spread of hypovirus within the pathogen populations (Liu and Milgroom 1996). While European chestnut is susceptible to *C. parasitica* infection, American chestnut is the most blight susceptible *Castanea* species (Graves 1950; Hebard 1982). The slight difference in susceptibility may be important for improving survivorship of trees with hypovirus-infected cankers, and further, the ability of hypoviruses to invade and control *C. parasitica* populations (Milgroom and Cortesi 2004). Despite these potential constraints, hypovirus has spread throughout a number of *C. parasitica* populations in Michigan (Day et al. 1977; Elliston et al. 1977; Fulbright et al. 1983), and demographic analyses have confirmed that the increase in growth and survivorship has resulted in ecological recovery in which chestnut trees are expected to remain as canopy trees in these populations (Davelos and Jarosz 2004).

The biocontrol of chestnut blight with hypovirus was undertaken at the West Salem stand because a number of features of the epidemic at this site were similar to what was found in recovering stands in Michigan: only a single strain of the pathogen was present, the stand is isolated, and the chestnuts were known to be growing vigorously (Cummings Carlson et al. 1998; McEwan 2006; Paillet and Rutter 1989). As with the North American chestnut epidemic a century ago, the disease progressed to the extent that almost all individuals were infected by 2014. Our initial goal was to restrain the epidemic with annual treatments of hypovirus on all cankers that could be accessed from the ground, using ladders and/or tree climbers. We successfully accomplished the goal of treating reachable cankers through 1999, but as the disease progressed, it was apparent that by the year 2000, the epidemic had overwhelmed our ability to treat all cankers in the stand (Fig 3).

The failure to contain the epidemic may have been due to the extreme debilitating action of the COLI hypovirus that was deployed initially in the stand. While the COLI hypovirus was successful at slowing the growth of virulent *C. parasitica* within a canker (M. L. Double and W. L. MacDonald, *unpublished data*), its ability to spread among trees was not documented by our bark isolations (Table 3). This led us to deploy a second hypovirus, EURO7, that had demonstrated its ability to spread among cankers within an infected trees, based on previous field studies (M. L. Double and W. L. MacDonald, *unpublished data*). However, spatial data also suggest that the EURO7 hypovirus did not spread as readily as hypovirus-free isolates (Jarosz et al. 2002).

For hypovirus-mediated biological control of chestnut blight to be successful four criteria have been proposed: (1) presence, persistence, and spread of hypoviruses; (2) reductions in disease incidence or disease virulence; (3) increase in tree growth and survival; and (4) increase in productivity of marketable products such as lumber or nuts (Milgroom and Cortesi 2004). Our results from West Salem are consistent with the first 3 criteria being met. We used three approaches to evaluate the success of our hypovirus treatments: (1) presence of hypovirus in cankers and spread of hypoviruses from cankers on treated to non-treated trees; (2) an evaluation of canker morphology (presence or absence of callus tissue; the presence of callus suggests reduced virulence of the pathogen); and (3) tree survivorship. Taken together, results from our evaluations provided insight into the success or failure of hypovirus treatment in this stand.

The presence, persistence, and spread of hypovirus in the stand (Table 3) is consistent with successful biological control of the blight. The increasing incidence of hypovirus over time, even when hypovirus introductions were suspended, indicates that hypoviruses are well established in the Disease Center portion of the stand. The dissemination of hypoviruses is supported by the spread of hypoviruses to cankers on untreated trees and to new cankers, but not until 2005, after the second deployment of the EURO7 hypovirus (Table 3). Of the 81 new, untreated cankers in the Disease Center observed from 2011 to 2014, 86% harbored hypovirus (M. L. Double and W. L. MacDonald, *unpublished data*). Further, when hypovirus frequency is considered, persistence and spread of hypovirus is even greater in non-treated cankers (Fig. 4). The percentage of isolates with hypovirus in treated cankers remained relatively stable from 2002 through 2014 (~80%), whereas in non-treated cankers, isolates with hypovirus increased dramatically from 2% in 2002 to 84% in 2014 (Table 3). The dissemination of the EURO7 hypovirus to cankers on treated trees eventually equaled that on non-treated trees, although that transition took more than a decade.

