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INTEGRATED USE OF RESISTANCE, HYPOVIRULENCE, AND FOREST MANAGEMENT TO CONTROL BLIGHT ON AMERICAN CHESTNUT

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Abstract: In the natural range of the species, the survival of most large American chestnut trees was associated with blight resistance, hypovirulence (reduced virulence) in the chestnut blight fungus, *Cryphonectria parasitica*, and favorable sites. Controlled intercrosses of these trees have resulted in progeny with acceptable levels of resistance, which have been used in further breeding. Some American chestnuts in a very large population (135,123 trees) derived from open pollinations of large survivors have shown promising levels of field blight resistance. In forest clearcuts and plantations, hypovirulence, associated with hypovirus infection in the blight fungus, develops naturally following blight epidemics. However, chestnut stems die due to: 1) the high blight susceptibility of American chestnut; 2) the rapid spread of vegetative compatibility-diverse, abundant, virulent inoculum; 3) the slow spread of hypovirulence; 4) high hardwood competition; and 5) low-temperature stress at high-altitude (> 2,500 feet) sites or drought. A long term (>20 years) and high level of blight control has been obtained on mesic, managed (control of competing hardwoods) sites, established with blight-resistant American chestnuts that were inoculated with a hypovirulent strain mixture. Cultural studies and nucleotide sequence analysis of two hypovirus regions (both >800 bp) indicated that blight control was associated with the spread of Italian *Cryphonectria hypovirus 1* (CHV1). Blight resistance may allow time for CHV1 to spread. Mesic shallow coves on lower altitude slopes are among the best sites to implement integrated blight management and restoration of American chestnut.

Key Words: American chestnut / Blight resistance / Hypovirulence / CHV1 / Forest management

INTRODUCTION

Following the chestnut blight pandemic, only a few timber-sized American chestnuts survived in the native range of the species, where it was once a dominant component of the former oak-chestnut and mixed mesophytic forest regions (Braun 1950). From the mid 1970s to the early 1980s, we investigated why these trees survived. The main focus was on tests for blight resistance, hypovirulence in the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, and site factors. Several large trees were found, with the largest being greater than 40 inches in diameter at breast height (dbh). This tree grows at the base of the Northern Blue Ridge Mountains in Virginia. Understanding why these trees survived may aid in attempts to control the blight and restore the American chestnut.

EVALUATION OF BLIGHT RESISTANCE AND HYPOVIRULENCE IN LARGE, SURVIVING AMERICAN CHESTNUT TREES

The concept of disease resistance in plants, as viewed by American chestnut workers, both scientists and non-scientists, is often very restricted. Some believe a plant almost has to be immune to be labeled "resistant" to a given disease and that resistant plants never die from disease. Scientists working in the field of disease resistance recognize that disease resistance is variable. For example, the grey scale shown in Fig. 1 shows how disease severity can vary from a very low level (represented by white) through increasing intensities of grey until the scale is black, representing high disease severity. These disease severity levels are then translated into disease resistance/susceptibility ratings, which, therefore, can vary from high disease resistance to high disease susceptibility. Some workers, such as disease-resistance scholar, J.E. Vanderplank (1982), have called the high disease resistance in Fig. 1 "full" or "complete resistance", and the high susceptibility "full" or "complete susceptibility". All in-between values were called "partial resistance" by Vanderplank. When chestnut stems of a different species or genotype were inoculated with a virulent strain of the chestnut blight fungus, disease severity ratings were continuously variable (Anagnostakis 1992; Bazzigher and Schmid 1962; Clapper 1952; Griffin et al. 1983; Hebard 1999), and thus resistance/susceptibility ratings can vary similarly, as represented in Fig. 1. The middle of the scale (see arrow) may be called partial resistance or moderate resistance (MR). The disease severity rating can be influenced by environmental factors, ontogenic factors, virulence of the pathogen, and the time interval over which the test is conducted (Bazziger and Schmid 1962; Griffin et al. 1986). Stressful environmental factors shown to be associated with more severe chestnut blight, such as early frosts (Berry 1951), growth in frost pockets or at high altitude (Headland et al.1976; Jones et al. 1980), drought (Goa and Shain 1995), and low light intensity (Uchida 1977), may increase disease severity (lower arrow by the scale). Factors favorable to the chestnut tree, such as optimal soil moisture (Goa and Shain 1995) over the growing season, a mesic site, and high intensity light (Uchida 1977), may lower disease severity (upper arrow by the scale) ratings (Fig.1). In the former oak-chestnut region, low temperatures at high altitudes and summer droughts are common events and can be stressful to chestnuts during blight resistance trials.

