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HYPOVIRULENCE: USE AND LIMITATIONS AS A CHESTNUT BLIGHT BIOLOGICAL CONTROL

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Abstract: The recovery of chestnut from chestnut blight in Italy and Michigan largely was responsible for the resurgence in chestnut research. The observed remission of disease now has been attributed to a biological control process called hypovirulence, whereby virulent strains are debilitated as a result of infection by fungal viruses (hypoviruses). Several species of hypoviruses now are known and each may impart unique effects on *Cryphonectria parasitica*. Lethal infections often are controlled by introducing the appropriate hypovirus into cankers. Unfortunately, at many locations within the native range of American chestnut, a complex system of vegetative incompatibility restricts hypovirus transmission among strains. Factors like vegetative incompatibility apparently regulate the widespread establishment of hypoviruses and presumably are, in part, responsible for our inability to artificially establish hypoviruses to the extent that has occurred naturally. Some of the factors that regulate hypovirus success or failure may be discovered as part of ongoing research at an isolated Wisconsin chestnut stand. Hopefully, understanding the phenomenon of hypovirulence eventually will allow it to be employed as part of the American chestnut restoration program.

INTRODUCTION

Cryphonectria parasitica was first recognized one hundred years ago as the fungus responsible for the cankers that resulted in the death of American chestnut (Merkel 1905). This brightly orange-pigmented organism was new to North America and by the time it was identified, its role in the tragedy that was about to unfold was cast. The remarkably detailed work of several early scientists unraveled the biological details of a host-pathogen relationship that would have unparalleled ecological and sociological impact (Anderson and Babcock 1913, Heald and Gardner 1913, Shear and Stevens 1913). Within ten years of the identification of *C. parasitica* as the causal fungus, their writings sadly were predictive of what was to ensue. As the blight spread, most of the research efforts turned to the strategy of breeding blight resistant trees (Fleet 1914). The early breeding programs met with limited success and never were designed to control blight in eastern forests. For almost fifty years, relatively little research attention was directed toward the causal fungus. If this same organism was introduced today, American chestnut undoubtedly would face the same fate.

We are fortunate that American chestnut was saved from extinction in its natural range by its propensity to sprout. The first one hundred years of chestnut blight is a blink in biological time. It may, however, be this surviving sprout population that over longer biological time periods allows for the expression of a disease of *C. parasitica* that may result in natural biological control of chestnut blight. A glimmer of hope that this was possible emerged from observations of "spontaneously healing" cankers that were noted on European chestnut growing in northern Italy (Grente 1965). The "recovery" phenomenon was confirmed when Jean Grente, a French mycologist, described a variety of unusual strains of *C. parasitica* that he isolated from the callusing cankers (Grente and Berthelay-Sauret 1978). The isolates he recovered often were lightly pigmented in contrast to the normal orange pigmented lethal strains. Moreover, Grente found that, like the infection from which they were isolated, when the isolates were inoculated to healthy chestnuts, lethal infections seldom resulted. Most significant was the observation that the cause of this debilitation was transmissible to virulent strains. Grente coined the term "hypovirulent" to describe the

reduced state of virulence and suggested that "cytoplasmic agents" were responsible for this phenomenon (Grente 1965). Remarkably, for the chestnut growing in northern Italy, this marked "recovery" was occurring within twenty-five years of the discovery of chestnut blight in Europe (Mittempergher 1978).

By the time recovery was detected in Italy, the spread of the chestnut blight fungus through the natural range of American chestnut was complete. There were few, if any, signs of resistance or recovery from infection. Grente's discovery and his research describing hypovirulence refocused attention on chestnut blight in this country, especially in the laboratories at The Connecticut Agricultural Experiment Station where a longstanding chestnut breeding program still was active. Further, the phenomenon of hypovirulence brought attention to the pathogen. It also was during this period of the early 1970s that a small stand of blighted chestnuts growing in Michigan was brought to the attention of the Connecticut research team (Elliston et al. 1977). They quickly discovered that the isolates from Michigan also carried a "cytoplasmic agent" that was transmissible and reduced the ability of *C. parasitica* to produce lethal cankers (Elliston 1985). Unlike the hypovirulent strains from Italy, the Michigan isolates retained their bright orange pigmentation. As the search for chestnut trees that were recovering from blight in Michigan intensified, more and more "recovering stands" were discovered, all outside the natural range of chestnut. Although chestnut blight is still the dominant stressing agent in most of the isolated Michigan stands, in some, the impact of the disease is minor, and the level of recovery mimics the expression of hypovirulence in some areas of Italy (Fulbright et al. 1983).

