HOST-PATHOGEN INTERACTIONS IN A SEGREGATING POPULATION OF BC_2F_2 HYBRID *CASTANEA DENTATA* AND OTHER CHESTNUT HYBRIDS AFTER EXPOSURE TO HYPOVIRUS-CONTAINING AND HYPVIRUS-FREE STRAINS OF *CRYPHONECTRIA PARASITICA*

Thesis Presented for the

Master of Science Degree

The University of Tennessee at Chattanooga

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December, 2003

To the Graduate Council:

I am submitting herewith a thesis written by Stephen Henry Alexander entitled "Host-Pathogen Interactions in a Segregating Population of BC_2F_2 Hybrid *Castanea dentata* and Other Chestnut Hybrids After Exposure to Hypovirus-Containing and Hypovirus-Free Strains of *Cryphonectria parasitica*." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science degree, with a major in Environmental Science.

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We have read this thesis and recommend its acceptance:

[redacted]

Accepted for the Council:

[redacted]

Dean of the Graduate School

HOST-PATHOGEN INTERACTIONS IN A SEGREGATING POPULATION OF BC_2F_2 HYBRID *CASTANEA DENTATA* AFTER EXPOSURE TO HYPOVIRUS-CONTAINING AND HYPVIRUS-FREE STRAINS OF *CRYPHONECTRIA PARASITICA*

Abstract

An F_2 population of second backcross interspecific hybrid chestnut trees (BC_2F_2) and the progeny of the Chattanooga Chestnut Tree Project (CCTP) breeding program were exposed to three isogenic strains of *Cryphonectria parasitica*. Strain Ep155 (virus-free) was used to screen the 4 – 7 year old chestnut trees for resistance to chestnut blight disease. Two virus-containing, hypovirulent strains, Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713), were used to investigate interactions of host disease resistance and expression of hypovirulence. The BC_2F_2 population varied from highly susceptible to highly resistant, as measured by Ep155 (virus-free) canker length at 95 days. Disease resistance in the CCTP population varied from highly susceptible to intermediately resistant. Ep155(CHV1-Ep713) cankers were significantly smaller than cankers caused by Ep155(CHV1-Euro7) and Ep155 (virus-free). Investigation of the interactions of host disease resistance and expression of hypovirulence is currently in progress at the CCTP breeding orchard.

Dedication

This thesis is dedicated to my father, Douglas R. Alexander (1950-1999), for being my best friend and greatest role model, and to my mother, Karen H. Alexander, for supporting, encouraging, and praying for me throughout my life. I also dedicate this thesis to Jesus Christ for blessing me with this wonderful opportunity and giving me the strength to complete it.

Acknowledgements

I wish to thank all those who helped me complete my Master of Science degree in Environmental Science. I am very grateful to Dr. J. Hill Craddock, the chairperson of my thesis committee, for his willingness to teach me, all his help in the orchards measuring cankers, and his friendship. I thank Dr. Fred Hebard for being on my thesis committee and for all of his help, advice, and time. I also thank Dr. David Aborn for being on my thesis committee and for his helpful insight. I thank Dr. Mark Double and Dr. Bill MacDonald for providing me with the fungal isolates used in this study and for their advice. I am thankful to my brother Mark Alexander for his priceless help and encouragement throughout graduate school and my chestnut research. Finally, I would like to thank my wife Carrie for being my number one supporter.

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I. INTRODUCTION

A. The Chestnut Tree

American chestnut (*Castanea dentata* [Marshall] Borkhausen), a member of the beech family (Fagaceae), once comprised up to 25 percent of canopy trees in some eastern hardwood forests of North America (Ashe, 1911). Its natural range included over 200 million acres of land extending from Maine and Southern Ontario to Alabama and Mississippi (Saucier, 1973). Mature American chestnut trees reached 60-120 feet in height with straight boles up to seven feet in diameter (Ashe, 1911). The leaves are thin, hairless, non-glossy, and are coarsely serrated with a pointed base (Fulbright, 2003). In addition to having had a faster growth rate than many of its associated hardwood species, the American chestnut formed sprouts from the root crown collar after cutting. They did not require replanting after they were harvested for timber, and the roots effectively prevented soil-erosion (Ashe, 1911; Anagnostakis, 1987).

The wood from American chestnut is easily split, seasons well, and is extremely resistant to decay possibly due to tannins found in both bark and wood. These characteristics made the American chestnut an extremely valuable species environmentally and economically. Some of the many uses for chestnut wood included construction, woodwork, furniture, fencing, boxes, barrel staves, railroad ties, ship masts, telegraph poles, mine timbers, and musical instruments. Chestnut extracts also provided tannin for leather processing (Saucier, 1973; Kuhlman, 1978; Anagnostakis, 1987).

The American chestnut blooms in June (later than oaks and hickories and after the danger of late spring frosts) and produces consistent nut crops. *C. dentata* seedlings usually begin bearing at about 10 years of age. Their nuts (3 per bur) are sweet tasting

and have a thin pellicle that is easily removed (Fulbright, 2003). This annual bearing provided a dependable cash crop for many families and also lent stability to forest wildlife populations (Burnham et al., 1986).

Related chestnut species were also important in other parts of the world. European chestnut (*Castanea sativa* Mill.) was introduced into the high mountains and orchards of Europe by the Romans from Minor Asia because of its many desirable qualities (Paglietta and Bounous, 1979; Bounous, 1999). Grafted, nut bearing cultivars of European chestnut can live productively for many hundreds of years (Tani and Canciani, 1993). *C. sativa* was highly valued because of its economic and aesthetic importance (Paglietta and Bounous, 1979; Bassi, 1990; Bourgeois, 1992; Bounous, 1999). *C. sativa*, like *C. dentata*, grows rapidly and forms a large, straight bole up to 100 feet tall and 4 feet in diameter (Fulbright, 2003). The trees have been cultivated throughout the mountainous areas of Southern Europe, the southern foothills of the Alps from Italy into Hungary, and along the Black Sea (Bassi, 1990; Bourgeois, 1992; Bounous, 1999). Similarly, Chinese chestnut (*Castanea mollissima* Bl.) and Japanese chestnut (*Castanea crenata* Sieb. & Zucc.) have been very important to the economies and cultures of East Asia (Rutter et al., 1991; Fulbright, 2003). These Asian species typically grow with a spreading, rounded crown, and mature at about 50 feet tall or less (Jaynes, 1979). Due to the relatively short stature of *C. mollissima* and *C. crenata*, they are not highly valued for their timber, but are good nut producers (Jaynes, 1979). Chinese and Japanese chestnuts have been cultivated as a prized food crop for thousands of years, and China ranks as the world's leading chestnut producer (Fulbright, 2003).

B. Chestnut Blight in America

At the turn of the twentieth century, both the American and European chestnut flourished. However, in 1904 a new fungus, (*Cryphonectria parasitica* (Murr.) Barr [*Endothia parasitica* (Murr.) P. J. and H. W. Anderson]) was discovered on dead and dying American chestnut trees in New York City (Merkel, 1906; Griffin, 2000). *C. parasitica* causes the devastating disease now known as chestnut blight. The fungus was also identified in Asia and many researchers believe that *C. parasitica* was possibly imported to the U. S. on Japanese nursery stock (Anagnostakis, 1987). Chinese (*Castanea mollissima* Blume) and Japanese chestnut (*Castanea crenata* Siebold & Zucc.) are resistant to chestnut blight; thus infections are easily unnoticed on these hosts (Clapper, 1952; Fairchild, 1913; Shear and Stevens, 1916; Anagnostakis, 1987; 1993). All attempts at controlling the spread of the fungal epidemic failed. It has been estimated that the disease progressed through the entire native range of *C. dentata* at the rate of about 23 miles per year. Within 50 years of the introduction of this pathogenic fungus to the United States, most *C. dentata* in the natural range were dead or dying from lethal blight wounds (Anagnostakis, 1987).

C. parasitica infects American chestnut trees through cracks and wounds in the bark, which often occur due to natural growth (Kuhlman, 1978). The infections result in sunken, necrotic bark cankers with exposed xylem that expand until they girdle the branch or trunk and kill all foliage distal to the infection. Epicormic sprouts usually arise from beneath the canker and maintain the vitality of the root system (Griffin, 1986). The high blight-susceptibly of *C. dentata* allows for fast fungal growth and formation of abundant sexual and asexual spore-producing bodies (stromata) on the bark surface

(Griffin, 2000). Imbedded within the stromata are flask-shaped perithecia containing ascospores (sexual spores) and pycnidia containing conidia (asexual spores). Ascospores are the product of sexual reproduction and thus are genetic individuals, different from the strains that produced them, and contain only a tiny amount of cytoplasm. Ascospores are mainly wind disseminated. The forcible expulsion of the ascospores from the ostiole, located at the end of the perithecial neck, can be triggered by a very small amount of rain. Wind can carry these spores to other surrounding American chestnut (Moore-Landecker, 1996; Griffin, 1986; Anagnostakis, 1987; Griffin, 2000). In moist weather, stromata initially contain pycnidia, which extrude conidia (asexual spores) in long chains or tendrils. Conidia are the product of non-sexual reproduction and thus are genetically identical to the strain that produced them, contain more cytoplasm than ascospores, and are embedded in a viscous matrix. They are more resistant to desiccation than ascospores and are commonly disseminated by the splashing of rain or by insect and other animal vectors (Roane et al., 1986; Griffin, 1986). Blight infection may be initiated when either ascospores or conidia come in contact with bark wounds (Garrod, 1985).

As an infection begins and the fungal spore starts to germinate, the chestnut responds by forming a wound periderm-induction barrier that is impermeable to water. This barrier limits the growth and elongation of individual hyphae, but not mycelia fans (Hebard, 1984). The hypha is the branching tube-like cell of the fungus; the mycelium is a mass of hyphae. The growth of mycelial fans in areas of chestnut bark where wound periderm has not fully developed causes the canker to expand (Hebard, 1984). Death of parenchyma cells in the bark of the chestnut tree has been observed at least 350 μm in advance of radiating mycelial fans (Hebard, 1984). When wound periderm of the

chestnut has fully formed, it effectively stops the growth of mycelial fans in chestnut bark. Unfortunately, optimal pH levels in the inner bark allow *C. parasitica* to produce enzymes that depolymerize the tree cell wall components at a rate that is faster than the formation of wound periderm (Anagnostakis, 1987). Polygalacturonase diffuses from the fungal mycelium into healthy tissue of the tree, causing the polypectate of the middle lamella to depolymerize (Albersheim, 1978; Goa et al., 1996). The degradation of the middle lamella exposes cell wall polysaccharides to the compound oxalic acid, which depolymerizes calcium salts of the polypectate (McCarrol et al., 1978; Vannini et al., 1993). This mycelial advancement in the bark leads to death of all foliage distal to the infected trunk or branch by girdling of the vascular cambium. Epicormic sprouts from beneath the canker keep the root system alive and perpetuate the disease cycle (Elliston, 1982; Kuhlman, 1983; Anagnostakis, 1987).

Today, the *C. dentata* populations in North America exist as small clusters of stump sprouts, mostly smaller than 3 cm diameter at breast height, dbh, in the understory and in clear cut areas of the eastern hardwood forests (Craddock, 1998; Griffin, 1989; Garrod, 1985). There is an abundance of virulent (lethal) blight spores in American forests (Hogan and Griffin, 2002). If a forest is clear-cut, blight incidence has been reported to reach 90-100% in the *C. dentata* population within 10 years. Once blight has developed, it only takes 1-2 years for almost all blighted stems to die (Hebard, 1982). Although the life of *C. dentata* is perpetuated by root sprouts, this rapid cycle of infection prevents most *C. dentata* from bearing nuts or out-competing the surrounding vegetation. Therefore, genetic diversity is being lost and there is danger of possible extinction due to habitat loss (Craddock, 1998).

C. Chestnut Blight in Europe

In addition to infecting American chestnut trees, *C. parasitica* also causes blight on the slightly more resistant *Castanea sativa* (European chestnut) (Graves, 1950; Berry, 1960; Hebard, 1982). Chestnut blight was officially recorded in Europe in 1938, near Genoa, Italy (Biraghi, 1946). The fungus proceeded to cause a blight epidemic in Europe that was much like the one in America, but not as severe. Antonio Biraghi, an Italian pathologist, followed the progress of the disease, discovering that it often went unnoticed due to death of chestnuts by ink disease caused by *Phytophthora* spp. (Bourgeois, 1992; Heiniger and Rigling, 1994). Chestnut blight reached France about 1946 and was identified in Canton Ticino in southern Switzerland in 1947 (Bazzigher, 1981; Heiniger and Rigling, 1994). By 1967, chestnut blight affected most, but not all areas where *C. sativa* grew in Europe (Paglietta and Bounous, 1979; Anagnostakis, 1987; Bourgeois, 1992; Heiniger and Rigling, 1994). In addition to socioeconomic changes due to World War II, the devastation caused by the introduction of *C. parasitica* resulted in a rapid decline in chestnut cultivation in many regions of Europe (Bounous, 1999).

D. Hypovirulence

A glimmer of hope came in 1951 when Biraghi discovered a noticeable improvement in the condition of blighted Italian chestnut stands (Biraghi, 1953). Then in 1964, the French mycologist Jean Grente isolated atypical strains of *C. parasitica* from cankers that appeared to be healing near Como, northern Italy and grew them on potato dextrose agar amended with methionine and biotin (Grente, 1965). He described the cultures as a variety of unusual strains of *C. parasitica* that, when compared to normal virulent strains of *C. parasitica* in culture, were reduced in pigmentation, sporulation, and

growth rate (Anagnostakis and Aylor, 1984; Fulbright and MacDonald, 1991). Grente (1965) called these abnormal strains "hypovirulent."

The swollen, callused cankers that are associated with these hypovirulent strains (h-strains) of *C. parasitica* are non-lethal to chestnut trees because they are superficial. This means they are swollen rather than sunken and restricted to the outer parts of the bark, leaving the vascular cambium unharmed (Elliston, 1985; Griffin, 1986). Some of the superficial cankers may have a central area of exposed xylem where *C. parasitica* successfully colonized and killed some of the bark tissue extending down to the vascular cambium. As tree resistance mechanisms restrict fungal growth, layers consisting of differentiated xylem and phloem form swollen ridges of callus around the exposed xylem (Griffin, 1986). Necrotic portions of these callused ridges colonized by *C. parasitica* often flake off and leave gnarly scars behind. Griffin describes some older trees that have been fighting fungal infection for many years as having cankers that are grotesque in appearance (Griffin, 1986).

In the mid 1970s, researchers were actively investigating the potential of these unique strains as biological control agents of chestnut blight. Hypovirulence was discovered to be associated with transmissible determinants in the cytoplasm of h-strains. Day et al. (1977) identified double-stranded RNA (dsRNA) segments located in the cytoplasm of several Italian, French, and American h-strains (Moffitt and Lister, 1975; Day et al., 1977). This dsRNA was not encapsidated in a protein coat like most fungal viruses (mycoviruses); rather, it was bound in pleiomorphic vesicles constructed of host-derived lipids (Hansen et al., 1985; Newhouse et al., 1990; 1983; MacDonald and Fulbright, 1991). Dodds (1980) reported that h-strains frequently contain multiple

segments of dsRNA which may vary in size and number within and among isolates. The dsRNA segments derived from a French hypovirulent strain have been characterized in great detail (Nuss, 1992). They consist of one large dsRNA (L-dsRNA) that contains two continuous open reading frames (ORF A and ORF B) and multiple defective interfering segments (Shapira et al., 1991; Nuss, 1992). Several studies analyzing the genetic organization, expression, and replication strategy of the L-dsRNA suggest that it is viral in origin (Choi, 1991a; Choi, 1991b, Fahima et al., 1993; Shapira et al., 1991). Fulbright (1984) demonstrated that elimination of dsRNA from h-strains of the fungus with cycloheximide was accompanied by a dramatic increase in fungal virulence. Then an experiment by Choi and Nuss (1992b.) involving the introduction of L-dsRNA into *C. parasitica* via DNA-mediated transformation established that the dsRNA is indeed the causal agent of hypovirulence (Choi and Nuss, 1992b.).

The International Committee on Taxonomy of Viruses has since established the family *Hypoviridae*, which is the first virus family without structural proteins (Hillman, 1995). To date, the family *Hypoviridae* contains the single genus Hypovirus and four hypovirus species, *Cryphonectria hypovirus* 1, 2, 3, and 4 (CHV1, CHV2, CHV3, and CHV4) (Hillman et al., 1995; 2000; Liu et al., 2002). The contribution of the defective interfering dsRNAs to the expression of the hypovirulent phenotype is not clear. Chen et al. (1993), however, found no phenotypic changes associated with the appearance of defective dsRNAs. The geographic origin of *Cryphonectria hypovirus* dsRNA is unknown (Elliston, 1982), but dsRNA containing strains of *C. parasitica* have been detected in parts of Asia and most likely were imported to the U. S. and Europe along with normal virulent strains (Elliston, 1982; Heiniger and Rigling, 1994).

While researching the histopathological development of cankers caused by h-strains, Hebard et al. (1984) reported that the formation of mycelial fans (an important step in virulence expression, as mentioned previously) as being reduced in hypovirulent strains. Molecular analysis of h-strains indicated that in dsRNA infected strains, the accumulation of specific mRNAs and polypeptides in the fungus is reduced (Powell and Van Alfen, 1987). Accumulation of the metabolite oxalate (oxalic acid depolymerizes calcium salts of the polypectate) has been found to be reduced in dsRNA-containing strains of *C. parasitica* (Havir and Anagnostakis, 1983). Some of the proteins that are less abundant in dsRNA-containing strains include an extra- and intracellular laccase (Rigling and Heiniger, 1989; Larson et al., 1992; Ringling and Van Alfen, 1993), a cell-surface protein (Carpenter et al., 1992), a cutinase (Varley et al., 1992), and a putative mating-type pheromone (Zhang et al., 1993). Expression of a specific viral coding domain (ORF A) was found to be the cause of reduced pigmentation, sporulation, and laccase accumulation of the fungus, but not the cause of reduced virulence (Choi and Nuss, 1992a.; Heiniger and Rigling, 1994).