Several approaches were used by us to evaluate blight resistance in large, surviving American chestnut trees, including *in situ* inoculations of branches on the large, surviving trees with standard virulent strains of the blight fungus, inoculation of seedling progeny from large surviving trees growing at the same location, inoculation of grafts of the large survivors, and inoculation of excised stems of the survivors in the laboratory (Griffin et al.1983). Canker length and, in some tests, evaluation of cambium colonization and necrosis following canker dissection with a knife, were used to determine disease severity. In later trials, necrosis at the cambium was evaluated by bark-core sampling of the cankers. To evaluate hypovirulence of *C. parasitica* isolates recovered from the large, surviving trees, the procedure was similar except that blight-susceptible, clearcut, or understory American chestnut trees were inoculated with several pathogen isolates from each large, surviving tree. Site factors evaluated included elevation, competition from other hardwoods, slope, aspect, and soil characteristics. The results indicated that survival of most large trees was associated with low to moderate levels of blight resistance, a low frequency of hypovirulence in the blight fungus population on the tree, and favorable sites that were relatively free from competition. In 15 trials of 13 surviving trees, 73% showed significant evidence of blight resistance when compared to blight-susceptible reference trees, following *in situ* or seedling inoculations in the field with standard virulent strains (Griffin et al. 1983). For 317 blight fungus isolates recovered from 19 surviving trees, 28% were hypovirulent or intermediate hypovirulent in pathogenicity trials on forest American chestnut trees. Several trees exhibited no evidence of blight resistance, and a few trees were growing in competition with other trees. Mites recovered from large surviving trees were associated with virulent and hypovirulent *C. parasitica* and may be agents of hypovirulence spread on surviving trees (Wendt et al. 1983; Griffin et al.1984).