Since these two remarkable settings have been described, hypovirulent strains have been identified at various locations within the natural range of American chestnut and some are associated with surviving trees (Griffin 2000). Unfortunately, the wide-spread recovery of chestnut as a result of the hypovirulence phenomenon is unknown in areas where sprout populations still persist. Several reviews of the recovery of chestnut blight and associated hypovirulence are published (Milgroom and Cortesi 2004, MacDonald and Fulbright 1991, Heiniger and Rigling 1994, Nuss 1992, Van Alfen 1992).

WHAT IS HYPOVIRULENCE?

A variety of factors control the level of virulence in *C. parasitica* including the genetic makeup of the fungus or a variety of nonviral, cytoplasmic agents, such as defective mitochondria or plasmids. However, the term hypovirulence most often refers to the reduction in virulence caused by fungal viruses. The first indication that virus-like agents might be involved came with the association of double-stranded (ds) RNA with the European and North American strains that were shown to be less virulent (Day et al. 1977). These dsRNAs eventually were shown to represent a unique group of viruses, now called hypoviruses (Hillman et al. 2000). The definitive proof of the cause and effect relationship and their infectious nature occurred through the application of molecular technology (Choi and Nuss 1992). Although fungal-virus associations have been know for decades, the hypoviruses associated with *C. parasitica* are unique; rather than being encapsulated in a protein coat, they are membrane bounded (Newhouse et al. 1983). As a result, a new virus family, the *Hypoviridae*, has been established for the four species (CHV1 though CHV4) of hypoviruses that have been discovered to date (Hillman and Suzuki 2004). Most studies have been of the CHV1 species, as it was the first hypovirus identified and is the hypovirus associated with biological control of chestnut blight in Europe (Shapira et al. 1991). This hypovirus also has been discovered infecting strains of *C. parasitica* in China and Japan, but it has never been identified as a natural component of *C. parasitica* in North America (Peever et al. 1998). The CHV3 hypovirus is associated with the recovering Michigan chestnut stands but its origin remains unknown as it has not been isolated in the Orient (Paul and Fulbright 1988). CHV2 is uncommon and known only from a site in New Jersey (Hillman et al. 1994). It also has been identified in *C. parasitica* populations in China (Peever et al. 1998). CHV4 is somewhat unique; unlike CHV1-CHV3, it has little or no observable effect on the virulence or other traits of *C. parasitica* (Enebak et al. 1994). It is

widespread in its association with isolates from the central Appalachians but its origin and role remain undiscovered.

The effects of hypovirus infection on the blight fungus are variable and appear to be a function of the *C. parasitica* strain as well as the infecting hypovirus (Chen and Nuss 1999, MacDonald and Double 1998). For those hypoviruses that reduce fungal virulence, infection often results in smaller non-lethal cankers and a corresponding reduction in the production of asexual spores and almost certainly the reduction or elimination of sexual sporulation. What currently is known about the molecular influence of the hypovirus on the physiological processes of the fungus has been reviewed (Nuss 1992, Nuss 1996).

EXPLOITING HYPOVIRULENCE

The discovery of hypovirulence and the observation of a notable level of disease control on American chestnut in Michigan brought hopes for the first time that some level of biological control was possible in North America. Procedures first employed by Grente to treat virulent infections were duplicated. Subsequently, modifications to Grente's treatment protocols and a variety of different inoculum types were used to introduce hypoviruses into virulent cankers on American chestnut sprouts (Hobbins et al. 1992, MacDonald and Double 1979). The results often were very encouraging as hypovirus transfer frequently occurred and the expansion of individual treated infections frequently was arrested as callus tissue formed at the margins of cankers. Even though many of the treatments were successful and the life of sprouts was prolonged, the sheer number of subsequent infections that developed on the same stem dramatically weakened the tree, and when some cankers were not arrested by treatment, trees died (MacDonald and Fulbright 1998). Further, there was little evidence that natural hypovirus spread on the same stem afforded any protection to other virulent infections that almost certainly would arise. With few exceptions, most hypovirulent introductions were unsuccessful if measured by the number of treated sprouts that remained alive several years after treatment (Milgroom and Cortesi 2004)).