Since dsRNAs are located in the cytoplasm of h-strains, they can be transmitted into the asexual conidia produced by *C. parasitica* (Turchetti and Maresi, 1991), but almost never into the sexual ascospores (Anagnostakis, 1988; Chen et al., 1993). Thus, sexual reproduction in *C. parasitica* populations has a negative impact on the dissemination of hypovirulence in nature (Anagnostakis, 1988). In a phenomenon described as "exclusive hypovirulence," hypovirulent strains can convert virulent strains of the same vegetative compatibility genotype (v-c type) to hypovirulent strains by the cytoplasmic transfer of dsRNA via hyphal anastamosis (Anagnostakis and Day, 1979;

Anagnostakis, 1981; Bazzigher et al., 1981; Griffin, 1986; Fulbright, 1999). Anagnostakis and Day (1979) paired virulent and hypovirulent strains from the same v-c group and showed that the virulent strains always converted easily to the hypovirulent phenotype. This included the transfer of the hypovirulence-associated traits such as reduced virulence, reduced pigmentation, and culture morphology to the virulent strain (Anagnostakis and Day, 1979).

Cryphonectria parasitica, like most ascomycetes, has a system of vegetative incompatibility controlled by allelic interactions in which two individuals are compatible only if they share the same alleles at all *vic* (vegetative incompatibility) loci (Cortesi and Milgroom, 1998). Therefore, individuals are vegetatively incompatible when alleles are different at one or more *vic* loci. The diversity of v-c types in a population is a function of allelic diversity and recombination among *vic* loci. A high number of v-c groups occur in North America on *C. dentata* (Anagnostakis and Day, 1979; Kuhlman and Bhattacharyya, 1984; Martin, 1991; MacDonald and Fulbright, 1991; Milgroom and Cortesi, 1999; Marra and Milgroom, 2001; Hogan and Griffin, 2002). The transfer of dsRNA is not always prohibited between strains in different v-c types, but it occurs more slowly and less frequently (Anagnostakis and Day, 1979). If the number of alleles in common between the two strains decreases, so does the frequency and duration of anastamoses and the frequency of transmission of hypovirulent agents (Liu and Milgroom, 1996; Milgroom and Cortesi, 1999). In this case, temporal anastamoses might allow the dsRNA to pass before the incompatibility reaction kills the fused cells (Heiniger and Rigling, 1994). When strains from differing v-c types interact on agar media, a barrage line that is densely packed with pycnidia and conidia forms between the

different strains, preventing anastamosis and successful formation of the heterokaryon (a cell containing genetically different nuclei) (Anagnostakis, 1977). Thus the population structure, with special regards to v-c types, is an important factor influencing the success of hypovirulence transmission in *C. parasitica* populations (Bissegger et al., 1997).

Wide variation has been observed in the expression of hypovirulence-associated phenotypes in dsRNA-containing strains (Peever et al., 2000). The typical white appearance of European dsRNA-containing strains grown in culture has been widely used to distinguish between v-strains and h-strains of *C. parasitica*. However, most naturally occurring North American h-strains are pigmented, making them indistinguishable from v-strains in culture; thus, culture morphology is not always a reliable means of identifying h-strains (Griffin et al., 1978; 1983; Jaynes and Elliston, 1982; Double et al., 1985; Griffin, 1999; 2000). In these cases, pathogenicity tests or analysis of dsRNA is required for identification of h-strains (Griffin, 1999). Variation is also reported in the amount of dsRNA present in different hypovirulent strains (Dodds, 1980). These characteristics give h-strains unique dsRNA banding patterns, observable after electrophoresis in polyacrylamide gels, and provide researchers with a means of tracking persistence and dissemination of individual h-strains released into the field (Dodds, 1980; Garrod, 1985).

Differences in the expression of hypovirulence in dsRNA-containing strains have been reported to range from avirulent to levels of almost normal virulence (Griffin et al., 1977; Elliston, 1978; Rigling and Heiniger, 1989; Peever et al., 2000). One can characterize and compare the virulence of field isolates by measuring canker size, reproductive capacity, or both (Macdonald and Fulbright, 1991). Chen et al. (2000a.)

described two European *Cryphonectria* hypoviruses that differ in their effect on the virulence of the fungus: French derived *Cryphonectria hypovirus* 1 strain Ep713 (CHV1-Ep713) and Italian derived *Cryphonectria hypovirus* 1 strain Euro7 (CHV1-Euro7). *C. parasitica* strains infected with CHV1-Ep713 formed small superficial cankers with little or no asexual spore-forming pycnidia and were severely compromised in their ability to expand on chestnut tissue. In contrast, CHV1-Euro7-infected strains exhibit an aggressive colonization of chestnut tissue early after inoculation that is comparable to normal v-strains. The resulting canker face has rigid margins and a significant level of spore-forming stromata. They are reported to attain sizes three to four times larger than cankers produced by CHV1-Ep713-infected strains before the canker expansion abruptly ceases (Chen et al., 2000a.). *C. parasitica* strains infected with hypoviruses like CHV1-Euro7 require field tests spanning more than one growing season for their reduction in virulence to be detectable (Elliston, 1982).

E. Hypovirulence as a Biological Control

Grente and Berthelay-Sauret initiated field trials from 1967 to 1972 in 12 chestnut orchards in southern France to test the potential for using hypovirulent strains as a biological control (Grente and Berthelay-Sauret, 1978). On an area of 20 hectares, 200 cankers were inoculated in the fall with one compatible hypovirulent strain. Most treated cankers did not expand until the next spring and none were lethal; this was not the case with the untreated cankers. After four years, nontreated cankers also showed signs of healing. Mortality decreased within a radius of 5 m from the treated cankers and new healing cankers appeared. These results suggested positive dissemination of h-strains among trees. Several successful field applications followed in different regions of France

(Heiniger and Rigling, 1994). The results were so promising that a biocontrol program, supported by the Ministry of Agriculture, was established in France to assist chestnut growers (Grente and Berthelay-Sauret, 1978; Heiniger and Rigling, 1994).

The French developed a protocol for field application of h-strains as a biocontrol and recommended it for commercial use by chestnut growers in France. Identification of the specific *C. parasitica* strain(s) infecting the proposed treatment area was necessary to determine the v-c type(s). Mixtures of compatible h-isolates were produced in the lab and packed into tubes for distribution and application. In the orchards, the edges of the cankers are defined by removing a thin layer of bark around the canker perimeters. Small plugs of bark (2-3cm apart) were removed from the exposed canker perimeter and the h-mixture was applied into the holes (Heiniger and Rigling, 1994).

Biological control and natural spread of hypovirulence is reported to control chestnut blight in Italy, France, and Switzerland (Turchetti and Maresi, 1993; Bissegger et al., 1997; Robin et al., 2000). Natural dissemination of hypovirulence has allowed many chestnut stands to recover, but field tests revealed that the persistence and dissemination of hypovirulent strains in forest stands is not reliable, especially when the forests are poorly managed (Griffin, 1986). Blight control, however, is possible in well-maintained orchards, where all cankers are treated with an appropriate mixture of hypovirulent strains (Heiniger and Rigling, 1994). Studies in chestnut coppices of Switzerland suggest that hypovirulence plays an important role in the decline of disease severity (Bissegger et al., 1997). Hypovirulence conversion studies revealed that vegetative incompatibility is not a major factor preventing the spread of dsRNA in Switzerland, Italy, or France where biocontrol efforts have been successful (Maresi et al.,

1995; Bissegger et al., 1997; Robin et al., 2000). Renewed interest in chestnut and its economic importance has led to a revival of chestnut cultivation in Europe (Bassi, 1990; Bourgeois, 1992; Tani and Canciani, 1993). Selected nut-producing varieties of *C. sativa* are again cultivated in plantations across the European landscape (Bassi, 1990; Bounous, 1999; Heiniger and Rigling, 1994). The Italian chestnut trees recovering from blight are again a source of timber and nuts for domestic use and export (Bourgeois, 1992; Heiniger and Rigling, 1994).

This European chestnut recovery is contradictory to what we see in the natural range of *C. dentata* in the U.S. where a high number of v-c types is considered a major barrier to the natural spread of h-strains in the wild (Kuhlman and Bhattacharyya, 1984). Despite extensive effort, long term biological control of chestnut blight using h-strains have not been successful within the natural range of the American chestnut (Dierauf et al., 1997). Work with hypovirulence as a biological control in North America began in 1972 when J. Grente sent European h-strains to the Connecticut Agricultural Experiment Station (Van Alfen et al., 1975). Initial work was restricted to laboratory and greenhouse testing only, under quarantine, but encouraging results led to permission for field testing (Anagnostakis, 1987). Jaynes and Elliston (1980) demonstrated that combinations of European and American h-strains, exhibiting varying pathogenicity levels, were more effective in controlling individual blight cankers than single h-strain treatments (Jaynes and Elliston, 1980). It was assumed necessary to include h-strains that resulted from conversion of a local v-strain in the mixtures. These mixtures of h-strains were also shown to convert incompatible v-strains more frequently in the trees than on agar in the lab (Anagnostakis and Day, 1979). Chestnuts in the plots treated by Jaynes and Elliston

generally grew and survived better than chestnuts in the surrounding untreated areas, and some blight control was reported as much as nine years after inoculation. Unfortunately, there has been no evidence that the original h-strains were able to spread and prevent new infections in the Connecticut treatment plots (Anagnostakis, 1987; 1990; 2001).

Wiley (1982) inoculated chestnut trees in West Virginia with h-strains before they were naturally infected by a local, wild v-strain (Wiley, 1982). Natural dissemination of h-strains was observed for several years, but all the trees have since died due to an overwhelming number of new infections (MacDonald and Fulbright, 1991; Li et al., 1997). Persistence of these strains in the forest sites was evaluated by Liu et al. (2002) who confirmed the presence of several dsRNA containing strains of *C. parasitica* in the treatment area, but not of the same v-c type as the original isolates released from 1978-1982 (Liu, et al., 2002).

Another study done on *C. dentata* in West Virginia conducted by Double et al. (1985) resulted in the isolation of several wild *C. parasitica* strains with abnormal culture morphology. Nine of twenty-one abnormal isolates examined via polyacrylamide gel electrophoresis contained dsRNA banding patterns. This discovery indicated that dsRNA-containing stains of *C. parasitica* may be common in the natural range of *C. dentata* (Double et al., 1985).

Amidst some encouraging results, many barriers need to be overcome before hypovirulence can be considered a useful tool for disease management within the natural range of *C. dentata*. Research involving the persistence and spread of hypovirulence among American *C. parasitica* populations is critically important to the development of

an effective biological control (Kuhlman, 1983). This is a daunting task due to the massive population decline of *C. dentata* in the United States.

Discoveries of American chestnuts outside the natural range have provided many unique opportunities for research using h-strains as a biological control. American chestnuts were transported all over the country by early settlers. After the blight epidemic reduced chestnut within the natural range to understory shrubs, large surviving American chestnut trees were identified in places like upper Michigan, Wisconsin, Minnesota and Iowa (Griffin, 1986). Many of these trees survived in isolation beyond the limit of the blight epidemic that was going on within the natural range of the American chestnut. Today blight has reached some of these locations, and they are sites for ongoing biological control research and experimentation.

One example of isolated populations of chestnut trees in the United States is the chestnut groves found in Michigan. To date, there have been more than 30 American chestnut stands containing large mature trees, saplings, and seedlings reported in Michigan (MacDonald and Fulbright, 1991). Hypovirulent strains have been found to occur naturally, causing superficial cankers and recovery from chestnut blight in some of these Michigan groves (Brewer, 1982; Fulbright et al., 1983). The natural biological control of blight and associated American chestnut survival appear to be much greater in Michigan than within the natural range of *C. dentata* (Fulbright et al., 1983; Garrod et al., 1985; Fulbright and MacDonald, 1991). Spread of virulent and hypovirulent strains within and between trees in the groves has been detected. In general, this spread involved the hypovirulent inoculum somehow moving at least 110 cm down a tree trunk, encountering a wound, and initiating a canker (Garrod et al., 1985).

A cytoplasmically transmissible hypovirulence has also been identified in dsRNA-free strains of *C. parasitica* isolated from healing cankers on American chestnut trees in southwestern Michigan (Fulbright, 1985; Baidyaroy et al., 2000). This unique form of hypovirulence is associated with a modification in the mitochondrial chromosomes. This study revealed that mitochondrial hypovirulence can occur spontaneously and spread within a natural *C. parasitica* population (Baidyaroy et al., 2000).

A large-scale experiment to deploy hypoviruses as biocontrol agents for blight is currently underway in a forest stand of American chestnut growing near West Salem, Wisconsin. The study began in 1992-1994 when cankers in designated treatment plots were treated with the hypovirulent strain CHV3-COLI. The virus was so debilitating to the *C. parasitica* that the fungus grew very slowly, had poor spore production, and persistence was limited (Double and MacDonald, 2002). The failure of CHV3-COLI to persist led to the use of another isolate that grew better in the bark of the tree. From 1995 to 1997, and again in 2003, cankers in designated treatment plots were treated with an h-strain containing CHV1-Euro7 (MacDonald et al., 2003). The original hypovirulent *C. parasitica* strain infected with CHV1-Euro7 was isolated in 1978 by Dr. William MacDonald (West Virginia University) from a superficial canker on a European chestnut coppice sprout in a forested area approximately 30 km north of Florence, Italy. This strain was the source of hypovirus CHV1-Euro7 RNA, which is widely used in biocontrol experiments (Chen and Nuss, 1999). Subjective canker ratings have shown that hypovirus treated cankers improved in canker morphology over the course of the study. Good dissemination has been reported from CHV1-Euro7 treated cankers to untreated

cankers on the same tree, but not to cankers on untreated trees in the West Salem stand (Liu et al., 1997; MacDonald et al., 2003).

As mentioned previously, few large American chestnut trees, greater than 10 inches diameter at breast height (dbh), have survived blight within the natural range. Griffin's research on the largest survivors in the natural range has identified three factors associated with survival: (1) low levels of blight resistance in the trees, (2) presence in cankers of low to high percentages of hypovirulent strains of the blight fungus (strains with reduced virulence) that are infected with hypoviruses (dsRNAs), and (3) favorable sites (Griffin et al., 1982; 1983). Survival of individual trees most often has been associated with two or all three of these factors (Griffin, 1999; 2000).

Some encouraging results within the natural range of *C. dentata* came from a study that began in 1980 when scions obtained from large, surviving American chestnut trees were used to establish grafted American chestnut trees at the Lesesne State Forest in Virginia (Elkins et al., 1980; Dierauf et al., 1997). In 1982 and 1983, natural blight cankers on the stems of these trees were inoculated with a mixture of European white and American pigmented hypovirulent strains of *C. parasitica*. The grafted trees at Lesesne State Forest have exhibited high levels of prolonged disease control, even in the presence of a large amount of virulent inoculum from the surrounding chestnut plantation (Dierauf et al., 1997; Griffin, 1999). In contrast to the adjacent stump sprouts, the grafted trees contained a high number of swollen, superficial cankers and consequently a low number of blight-killed branches. Bark cores that were extracted from the superficial cankers exhibited a high ratio of healthy to necrotic tissue (Dierauf et al., 1997). The white h-strains were reported to have spread to other parts on the grafted trees, but no evidence

supporting spread of the pigmented h-strains has been found (Dierauf et al., 1997). Hogan and Griffin (2002) reported dissemination of the white h-strains throughout the grafted trees and into 45 different v-c types of *C. parasitica*. This type of hypovirulence dissemination is an extremely rare occurrence in the natural range of the American chestnut. Further research involving the mechanisms that affect the spread of hypovirulent strains in and around these grafted trees may help explain the unusually high level of observed disease control (Dierauf et al., 1997; Griffin, 1999).

F. Successful Biocontrol of Blight in Europe and not North America

Several mechanisms are considered to play a role in the failure of h-strains to control blight in North America on *C. dentata* as they do in Europe on *C. sativa*. These factors include the difference in abundance of natural virulent *C. parasitica* inoculum, differing environmental and management conditions, different numbers of v-c types present, differences in the viral dsRNAs, and differences in host resistance to *C. parasitica* between *C. dentata* and *C. sativa* (Griffin, 1986; Robbins and Griffin, 1999).

The naturally occurring h-strains that have been found in America are reported to be highly variable in their expression of hypovirulence, and not readily disseminated in the *C. parasitica* populations (Double et al., 1985; Peever et al., 1997; Griffin, 1999). In contrast, the rate of the natural spread of hypovirulent strains through blighted European chestnut stands in France has been calculated as one to two meters per year (Kuhlman, 1978; Anagnostakis, 1987).

The amount of dsRNA present in European and American h-strains generally varies (Dodds, 1980). White hypovirulent strains in Italy, such as the reference strain CHV1-Ep713, contain high concentrations of dsRNA (Dodds, 1980). Most pigmented

American hypovirulent strains have been shown to contain dsRNA, but in much lower concentrations (Dodds, 1980). Some American hypovirulent isolates of *C. parasitica* have been found not to contain any detectable levels of dsRNA (Jaynes and Elliston, 1982; Griffin et al., 1983). It is possible that dsRNA was absent from the part of the thallus that was subcultured for assay in these trials, or there were other hypovirulence determinants at work (Jaynes and Elliston, 1982; Griffin et al., 1983).