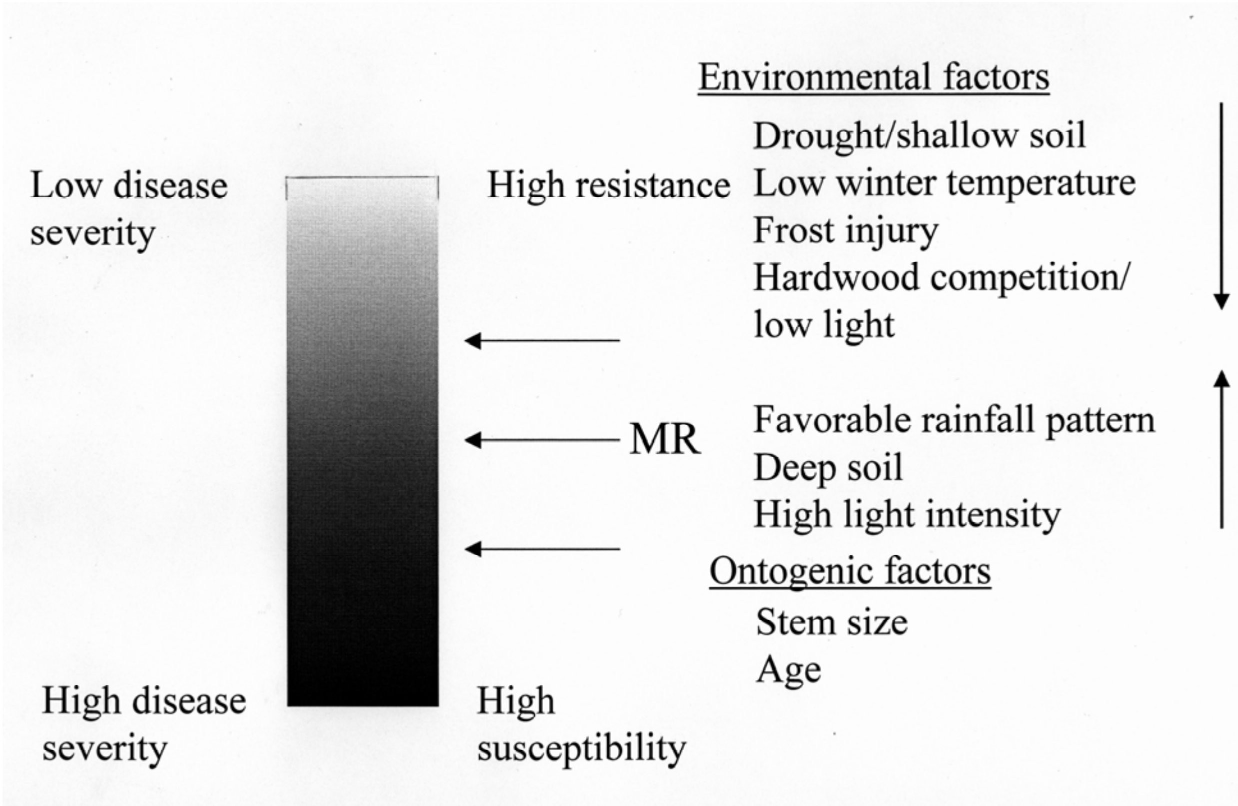

Figure 1. Concept of plant disease or chestnut blight resistance in relation to observed continuously variable disease severity, as represented by the grey scale bar. Low disease severity (whitish top) may be called high or complete disease resistance (*sensu* Vanderplank 1982), high disease severity (blackish bottom) called high or complete susceptibility, and intermediate disease severity (medium grey) may be called moderate resistance (MR) or partial resistance. For chestnut blight, in any given year or test, environmental and ontogenic factors can shift the disease severity rating and resistance rating up or down as shown (see arrows).

BREEDING AMERICAN CHESTNUT FOR BLIGHT RESISTANCE

Initially, four large, surviving trees (Fig. 2) were selected from this group of surviving trees to start an American chestnut blight-resistance breeding program. Controlled intercrosses were made among these trees. In this American Chestnut Cooperators' Foundation (ACCF) program, we are testing the hypothesis that additive blight resistance can be obtained through two or three cycles of selection. Vanderplank (1982) has documented that, in practice, gains of resistance through selection have been common and rapid in other host-pathogen systems. The evidence was that this additive resistance is mostly oligogenic. In our program, the progeny were grown at high elevation (> 2,500 feet), and after 7 years, these progeny were evaluated for blight resistance as described above. Two of the crosses, F x M and F x G, or the reciprocal cross, produced a high percentage of progeny that had acceptable disease severity ratings for further breeding. After one year of canker growth in these crosses, about one-half of the progeny trees had relative canker disease severity indexes (canker length x percent necrosis at the cambium) that indicated resistance when compared to results obtained for the blight-susceptible clone used in the progeny test. Also, this conclusion agrees with data obtained in the *in situ* tests mentioned above (Griffin et al. 1983), although strict comparisons to the latter cannot be made. The canker length and percent cambium necrosis components of the canker disease severity index for several progeny trees and their parents, obtained earlier, are shown in Fig. 2. The M (McDaniel) surviving tree, which had the highest canker disease severity index of the four parent trees, combined well with the F (Floyd) surviving tree, as did the G (Gault) surviving tree. The WY (Weekly) surviving tree yielded low percentages of progeny that had acceptable disease severity ratings for further breeding. As these trials lasted one year at high altitude, they included the winter dormant season, which has been associated with low-temperature stress, canker development or expansion toward the vascular cambium, and possible breakdown of blight resistance at high elevations (see later sections). Using individual F_1 trees, F_2 progeny from the F x M and F x G crosses are now being grown. Backcrosses have been made to maintain some of the desirable traits of these locally-adapted parent trees. In addition, the progeny of several new intercrosses from trees showing high vigor and/or adaptation to different elevations are being gown as additional resistance sources.