A second approach used to evaluate the success of biocontrol using hypoviruses was canker morphology, the response to reduced pathogen virulence. Cankers rated 1 or 2 (indicating less sporulation and greater callus production) had a lower frequency of virulent *C. parasitica* and a higher frequency of hypovirulent *C. parasitica* compared to cankers rated 3 or 4 (Table 5). The majority of new cankers were rated as 3 or 4, indicating that new cankers are predominately virulent (M. L. Double and W. L. MacDonald, *unpublished data*). Two-year-old cankers were tested by ANOVA and the history of treatment had a significant effect on the morphology of subsequent cankers (Table 7). However, the proportion of all cankers rated 1 or 2 increased from 58% in 2000 to 78% in 2014 (Table 5). This difference in canker rating over time indicates that, in the Disease Center region of the stand, there was a shift from more virulent, callus-free cankers to those with high amounts of callus and decreased sporulation (i.e. reduced virulence); this result is consistent with successful biological control.

The third approach used to evaluate the success of biological control was tree survival. The landowners required inoculation of all reachable cankers on every infected tree in exchange for allowing hypovirus introduction and annual monitoring of the stand. Thus, no control trees were available to compare survival of trees with and without hypovirus treatments from 1992 to1997. This shortcoming was somewhat offset with a cohort of trees that were not treated between 1998 and 2003. Thus, a comparison was possible between a cohort of trees initially infected and treated with hypovirus between 1992 and1997 and a cohort of trees that were infected between 1998 and 2003 and not treated with hypovirus until 2004. The increased survival of trees from the 1992 to 1997 cohort compared to the 1998 to 2003 cohort (51% and 31%, respectively) suggests that hypovirus treatments had a positive effect on the tree survival. Indeed, several trees in the Disease Center appear to be rebuilding their crowns (developing new foliage) and producing burrs over the past two-to-three years (A. L. Davelos Baines, *personal observation*).

The final criterion proposed by Milgroom and Cortesi (2004) for successful biological control is the increase in productivity of marketable products such as lumber or nuts. In an ecological context, this criterion is not relevant. A more valuable criterion would be the successful recruitment of new seedlings that would allow the population to persist in the long-term (Silvertown et al. 1996). While data on seedling recruitment is not yet available, Gilland et al. (2012) estimated 1.043 x 106 seeds would be produced per hectare from mature chestnut trees in the stand. Whether or not these nuts are marketable does not influence seedling recruitment and thus, the long-term persistence of this stand. However, these expectations of seed production are a positive sign of biological control and survival of the stand.

A final observation is that the majority of cankers in this stand have a complex fungal component; they contain mixtures of *C. parasitica* without hypovirus (V), *C. parasitica* with hypovirus (HV), and other fungi (NCP) (Tables 4 and 5; Fig. 4). Cankers that contain a mixture of virulent and hypovirulent components have been noted in European (Heiniger and Rigling, 1994; Turchetti et. al, 2008) and U.S. chestnut populations (Hogan and Griffin, 2002). Mixed cankers also have been found in recovering stands in Michigan (Schaupp, et al. 1997; A. M. Jarosz, *unpublished data*).

All cankers, whether mainly virulent or hypovirulent, are a mixture of *C. parasitica* and other fungi. However, the amount of non CP isolated from cankers is much higher for hypovirulent than virulent cankers (cankers rated 1 and 2 versus 3 and 4) (Table 6). It is our contention that hypoviurlent cankers simply survive longer, allowing for colonization by other organisms. There does not appear that there is an upper limit to the amount of hypovirus acquisition within a canker. Our data indicate that cankers with hypovirus are slowly invaded by secondary fungal colonists, mainly *Trichoderma* sp. along with *Botryosphaeria* sp. *Didymostilbe* sp., *Penicillium* sp. *Paraconiothyrium* sp., *Epicoccum niger, Umbelopsis isabllina* and *Pestalotia* sp (Double et al. 2013). Similar results have been found for cankers on chestnuts growing in the Black Sea region of Turkey (Akilli et al. 2009), where a *Trichoderma* isolate was found to be as effective as hypovirulent isolates in controlling virulent cankers. The full containment of *C. parasitica* within a canker may involve both the action of hypovirus and these secondary invaders. Earlier work in the eastern United States reported that *Trichoderma* spp. was used to suppress *C. parasitica* within cankers (Tattar et al. 1996). At this point, the role of secondary invaders is unclear as to whether they are necessary for full biological control of *C. parasitica* when hypoviruses are being utilized. Our sampling was restricted to living trees. As such we did not sample cankers on dead trees that may harbor a vastly different constituency of secondary fungi.