Although environmental factors affect life-cycle of *C. parasitica*, Anagnostakis and Aylor (1984) showed that the differences in temperature between North American and European climates are not sufficient to explain why h-strains do not spread in the field under northeastern U. S. temperature conditions. American chestnut trees in the central and southern section of the natural range grow at relatively high altitudes. Griffin et al. (1993) and Griffin and Griffin, (1995) suggested that low temperatures, when combined with high altitudes, may further stress chestnut trees. The resulting lowered resistance may cause them to become even more susceptible to virulent and some hypovirulent strains of *C. parasitica*. Severe cankers have even been found on Chinese chestnut, which is normally highly blight resistant, growing in high altitude locations that have low temperatures (Jones et al., 1980).

Another environmental factor differing between *C. sativa* in Europe and *C. dentata* in North American is management practice. European chestnut trees are typically grown in orchards that are well managed. This seldom is the case within the natural range of the American chestnut (Griffin, 1986). Griffin et al. (1983, 1984) hypothesized that clearing all hardwood competition from around American chestnut in forest clearcuts may allow a natural succession of hypovirulence to start before all trees

in an open area are killed by blight. This hypothesis is supported by reports from Michigan and Italy where regeneration of the chestnut population and expression of hypovirulence has occurred in orchards where little or no competition from other plant species is a factor (MacDonald and Fulbright, 1991).

The high level of v-c type diversity found in North American *C. parasitica* populations is believed to significantly contribute to the reduced levels of hypovirus transmission between individual *C. parasitica* strains (Nuss, 2000). In Europe, v-c type diversity is low compared to v-c type diversity in the natural range of the American chestnut. This is due to a combination of lower v-c type diversity and limited sexual recombination of polymorphic *vic* loci in Europe (Anagnostakis, 1986; Bissegger et al., 1997; Heiniger and Rinling, 1994; Griffin, 2000). The successful transmission of hypovirulence in Switzerland has been attributed to a low number of v-c types and a low amount of sexual recombination among the *C. parasitica* population (Bissegger et al., 1997).

Possibly the most significant factor contributing to the survival of European and the few large surviving American chestnut trees is host resistance to *C. parasitica*. A study in Switzerland revealed that some cankers on trees in the Swiss research plots contained only orange isolates, but did not kill their host. This suggests that hypovirulence is not the only factor in disease control (Bissegger et al., 1997). The moderate host resistance difference between *C. sativa* and *C. dentata* is suspected to be responsible for the non-lethal affects of these orange isolates (Bissegger et al., 1997; Griffin, 1986; Griffin, 1999). Several studies on the relative resistance of different chestnut species to blight reported C*astanea sativa* as slightly higher in blight resistance

than *C. dentata* (Graves, 1950; Berry, 1960). If a higher level of host resistance could be integrated into the American *C. dentata* population, slower canker development and delayed mortality may allow more time for the hypovirulent strains to infect and subsequently convert cankers in areas where virulent inoculum is abundant (Brewer, 1982; Griffin, 1986; Heiniger and Rigling, 1994). Since the onset of blight in the United States, work has been in progress involving the incorporation of the blight resistant genes from Chinese and Japanese chestnut into the American chestnut genotype.

G. Breeding for Host-Resistance

Chestnut breeding in the United States began in the 1800s, the time of the first real interest there in chestnut as a nut tree (Jaynes, 1979). European chestnut cultivars were widely planted and grafted on native American sprouts (Bounous et al., 1993). Burbank in California and Van Fleet in Maryland had begun hybridization to improve the then available nut-bearing selections (Berry, 1978). Introduction of blight ended the American chestnut orchard industry (Jaynes, 1978). Breeding for timber types (forest trees like *C. dentata* plus blight resistance of *C. mollissima*) was begun in the early 1920s at the USDA, Beltsville, Maryland, where plant introduction, especially from East Asia, was actively pursued by the sponsoring of a series of important plant exploration missions to China (Thor, 1978; Wallace, 1987). Thousands of seeds and hundreds of cultivars of *Castanea mollissima*, *Castanea crenata*, *Castanea henryi* and related *Castanopsis* species were introduced into the U.S.A. in hope to attain blight resistance (Bounous et al., 1993).

Today The American Chestnut Foundation (TACF) uses a backcross breeding strategy in a continued effort to breed blight resistant American-type chestnuts for forest

outplanting (Hebard, 1994). TACF has breeding programs located throughout the natural range of *C. dentata* including Pennsylvania, Indiana, Maine, Tennessee and Virginia. Artificial inoculation with virulent strains is used in progeny tests to identify blight resistance (Hebard and Shain, 1989; Griffin, 2000). Ep155, the standard virulent isolate used in resistance screening, was originally obtained by Anagnostakis (Connecticut Agricultural Experiment Station) in 1977 from a canker on *Castanea dentata* in a field plot in Connecticut (Anagnostakis, 1992). The backcross method used by Dr. Fred Hebard (TACF staff scientist) in Meadowview, VA, entails crossing American and Chinese chestnut to obtain interspecific hybrid trees which are 1/2 American and 1/2 Chinese chestnut (F_1s). The F_1 hybrids are then backcrossed to the American chestnut to produce populations of seedling trees which are on average 3/4 American, 1/4 Chinese (BC_1s). The most blight resistant BC_1 trees are selected for a second cycle of backcrossing and selecting to produce trees which are on average 7/8 American, 1/8 Chinese chestnut (BC_2s). A third cycle produces populations of seedlings which are on average 15/16 American (BC_3s). The next step is to intercross the BC_3s to produce lines which breed true for high levels of blight resistance (BC_3F_2s). Selected BC_3F_2 trees will produce seed for reforestation. Based on experience with other crops, the 15/16 American chestnut trees are expected to be indistinguishable from pure American chestnut, except for their blight resistance (Hebard et al., 1991; Hebard, 1994). The American Chestnut Foundation has a goal of breeding genetically diverse blight resistant nuts for initial distribution and reintroduction into the forest by the end of the decade (Hebard and Sisco, 1999).

For successful reintroduction of *C. dentata* into its natural range, trees must be bred with germplasm local to the regions proposed for reforestation (Griffin, 2000). The goal of the Chattanooga Chestnut Tree Project is the restoration of the American chestnut to the Southern Appalachian and Cumberland Plateau Regions. This goal is currently being pursued via research that is focused on the integration of biological control using hypovirulent strains and selective breeding of resistant chestnut trees with local germplasm. The Chattanooga Chestnut Tree Project breeding orchard, Bendabout Farm, is located about 40 miles east of Chattanooga in the Ridge and Valley domain, near Cleveland, TN. Bendabout Farm is maintained by cooperative efforts of the owners with The University of Tennessee at Chattanooga and The American Chestnut Foundation. The plantation at Bendabout Farm includes hundreds of hybrid chestnut trees and improved cultivars. In one experiment, begun in 1996 and 1997, 126 seeds were planted in six rows, each containing twenty-one trees. Not all of the trees have survived. As of 5 June, 2003, 102 of the original 126 hybrid chestnut trees for this experiment were recorded living in the orchard. The seed nuts planted were the fruit of open pollination of second backcross (BC_2) trees at TACF Research Farm, Meadowview, VA. The resulting seedlings are designated BC_2F_2s. Trees located in the Meadowview breeding orchard were screened for resistance according to TACF guidelines. The Bendabout Farm orchards also include native chinquapins (*C. pumila*), *C. dentata* grafted clones, *C. sativa*, Henry Converse hybrid seedlings, grafted *C. mollissima* and *C. crenata* clones, and hybrid seedling progeny from crosses made by Dr. Hill Craddock at Bendabout Farm. The hybrids from these crosses made at Bendabout Farm include F_1s (American x Chinese), BC_1F_1s, BC_2F_2 grafted clones from the TACF, BC_3F_1s, Euro-Chinese hybrids,

and Euro-Japanese hybrids. Family pedigrees for trees included in the 2003 resistance screening at Bendabout Farm are reported in Table 1.1. [The trees in this experimental population had a trunk diameter > 3cm.]

The success of the breeding work depends on the continued availability of American chestnut trees, that are locally adapted to the area, to use as parents. The incorporation of southern germplasm increases the likelihood that the hybrids produced in the future will grow well in the surrounding forests. When potential parent trees are located, forest management techniques are applied, if possible, to enhance the probability of survival, nut production, and incorporation into the breeding program. The hypovirulence work involves maintaining biological control of local v-strains in the Chattanooga breeding orchards, screening hybrids for resistance, and researching the interactions of different h-strains of host trees varying levels of resistance.

H. Construction of cDNA

In an attempt to bypass some of the possible factors limiting spread of hypoviruses on American chestnut trees, *C. parasitica* strains have been genetically modified so that the sexual spores will give rise to strains containing dsRNAs of European origin (Anagnostakis et al., 1998). Infectious cDNA clones of mild (CHV1-Euro7) and severe (CHV1-Ep713) hypovirus strains responsible for virulence attenuation (hypovirulence) of the chestnut blight fungus *C. parasitica* were used to construct viable chimeric viruses (Chen et al., 2000b.). A 'mild' h-strain is only mildly debilitated in the fungal growth and processes in the tree and is thus still an aggressive pathogen. A 'severe' h-strain is severely debilitated to the fungal growth in the tree and is a weak pathogen. When this cDNA was introduced into virulent strains of *C. parasitica* through

Table 1.1. Family pedigrees for chestnut trees located at The Chattanooga Chestnut Tree Project Breeding Orchard included in the 2003 blight resistance screening.

Type of Tree	Number of Trees
Controls:	
Castanea dentata (from Lookout Mt., GA)	1
Marrone di Castel del Rio' open pollinated x *Castanea sativa*	1
Marrone di Marradi' open pollinated	2
Grafted tree of 'Norris' on *Castanea mollissima* seedling	2
Castanea pumila (native chinquapins)	2
Henry Converse hybrid #2 (American x Chinese: F_1) of unknown origin	2
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}: BC_2-F_2	
open pollenated CL160	16
open pollenated CL283	40
The seed sources are opCL160 and opCL283. These are progeny of Clapper BC_2s by open pollintion. Many should be BC_2-F_2s. Pedigree for Clapper: (M16, PI# 34517 x FP 555) x FP 555. M16 was a Chinese and FP 555 was an American	
{Am x [Am x (Ch x Am)]} x {Am x [Am x (Ch x Am)]}: BC_2-F_2 grafted clones	
SA-18 / Sweethart seedling	2
SA-319 / Sweethart seedling	1
SA-433 / Sweethart seedling	1
SA-537 / Sweethart seedling	3
SA-606 / Sweethart seedling	1
American x Chinese: F_1	
D5xFF2-1	2
D10xFF2-1	2
{[Am x (Ch x Am)] x (Am x Ch)}: BC_1F_1	
A5xAG 1-1 (*Castanea dentata* x Nanking)	1
B1xAG 1-1	1
D1xAG 1-1	1
D5xAG 1-1	2
E10xAG 1-1	2
Am x {Am x [Am x (Ch x Am)] x (Am x Ch)}: BC_3-F_1	
E10xBE331	3
Pedigree for BE331: Bu31C1 x Graves, a BC_2 (Bu31C1 is American) Graves is CT Ag Expt Stn Sleeping Giant State Park West Red Pine Lot R13T1, Cross #37-53 Chinese-American B_1; R13Y1 is South Lot R2T8 F_1 x Bowman tree, Clinton Corner, NY, American in 1953; R2T8 is Mahogony Chinese (South Lot R1T15) x Wash DC dentata (FP551) in 1934, cross #105B-34. Graves was selected from 9 nuts	
E10xAB229	13
Pedigree for AB229: Musick x opMusick-91 (open pollinated by MusickChinese), an F_1 (Musick is American)	
E10xAB39	2
Pedigree for AB39: RCW2C x Clapper, a B_2 (RCW2C is American)	
A10xGR210	1
***Castanea mollissima* x *Castanea sativa* backcrossed to *Castanea sativa*: Euro-Chinese**	
'Skookum' open pollinated x *Castanea sativa*	2
***Castanea crenata* x *Castanea sativa* backcrossed to *Castanea sativa*: Euro-Japanese**	
'Marigoule' open pollinated x *Castanea sativa*	1
'Colossal' open pollinated x *Castanea sativa*	1
'Bouche de Betizac' open pollenated seedling	2

DNA-mediated transformation, the virulent strain was converted to the hypovirulent phenotype (Choi and Nuss, 1992b.). Chen et al. (1993) demonstrated that the introduced cDNA is transferred through asexual sporulation and is effectively transmitted to ascospores; an event that has never occurred with natural hypovirus dsRNA (Anagnostakis, 1988). According to Nuss, this advancement in hypovirulence research represents a method of hypovirulence transmission that is expected to bypass existing barriers, including vegetative incompatibility (Chen et al., 2000b.; Nuss et al., 2002).

II. RESEARCH ON THE HOST-PATHOGEN INTERACTIONS OF HYBRID CHESTNUT TREES AFTER EXPOSURE TO VIRULENT AND HYPOVIRULENT *CRYPHONECTRIA PARASITICA* STRAINS

A. General Observations

We have seen the effective biological control of chestnut blight in Italy, France, and Switzerland on *C. sativa* (Heiniger and Rigling, 1994; Turchetti and Maresi, 1993; Bissegger et al., 1997). Biological control via hypovirulence has also been observed in the natural range of *C. dentata* on graft-propagated clones of large surviving American chestnut trees in the Lesesne State Forest, VA (Robbins and Griffin, 1999; Griffin, 1999). Hypovirulence has generally not been effective as a biocontrol within the natural range of *Castanea dentata* in North America (MacDonald and Fulbright, 1991). Hypovirulence was not an effective biological control on wild-type (susceptible) American chestnut trees in the same plots as the grafted clones of the large surviving Americans (Robbins and Griffin, 1999; Griffin, 1999).

B. General Hypotheses and Predictions

It has been hypothesized that some low level of host resistance to chestnut blight found in *C. sativa* and not in *C. dentata* is a significant factor contributing to the successful biological control of *C. parasitica* in Europe and not in North America. The presence of low levels of host resistance in the large surviving grafted Americans has also been considered a possible factor contributing to the successful disease control on the grafts and not the surrounding wild-type *C. dentata* stump sprouts (Robbins and Griffin, 1999). The integration of Asian blight resistance into the American genotype through the

backcross breeding (introgression) program has led to the production of hybrid progeny with a broad range of host resistance to chestnut blight (Hebard et al., 1991; Hebard, 1994). When populations of backcross breeding progeny that are segregating for resistance to blight are exposed to lethal strains of *C. parasitica,* canker sizes are expected to vary from small superficial cankers (on resistant trees) to large girdling necroses (on susceptible trees).

C. Observations

1. BC_2F_2s and other hybrid chestnut progeny of the TACF backcross breeding program vary in blight resistance from susceptible to resistant (Hebard et al., 1991; Hebard, 1994).

2. The standard virulent dsRNA-free *C. parasitica* strain Ep155 is lethal to susceptible chestnut trees and may be lethal to trees of intermediate resistance as well. However, the isogenic strain Ep155 infected with the *Cryphonectria hypovirus* 1-Euro7 (CHV1-Euro7) initially produces a canker similar to that of the dsRNA-free Ep155 strain for approximately the first year of infection. Then canker expansion slows markedly, and the expression of hypovirulence is observed (Chen et al., 2000a.; Elliston, 1982; Hebard and Shain, 1989).

3. Ep155(CHV1-Ep713) is severely disabled.

D. Problems

1. How do BC_2F_2 hybrids having different resistance levels vary in their response to dsRNA-containing and dsRNA-free strains of *C. parasitica*?

2. How do Bendabout Farm hybrid chestnut progeny selected for the 2003 blight resistance screening, having different levels of resistance, vary in their response to dsRNA-containing and dsRNA-free strains of *C. parasitica*?

3. Can Ep155(CHV1-Euro7) be used to screen progeny of the backcross breeding program for resistance instead of the standard v-strain Ep155 (virus-free)?

4. Does Ep155(CHV1-Ep713) expression differ from that of Ep155(CHV1-Euro7) on hosts having different levels of disease resistance?

E. Hypotheses

1. Individual BC_2F_2 hybrid chestnut trees in a half sibling population possess different levels of host resistance to chestnut blight as measured by Ep155 (virus-free) canker length.

2. The Chattanooga Chestnut Tree Project hybrid progeny have a range of host resistance to chestnut blight that varies from susceptible to intermediate levels resistance as measured by Ep155 canker length.

3. Hypovirulent strain Ep155(CHV1-Euro7) is as effective at screening chestnut trees for resistance to chestnut blight as Ep155 (virus-free).

4. Ep155(CHV1-Ep713) severely debilitates the strain Ep155.

5. Expression of hypovirulence by strain Ep155(CHV1-Ep713) will vary in a way that is similar to strain Ep155(CHV1-Euro7).

F. Predictions

1. If we screen a population of BC_2F_2 hybrid chestnut trees for resistance to chestnut blight using Ep155 (virus-free), then variation in blight resistance will be observed among the population. Resistant trees will form small superficial cankers at the

site of inoculation and survive, some intermediately resistant trees may form small superficial cankers and survive, but others will be girdled and die. Susceptible trees will all be girdled by the canker and die.

2. If we screen the Chattanooga Chestnut Tree Project hybrid chestnut progeny for resistance to chestnut blight using Ep155 (virus-free), then variation in blight resistance will be observed among the progeny.

3. If we inoculate BC_2F_2 hybrids and the CCTP progeny that have different levels of host resistance to chestnut blight, with Ep155 (virus-free) and Ep155(CHV1-Euro7), then there will be no significant difference in the resulting canker sizes of the two isolates.

4. If we inoculate BC_2F_2 hybrids and the CCTP progeny that have different levels of host resistance to chestnut blight with Ep155(CHV1-Ep713), then the resulting cankers will be small and superficial.

5. If we inoculate BC_2F_2 hybrids and the CCTP progeny that have different levels of host resistance to chestnut blight with Ep155(CHV1-Ep713) and Ep155(CHV1-Euro7), then trees with higher resistance levels will have smaller Ep155(CHV1-Ep713) and Ep155(CHV1-Euro7) cankers, and trees with high levels of susceptibility will have relatively larger Ep155(CHV1-Ep713) and Ep155(CHV1-Euro7) cankers.