As a result of the early releases, several factors were discovered that may influence the effectiveness of the hypovirulent treatments. When additional hypovirulent strains were discovered and their infecting hypoviruses investigated, the variation in their effects on *C. parasitica* became apparent. Some virulent strains were so debilitated by hypovirus infection that they grew poorly in bark and almost completely failed to produce hypovirulent inoculum (Double and MacDonald 1995). Therefore, concern arose that highly debilitating hypoviruses have such an extreme effect on their fungus host that there is little potential for the strains to grow in bark and produce inoculum to perpetuate themselves. A sense developed that hypoviruses that do not debilitat*e C. parasitica* as significantly may be more useful biological control agents (MacDonald and Fulbright 1998). Logically, if strains are more capable of invading bark and generating hypovirulent inoculum without killing their hosts, they may be more capable of disseminating their hypoviruses and thus potentially better biological control agents.

ROLE OF VEGETATIVE COMPATIBILITY

Early laboratory and field experimentation also revealed that an incompatibility system existed in *C. parasitica* (Anagnostakis 1977). When strains are incompatible, their hyphal elements fail to fuse (anastomose), restricting cyptoplasmic and hypovirus exchange. Unlike many plant and animal viruses, viruses that infect fungi have no extracellular phase and therefore must be transmitted to progeny in spores during reproduction or via hyphal anastomosis and cytoplasmic mixing. The system of vegetative compatibility in *C. parasitica*, as in other fungi, represents a self-recognition system that prevents incompatible strains from fusing. Essentially, as the hyphal filaments of the fungus approach each other, cell death occurs, restricting the fusions necessary for hypovirus transmission. The system of vegetative

incompatibility in *C. parasitica* is controlled by at least six genes (Huber 1996, Cortesi and Milgroom 1998). The probability of hypovirus transmission is high when strains share identical genes. Transmission is less likely if gene differences exist with probabilities of transmission related to the number of gene differences and the specific genes present.

Considerable research on vegetative compatibility has been conducted (Cortesi and Milgroom 1998, Milgroom 1995, Milgroom and Cortesi 1999). One interesting relationship that has been discovered relates to the diversity of vegetative compatibility types and hypovirus transmission in field settings. In general, sites where biological control generally is more successful have a less diverse population of vegetative incompatibility genes (Milgroom et al. 1996). This appears to be the situation in Italy and Michigan (Cortesi et al. 1996) where the number of vegetative compatibility genes that are expressed is quite low when compared to the diversity that occurs in Asia or the central Appalachian region (Figure 1). Whether the lack of diversity is responsible for the widespread distribution of hypoviruses and biological control that has occurred is unknown.

Table 1. Diversity of vegetative compatibility types in four chestnut areas (two in the U.S. from *Castanea dentata* stands, one in Italy from a *C. sativa* stand, one in China from a *C. mollissima* stand and one in Japan from a *C. crenata* stand).

Population	Number of isolates tested	Number of VC types
Finzel, MD	57	25
Bartow, WV	61	29
Italy*	716	20
China*	79	71
Japan*	30	29

*Data from Milgroom

A second interesting relationship between hypovirus infection and the diversity of vegetative compatibility types is the effect hypovirus infection may have on the diversity of vegetative compatibility types at a site. Sexual reproduction is responsible for maintaining diversity (Marra and Milgroom 2001). One must therefore consider whether the low diversity that exists at some recovering sites is because hypovirus infections have reduced sexual reproduction or the reduced diversity has been a longstanding feature of the stand and has permitted hypoviruses to be disseminated successfully.

Although vegetative compatibility diversity appears to influence the success of biological control, other factors also may be involved. Certainly the role of the host cannot be overlooked in the expression of the hypovirulence phenomenon. In tests of susceptibility, European chestnuts consistently have been shown to be slightly more resistant than American chestnut (Bazzigher 1981). A more resistant host almost certainly would provide a longer time period for infections to acquire hypoviruses, perhaps enough time for the successful expression of the hypovirulence. Similarly, the ecosystems in which the two species typically grow are quite different. In its North American range, chestnut grows among a diverse mix of competing hardwoods. This often is not the case in Europe, especially in areas where European chestnut is cultivated for nut or coppice production (Bounous 1999). Likewise, at many recovering sites in Michigan, chestnuts grow in the absence of significant competition from other species. These settings permit the constant regeneration of chestnut biomass and may in turn foster the dissemination of hypoviruses. Unfortunately, in eastern North America, chestnut is largely a relic in the understory with little opportunity to grow and develop significant numbers of canker to even acquire hypoviruses. One site where many of these limitations do not exist is in a stand of American chestnut growing near West Salem, Wisconsin.

