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Biological Control of Chestnut Blight: Use and Limitations of Transmissible Hypovirulence

The introduction of *Cryphonectria (Endothia) parasitica* (Murrill) Barr into North America at the turn of this century created one of the first major challenges to the relatively young science of plant pathology. This brightly pigmented orange ascomycete, introduced into the New York City area on Oriental chestnuts, did not remain a curiosity for long. Scientists quickly unraveled the details of a host-parasite interaction that would have unparalleled ecologic, economic, and sociologic impact on the eastern United States (Fig. 1). Sadly, within 10 years after the discovery of the causal fungus, most of these researchers admitted that little could be done to slow the epidemic. The frustration they felt is evident in their early writings (4). To them, the only remaining control strategy was to initiate breeding programs to preserve the best traits of the American chestnut (*Castanea dentata* (Marsh.) Borkh.) and incorporate resistant germ plasm from Chinese (*C. mollissima* Blume) and Japanese (*C. crenata* Siebold & Zucc.) chestnut. These undertakings, which met with limited success, were never designed to control chestnut blight in our eastern forests.

Fortunately, the American chestnut was saved from extinction in its natural range by its propensity to sprout from the roots (Fig. 2). Ironically, this perpetual sprouting also may have provided for the development or expression of a natural biological control phenomenon that we may be able to exploit to regulate *C. parasitica* in our forests.

Discovery and Description of Hypovirulence

The first glimmer of hope for the American chestnut dates to the 1950s when an Italian plant pathologist, Antonio Biraghi, observed "spontaneous healing" of cankers on European chestnut (*C. sativa* Mill.) in northern Italy (5). His observations aroused the curiosity of Jean Grente, a French mycologist, who described a variety of unusual strains of *C. parasitica* associated with the healing cankers on these European chestnuts. The isolates of *C. parasitica* that Grente obtained from these cankers were lightly pigmented in contrast to the normal, bright-orange strains. Furthermore, he found that these strains could infect European chestnut but seldom produced lethal infections. These observations prompted him to call them "hypovirulent." This observation was significant, but, more important, Grente and his co-workers found that the factors responsible for hypovirulence were transmissible. Using in vitro and in vivo tests, they demonstrated that normal strains became hypovirulent after hyphal anastomosis with hypovirulent strains (Fig. 3). They therefore considered the determinants of hypovirulence to be potentially useful as biocontrol agents (20).

Fewer than 25 years passed from the time chestnut blight was discovered in Europe until recovery was first observed in Italian chestnut stands (5). By this time the disease had been present in North America for more than 50 years, with few if any signs of resistance to or recovery from infection. The Italian situation, however, refocused attention on chestnut blight in the United States and led to experimentation by Van Alfen and his colleagues at The Connecticut Agricultural Experiment Station (36). In greenhouse tests they confirmed Grente's findings by demonstrating that European hypovirulent isolates of *C. parasitica* could be used successfully to stop the expansion of individual cankers initiated by North American virulent isolates. The introduction of hypovirulent isolates into expanding cankers induced the formation of callus tissue at the edges of cankers on young stems. Descriptions of their research and of the resulting nonlethal cankers were widely publicized.

As a result of the attention this work received, one observant naturalist sent bark samples to The Connecticut Agricultural Experiment Station from a small stand of blighted but surviving trees in Michigan. These trees had been planted by early settlers and, although severely damaged by blight, were still alive and had many nonlethal cankers, similar to those described in Italy (Fig. 4). Elliston et al (15) found that although the cultures obtained from these cankers retained the normal orange pigmentation of virulent strains, they fit many of the criteria then used to define hypovirulence; they had abnormal culture morphology, were less virulent than normal isolates, and transmitted these traits to virulent isolates.

Brewer (6) later determined that surviving blighted American chestnut trees in Michigan were common, and Fulbright et al (18) found hypovirulent isolates in several of these blighted chestnut stands. Today, more than 30 American chestnut stands that are surviving infection have been identified in Michigan. They consist of large mature trees, saplings, and seedlings. In many of these stands, blight is still the dominant biological stress, but in a few, almost all signs of *C. parasitica* have disappeared. Even though the natural range of the American chestnut reached into southeastern Michigan, the recovering stands all are located in western or central Michigan, outside the natural range.