G. Experiments

1. To test for variation in blight resistance among the half-sib BC_2F_2 population we screened the trees for blight resistance using the standard virulent *C. parasitica* strain Ep155 (virus-free). Next, we classified the BC_2F_2s based on canker size as susceptible, intermediately resistant, or resistant (as per TACF guidelines).

2. To test for variation in blight resistance among the Chattanooga Chestnut Tree Project hybrid progeny we screened the trees using the standard virulent *C. parasitica* strain Ep155 (virus-free). Next, we classified the CCTP progeny based on canker size as susceptible, intermediately resistant, or resistant (as per TACF guidelines).

3. To test whether or not Ep155(CHV1-Euro7) can be used to screen progeny for blight resistance, we inoculated each tree in a segregation population of BC_2F_2 hybrid chestnut trees and most of the CCTP progeny with Ep155 (virus-free) and Ep155(CHV1-Euro7) and looked for significant differences in the near-term canker sizes between the two isolates.

4. To test whether Ep155(CHV1-Ep713) and Ep155(CHV1-Euro7) canker sizes vary in size on trees having differing levels of host resistance, we inoculated each tree in a segregation population of BC_2F_2 hybrid chestnut trees and most of the CCTP progeny with Ep155(CHV1-Ep713) and Ep155(CHV1-Euro7) and looked for significant differences in the near-term canker sizes between the two isolates on the susceptible, intermediate, and resistant trees.

H. Research Objectives

My research investigated two aspects of the Chattanooga Chestnut Tree Project: breeding for blight resistance and the use of hypovirulence as a biological control. The four objectives of this current research are as follows: (1) to determine levels of blight resistance among individuals in a population of half-sib BC_2F_2 hybrid chestnut trees (2) to determine levels of blight resistance among the Chattanooga Chestnut Tree Project hybrid progeny, (3) to determine if the hypovirulent strains Ep155(CHV1-Euro7) or Ep155(CHV1-Ep713) can be used to screen hybrid chestnut progeny for blight resistance

instead of Ep155 (the standard v-strain used in resistance screening), (4) and to develop protocol for production of *C. parasitica* strains.

III. MATERIALS AND METHODS

A. Introduction to Materials and Methods

The successful biological control of chestnut blight has been observed among the *Castanea sativa* populations in Europe (Heiniger and Rigling, 1994). Hypovirulence has not controlled chestnut blight within the natural range of the blight susceptible *Castanea dentata* (MacDonald and Fulbright, 1991). A rare occurrence of biological control via hypovirulence in the natural range of *C. dentata* has been observed on large surviving American grafts in the Lesesne State Forest, VA, which have been reported to exhibit some levels of natural host resistance to chestnut blight (Robbins and Griffin, 1999; Griffin, 1999). TACF backcross breeding progeny vary in blight resistance from susceptible to resistant. The expression of hypovirulence among trees of varying susceptibility has been observed to range from avirulent to almost virulent (Griffin et al., 1977; Elliston, 1978; Rigling and Heiniger, 1989; Peever et al., 2000). The standard v-strain used for screening progeny of the backcross breeding program for blight resistance is Ep155 (virus-free), which has been observed to have similar affects on chestnut trees as Ep155 infected with the *Cryphonectria hypovirus* 1-Euro7 [Ep155(CHV1-Euro7)] dsRNA for up to one year after infection when the Ep155(CHV1-Euro7) canker expansion abruptly ceases (Chen et al., 2000a.).

The general problems that led to the questions addressed in the present study include the following: Why does hypovirulence work as a biological control in Europe on *C. sativa* and not in North America on *C. dentata*? Why does hypovirulence work as a biological control in the Lesesne State Forest, VA, on large surviving American chestnut grafts and not the surrounding native *C. parasitica* population? How do TACF BC_2F_2

hybrids having different resistance levels vary in their expression of hypovirulence? Could Ep155(CHV1-Euro7) be effectively used to screen hybrid chestnut trees for disease resistance? Does *Cryphonectria hypovirus* 1 - Ep713 severely debilitate *C. parasitica* strain Ep155? Does Ep155(CHV1-Ep713) canker size vary on trees having different resistance levels?

Some of the general hypotheses contributing to the development of this study were as follows: some intermediate level of host resistance to chestnut blight found in *C. sativa* but not in *C. dentata* is a significant factor contributing to the successful biological control of *C. parasitica* in Europe but not in North America; some level of host resistance to chestnut blight found in the grafted large surviving American chestnut trees but not in the wild-type *C. dentata* in the Lesesne State Forest allows for the successful expression and persistence of hypovirulence as a biological control for chestnut blight on these trees but not on the wild-type trees; variation in the expression of hypovirulence may occur in BC_2F_2 hybrid chestnut trees having differing levels of host resistance to blight; and that the hypovirulent strain Ep155(CHV1-Euro7) may be as effective at screening chestnut trees for resistance to chestnut blight as the standard virulent strain Ep155.

I hypothesized that BC_2F_2 and other chestnut hybrids have different levels of host resistance to chestnut blight and that the hypovirulent strain Ep155(CHV1-Euro7) is as effective at screening chestnut trees for resistance to chestnut blight as Ep155 (virus-free).

I began testing the hypotheses by screening hybrid chestnut progeny of the TACF and CCTP backcross breeding program with Ep155 (the standard v-strain of *Cryphonectria parasitica*) for resistance to chestnut blight. I also exposed these BC_2F_2

hybrids to the isogenic dsRNA-containing isolates Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713). The progeny were classified as susceptible, intermediately resistant, or resistant to chestnut blight. Canker sizes of the three isolates on all of the trees were measured and compared.

B. The Trees

As mentioned previously, the Chattanooga Chestnut Tree Project breeding orchard, Bendabout Farm, is located about 40 miles east of Chattanooga in the Ridge and Valley domain, near Cleveland, TN. One plot of seed nuts planted at Bendabout Farm in 1996 and 1997 were the fruit of open pollination of second backcross (BC_2) trees at TACF Research Farm, Meadowview, VA. The resulting seedlings are designated BC_2F_2s. The trees chosen for resistance screening and exposure to virus-containing strains included *C. dentata*, *C. sativa*, Henry Converse hybrid seedlings, grafted *C. mollissima* and *C. crenata* clones, and hybrid seedling progeny from crosses made by Dr. Hill Craddock at Bendabout Farm. The hybrids from these crosses made at Bendabout Farm included F_1s (American x Chinese), BC_1F_1s, BC_2F_2 grafted clones from the TACF, BC_3F_1s, Euro-Chinese hybrids, and Euro-Japanese hybrids. Native Allegany chinquapins (*C. pumila* Mill.) were also included in the resistance screening. Chinquapins are usually described as a shrub growing 6 to 15 feet tall and are distinguished by having only one nut per bur (Jaynes, 1979). The trees in this experimental population that had a trunk diameter > 3cm were chosen for screening in 2003. Family pedigrees for trees included in the 2003 screening at Bendabout Farm are reported in Table 1.1.

C. Obtaining the Isolates

Dr. Mark Double of Morgantown, WV provided agar slants of three isogenic

isolates used in this present study. These isolates were dsRNA-free Ep155 (virus-free), *Cryphonectria hypovirus* 1-Euro7-infected Ep155 [Ep155(CHV1-Euro7)], and *Cryphonectria hypovirus* 1-Ep713-infected Ep155 [Ep155(CHV1-Ep713)]. The two hypovirus-containing fungal isolates, Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713), were transfected 3 October 2003 by the laboratory of Dr. Don Nuss of the University of Maryland Biotechnology Institute. The stock cultures were all stored in an incubator at around 5°C until needed for transfer to PDAmb.

D. Preparation of PDAmb

To make enough PDAmb for approximately 50 sterile Petri plates (Fisherbrand® 95x15mm), I added 39g of potato dextrose agar (Difco), 100mg of D-methionine, and 1.0mL of biotin stock solution to a 2L Erlenmeyer flask containing 1L of purified water. While stirring, the mixture was brought to a boil on a hot plate and then immediately removed from the heat source. The mouth of the flask was covered with aluminum foil and then the flask was autoclaved. Using sterile techniques (alcohol and flames) appropriate levels of the autoclaved mixture were poured into 50 Petri plates under a sterile hood. The 50 plates were then carefully stacked in three columns and pushed closely together to allow the medium to cool for at least an hour.

To make 10mL of biotin stock solution for the PDAmb, I added 0.01g of Biotin powder to 10mL of purified water in a small vial and shook the mixture well.

E. Transfer and Growth

All transfers were performed using sterile procedures under a sterilized hood and next to flaming Bunsen burners. On 4 May 2003 transfers of each isolate from the agar slant test tubes to sterile Petri plates containing PDAmb were made using a new

disposable plastic Fisherbrand® sterile loop for each transfer. With the plastic loop I removed a small piece of the mycelium from the agar slant and placed it into the center of the PDAmb on the Petri plate. The perimeters of the Petri plates were sealed using parafilm, and labeled with the date and name of isolate.

The isolates were then incubated on shelves in the lab at room temperature under fluorescent lights for 8 days before transfer to new PDAmb Petri plates (Scibillia et al., 1992). On 12 May 2003, after eight days of growth in the growth chambers, (before the cultures had reached the edged of the PDAmb) the first plate to plate transfers were performed. This was done using a flame/alcohol sterilized scalpel to cut 1cm cubes about 0.5-1cm behind the leading edge of the culture (this reduces the possibility of inadvertently subculturing a virus-free portion from the hypovirulent isolates). Each cube was transferred, mycelial side down, to the center of a new Petri plate of PDAmb. The perimeter of each plate was then sealed with parafilm and labeled. The plates were then placed in the growth chamber (under conditions mentioned above) for 7-10 days. The 7-10 day old isolates were transferred two more times using the same methods. (20 May 2003 and 28 May 2003).

F. Experimental Design:

We chose a fixed pattern of inoculations on the trees in the experiment. All inoculations faced northeasterly and were arrayed in the same manner on each tree. First, we inoculated all the BC_2F_2 hybrids involved in the experiment with Ep155 (virus-free) approximately 20cm from the ground. Next, we inoculated all the BC_2F_2 hybrids with Ep155(CHV1-Euro7) approximately 15cm above the Ep155 (virus-free) inoculation site. Finally, we inoculated all the BC_2F_2s with Ep155(CHV1-Ep713) 15cm above the

Ep155(CHV1-Euro7) site. Inoculated sites were then covered with masking tape to retard drying. All wounds were spaced approximately 15cm apart and faced northeasterly. The minimum trunk/branch size inoculated was 3cm in diameter.

G. Field Inoculations

On 2 June 2003 five to ten plates of each of the three rapidly growing isolates [Ep155 (virus-free), Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713)] were chosen for inoculation of chestnut hybrids located at Bendabout Farm, MacDonald, TN. I used the "direct inoculation technique" to inoculate the trees (Griffin, 1983). This entailed removing a plug of bark with a alcohol-flame sterilized #2 cork borer (6 mm diameter) from a tree and inserting a disk of agar (6 mm), cut from 0.5-1.0 cm within the advancing margin of plated colonies, into the wound (Griffin, 1983). All inoculations were made on 2 and 3 June 2003 (day 0-1). All 56 BC_2F_2 hybrids included in this study were inoculated with Ep155 (virus-free), Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713). Three F_1 hybrids were inoculated with the virus-free strain and both virus-containing strains, and one F_1 hybrid was only inoculated with the two virus-containing strains. Four BC_1F_1 hybrids were inoculated with the virus-free strain and both virus-containing strains, and two BC_1F_1 hybrids were only inoculated with the two virus-containing strains. Eleven BC_3F_1 hybrids were inoculated with the virus-free strain and both virus-containing strains, seven BC_3F_1 hybrids were only inoculated with the two virus-containing strains, and one BC_3F_1 was inoculated with Ep155(CHV-1Euro7) only. Eight TACF BC_2F_2 grafted clones were inoculated with the virus-free strain and both virus-containing strains. The Euro-Chinese hybrids were only inoculated with the two virus-containing strains. One Euro-Japanese hybrid was inoculated with the virus-free strain and both virus-

containing strains, two were inoculated with both virus-containing strains only, and one was just inoculated with Ep155(CHV1-Euro7). The cork borer was sterilized using alcohol and a flame before and after each plug was removed from a tree.

H. Measurement of Canker Expansion

I measured the length and width of each canker along the perpendicular axis using a dial caliper (accurate to 0.1mm). Measurements of the discolored bark tissue were taken on 23 - 24 June (21 - 22 days after inoculation), 29 - 30 July (57 - 58 days after inoculation), and 4 - 5 September (94 - 95 days after inoculation). For most measurements the bark was stripped on the perpendicular axes of each canker (using an alcohol/flame sterile blade) to confirm that the discolored tissue was the same size at the surface as it was at the cambium (Anagnostakis, 1992). For each tree we first measured the Ep155(CHV1-Ep713) canker, then we measured the Ep155(CHV1-Euro7) canker, and lastly we measured the Ep155 canker.

I. Hypovirulence Conversion of Ep155

On 4 - 5 September 2003 (day 94-95) a hypovirulent slurry containing the h-strains Ep155(CHV1-Ep713) and Ep155(CHV1-Euro7) was prepared. This was done by adding four cultures (8 days-old) of each h-strain along with its PDAmb medium to a blender. Next, water was added until the mixture reached the consistency of applesauce by blending (Jaynes and Elliston, 1978). The hypovirulent slurry was transferred into a sterile container and taken to the Bendabout Farm orchard for application that day.

Before the h-slurry was applied to the Ep155 (virus-free) canker area on each tree, the entire Ep155 canker was carefully removed by hand carving with pocket knives. The h-slurry was then transferred into a large beaker and liberally applied to the entire Ep155

(virus-free) canker area using a paint brush. Once application was complete, the treated area was loosely covered with masking tape to slow drying.

J. Model for Analysis of Interactions

I tested for interactions between the isolates and resistance classes using a two-way ANOVA. The statistical model used for analysis of the interactions among resistance class and isolate was: Canker length = resistanceClass + isolate + resistanceClass * isolate + error.

IV. RESULTS

A. Results of the BC_2F_2 Resistance Screening

A population of 56 BC_2F_2 half sibling chestnut trees was screened for resistance via inoculations with the standard v-strain Ep155 (virus-free). Measurements of canker length and width were taken at the perpendicular of each axis in June (day 21-22 after inoculation), July (day 57-58) and September (day 94-95). In order to observe the canker size data in different dimensions July and September canker sizes for the BC_2F_2 and control trees was plotted in three ways; as canker length (mm) (Figure 3.1 and 3.2), as the sum of the length and width (mm) (Figure 3.3 and 3.4), and as the canker area (mm^2) (Figure 3.5 and 3.6). Canker area (mm^2) was calculated for July and September using the formula for an ellipse: (π * L * W) / 4 (Peever et al., 2000). As measured by canker length (mm), canker length + width (mm), and canker area (mm^2) the two half-sib BC_2F_2 families exhibited a broad range of host resistance. As measured by canker length (mm) in September on the BC_2F_2s the Ep155 canker size ranged from 57.6mm – 127.2mm, Ep155(CHV1-Euro7) canker size ranged from 22mm – 127.3mm, and Ep155(CHV1-Ep713) canker size ranged from 15.1mm – 62.5mm (Fig. 3.1). As measured by the sum of the canker length and width (mm) in September on the BC_2F_2s the Ep155 canker size ranged from 92mm – 199.8mm, Ep155(CHV1-Euro7) canker size ranged from 36.5mm – 185.5mm, and Ep155(CHV1-Ep713) canker size ranged from 26.7mm – 113.2mm (Fig. 3.3). As measured by canker area (mm^2) in September on the BC_2F_2s the Ep155 canker size ranged from 1318mm^2 – 7461mm^2, Ep155(CHV1-Euro7) canker size ranged from 250.4mm^2 – 6104mm^2, and Ep155(CHV1-Ep713) canker size ranged from 137.5mm^2 – 294.9mm^2 (Fig. 3.5).