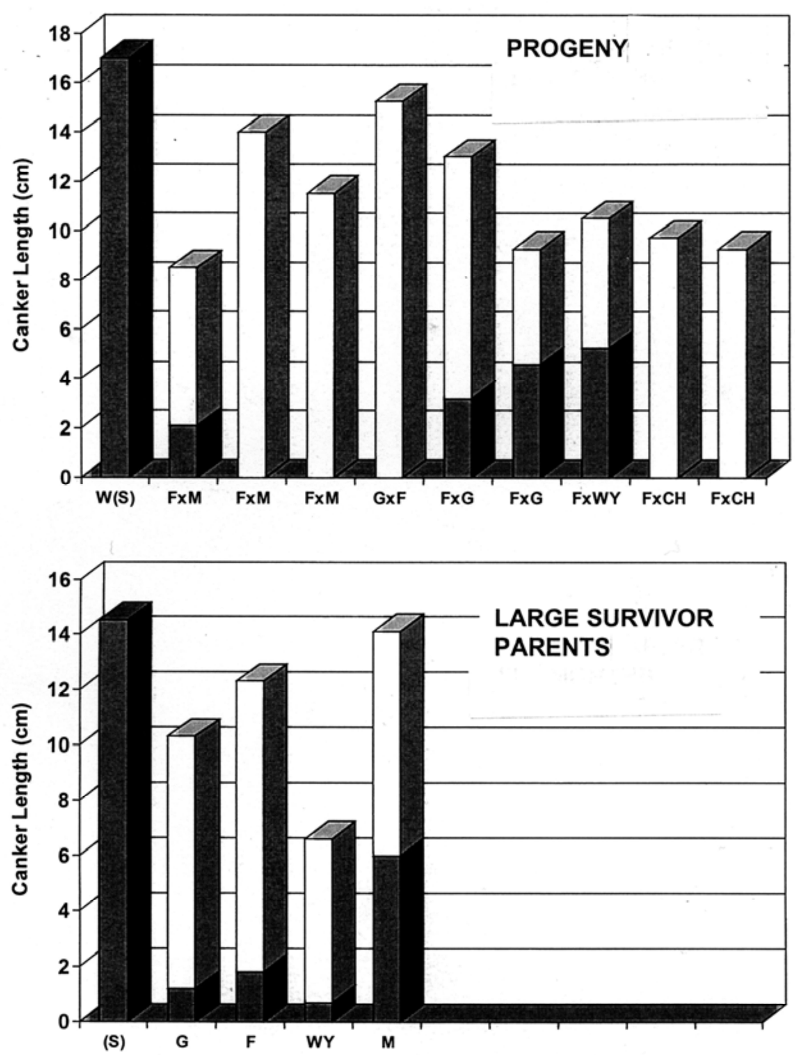

Figure 2. Canker lengths and percent of cambium areas beneath the cankers that are necrotic (shaded portion of bars) in 1-year duration blight resistance trials, with a standard virulent strain of *C. parasitica*, for (below) *in situ* inoculations on branches of the G, F, WY, and M large, surviving American chestnut parents, and (above) on main stems for 7-year-old intercross progeny of these surviving trees. The mean of seven susceptible reference trees (S) used is shown for the parents and the mean of one W(S) susceptible reference tree used for the progeny is shown. CH indicates Chinese chestnut reference parent.

A large population of American chestnuts with a potential for blight resistance are being grown by ACCF cooperators. These trees are progeny from open pollinations of a population of trees derived from large survivors and grown in the same breeding orchard. These breeding-orchard trees have exhibited low to moderate levels of blight resistance, and many are related to the parent trees used in the control pollinations. These trees likely share some of the same alleles that may be responsible for blight resistance and other desirable traits. The blight-resistant progeny of these open-pollinated trees should be good sources for increasing genetic diversity during later American chestnut restoration efforts. As of February, 2004, very large numbers of seedling transplants (93,643) and seed nuts (41,480) have been planted, and some have shown promising levels of field resistance to chestnut blight. The best of these trees, in terms of durable field blight resistance, can be incorporated into the controlled-cross breeding program described above. From these open pollinations, the National Park Service is now growing 4,500 seedling transplants and 462 trees from nuts.