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Jaynes and Elliston (26) and Griffin et al (23) have tested isolates from surviving American chestnut in other states and found that many surviving trees were infected with hypovirulent strains. Other hypovirulent isolates have since been recovered from trees in Maryland, New York, Virginia, Tennessee, and West Virginia. Infected, surviving trees with hypovirulent isolates of *C. parasitica* also are present in southern Ontario (Colin McKeen, *personal communication*). It is now apparent that Europe does not have a monopoly on surviving chestnut trees or hypovirulent strains of the fungus, but the recovery of chestnut in Europe has been so successful that the reestablished European chestnut industry is now exporting chestnuts to the United States. The relatively few American chestnut trees producing nuts in North America provide a valuable source of American chestnut seed for scientists and chestnut enthusiasts. However, a chestnut industry based on the American chestnut tree still would be risky, if not impossible.

What Is Hypovirulence?

Grente first coined the term "hypovirulent" to describe the isolates of *C. parasitica* recovered from surviving European chestnuts in Italy. Many phenotypic traits of those hypovirulent isolates were used to define hypovirulence, but because many traits are variable, hypovirulence is not easily defined as "less virulent" or "attenuated."

What criteria must a pathogen possess to be termed hypovirulent? The first and primary trait is low virulence. Although there is no "normal" virulence level, there is an "expected" level, which can be determined after observing the disease in situ. Pathogens that induce small cankers or sporulate less in a given time period may be less virulent. Elliston (12) demonstrated that hypovirulent isolates of *C. parasitica* show a wide range of virulence and that any given hypovirulent isolate may cause large cankers, whereas others are unable to grow when introduced into living trees. By measuring canker size, reproductive capacity, or both, one can characterize and compare the virulence of field isolates.

Hypovirulent isolates of *C. parasitica* often show unusual culture morphology on agar media (Fig. 5). The first hypovirulent isolates recovered from Italy and France were nonpigmented and posed problems for species identification. Hypovirulent isolates from Michigan and other locations in North America maintain pigment production, but some lack expected zonation patterns when grown on media under alternating light and darkness. Culture morphology has been used widely for identification of hypovirulent isolates. However, the use of this feature should be limited to well-characterized strains that have distinct, recognizable morphologies. Culture morphology should never be the sole criterion used for screening unknown isolates for hypovirulent phenotypes; hypovirulent isolates with normal culture morphology may be overlooked or virulent strains with unusual morphology may be improperly classified as hypovirulent.

An important characteristic of hypovirulent *C. parasitica* that makes the biological control of blight possible is the transmissible nature of the cytoplasmic agents of hypovirulence. Hypovirulent isolates can convert virulent strains to hypovirulent after hyphal anastomosis. When conversion occurs, the recipient strain demonstrates all or most of the hypovirulent characteristics associated with the donor strain, including virulence, pigmentation, sporulation, and culture morphology (13,14). Therefore, when an orange North American virulent isolate is converted by a less pigmented European hypovirulent one, the American isolate becomes hypovirulent with reduced pigmentation (Fig. 3).

What genetic factor or factors code for these abnormal phenotypic characteristics in *C. parasitica*? The transmissible nature of hypovirulence demonstrates that genetic factors are cytoplasmic. Indeed, almost all hypovirulent strains of *C. parasitica* contain molecules of double-stranded ribonucleic acid (dsRNA) (7). Most fungal viruses have a single or multisegmented dsRNA genome and a protein coat. The dsRNA molecules found in *C. parasitica*, whether from European or North American isolates, do not appear to be associated with a protein coat. Rather, they appear to be associated with vesicles of host origin (9,30). Viruslike replicase activity has been found in one hypovirulent strain (35). While evidence strongly supports dsRNA as the genetic factor involved in hypovirulence, its causal nature has not been proved because Koch's postulates have not been fulfilled. Three lines of evidence, however, indicate that dsRNA is reponsible for the hypovirulent phenotype in most isolates of *C. parasitica*. First, nearly all hypovirulent isolates contain dsRNA; virulent ones usually do not. Second, a virulent isolate converted to hypovirulent will usually obtain the characteristics of the hypovirulent isolate with which it was paired and it generally will contain the same dsRNA molecule (based on size and number of dsRNA segments) as the hypovirulent isolate involved in the conversion. Third, when the dsRNA is eliminated from an isolate either by single-conidium isolation or treatment with cycloheximide (16), the resulting culture changes to a virulent phenotype.