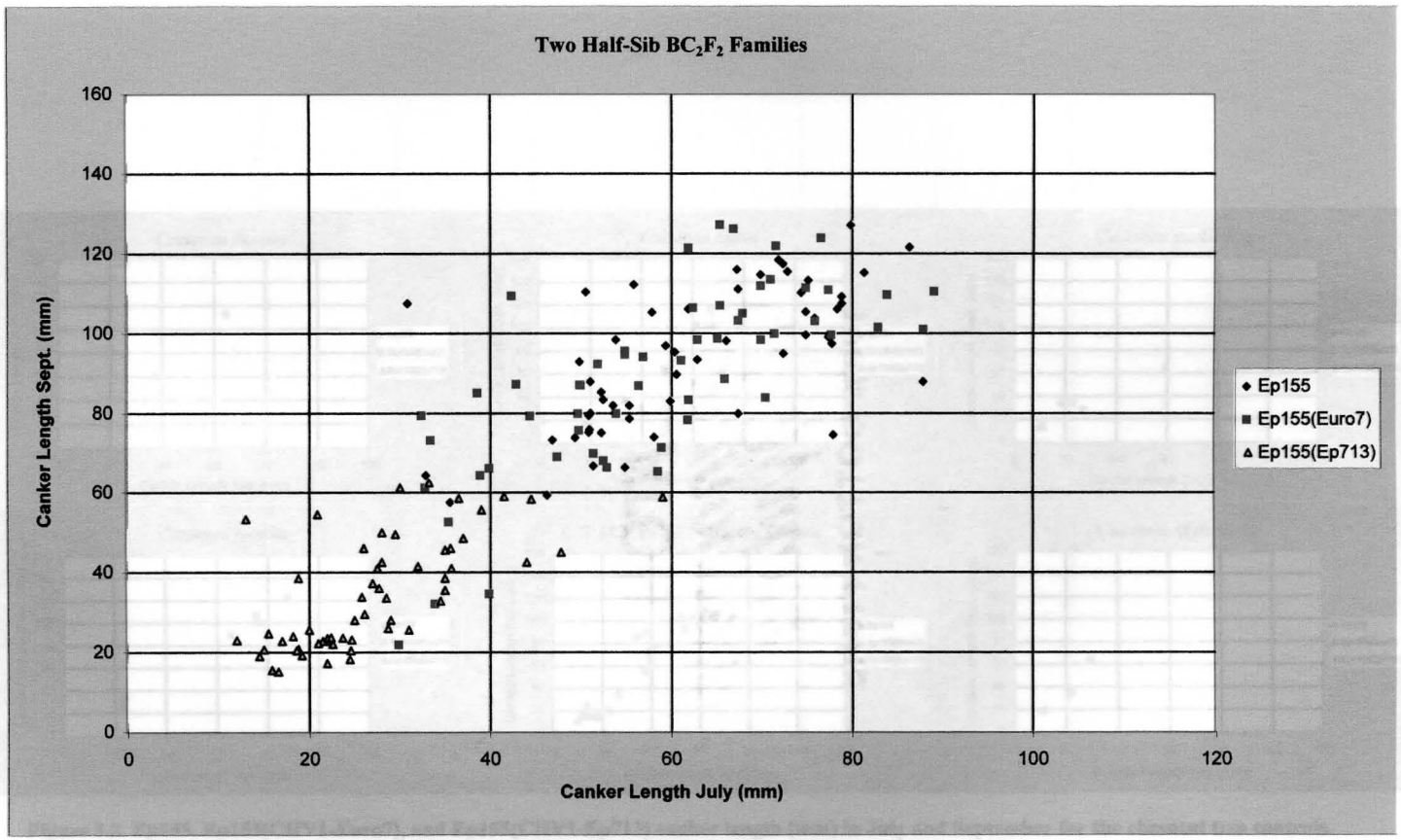


Figure 3.1. Ep155, Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker length (mm) in July and September for BC_2F_2s.

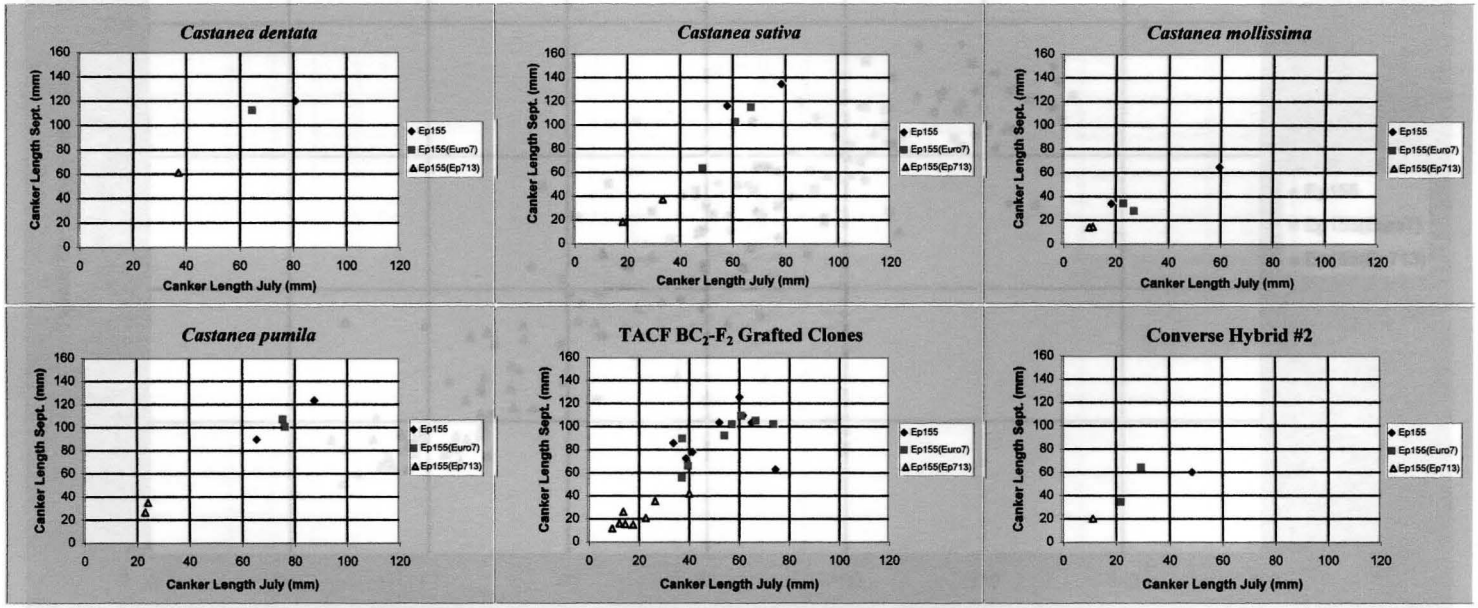


Figure 3.2. Ep155, Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker length (mm) in July and September for the chesntut tree controls.
- The one *C. dentata* control received all three inoculations.
- Of the two Converse Hybrid #2s, one received inoculations of all three strains and the other received one Ep155(CHV1-Euro7) inoculation.

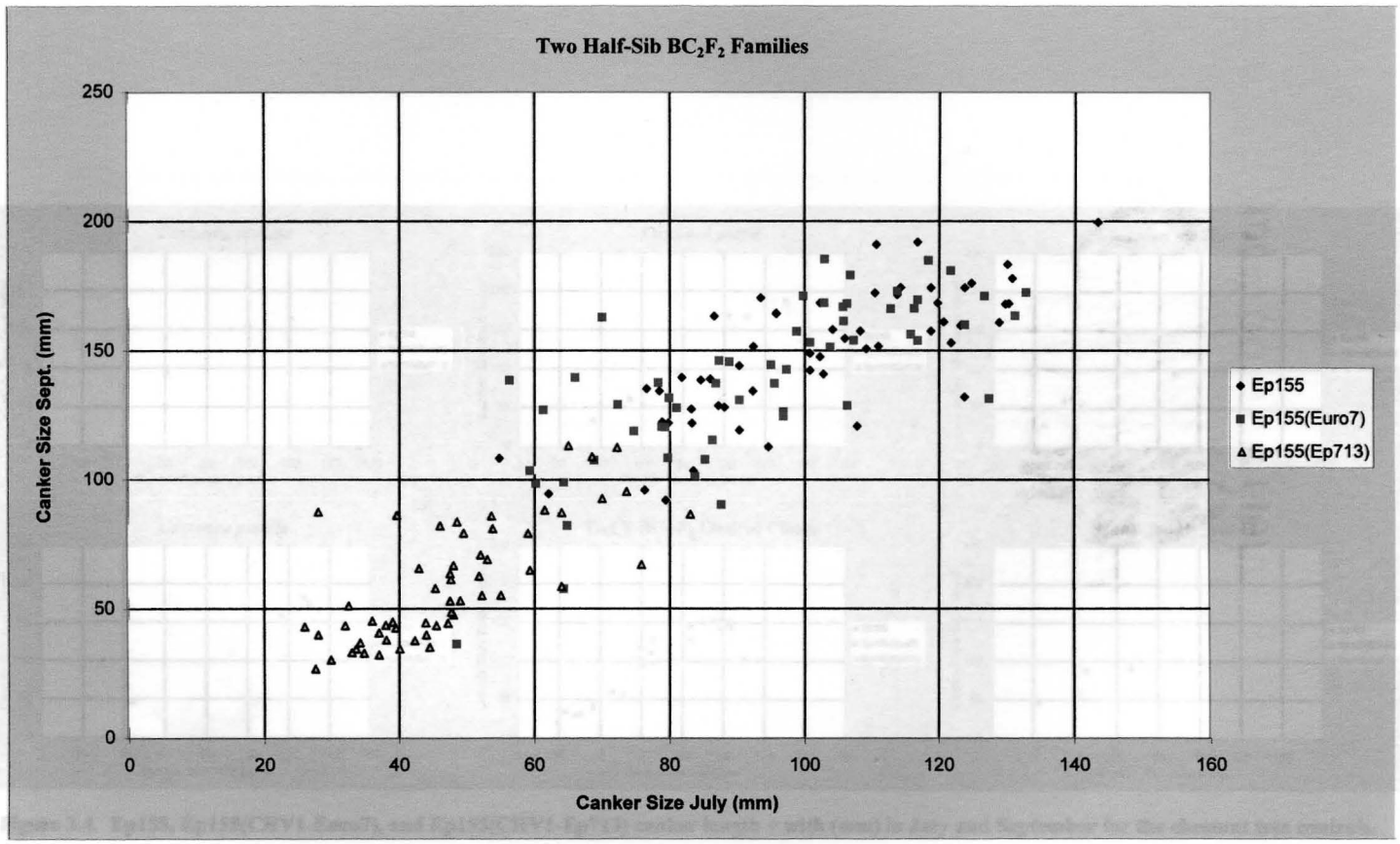


Figure 3.3. Ep155, Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker length + with (mm) in July and September for BC_2F_2s.

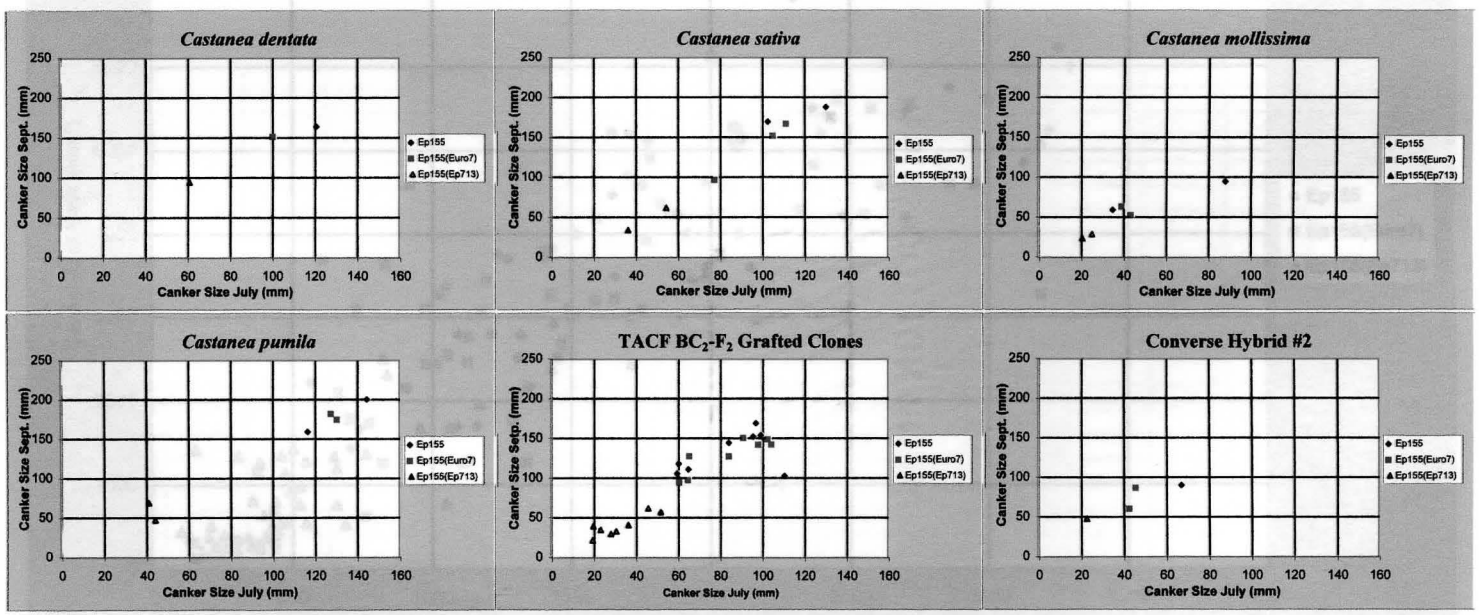


Figure 3.4. Ep155, Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker length + with (mm) in July and September for the chestnut tree controls.

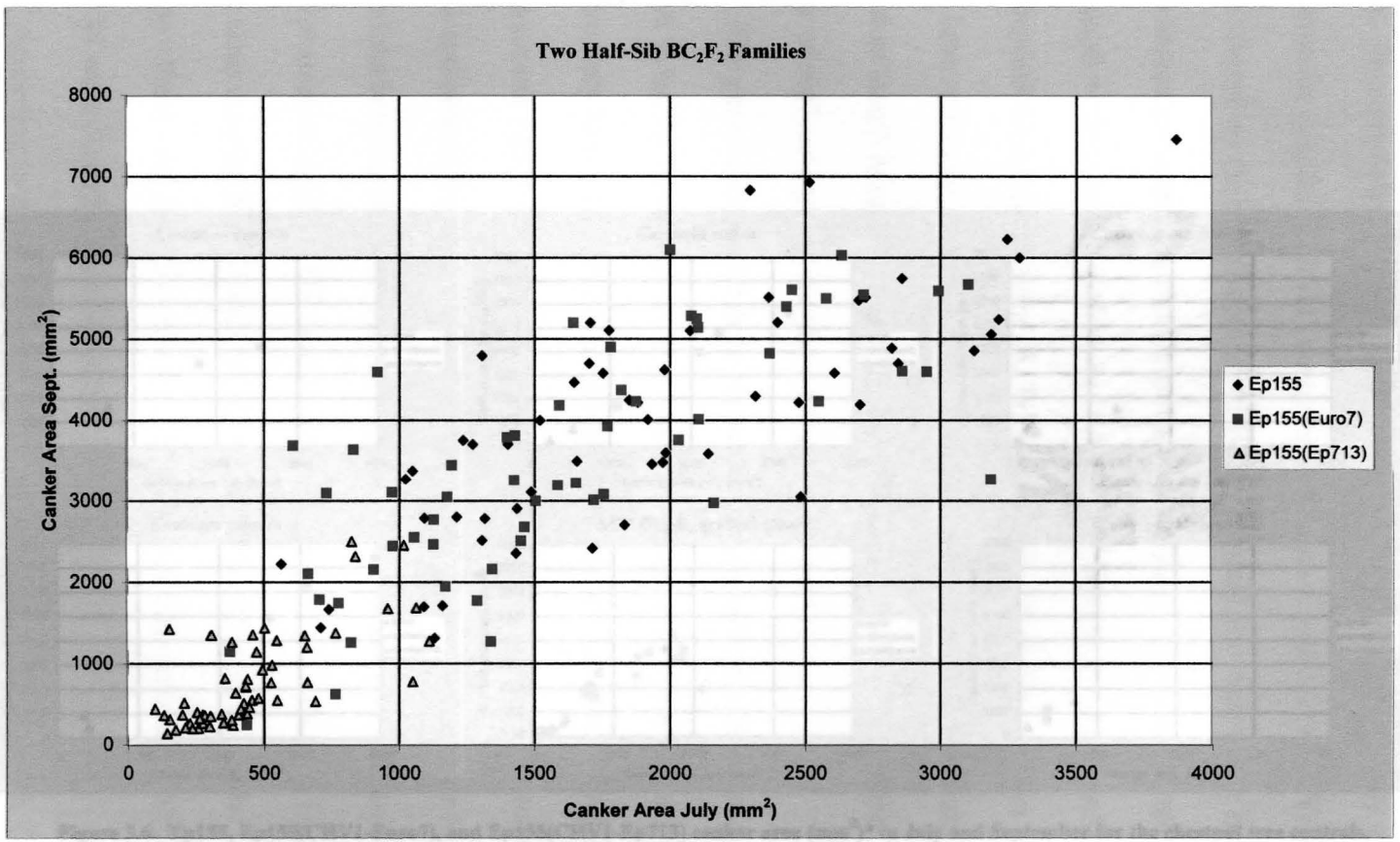


Figure 3.5. Ep155, Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker area (mm^2)* in July and September for BC_2F_2s.
*** Canker area was calculated using the formula for an ellipse: (π * L * W) / 4.**

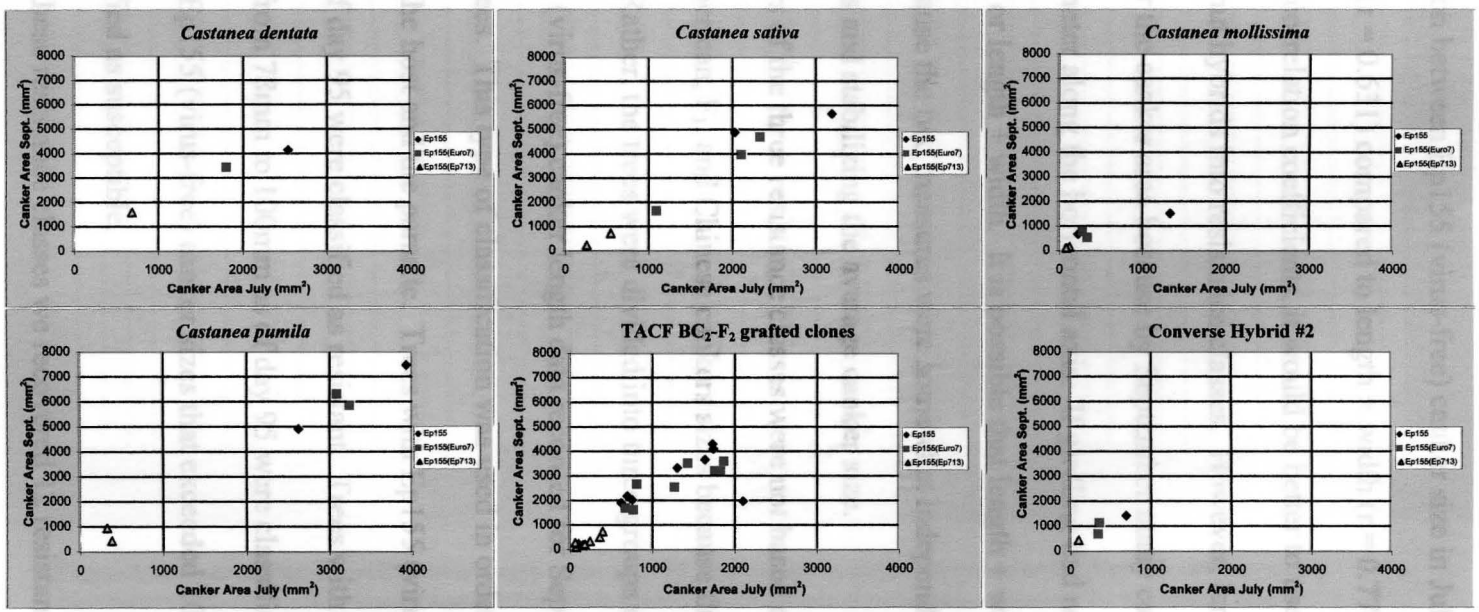


Figure 3.6. Ep155, Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker area (mm^2)* in July and September for the chestnut tree controls.
*** Canker area was calculated using the formula for an ellipse: (π * L * W) / 4.**

We used the length dimension of the September Ep155 (virus-free) canker measurement for classification of the hybrid chestnut trees into the three blight resistance classes. The correlation between Ep155 (virus-free) canker size in July and September was worse for length (r = 0.631) compared to length + width (r = 0.774) or area (r = 0.770). Based on the correlation coefficients, it would be better to use length + width or area to separate chestnut hybrids into resistance classes. However, length was chosen over length + width or the canker area because by September many cankers girdled the trunk beyond the diameter along the horizontal axis. If girdling had not occurred, I would have used area or length + width. It is possible that length + width or area were better than length because the two measures were somewhat independent, increasing the number of replications and stabilizing the average canker size.