The role of ve may have impacted the success of biological control in the stand. One reason for the successful spread of hypoviruses may be the result, in part, of the relatively low diversity of the *C. parasitica* population in the stand. The first vc type found, WS-1, has remained the dominant vc type in the stand for 19 years (Table 2) while other areas of the stand had a more diverse vc structure. Robin et al. (2009) state that a low diversity in vc types provides good opportunities for biocontrol with CHV-1 in areas of France and Spain. Our results in the Disease Center are in agreement with the conclusion by Robin et al (2009).

This long-term study of hypoviruses has shown that time is a critical factor in the development of a successful biological control program of chestnut trees. Nearly 3,200 cankers were treated over the course of 20 years; this revealed robust trends that are consistent with successful biological control. Despite the fact that the *C. parasitica* population was clonal in the early 1990s, nearly two decades were necessary for dissemination of hypoviruses, significant number of callusing cankers and biological control to be evident in the Disease Center. Since hypovirus-infected isolates sporulate at such a reduced amount in comparison with virulent isolates, biological control within the *C. parasitica* system is very difficult in many areas of North America. Thus, continuous applications of hypovirus inoculum seems to be necessary to combat the advantage that virulent isolates possess. We still have little understanding of the role of secondary invaders in the phenomenon of biological control. The combination of extensive application of hypovirulent isolates over time, coupled with invading secondary organisms, may be the key to biological control of chestnut blight.

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Table 1.Percent of *C. parasitica* isolates in eachof the three major vegetative compatibility (vc) groups from 1994 to 2014 in the Disease Center plots. Total number of cankers tested in each year is presented. Trees within the Disease Center plots were not sampled in 2003, 2008, or 2010.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Year | | | | | | | | | | | | | | | | | |
| VC group | 94 | 95 | 96 | 97 | 98 | 99 | 00 | 01 | 02 | 04 | 05 | 06 | 07 | 09 | 11 | 12 | 13 | 14 |
| WS-1 | 100 | 100 | 100 | 100 | 100 | 95 | 94 | 97 | 99 | 98 | 92 | 96 | 95 | 84 | 94 | 89 | 92 | 88 |
| WS-2 | 0 | 0 | 0 | 0 | 0 | 5 | 3 | <1 | 1 | <1 | 1 | 3 | <1 | 6 | 1 | 3 | 2 | 1 |
| WS-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 2 | 2 | <1 | 1 |
| Number tested | 49 | 193 | 207 | 236 | 85 | 253 | 124 | 507 | 464 | 380 | 281 | 207 | 218 | 191 | 153 | 151 | 187 | 137 |

Table 2. Hypovirus incidence (cankers that had at least one out of 12 isolates from bark plugs showing infection by hypovirus) in percent from treated and non-treated cankers on treated trees and non-treated cankers on non-treated trees in the Disease Center over time.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Canker  treatment | 1994a | 1997a | 2002 | 2005 | 2007 | 2011 | 2014 |
| % (N) | % (N) | % (N) | % (N) | % (N) | % (N) | % (N) |
| Treated cankers on treated trees | 55 (66) | 78 (252) | 80 (147) | 78 (156) | 86 (10) | 84 (93) | 83 (47) |
| Non-treated cankers on treated trees | 29 (31) | 55 (120) | 30 (167) | 77 (111) | 83 (53) | 77 (49) | 72 (47) |
| Non-treated cankers on non-treated trees | 15 (7) | 10 (72) | 2 (58) | 53 (34) | 51 (53) | 64 (54) | 84 (46) |