Hypovirulent isolates of *C. parasitica* frequently harbor more than one segment of dsRNA, and the number and sizes of these segments usually are different and vary from one isolate to another (Fig. 6). The molecules range in size from approximately 12 kb to less than 1 kb. Most hypovirulent strains contain a large dsRNA molecule in the range of 8 to 12 kb that is frequently associated with few to several smaller molecules (8). Most of these smaller molecules are related by nucleic acid sequence to the larger molecules present in the fungus but lack portions of nucleic acid sequences present in the largest molecule. Although dsRNA molecules examined

Fig. 1. Young pole-size chestnut stand devastated by chestnut blight (West Virginia, circa 1925).

Fig. 2. Proliferation of chestnut sprouts at the base of a blight-killed tree.

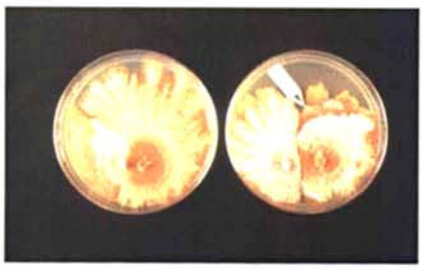

Fig. 3. Pairings of virulent (right) and hypovirulent (left) isolates of *Cryphonectria parasitica*. Conversion has occurred in the virulent strain (arrow), and the pattern of mycelial growth has changed.

from European and North American isolates of *C. parasitica* are associated with hypovirulence, they do not share extensive genetic similarity (29,31); however, terminal sequence analysis showed these dsRNA molecules share some common terminal sequences (24,34). This indicates that although there are similarities among the dsRNA molecules affecting *C. parasitica*, these viruslike agents are not necessarily genetically identical.

The role of dsRNA in hypovirulence remains unknown. Recent studies indicate that dsRNA produces protein products within the fungus (33). These proteins or others may be affecting fungal gene expression, as hypovirulent isolates apparently do not accumulate certain proteins found in virulent dsRNA-free isolates (32). Such studies will help our understanding of how dsRNA causes hypovirulence and also may help discover virulence determinants in *C. parasitica*.

There also are isolates of *C. parasitica* having low virulence without detectable levels of dsRNA. Jaynes and Elliston (26) and Fulbright (17) have found in recovering stands of chestnut trees isolates that do not contain dsRNA. Little work has been done to determine the cause of the reduced virulence phenotype in these isolates, however. Fulbright (17) demonstrated the transmissible nature of hypovirulence in one isolate lacking dsRNA and showed its usefulness in biocontrol of chestnut blight.

Exploiting Hypovirulence

For the first time since the devastation of the American chestnut, there are prospects of a unique biological control for this disease. Based on assumptions that the survival of trees in Michigan and Italy resulted from the natural buildup of hypovirulent strains, one approach to the exploitation of hypovirulence has been to investigate ways to artificially introduce these strains into the forest. Grente's procedure of treating infections by exposing cankers to some form (inoculum plugs, conidial/mycelial slurries in sprays) of hypovirulent inoculum at first was promising, because expansion of individual cankers often was arrested and callus tissue formed at the margins (25). However, the procedure failed to control some infections or to influence the development of subsequent cankers on the same stem. In some instances 15 or more cankers on a single tree were arrested, but as new cankers developed over time, trees died from the sheer number of infections they supported. Sprouts that remained alive after successive years of canker treatment were the exception (25,37).

The strains first used for canker treatment were highly curative but grew and sporulated so poorly that their persistence was limited. As infections ceased to expand and periderm tissue formed, the amount of colonized bark containing the agents of hypovirulence diminished rapidly. Failure of strains to persist led to the consideration of isolates with greater potential to grow in bark and provide a persistent source of inoculum.

Early tests in vivo and in vitro also revealed that virulent and hypovirulent strains often failed to interact if the two strains were vegetatively incompatible (limited hyphal anastomosis restricted dsRNA transmission) (1,3). This ex-

Fig. 4. Symptoms of virulent and hypovirulent strains of *Cryphonectria parasitica*: (A) Virulent infection on a young chestnut sprout in West Virginia. (B) Hypovirulent infection on a young chestnut sprout in Michigan. (C) Virulent infection converted to callus-forming canker 3 months after application of hypovirulent inoculum through punch holes, which are still evident.

planation of unsuccessful treatment of some virulent infections led to an appreciation of the complexity of vegetative compatibility in eastern forests. More than 70 compatibility types have since been described and the existence of as many as 128 hypothesized (3,10). Although vegetative incompatibility may be a significant barrier to successful interaction of virulent and hypovirulent strains, some hypovirulent isolates successfully transmit the agents of hypovirulence to virulent isolates from a variety of vegetative compatibility groups (2,28). Combinations of these highly interactive hypovirulent strains have successfully converted virulent strains from most known vegetative compatibility groups both in vivo and in vitro (27). Yet, even with this information, field treatment strategies have not dramatically influenced the course of disease or significantly prolonged tree life.