The parameters of the three resistance classes were not based on a discriminate analysis using the American, F_1, and Chinese cankers sizes because the cankers on the F_1s were very large. Rather, the trees were divided into three groups using the minimum and maximum Ep155 (virus-free) canker length data recorded for September among the BC_2F_2s and control trees. This type of classification was used in order to detect any interactions between the host and the parasite. Trees with Ep155 (virus-free) canker sizes 77mm or smaller as of day 95 were classified as resistant. Trees with Ep155 (virus-free) canker sizes ranging from 78mm to 100mm as of day 95 were classified as intermediately resistant. Trees with Ep155 (virus-free) canker sizes that exceeded 100mm in length as of day 95 were classified as susceptible.

According to these resistance classes we recovered 12 resistant, 22 intermediately resistant, and 22 susceptible BC_2F_2 half sibling hybrids from the population. Figure 3.7a

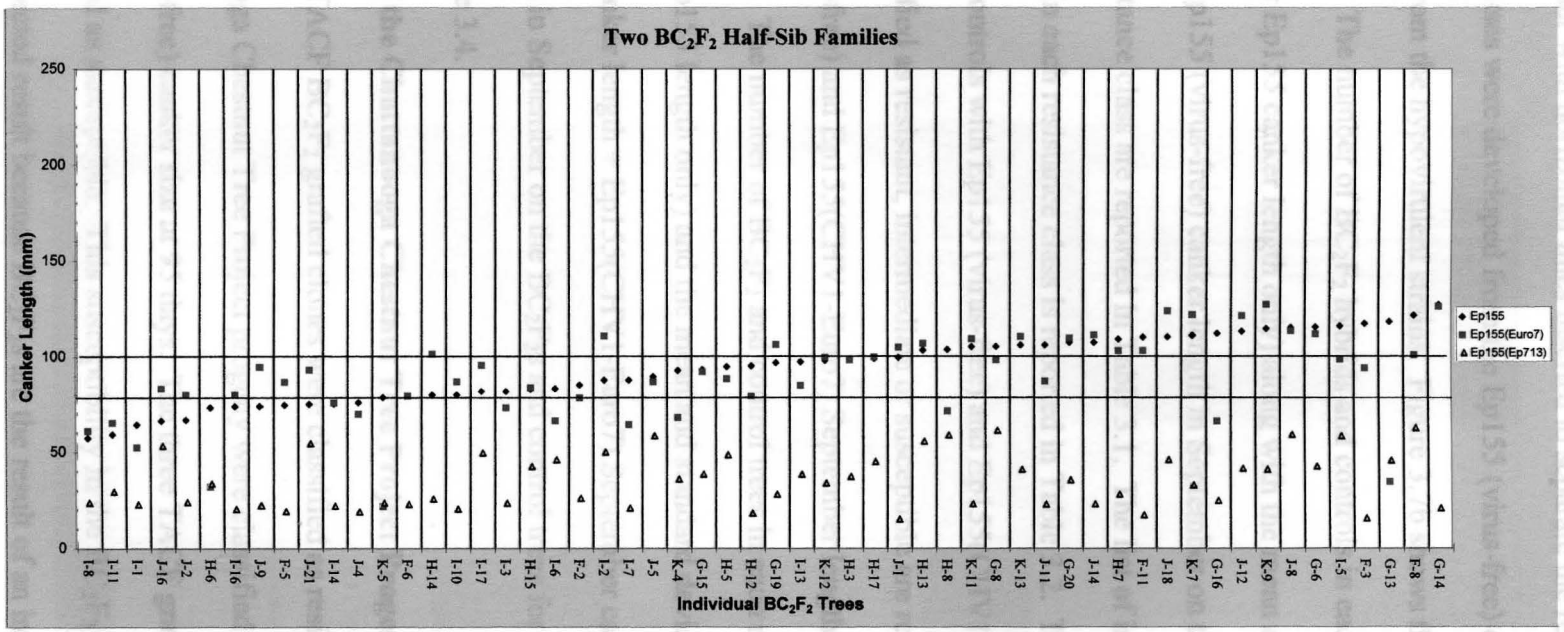


Figure 3.7a. Scatter plot of Ep155 canker length (mm) after 95 days ranked in ascending order for BC_2F_2s with corresponding Ep155(CHV1-Ep713) canker lengths and Ep155(CHV1-Euro7) separated into three resistance classes* by horizontal lines.
*** Resistance classes developed from the Ep155 canker size data**
*** Resistant ≤ 77mm; 100mm ≥ Intermediate > 77mm; Susceptible > 100**

shows the Ep155 (virus-free) canker length in September on the BC_2F_2s plotted in ascending order with horizontal lines inserted to separate the resistance classes. The resistance classes were developed from the Ep155 (virus-free) canker length data and not canker data from the hypovirulent strains. Figure 3.7b shows the controls plotted in the same format. The number of BC_2F_2 hybrids and controls in each resistance class (based on September Ep155 canker length only) along with the mean and standard deviation for the average Ep155 (virus-free) canker length in September on the BC_2F_2 and control trees for each resistance class are reported in Table 3.1. The list of individual BC_2F_2 and control trees in each resistance class is reported in Table 3.2. The number of BC_2F_2 hybrids and controls with Ep155 (virus-free) and Ep155(CHV1-Euro7) September canker lengths classified as resistant, intermediate or susceptible are reported in Table 3.3. Ep155 (virus-free) and Ep155(CHV1-Euro7) September lengths were averaged together and analyzed. The number of BC_2F_2 and control trees in each resistance class (based on September Ep155 length only) and the mean and standard deviation for [(Ep155 September canker length + Ep155(CHV1-Euro7) September canker length) / 2] average canker length in September on the BC_2F_2s and control trees for each resistance class are listed in Table 3.4.

B. Results of the Chattanooga Chestnut Tree Project Progeny Resistance Screening

Two TACF BC_2F_2 grafted clones were classified as resistant and all but three of the Chattanooga Chestnut Tree Project progeny were classified as susceptible by the Ep155 (virus-free) canker size at 95 days. The three TACF grafted clones of SA-537 were classified as susceptible. This susceptibility in the BC_2F_2 grafted clones of SA-537 was an unexpected result because BC_2F_2s are the result of an intercross between two

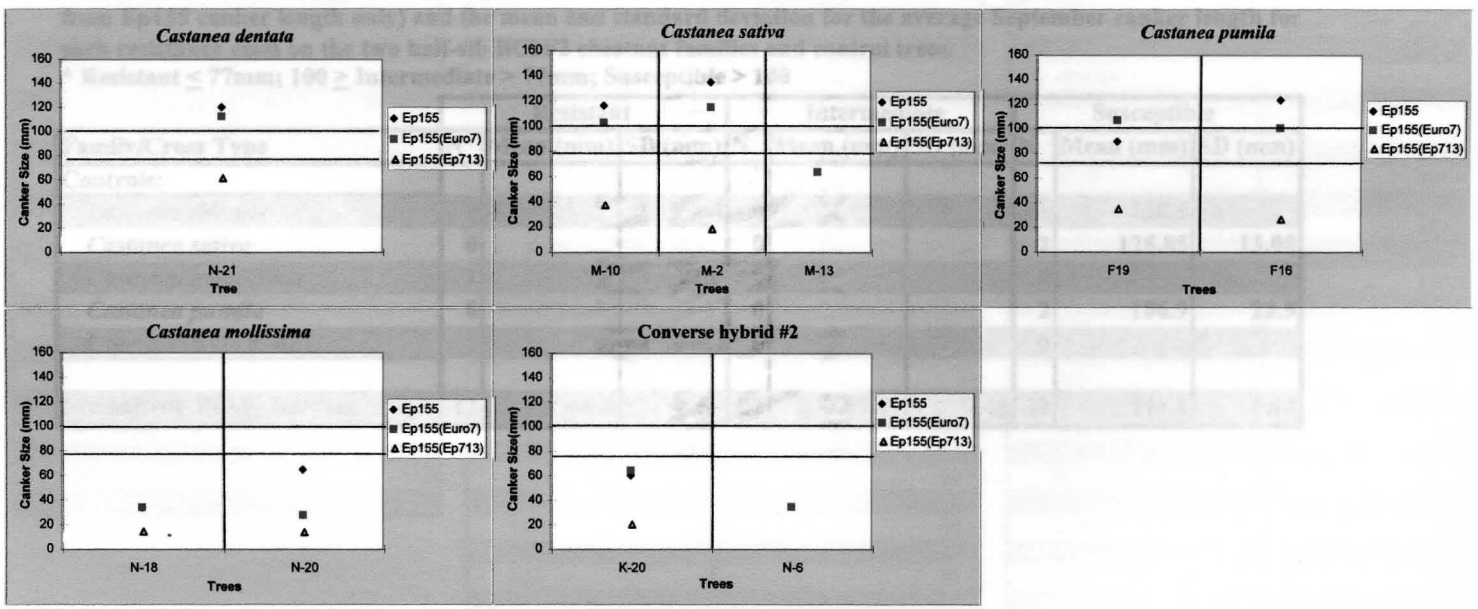


Figure 3.7b. Scatter plots of Ep155 canker length (mm) after 95 days ranked in ascending order for the control trees with corresponding Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713) canker lengths and separated into three resistance classes* by horizontal lines.
*** Resistant ≤ 77mm; 100mm ≥ Intermediate > 77mm; Susceptible > 100**

Table 3.1. Number of half-sib BC_2F_2 hybrids and control trees separated into three resistance classes* (developed from Ep155 canker length only) and the mean and standard deviation for the average September canker length for each resistance class on the two half-sib BC2F2 chestnut families and control trees.
*** Resistant ≤ 77mm; 100 ≥ Intermediate > 77mm; Susceptible > 100**

	Resistant			Intermediate			Susceptible		
Family/Cross Type	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**
Controls:									
Castanea dentata	**0**			**0**			**1**	**120.5**	**na**
Castanea sativa	**0**			**0**			**2**	**125.85**	**13.08**
Castanea mollissima	**2**	**49.7**	**21.9**	**0**			**0**		
Castanea pumila	**0**			**0**			**2**	**106.9**	**23.9**
Converse hybrid #2	**1**	**60.6**	**na**	**0**			**0**		
two half-sib BC_2F_2 families	**12**	**69.8**	**6.57**	**22**	**89.41**	**7.48**	**22**	**111.8**	**6.13**

Table 3.2. The list of individual BC_2F_2 and control trees in each resistance class. - The letter refers to the row of the tree and the number refers to the placement order of the tree in the orchard row.

Half-sib BC_2F_2 and control trees classified as 'Resistant' (Resistant ≤ 77mm) based on the September Ep155 canker length	
Type of Tree	**Ep155 length (mm)**
Controls	
C. mollissima	
N-18*	34.2
N-20	65.2
Converse hybrid #2	
K-20	60.6
Half-Sib BC_2F_2	
I-8	57.6
I-11	59.4
I-1	64.5
J-16	66.5
J-2	66.9
H-6	73.4
I-16	74
J-9	74.1
F-5	74.7
J-21	75.2
I -14	75.3
J-4	76

Half-sib BC_2F_2 and control trees classified as 'Intermediate' (100 ≥ Intermediate > 77mm) based on the September Ep155 canker length only	
Type of Tree	**Ep155 length (mm)**
Controls	
C. pumila	
F-19	90
Half-Sib BC_2F_2	
K-5	78.7
F-6	79.5
H-14	80
I-10	80.1
I-3	82
I-17	82
H-15	83.1
I-6	83.5
F-2	85.4
I-2	88
I-7	88
J-5	89.8
K-4	93
G-15	93.5
H-5	95
H-12	95.4
G-19	97
I-13	97.5
K-12	98.2
H-3	98.5
H-17	99.2
J-1	99.7

Half-sib BC_2F_2 and control trees classified as 'Susceptible' (Susceptible > 100) based on the September Ep155 canker length only	
Type of Tree	**Ep155 length (mm)**
Controls	
C. dentata	
N-21	120.5
C. sativa	
M-10	116.6
M-12	135.1
C. pumila	
F-16	123.8
Half-Sib BC_2F_2	
H-13	103.6
H-8	103.9
K-11	105.4
G-8	105.5
J-11	106.2
K-13	106.2
G-20	107.6
J-14	107.6
H-7	109.3
F-11	110.3
J-18	110.5
K-7	111.2
G-16	112.3
J-12	113.4
K-9	114.8
J-8	115.3
G-6	115.6
I-5	116.1
F-3	117.5
G-13	118.5
F-8	121.7
G-14	127.2

Table 3.3. Number of BC_2F_2 hybrids and control trees separated into three resistance classes* (based on Ep155 and Ep155(CHV1-Euro7)). Resistance classes determined by September (Day 95) Ep155 and Ep155(Euro7) canker lengths.

*** Resistant ≤ 77mm; 100 ≥ Intermediate > 77mm; Susceptible > 100**

- Individual trees were classified as Resistant, Intermediate, or Susceptible only in both Ep155 and Ep155(CHV1-Euro7) cankers were within the size parameters of the class.

- If Ep155 and Ep155(CHV1-Euro7) cankers from one tree were represented in different classed then the tree was placed in one of the following categories:

Ep155 Resistant and Ep155(Euro7) Resistant, Intermediate, or Susceptible

Ep155 Intermediate and Ep155(Euro7) Resistant, Intermediate, or Susceptible

Ep155 Susceptible and Ep155(Euro7) Resistant, Intermediate, or Susceptible

	Ep155 resistant			Ep155 intermediate			Ep155 susceptible		
Family/Cross Type		Euro7			Euro7			Euro7	
Controls:	resistant	intermediate	susceptible	resistant	intermediate	susceptible	resistant	intermediate	susceptible
Castanea dentata									1
Castanea sativa									2
Castanea mollissima	1			1					
Castanea pumila			1						1
Converse hybrid #2					1				
two half-sib BC_2F_2 families	6	6		5	13	4	3	4	15

Table 3.4. Number of BC_2F_2 hybrids and control trees separated into three resistance classes* and the mean and standard deviation for average of the Ep155 and Ep155(CHV1-Euro7) canker lengths in September (Day 95) for all three isolates on two half-sib BC_2F_2 families and control trees.

***Resistance classes developed from September (Day 95) Ep155 canker lengths:**
Resistant ≤ 77mm; 100 ≥ Intermediate > 77mm; Susceptible > 100

	Resistant			Intermediate			Susceptible		
Family/Cross Type	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**
Controls:									
Castanea dentata	0			0			1	116.65	na
Castanea sativa	0			0			2	117.55	10.89
Castanea mollissima	2	40.52	8.73	0			0		
Castanea pumila	0			1	98.7	na	1	112.4	na
Converse hybrid #2	1	62.65	na	0			0		
two half-sib BC_2F_2 families	13	68.11	9.86	23	88.63	6.79	20	110.54	6.71

BC_2F_1s and should all have inherited dominance for resistance from their Chinese ancestor. The susceptibility found in these BC_2F_2s grafted clones could be a result of physiological stress due to the graft union, or possibly they were originally susceptible to blight. The parameters used to judge resistance at Meadowview, VA may have also been slightly different than those used at Bendabout Farm. The number of CCTP progeny and control trees in each resistance classes (developed from Ep155 canker length only), and the mean and standard deviation for the average Ep155 (virus-free) canker length in September (95 days after inoculation) on the CCTP progeny and control trees for each resistance class are listed in Table 3.5. The list of individual CCTP progeny and control trees in each resistance class is reported in Table 3.6. The number of Chattanooga Chestnut Tree Project progeny and control trees in each resistance classes (developed from Ep155 canker length only) mean and standard deviation for Ep155 + Ep155(CHV1-Euro7) / 2 average canker length in September on the CCTP progeny and control trees for each resistance class are listed in Table 3.7. Individual trees (half-sib BC_2F_2, CCTP progeny and controls) either resistant to Ep155 (virus-free) or Ep155(CHV1-Euro7), but not both strains are listed in Table 3.8.