INTEGRATION OF BLIGHT RESISTANCE AND HYPOVIRULENCE FOR CHESTNUT BLIGHT CONTROL

In 1980, John Elkins and Bruce Given (West Virginia Department of Agriculture), of the ACCF, worked with Tom Dierauf, of the Virginia Department of Forestry, to graft large, surviving American chestnuts on American chestnut rootstocks. These tree rootstocks were established 11 years earlier by Al Dietz (1978), founding officer of the ACCF, at the Lesesne State Forest, for the purpose of breeding a blight-resistant American chestnut by radiation breeding. The site in the Lesesne State Forest was 1,350 feet in elevation and mesic. Most of the trees in this radiation breeding program were dying from chestnut blight, but stump sprouts and the rootstocks survived. In 1982 and 1983, blight cankers on the American chestnut grafts were inoculated with a mixture of American and European dsRNA-infected, hypovirulent strains obtained from J.E. Elliston of the Connecticut Agricultural Experiment Station. Over the next two decades, these trees exhibited a high level of blight control, with blight cankers exhibiting a high degree of superficiality (Dierauf et al. 1997). By 1999, the largest tree had attained a height of 61 feet and a dbh of 15.7 inches (Griffin 2000). This same tree had a dbh of 19.0 inches in March, 2004 (Fig. 3). This blight control occurred in the presence of an abundant, virulent, ascospore inoculum generated by numerous perithecia in the thousands of cankers on the 5,000 American chestnut trees planted by Dietz in the Lesesne plantation. Inoculation trials with a standard virulent strain provided evidence for blight resistance in these grafts, and the virulent strain was later recovered from the superficial cankers resulting from the inoculations (Robbins and Griffin 1999). Some seedling American chestnut trees from the blight-resistant, large survivor intercrosses described above, also have exhibited a high to moderate level of blight control after 20 years when inoculated with a European hypovirulent strain mixture early or after several years of tree growth.

Extensive research indicated that dsRNA hypoviruses from the European hypovirulent strains had spread into 34-41% of isolates of the blight fungus recovered from the grafted trees at Lesesne, based on colony morphologies of over 800 *C. parasitica* isolates recovered from cankers (Griffin 1999; Robbins and Griffin 1999; Hogan and Griffin 2002 a and b). Colonies of European hypovirulent strains commonly have a predominantly white phenotype versus the orange-pigmented phenotype of dsRNA-free virulent, normal strains or many American hypovirulent strains. Assays of over 70 isolates of *C. parasitica* from the grafts, all with a predominantly orange-pigmented phenotype, indicated that most were free of hypovirus dsRNA. In white isolates, the European hypovirus, *Cryphonectria hypovirus 1* (CHV1, Hillman et al. 1995), had spread 642 cm from the hypovirulent–strain-inoculated zone and into a very large number (> 45) of vegetative compatibility types of the chestnut blight fungus (Hogan and Griffin 2002a). Blight resistance may have allowed time for CHV1 to spread. Vegetative incompatibility between different strains of the blight fungus was believed to be a major barrier to hypovirus transmission and biocontrol of chestnut blight in the United States, where a similar large number of vegetative compatibility types of *C. parasitica* had been identified in research plots (Anagnostakis and Kranz 1987; Anagnostakis and Day 1979; Kuhlman and Bhattyacharyya 1984; Lui and Milgroom 1996). Within a canker, however, incomplete movement of CHV1 within a vegetative compatibility type was found for the natural cankers on the grafts (Hogan and Griffin 2002b).

A few predominantly orange-pigmented isolates from the grafts had high dsRNA content, and high dsRNA is characteristic of European hypovirulent strains (Dodds 1980). The European hypovirulent strains inoculated on the grafts were of French and Italian origin. The French strain inoculated on the

Figure 3. Integrated blight control on American chestnut with resistance and hypovirulence. Photo taken in March, 2004 of a 19.0-inch dbh stem (left) and an 18.5-inch dbh stem (right) of a two-stem American chestnut tree grafted in 1980 from a large survivor and later inoculated with a mixture of hypovirulent strains of the blight fungus. Arrow points to a highly superficial (nonkilling) canker. This high level of blight control is typical throughout this tree, which is over 60 feet tall. Bars on scale to right are 1 foot long.

grafts had a predominantly orange-pigmented phenotype, but was derived from a predominantly white hypovirulent strain (Elliston 1985). The inoculated Italian hypovirulent strains had predominantly white phenotypes. Single-spore analysis of the white isolates from the grafts suggested that hypovirus from the Italian inoculated strains had spread on the grafts (Hogan and Griffin 2002a). Colony morphologies of hypovirulent strains are variable in subculture, however. Therefore, nucleotide sequence identification was used to determine the identity of CHV1 in the white isolates and to determine the identity of CHV1 in the predominantly pigmented isolates as French or Italian.