The assumption is that for hypovirulence to be successful, a constant reservoir or source of hypovirulent inoculum must be present to spread and interact with the natural population of *C. parasitica*. In the native chestnut forest this interaction would have to occur on young chestnut sprouts or other related species. One approach in West Virginia has been to initiate hypovirulent infections on healthy stems before natural infection by virulent strains (37). In one study, large scratched-wounded areas of the bark were inoculated with hypovirulent isolates that were able to grow and reproduce but did not kill their hosts. *C. parasitica* then was isolated from new lesions as they arose on other areas of the bark. Natural dissemination was measured from assays of these new infections. Dissemination was observed in the first year of the experiment and increased in subsequent sampling periods up to 4 years. Infections that initially yielded only virulent isolates, when sampled again 1 year later, often yielded one or more hypovirulent isolates and frequently a complex variety of strains. Although this detection of natural dissemination was very encouraging, almost all trees in these study plots have died because of an overwhelming number of infections caused by virulent strains of *C. parasitica*.

Another study of dissemination in Michigan utilized a genetically marked hypovirulent strain and specific dsRNA molecules (19). Open petri dishes containing asexually sporulating cultures of the hypovirulent fungus were placed on trees for 6 months above a series of small wounds made on the trunk of 20 trees. The hypovirulent strain was found on treated trees and also on control trees nearby. The finding of dsRNA molecules in isolates from natural infections demonstrated dsRNA transfer in situ. Three years later, more than one-half of the trees harbored dsRNA-containing isolates. Therefore, hypovirulent strains and dsRNA may be disseminated by conidia produced from small cankers initiated by hypovirulent strains. Whether or not a hypovirulent epidemic is established in this plot will be determined by periodic evaluation of new and existing cankers.

The premise that either canker treatment or various other methods of introducing hypovirulent strains can control chestnut blight over a short time is probably unrealistic. In the American chestnut range, survival of small stems is directly related to their circumference and the rate with which they are girdled by a virulent strain. Openings in the Appalachian Mountains where chestnut sprouts abound often have been created by some forest harvesting practice. When released, existing sprouts thrive and many new sprouts develop, and as host substrate increases so does the virulent population of *C. parasitica*. This rapid buildup of host and pathogen populations eventually results in a 5- to 10-year epidemic and the death of most sprouts. This interval of time may not be adequate for agents of hypovirulence to become established or for their effects of prolonging tree survival to become evident. As the epidemic ensues, chestnut is eliminated by competing vegetation and once again becomes a minor understory shrub (22).

In retrospect, many field studies that were originally designed to test the hypothesis that hypovirulent strains can serve as biocontrol agents were premature. Yet, such studies provided insight into a variety of research needs. The mode by which hypovirulent strains become established to influence virulent populations of *C. parasitica* remains unclear. Most significant is the need for fuller understanding of the phenomenon of hypovirulence in nature, particularly the origin of the dsRNA associated with this phenomenon. We mistakenly assumed dsRNA is not a common component of the chestnut blight fungus in eastern forests. Recent examination of isolates from Maryland, Virginia, and West Virginia has shown dsRNA is present in more than one-half of the isolates from some locations (11).

If dsRNA is a common component of *C. parasitica* in eastern North America, then one must ask why this (these) dsRNA(s) has not enabled recovery of the American chestnut within its native range as it presumably has in Michigan and Italy. Several explanations exist. Perhaps the dsRNAs carried by *C. parasitica* in the native range of Amer-

Fig. 5. Virulent (single culture) and various hypovirulent isolates of *Cryphonectria parasitica* from Europe (top row) and North America (bottom row) showing variations in pigmentation and culture morphology.