C. dentata was highly susceptible. *C. mollissima* and the Henry Converse Hybrid seedlings were highly resistant. *C. sativa* was not more resistant to Ep155 (virus-free) than the *C. dentata* in this experiment. The F_1s in this experiment were all classified as susceptible. Figure 3.8 show scatter plots of Ep155 (virus-free) canker length (mm) after 95 days ranked in ascending order on all of the grafted trees included in this study with corresponding Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713) canker lengths. The Ep155 (virus-free), Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker length (mm)

Table 3.5. Number of Chattanooga Chestnut Tree Project progeny and control trees separated into three resistance classes* (developed from Ep155 canker length only) and the mean and standard deviation for the average September canker length for each resistance class on the CCTP progeny and control trees.

*** Resistance classes determined by September (Day 95) Ep155 canker lengths: Resistant ≤ 77mm; 100 ≥ Intermediate > 77mm; Susceptible > 100**

	Resistant			Intermediate			Susceptible		
Family/Cross Type	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**
Controls:									
Castanea dentata	0			0			1	120.5	na
Castanea sativa	0			0			2	125.85	13.08
Castanea mollissima	2	49.7	21.9	0			0		
Castanea pumila	0			0			2	106.9	23.9
Converse hybrid #2	1	60.6	na	0			0		
CCTP progeny:									
F_1	0			0			3	124.1	18.22
BC_1F_1	0			0			4	114.6	8.83
BC_3F_1	0			1	97.1	na	10	122.37	13.76
TACF BC_2F_2 grafted clones	2	68.1	6.65	2	82.3	51.52	4	110.98	10.41
Euro-Japanese	0			0			1	106.7	na

Table 3.6. The list of individual CCTP progeny and control trees in each resistance class.

CCTP progeny and control trees classified as 'Resistant' (Resistant ≤ 77mm) based on the September Ep155 canker length	
Type of Tree	**Ep155 length (mm)**
Controls	
C. mollissima	
N-18	34.2
N-20	65.2
Converse hybrid #2	
K-20	60.6
Half-Sib BC_2F_2	
I-8	57.6
I-11	59.4
I-1	64.5
J-16	66.5
J-2	66.9
H-6	73.4
I-16	74
J-9	74.1
F-5	74.7
J-21	75.2
I -14	75.3
J-4	76
TACF BC_2F_2 grafted clones	
O-27 (SA433/Sweetheart)	63.4
O-29 (SA18/Sweetheart)	72.8

CCTP progeny and control trees classified as 'Intermediate' (100 ≥ Intermediate > 77mm based on the September Ep155 canker length	
Type of Tree	**Ep155 length (mm)**
Controls	
C. pumila	
F-19	90
BC_3F_1	
M-26	97.1
TACF BC_2F_2 grafted clones	
O-28 (SA18/Sweetheart)	78.4
P-34 (SA606/Sweetheart)	86.2

CCTP progeny and control trees classified as 'Susceptible' (Susceptible > 100) based on the September Ep155 canker length only	
Type of Tree	**Ep155 length (mm)**
Controls	
C. dentata	
N-21	120.5
C. sativa	
M-10	116.6
M-12	135.1
C. pumila	
F-16	123.8
BC_1F_1	
L-1	106.2
L-4	109.2
L-14	117.1
L-12	125.9
BC_3F_1	
M-27	102.7
M-29	110.7
L-23	114.1
M-21	114.4
L-30	118.9
L-21	120.7
L-31	121.6
L-34	134
L-27	139.9
L-37	146.7
TACF BC_2F_2 grafted clones	
O-24 (SA537/Sweetheart)	103.9
N-22 (SA537/Sweetheart)	104
N-29 (SA391/Sweetheart)	110
N-23 (SA537/Sweetheart)	126

Table 3.7. Number of CCTP progeny and control trees separated into three resistance classes* and the mean and standard deviation for average of the Ep155 and Ep155(CHV1-Euro7) canker lengths in September (Day 95) for all three isolates on the CCTP progeny and control trees.

*** Resistance classes determined by September (Day 95) Ep155 canker lengths:**
Resistant ≤ 77mm; 100 ≥ Intermediate > 77mm; Susceptible > 100

	Resistant			Intermediate			Susceptible		
Family/Cross Type	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**	**N**	**Mean (mm)**	**SD (mm)**
Controls:									
Castanea dentata	0			0			1	116.65	na
Castanea sativa	0			0			2	117.55	10.89
Castanea mollissima	2	40.52	8.73	0			0		
Castanea pumila	0			1	98.7	na	1	112.4	na
Converse hybrid #2	1	62.65	na	0			0		
CCTP progeny:									
F_1	0			0			3	124.48	23.87
BC_1F_1	0			0			4	109.21	5.91
BC_3F_1	0			1	96.6	na	10	115.97	11.37
TACF BC_2F_2 grafted clones	2	68.43	5.69	2	85.55	3.61	4	106.8	6.42
Euro-Japanese							1	105.9	na

Table 3.8. Trees (half-sib BC_2F_2, CCTP progeny and controls) either resistant to Ep155 or Ep155(CHV1-Euro7), but not both strains.

Type of Tree	Resistant to Ep155 but not Ep155(CHV1-Euro7)	Resistant to Ep155(CHV1-Euro7) but not Ep155
Half-sib BC_2F_2	J-16	I-3
	J-2	I-6
	I-16	I-7
	J-9	K-4
	F-5	H-8
	J-4	G-16
		G-13
CCTP progeny		
TACF BC_2F_2 grafted clones	O-27 (SA433/Sweetheart)	O-28 (SA18/Sweetheart)

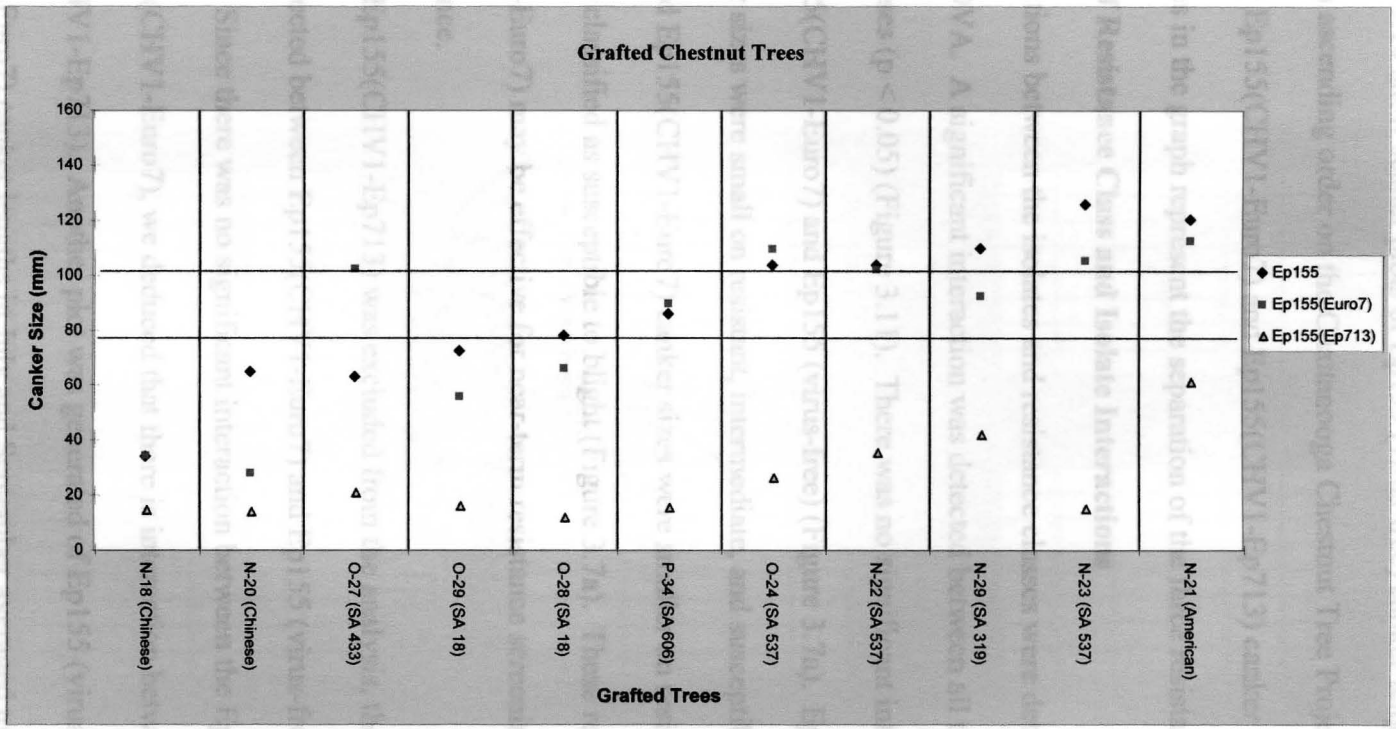


Figure 3.8. Scatter plots of Ep155 canker length (mm) after 95 days ranked in ascending order on all of the grafted trees included in this study with corresponding Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713) canker lengths and separated into resistance classes* by horizontal lines.

*** Resistant ≤ 77mm; 100mm ≥ Intermediate > 77mm; Susceptible > 100**

- O-29 and O-28 are grafted clones of SA18/Sweetheart

- O-24, N-22, and N-23 are grafted clones of SA 537/Sweetheart

in July and September for the Chattanooga Chestnut Tree Project progeny is plotted in Figure 3.9.

Figure 3.10 show scatter plots of Ep155 (virus-free) canker length (mm) after 95 days ranked in ascending order on the Chattanooga Chestnut Tree Project progeny with corresponding Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713) canker lengths. The horizontal lines in the graph represent the separation of the three resistance classes.

C. Analysis of Resistance Class and Isolate Interactions

Interactions between the isolates and resistance classes were determined using a two-way ANOVA. A significant interaction was detected between all three isolates and resistance classes ($p < 0.05$) (Figure 3.11). There was no significant interaction detected between Ep155(CHV1-Euro7) and Ep155 (virus-free) (Figure 3.7a). Ep155(CHV1-Ep713) canker sizes were small on resistant, intermediate, and susceptible trees. Ep155 (virus-free) and Ep155(CHV1-Euro7) canker sizes were smaller on resistant trees and larger on trees classified as susceptible to blight (Figure 3.7a). These results indicate that Ep155(CHV1-Euro7) may be effective for near-term resistance screening of chestnuts for disease resistance.

When Ep155(CHV1-Ep713) was excluded from the analysis, there was no interaction detected between Ep155(CHV1-Euro7) and Ep155 (virus-free) ($p < 0.05$) (Figure 3.12). Since there was no significant interaction between the Ep155 (virus-free) and the Ep155(CHV1-Euro7), we deduced that there is interaction between either one and Ep155(CHV1-Ep713). Another plot was generated of Ep155 (virus-free) and Ep155(CHV1-Euro7) canker lengths in July and September averaged together for the BC_2F_2s and is displayed in Figure 3.13.

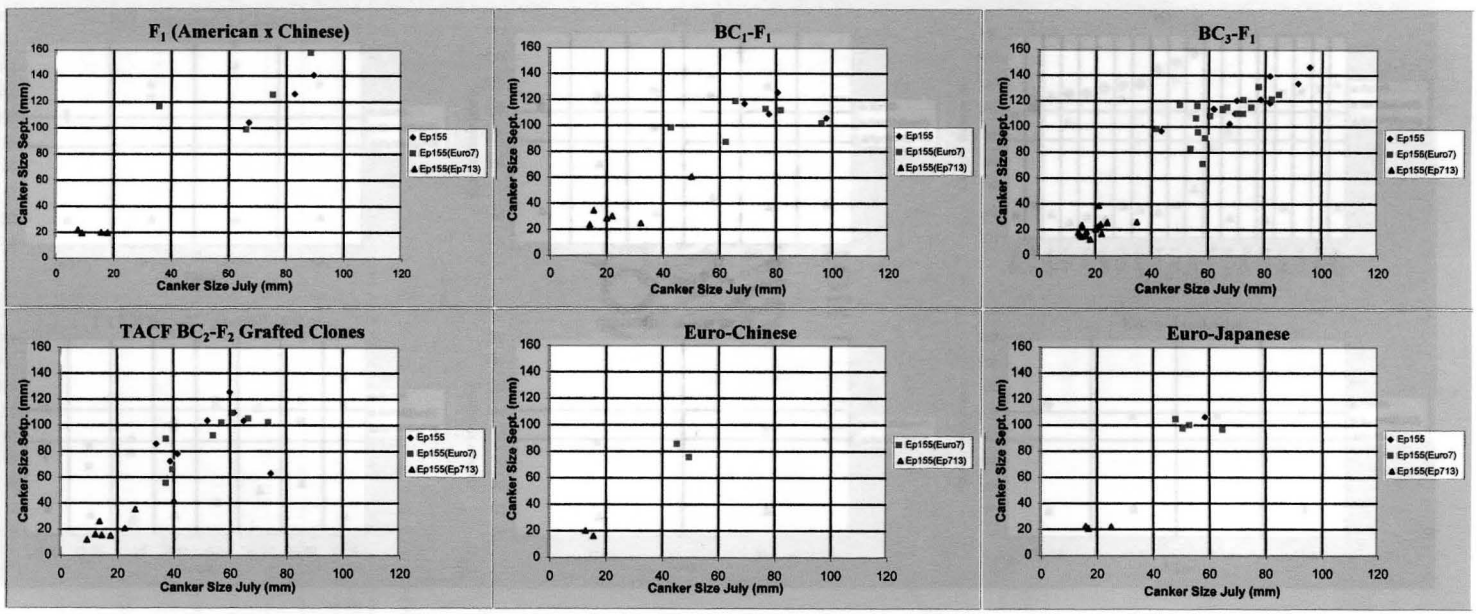


Figure 3.9. Ep155, Ep155(CHV1-Euro7), and Ep155(CHV1-Ep713) canker length (mm) in July and September for Chattanooga Chestnut Tree Project hybrids.

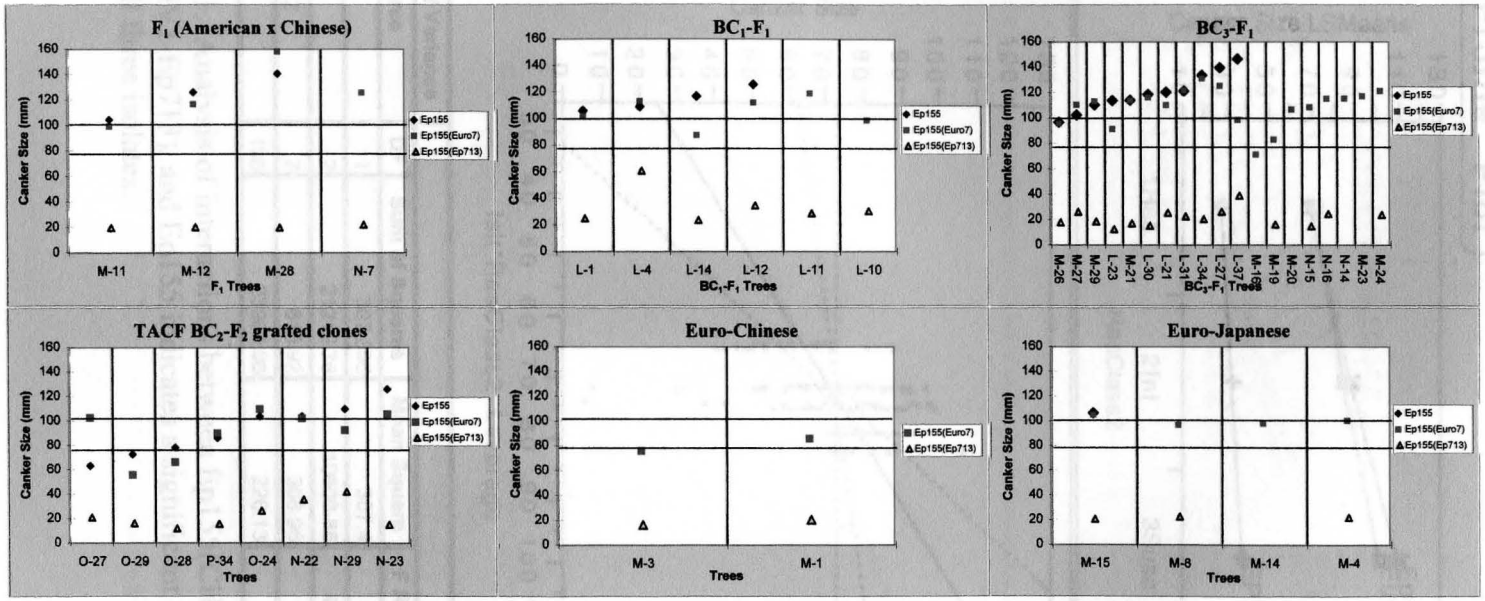
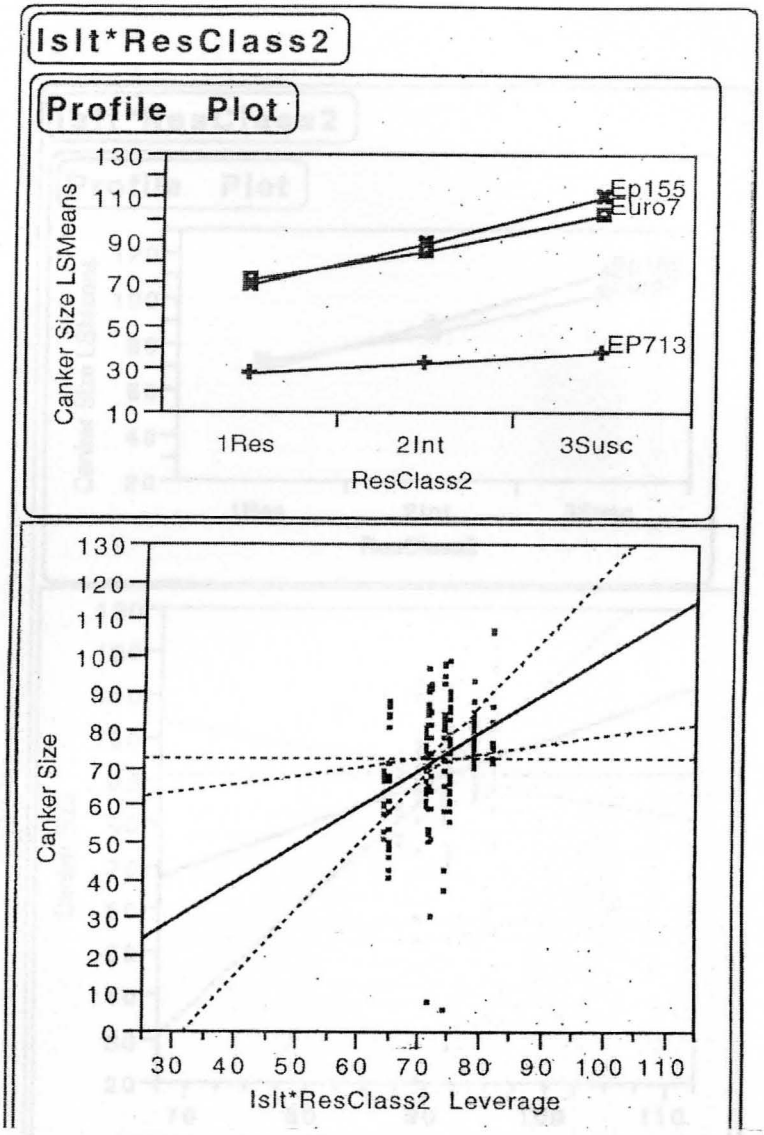