To identify CHV1 as French or Italian, hypovirus dsRNA was extracted from predominantly white and predominantly pigmented *C. parasitica* isolates recovered from cankers on the grafts. cDNAs were then made by reverse transcriptase-polymerase chain reaction (RT-PCR) for two hypovirus regions: (1) an 844-bp region in the helicase domain of open reading frame B (ORF B), and (2) an 894-bp region that included part of the 5′ non-coding region and part of p29 of ORF A. Nucleotide sequence analysis indicated that all pigmented and white *C. parasitica* hypovirus isolates from the grafted trees and Italian inoculated strains had high identities to each other and high identities (98.7-99.9%) to the Italian reference hypovirus, CHV1-Euro7 (Griffin et al. 2004). Identities to French reference hypovirus CHV1-EP713 were low (<89.8%). Thus, no evidence was found for the presence of French hypovirus, except for the hypovirus in the predominantly orange-pigmented French hypovirulent strain inoculated on the trees.

FOREST MANAGEMENT IN CLEARCUTS AND PLANATIONS FOR INTEGRATED CHESTNUT BLIGHT CONTROL AND RESTORATION OF AMERICAN CHESTNUT

American chestnut presently may be found sparsely to frequently as an understory tree throughout the native range of the species in both the former oak-chestnut and mixed mesophytic forest regions (Braun 1950). Xeric and intermediate sites may have very high population densities of understory American chestnuts, especially at elevations of about 3,000 feet or higher in the Mid-Atlantic area of the former oak-chestnut region. Highly mesic sites may have little or no understory American chestnut survival (Griffin 1992a). Chestnut blight is endemic in these understory trees with about 15-20% blight incidence. When these areas are clearcut, American chestnut grows as rapidly as any hardwood in the clearcut (Smith 1977). This rapid growth is followed by a chestnut blight epidemic over a 10-year period when 90-100% of the chestnut trees are blighted. A great abundance of virulent ascospore inoculum develops from perithecia on hundreds of American chestnut stems in these clearcuts. Similar epidemics occur in blight-susceptible American chestnut plantations. Near the end of the epidemic, some trees exhibit superficial cankers which are associated with hypovirulent strains, some possibly originating from cankers in the understory American chestnuts (Griffin et al. 1983; Griffin et al. 1984). On xeric and intermediate sites, following stem death from blight, numerous stump sprouts develop, some of which are browsed by deer. On mesic sites, with high competition from hardwoods, few stump sprouts develop, almost all of which are browsed by deer. On these sites with great tree-growth potential, American chestnut rootstocks are completely lost. Survival of American chestnut over all sites was inversely related to basal area of competing hardwoods (Griffin et al. 1991). Light intensities were very low on sprouts at the base of the stump on the mesic sites (Griffin 1992b).

Forest management involving removal of competing hardwoods resulted in the development of superficial cankers that were associated with dsRNA-infected hypovirulent strains of the chestnut blight fungus (Griffin et al. 1991; Griffin et al. 1993). This management practice increased stem size, promoted mast production, and maintained the survival of chestnut stems for several years beyond that found in check plots having no removal of competing hardwoods. The greatest blight control was found on a mesic site that was clearcut and a mesic plantation site (Griffin et al. 1991). However, at all locations blight control eventually broke down. Blight control also broke down in clearcuts where hypovirulent strains were artificially introduced. Research indicated that this breakdown of biocontrol was associated with the following: 1) the high blight susceptibility (quick kill) of forest American chestnuts; 2a) the secondary colonization of superficial (hypovirulent) cankers by virulent strains in diverse vegetative compatibility types that were generated in the clearcut or plantation; 2b) the development of new killing cankers elsewhere on the chestnut stem by virulent strains; 3) the slow spread of hypovirulence; and 4) breakdown at high altitude of superficial (hypovirulent) cankers over winter (Griffin et al 1993; Griffin and Griffin 1995). In the absence of hardwood management, even in unmanaged plantations, factor 5) is high hardwood competition and the associated reduced light. All lead or contribute to chestnut stem death. Using artificially introduced hypovirulent strains in forest situations, others have also found blight control to be either unsuccessful (Liu et al. 2002) or partial (Anagnostakis 2001). Further, introduced European hypoviruses (CHV1) did not persist at sites where they were introduced (Liu et al. 2002; Peever et al. 1997).