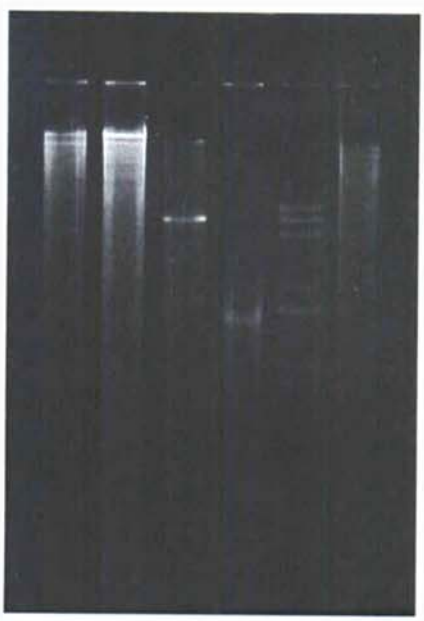

Fig. 6. Polyacrylamide gel with dsRNA molecules from *Cryphonectria parasitica* stained with ethidium bromide. Sources are (from left to right, in pairs) Europe, Michigan, and West Virginia.

ican chestnut do not affect pathogenicity or may not be readily transmitted, or both. Many of the Michigan and Italian strains, even though significantly debilitated, appear to have spread in the virulent population. If the dsRNAs are common components of the *C. parasitica* population in the East and if some are appropriate for biocontrol, perhaps the opportunity for expression has not been achieved. One explanation for the lack of expression might come from a major difference in the ecosystems (21). In both Michigan and Italy where expression of hypovirulence occurs, chestnut regeneration has continued with little or no competition from other plant species. This seldom is the case in the Appalachian region. Finding dsRNA-infected *C. parasitica* isolates in the absence of biological control indicates that dsRNA from various regions should be studied to understand the role of these molecules in hypovirulence and biological control.

An important shortcoming in our knowledge is the lack of understanding of "hypovirulence epidemics." The mechanism for such debilitated strains to become well established among a highly virulent population may relate to the source of hypovirulence and its contagious nature. We know little of the epidemiology of the interaction between virulent and hypovirulent strains. For example, exposure of a canker containing a virulent strain to hypovirulent inoculum not only may cause conversion of the virulent thallus but also may reduce the amount of virulent inoculum and initiate hypovirulent inoculum production. The contagious nature of hypovirulence thus may decelerate a virulent epidemic but increase a hypovirulent one. A gradual transition from virulence to hypovirulence appears to be responsible for the survival of chestnut in a small woodlot at The Connecticut Agricultural Experiment Station, where hypovirulent strains were introduced over a 4-year period more than a decade ago (J. E. Elliston, *personal communication*). Isolates containing dsRNA are commonly recovered from the cankers in this planting, but the effect of dsRNA on virulence is not clear. The Connecticut setting is a pure stand of chestnut.

Outlook

For most of this century we have only been able to describe *C. parasitica* and its effects and to perform some breeding experiments. Now, however, nature has provided a novel approach for control of this devastating disease. Yet biological control has not been achieved by simply adding hypovirulent strains to the normal disease situation. Clearly, to utilize hypovirulence we must first understand hypovirulence, and to achieve success, researchers in plant pathology, ecology, epidemiology, genetics, molecular biology, and other disciplines must coordinate efforts if the remaining chestnuts are to revive. The naturally recovering stands in Europe and Michigan indicate that success is possible. With coordinated research efforts we may be able to do more than entertain thoughts of controlling this destructive pathogen.

Acknowledgments

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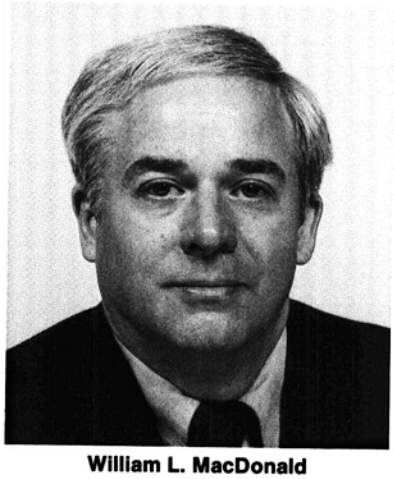

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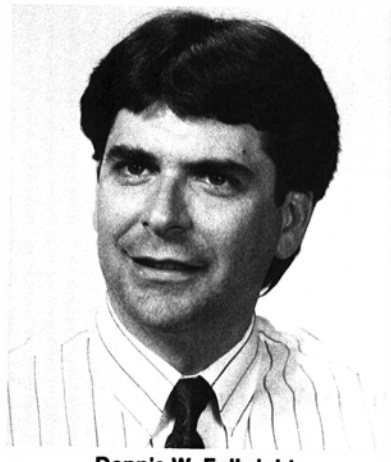

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