aIncludes trees before the establishment of permanent plots

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Table 4. Percentage of isolates from individual cankers followed over time on treated and non-treated American chestnut trees that were virulent *C.. parasitica* (V), *C. parasitica* with hypovirus (HV), and fungi other than *C. parasitica* (NCP). Samples sizes are presented. Previously Treated Cankers (from 1992-2000) Sampled Over Time (same data set over time) | | | | | |
| Year | N | %V | %HV | %NCP |  |
| 2001 | 343 | 35% | 37% | 38% |  |
| 2004 | 145 | 20% | 35% | 45% |  |
| 2007 | 61 | 20% | 33% | 46% |  |
| 2011 | 44 | 18% | 32% | 50% |  |
| 2014 | 32 | 29% | 42% | 29% |  |
|  |  |  |  |  |  |
| New Cankers in 2001 on Non-Treated Trees Sampled Over Time (same data set over time) | | | | | |
| Year | N | %V | %HV | %Non C.p. |  |
| 2001 | 271 | 78% | 6% | 16% |  |
| 2004 | 93 | 47% | 21% | 32% |  |
| 2007 | 39 | 31% | 21% | 48% |  |
| 2011 | 21 | 22% | 34% | 43% |  |
| 2014 | 18 | 28% | 36% | 36% |  |

Table 5.  Canker ratings, percentage of cankers in each rating class by year, hypovirus incidence (percentage of cankers having at least one of twelve isolates with hypovirus), and frequency of virulent, hypovirulent and non-*C. parasitica* (based on twelve samples/canker) for years 2000, 2009 and 2014 in the Disease Center.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Year** | **Canker**  **rating** | **% Total Cankersa** | **Hypovirus incidence (%)** | **Percent bark samples based on 12 samples/canker** | | |
| **V** | **HV** | **NCP** |
| 2000 | 1 | 37 | 70 | 20% | 28% | 52% |
| 2 | 21 | 59 | 56% | 17% | 27% |
| 3 | 20 | 52 | 59% | 13% | 31% |
| 4 | 22 | 64 | 59% | 13% | 28% |
| 2009 | 1 | 48 | 76 | 29% | 25% | 55% |
| 2 | 18 | 59 | 36% | 18% | 46% |
| 3 | 14 | 71 | 53% | 16% | 31% |
| 4 | 20 | 61 | 49% | 12% | 39% |
| 2014 | 1 | 48 | 66 | 25% | 40% | 35% |
| 2 | 30 | 65 | 41% | 42% | 17% |
| 3 | 14 | 72 | 42% | 38% | 20% |
| 4 | 8 | 29 | 67% | 11% | 23% |

a Total canker by year: 2000 (N=126); 2009 (N=151); 2014 (N=

Table 7. Analysis of variance for two-year old cankers based on the influence of the canker rating in year one (Y1R), treatment of the canker in year one (TY1), and the history of hypovirus treatment for tree (TTH).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Source of Variance** | **Degrees of Freedom** | **Mean Square** | **F Value** | **Significance** |
| Year 1 Rating (Y1R) | 3 | 13.49 | 17.93 | <0.0001 |
| Treatment in Year 1 (Ty1: yes or no) | 1 | 6.12 | 8.26 | 0.0043 |
| Y1R by TY1 interaction | 3 | 0.68 | 0.91 | 0.4380 |
| Tree previously treated with hypovirus (TTH: yes or no) | 1 | 3.05 | 4.06 | 0.0447 |
| Y1R by TTH interaction | 3 | 0.41 | 0.54 | 0.6528 |
| TY1 by TTH interaction | 1 | 2.31 | 3.07 | 0.0806 |
| Y1R by TY1 by TTH interaction | 3 | 1.37 | 1.81 | 0.1439 |
| Error | 400 |  |  |  |

Figure Captions

**Fig. 1**. Map of chestnut stand at West Salem, Wisconsin (from Palmer et al. 2008). The twelve plots that were initiated in 2001 are indicated. The Disease Center encompasses plots 2, 3, 4, and 6.

**Fig. 2**. Morphology of Wisconsin 25-1 virulent (top), Wisconsin 25-1 (CHV1-Euro7) (lower left) and Wisconsin 25-1 (CHV3-COLI 11-1) (lower right) isolates of *C. parasitica* grown on PDA.

**Fig. 3**. Cumulative number of cankers (Black bars) and number of cankers treated with hypovirus (Grey bars) in the Disease Center over time.

**Fig. 4**. Percentage of virulent *C. parasitica* (CP-V), hypovirulent *C. parasitica* (CP-HV) and non-*C. parasitica* (Non-CP) isolates from bark plugs in different years. Cankers from treated trees are on the left, cankers from non-treated trees are on the right.