UTILIZING HYPOVIRUSES AT WEST SALEM

The West Salem stand is the largest stand of American chestnut in the U.S. The origin of the stand dates to the late 1800s when a few chestnuts were planted at the site by the landowner who had moved there from the eastern U.S. (Cummings Carlson et al. 2002). Chestnut now is the dominant species on about 50 acres of land. Unfortunately, in 1986, *C. parasitica* was discovered at the site and now threatens the future of this magnificent stand. Attempts from 1988-90 to eradicate the fungus failed. A biological control program was initiated in 1992. At that time, the stand seemed to present an excellent opportunity to exploit hypoviruses for two reasons. First, there were few trees infected so the disease was at the very early stage of the epidemic. Second, the stand was infected by a single clone of *C. parasitica*; hence, the barriers imposed by vegetative compatibility did not exist (McGuire and Milgroom 2002). Over the next six years, two hypoviruses were introduced into the resident West Salem strain (Double and MacDonald 2002). These were deployed by introducing inoculum into small holes made around the margin of the canker. The first hypovirus (CHV3 type) deployed (1992-94) was obtained from a recovering grove of chestnuts near Cadillac, Michigan. The second was a hypovirus (CHV1 type) associated with an Italian hypovirulent strain and was used for treatment from 1995-97. As cankers were treated, they routinely acquired hypovirus and the treated chestnuts responded by producing significant callus growth to close the infection.

Between 1992-1997, about 650 cankers on 138 trees were treated. To assess hypovirus spread each season, 8-12 small bark plugs were removed from the treated cankers and also from new cankers that had formed. An evaluation of hypovirus infection was made when the plugs were cultured and the cultural appearance of the resulting colonies was compared to that of virulent strains.

From 1998-2002, no additional hypovirus introductions were made, so that an evaluation of the level of natural spread could be made over several seasons. The results have been mixed. Treated cankers have retained hypoviruses and many are heavily callused and blight is no longer damaging (Table 1). Likewise, new cankers that have developed on trees with treated cankers have acquired hypovirus at high levels. Many of these stems are still alive almost ten years after initial treatment. Unfortunately, hypoviruses have not spread significantly to cankers on nearby trees that never received hypovirus treatment (Double and MacDonald 2002). Because the number of new virulent infections continues to increase rapidly, the decision was made in 2003 to once again introduce hypoviruses. If biological control can be initiated on individual trees by canker treatment, as seems to be indicated, the additional treatments may help save some trees and also help determine why viruses do not disperse to new cankers on trees that are untreated.

Table 2. Classification of cankers at West Salem, WI based on cultural morphology of *Cryphonectria parasitica* isolates removed from treated cankers, non-treated cankers on treated trees and non-treated cankers on non-treated trees from 1994-2000.

	Hypovirus-treated cankers		Non-treated cankers on treated trees		Non-treated cankers on non-treated trees	
Year Sampled	Hypovirulent	Virulent	Hypovirulent	Virulent	Hypovirulent	Virulent
1994	55%	45%	18%	82%	29%	71%
1995	55%	45%	22%	78%	0%	100%
1996	80%	20%	58%	42%	33%	67%
1997	82%	18%	42%	58%	9%	91%
1998	83%	17%	71%	29%	22%	78%
1999	81%	19%	71%	29%	28%	72%
2000	60%	40%	46%	54%	9%	91%

CONCLUSIONS

After its initial discovery, the prospects of utilizing hypoviruses to biologically control chestnut blight seemed reasonably straightforward and exploitable. The early successes achieved by treating infections on stems were in themselves remarkable. Unfortunately, hypovirus populations have not been perpetuated or disseminated adequately at sites where they have been artificially released, as has happened naturally at some locations. Admittedly, the phenomenon of hypovirulence, like most biological issues, is wrought with complexity. Whether we can duplicate artificially what has happened naturally remains a significant question. Clearly, major details of the epidemiology of chestnut blight and the infecting hypoviruses need to be unraveled. The successful transition of a virulent *C. parasitica* to one that is laden with debilitating hypoviruses is not regulated by a single factor. A summary of some of the components involved in the expression of hypovirulence that require further research include:

- an understanding of the contribution of chestnut genotype to the expression of hypovirulence;
- an appreciation of the role environmental factors play in contributing to the success or failure of hypovirulence;
- an evaluation of the pathogen population relative to its ability to cause disease, reproduce and acquire hypoviruses;
- an assessment of the influence specific hypovirus species have on *C. parasitica*;
- a consideration of potential vector relationships that might influence hypovirus dissemination; and,
- an evaluation of strategies for the deployment of hypoviruses.

We remain encouraged by the levels of recovery from blight that has occurred naturally at some locations. Over long biological time periods, hypovirulence may emerge on its own within the American chestnut's natural range. But, if man is to influence the process of biological control, a more complete understanding of the biology of the hypovirulence phenomenon is required.

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