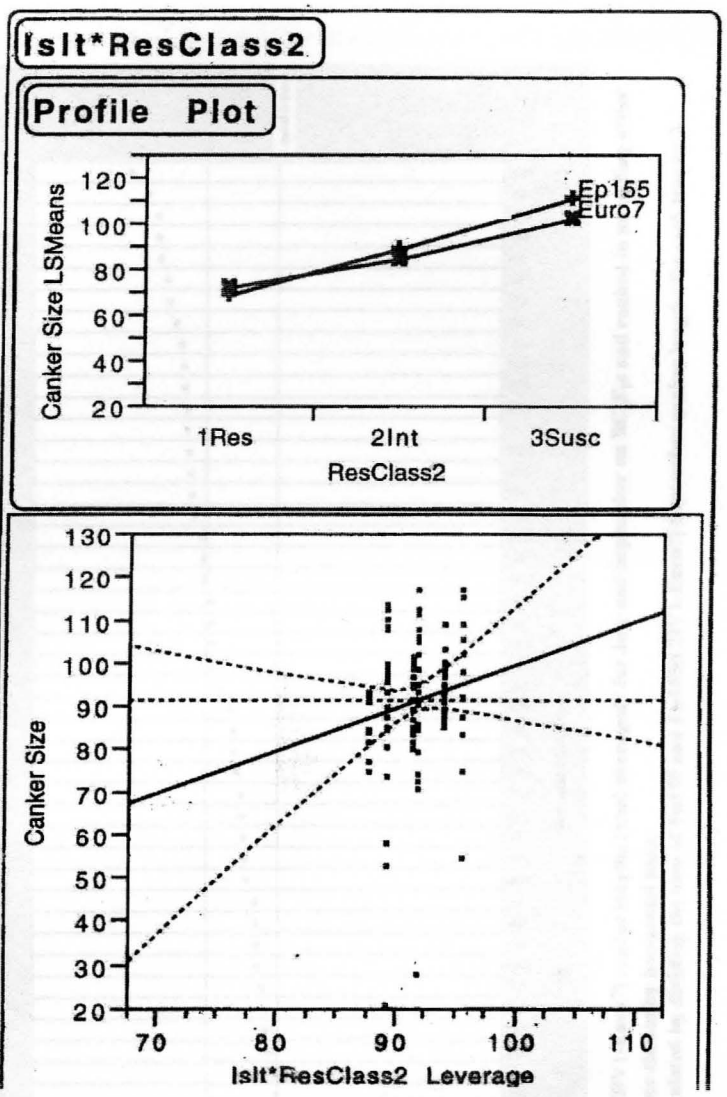
Figure 3.10. Scatter plots of Ep155 canker length (mm) after 95 days ranked in ascending order for Chattanooga Chestnut Tree Project hybrids with corresponding Ep155(CHV1-Euro7) and Ep155(CHV1-Ep713) canker lengths and separated into three resistance classes by horizontal lines.

- If there was no Ep155 inoculation then data was sorted ascending by Ep155(CHV1-Euro7) canker length.



Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
IsIt	1	307.395	307.4	1.359	0.2463
ResClass2	2	21219.76	10609.88	46.919	<.0001
IsIt*ResClass2	2	613.92	306.96	1.357	0.2618
Error	106	23969.96	226.13		

Figure 3.11. Analysis of interactions between Ep155(CHV1-Euro7), Ep155(CHV1-Ep713), and Ep155 indicates a significant interaction between all three isolates.



Analysis of Variance					
Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
IsIt	2	103511.62	51755.82	1.3594	0.2463
ResClass2	2	18381.72	9190.86	46.919	<.0001
IsIt*ResClass2	4	4288.93	1072.23	1.3574	0.2618
Error	159	33980.83	213.7		

Figure 3.12. Analysis of interactions between Ep155(CHV1-Euro7) and Ep155, excluding Ep155(CHV1-Ep713), indicates no interaction between Ep155(CHV1-Euro7) and Ep155.

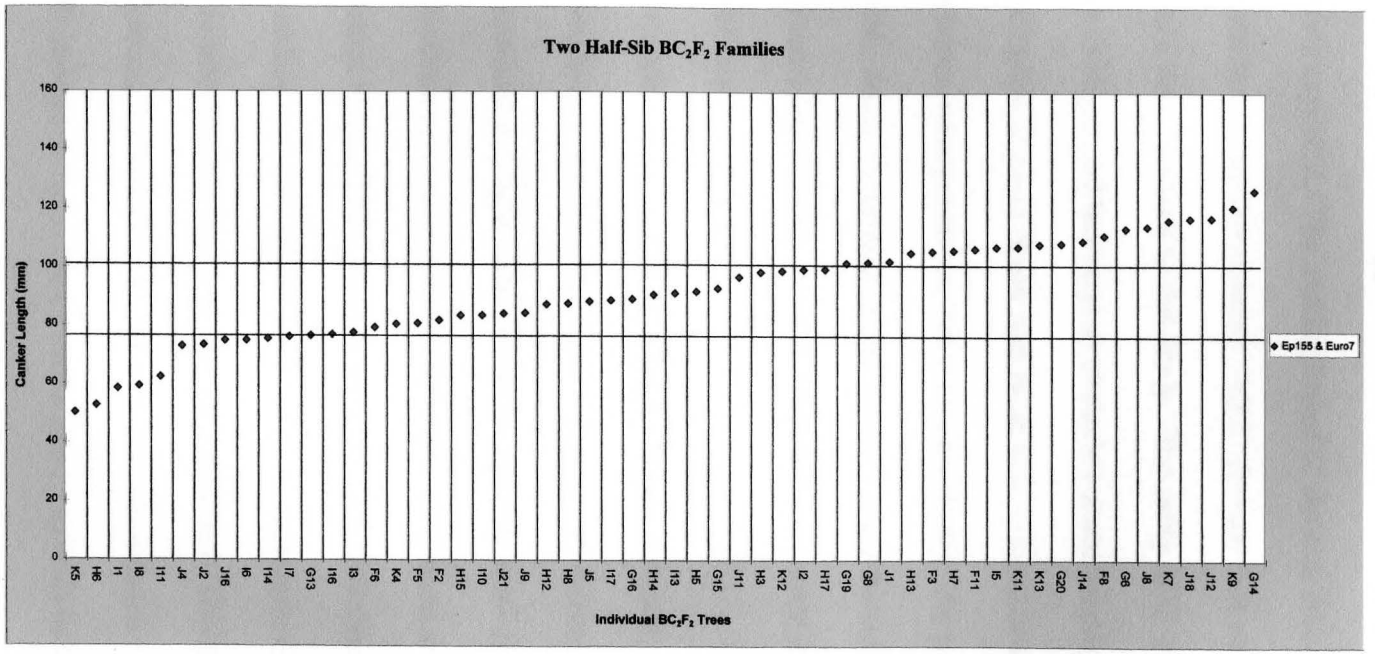


Figure 3.13. Ep155 and Ep155(CHV1-Euro7) canker lengths (mm) averaged* for July and September on BC_2F_2s and ranked in ascending order and separated into three resistance classes by horizontal lines.

*** Average canker length was calculated by dividing the sum of Ep155 and Ep155(CHV1-Euro7) September canker lengths for each tree by 2.**

D. Near-Term Results of the Hypovirulence Conversion

Data concerning the results of the hypovirulence conversion will be collected and updated each year as expression of hypovirulence and survivors are recorded.

V. DISCUSSION

A. Controls

The one American chestnut control in this study was classified as susceptible to chestnut blight as we expected. The availability of *C. dentata* controls was a problem encountered in these screening experiments. All *C. dentata* that were planted in the orchard had died at a time previous to this study. The rarity of *C. dentata* is due to its high susceptibility to blight and has been an obstacle encountered by chestnut researchers since the *C. dentata* population in North American was reduced to understory stump sprouts by *C. parasitica* (Anagnostakis, 1987; Griffin, 1986). As genetic resources continue to diminish, due to the abundance of virulent blight inoculum in North America, *C. dentata* may become increasingly more difficult to incorporate into field studies with hybrid chestnut trees such as this one.

The *C. mollissima* were classified as highly blight resistant as we expected. The one Henry Converse hybrid seedling that received an Ep155 (virus-free) inoculation was also classified as resistant to blight. The European chestnut trees and the F_1 hybrids included in the resistance screening with Ep155 (virus-free) were all classified as susceptible to chestnut blight. The *C. sativa* were not more resistant than *C. dentata* or *C. pumila* as we had anticipated they would have been. In most tests *C. sativa* and F_1 hybrids have low to intermediate levels of disease resistance. This test did not distinguish well between intermediate and low levels of resistance. But, the relatively high level of blight susceptibility of the *C. sativa* and F_1 hybrids in this study is within a range of variation that we expected. Data collection for the different species and cross-types was limited due to the small sample sizes available at the Bendabout Farm breeding

orchard. One of the *C. pumila* in the study (F-19) was classified as intermediately blight resistant and the other *C. pumila* was classified as blight susceptible.

B. BC_2F_2 Half Siblings

As measured by September Ep155 (virus-free) canker length, the sum of length and width, and canker area (mm^2) the two half-sib BC_2F_2 families exhibited a broad range of host resistance to blight. These results support our hypothesis that individual chestnut trees in a BC_2F_2 hybrid population possess different levels of host resistance to chestnut blight. We will continue to observe the twelve resistant trees to see if they allow for expression and persistence of hypovirulence. It will be interesting to see how hypovirulence is manifested among the 22 intermediately resistant chestnut trees. The intermediate levels of blight resistance may provide time needed for the introduced hypovirulent strains to establish themselves in the trees. It is too soon to judge the expression of hypovirulence in the trees. It is also too soon to judge survivorship of the trees. One potential future study involving the BC_2F_2s classified as resistant could involve the cloning of the individual trees by graft propagation and the screening of multiple replicates of each tree and outlier. This type of study would help identify any unique trends in the individual trees. To further confirm that these trees are resistant, one could perform a test cross by crossing one of the BC_2F_2s, classified as resistant, with an American chestnut, which is homozygous recessive for susceptibility, and look for any susceptible trees among the progeny. Intercrossing two of the BC_2F_2s, classified as resistant, and looking for any susceptibility among the progeny would also be a helpful test to further confirm resistance.

C. Chattanooga Chestnut Tree Project Progeny

Previous breeding experiments revealed that the progeny of Chinese x American crosses (F_1s) have a level of resistance intermediate between the two species, although canker development ranges from American-like to Chinese-like, but are often closer in size to the American canker than the Chinese canker (Berry, 1960; Burnham et al., 1986; Hebard et al., 1991; Hebard, 1994). The three Chattanooga Chestnut Tree Project F_1 hybrids that received an Ep155 (virus-free) inoculation were classified as susceptible and had canker sizes similar to that of the American control. All four of the BC_1F_1s that received an Ep155 (virus-free) inoculation were classified as susceptible as well. Of the eleven BC_3F_1s that were screened with Ep155 (virus-free), ten were classified as susceptible and one tree (M-26) was classified as intermediately resistant to blight. That one tree (M-26) will be advanced in the breeding program and Dr. Craddock plans to clone it by grafting.

D. Grafted Clones

There were eight TACF BC_2F_2 grafted trees screened for resistance using Ep155 (virus-free). Three trees were classified as resistant, two of which were the same clone, SA18. SA606 was classified as intermediately resistant. The three SA537 trees and the one SA319 were classified as susceptible. Possible explanations for the susceptibility in these BC_2F_2 grafted clones is discussed previously on page 50.

E. Ep155 and Ep155(CHV1-Euro7)

There was a good correlation (no significant difference) between the grand means of Ep155 (virus-free) and Ep155(CHV1-Euro7) September canker sizes (Figure 3.7a). There were several Ep155(CHV1-Euro7) outliers that gave a misleading reading of blight

resistance when compared to Ep155 (virus-free) on the same tree (Figure 3.7a.). Used in combination with Ep155 (virus-free), Ep155(CHV1-Euro7) may be an effective tool for the screening of chestnut trees for resistance to blight. If Ep155(CHV1-Euro7) was used alone to screen for resistance to blight, then it would most likely lead to the selection of trees that seemed to have moderate to high levels of blight resistance, but in reality are susceptible to lethal strains of *C. parasitica*. Most of the Ep155(CHV1-Euro7) September canker sizes that differed from the Ep155 (virus-free) canker sizes were smaller. It may be possible that the early activation of the virus in some trees and not others is the cause for the Ep155(CHV1-Euro7) outliers. Some of the Ep155(CHV1-Euro7) cankers were larger than the Ep155 (virus-free) cankers. For example, three half-sib BC_2F_2 hybrid chestnut trees (H-8, G-16, and G-13) were classified as blight susceptible by September Ep155 (virus-free) canker length, but had Ep155(CHV1-Euro7) cankers that were in the blight resistant size range. In these cases if you were only using Ep155(CHV1-Euro7) as a resistance screener it would give a false positive for a susceptible tree. Therefore Ep155(CHV1-Euro7) may be better used to screen for resistance in combination with Ep155 (virus-free) so that outliers inconsistent with their corresponding Ep155 cankers could be identified and disregarded.

F. Ep155(CHV1-Ep713)

Ep155(CHV1-Ep713) canker sizes were significantly smaller than the Ep155(CHV1-Euro7) and the Ep155 (virus-free). Ep155(CHV1-Ep713) was not a good predictor of blight resistance in this study because cankers were small on trees regardless of disease resistance levels. It may prove to be a good h-strain for treatment of individual virulent cankers that need to be contained.

G. Conversion of Ep155 to Hypovirulence

The present study was not an experiment on hypovirulence conversion. The motivation behind the conversion of the Ep155 (virus-free) cankers to hypovirulence was solely to keep the trees alive. If the trees died because of susceptibility to blight, then we would not be able to compare the canker sizes of Ep155 (virus-free), Ep155(CHV1-Euro7), or Ep155(CHV1-Ep713) on the hybrids segregating for blight resistance. Death of the intermediately resistant and susceptible trees would also prevent the observation of hypovirulence expression on a population of chestnut trees having a broad range of blight resistance. Screening the trees with Ep155 (virus-free) and classifying them as resistant, intermediate, or susceptible to blight was a necessary step to allow for future observation of h-strains on chestnut trees with varying levels of host resistance to blight.

H. Problems Encountered in the Study

We had difficulty measuring the Ep155(CHV1-Ep713) cankers. The small, callused, superficial cankers caused by Ep155(CHV1-Ep713) often required carving away some of the bark along the canker perimeter to reveal the extent of the necrotic tissue. The type of bark on the hybrid chestnuts ranged from thick and rough in texture to thin and smooth. As reported by Anagnostakis (1992) fungal growth in trees with relatively thick bark was not uniform from surface to cambium. It was usually uniform in thin bark, but discolored tissue must be measured both on the surface and at the cambium at the end of experiments to confirm this (Anagnostakis, 1992). Extreme caution was taken while carving away of the bark along the perimeter of Ep155(CHV1-Ep713) cankers. Sometimes, even removal of a thin layer of bark resulted in the removal of some of the mycelial colony and the accidental reduction in canker diameter. This was because

of the extreme superficiality of the Ep155(CHV1-Ep713) cankers. Bark was also removed along the perimeters of the Ep155 (virus-free) and the Ep155(CHV1-Euro7) cankers, but did not affect the canker diameters because of the depth of the mycelial colony and subsequent necrotic zone in the bark.

I. Conclusions

The two-part approach of the Chattanooga Chestnut Tree Project to solving the problem of chestnut blight involves breeding blight resistant chestnut trees and the biological control of blight using hypovirulent strains of *Cryphonectria parasitica*. The successful biological control of chestnut blight among the Chattanooga Chestnut Tree Project breeding orchard progeny is critical if local germplasm is to be conserved. The Project breeding work depends on the availability of locally adapted American chestnut trees to use as parents. An implementation of the biological control techniques, as seen in European orchard plantations, in the Project breeding orchard may aid in the effort to establish hypovirulence among the progeny. Future research will be needed to further investigate the host-pathogen-parasite relationships and shed light on the many unanswered questions that surround this topic.

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