In our study, the clearcut and plantation blight-susceptible trees grew at altitudes ranging from 2,000 to 3,500 feet. In contrast, some blight-susceptible American chestnuts naturally infected with hypovirulent strains at low altitudes (< 1,000 feet elevation) have exhibited durable blight control, even in the absence of blight resistance (Griffin et al. 1983; Griffin and Griffin 1995). When pure cultures of hypovirulent strains and bark plugs from a low altitude tree were inoculated into American chestnut trees at high altitude (3,500 feet in elevation), superficial cankers were produced during the growing season. However, the hypovirulent strains colonized the cambium over the winter, causing a breakdown in the canker superficiality rating and biocontrol (Griffin and Griffin 1995). Tests indicated that the hypovirus in the hypovirulent strains survived the winter. These trees died as the cambium was completely killed. Conversely, the original low-altitude American chestnut tree (hypovirulence source) still exhibited stable blight control as of March, 2004 (unpublished).

As indicated above, physiological stress in chestnut species may occur at high altitudes, from early frosts, and in frost pockets. At these sites, blight severity can be very high even on the highly blight-resistant Chinese chestnut and some stems can be killed (Berry 1951; Headland et al 1976; Jones et al. 1980). Other studies have indicated that American chestnut at high altitude sites are under physiological stress. This is indicated by greatly increased electrolyte leakage from bark tissues collected from high altitude (3,900 feet) versus low altitude (530 feet) sites (Griffin 2000). The above findings suggest that the blight control breakdown at high-altitude clearcuts described above may be related in part to physiological stress. Some large-surviving American chestnut trees have been found at elevations higher than 4,000 feet, and these trees, adapted to blight at these elevations, may be useful for breeding and integrated blight control at higher elevations. High elevation sites (>2,500 feet elevation) may account for the bulk of the surviving population of understory American chestnuts in Virginia

Physiological stress on American chestnuts may also occur during the summer drought periods commonly encountered in the former oak-chestnut forest region (Braun 1950). Gao and Shain (1995) found drought stress may contribute to blight susceptibility. Additionally, Anagnostakis (2001) found that in Connecticut forest plots, where hypovirulence was artificially introduced, American chestnut trees with many cankers often died in the summer following drought the previous year. As indicated above, biocontrol associated with natural hypovirulence was less on managed, xeric clearcut sites than on managed, mesic clearcut sites (Griffin et al. 1991). Xeric slope sites frequently have small American chestnut stumps and very high population densities of understory American chestnuts that are associated with a less dense canopy. This can lead to the false conclusion that they are the best chestnut restoration sites. In contrast, mesic shallow cove sites on slopes had large American chestnut stumps (up to 3-4 feet in diameter) with moderate numbers of understory American chestnuts (Griffin 1992a). Deep cove sites had medium-sized stumps and little or no understory American chestnuts. Mesic shallow cove sites on slopes have great potential for chestnut growth, integrated blight control with resistance, hypovirulence, and forest management, chestnut restoration, and natural regeneration of American chestnut. Often they have deep, fertile soils. Large-surviving American chestnuts at high altitude (>2,500 feet) may be useful for breeding and integrated control at higher elevation, mesic sites.

CONCLUSIONS

Research indicated that the survival of most large surviving American chestnut trees was associated with resistance, hypovirulence, and favorable sites. Our ACCF breeding program utilizes controlled intercrosses of these trees, which has resulted in progeny with acceptable levels of resistance. These progeny trees have been used in further breeding. This may result in trees with additive blight resistance. Some American chestnuts in a very large population (135,123 trees), derived from open pollinations of large survivors, have shown promising levels of field blight resistance. This large population may serve as a source of genetic diversity in future restoration efforts. The high blight susceptibility of forest American chestnuts, along with the abundance of a vc-diverse, virulent inoculum of *C. parasitica* has severely limited blight control and the use of hypovirulence in forest clearcuts and plantations. High altitude, low temperature stress, drought stress, and hardwood competition are additional factors that inhibit blight control and the use of hypovirulence. However, a long term (> 20 years) and high level of blight control has been obtained on mesic, hardwood-managed sites. These sites were established with blight-resistant American chestnut trees that were inoculated with hypovirulent strains of *C. parasitica*. Nucleotide sequence analysis indicated that blight control was associated with the spread of Italian CHV1. Some blight resistance may be needed in American chestnut to allow time for hypoviruses to spread. Site selection and removal of competing hardwoods may be critical forest management practices needed for blight control. Mesic shallow coves on lower altitude slopes are among the best sites to implement integrated use of resistance, hypovirulence, and forest management for blight control and restoration of American chestnut. High-altitude (>2,500 feet), large-surviving American chestnuts may be useful for breeding and integrated control at higher elevation, mesic sites.

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