SPREAD OF WHITE HYPOVIRULENT STRAINS OF *CRYPHONECTRIA PARASITICA* AMONG AMERICAN CHESTNUT TREES AT THE LESESNE STATE FOREST

by

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Spread of white hypovirulent strains of *Cryphonectria parasitica* among American chestnut trees at the Lesesne State Forest

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(ABSTRACT)

Sixty-two natural cankers on branches and main stems of three 16-year-old grafted American chestnut trees at the Lesesne State Forest were sampled for *Cryphonectria parasitica*. Cankers were sampled in 1996 and 1997 at various distances from the main stem zone on the grafts (ground to 183 cm) that was inoculated in 1982 and 1983 with a mixture of dsRNA-containing white and pigmented hypovirulent strains. Grafted trees exhibited a high level of blight control, and all bark cores extracted from cankers on the grafted trees showed superficial necrosis. Bark cores extracted from these cankers yielded 156 isolates of *C. parasitica*. Fifty-three of these isolates were white, and 103 were pigmented. The farthest canker containing a white isolate was located 564 cm from the zone inoculated with hypovirulent strains (H-inoculated zone). The number of white isolates recovered per canker on the grafted trees near the H-inoculated zone (< 0.5 maximum sampling distance) was significantly greater (P=0.0039) than the number of white isolates recovered per canker on the grafted trees far from the H-inoculated zone (>0.5 maximum sampling distance). Lloyd's index of patchiness value for the frequency of white isolates in cankers was 1.36, indicating that white isolates were slightly aggregated in cankers. White isolates of *C. parasitica* were found in two of seven artificially established cankers 5 months after inoculation with a pigmented virulent strain (WK). Thirteen of 14 pigmented isolates collected from these cankers after 5 months were compatible with WK in vegetative compatibility (VC) tests. Eight of 25 white isolates recovered 5, 11, and 50 months after WK inoculation converted the pigmented WK strain to the white hypovirulent phenotype *in vitro*. Sixty-five pigmented isolates collected from natural cankers were paired in VC assays, revealing 28 VC groups. All 11 white isolates of *C. parasitica* assayed contained a 12.7 kb dsRNA in high concentrations. None of 48 pigmented isolates assayed contained dsRNA. All white isolates tested in virulence trials on American chestnut stems in a forest clearcut were hypovirulent, based on low canker severity indices. Little or no dissemination of white strains to cankers on the American chestnut stump sprout clusters, which surround the grafted trees, was found. In the future, to maximize spread of white hypovirulent strains on American chestnut trees, it may be beneficial to re-inoculate trees with hypovirulent strains farther up the main stem after substantial tree growth has occurred.

DEDICATION

This thesis is dedicated to the American chestnut.

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CHAPTER 1
INTRODUCTION

Cryphonectria parasitica (Murr.) Barr[=*Endothia parasitica* (Murr.) P.J. and H.W. Anderson], the causal agent of chestnut blight, killed 3.5 billion American chestnut (*Castanea dentata* (Marsh.) Borkh.) trees within a 40-year period (62). The fungus enters trees through wounds, causing the formation of cankers on tree branches and trunks, which invade the vascular cambium and eventually kill the tree.

The European chestnut (*C. sativa*, Mill.) is less susceptible to blight (30, 10, 11), and survived the epidemic more successfully than the American chestnut. Many surviving stump sprout clusters in Italy were infected with *C. parasitica* , but, upon observation, had cankers which appeared to be healing. These cankers were found to be superficial, as the fungus did not invade the vascular cambium. In 1964, Grente (32) isolated fungal samples from these abnormal cankers, and found atypical strains of *C. parasitica* which exhibited a change in pigmentation. Normal colonies of the fungus have a distinct yellow-orange color, which results from sporulation. The atypical strains lacked pigmentation, appearing white when grown in culture. In addition, most white strains exhibited reduced virulence, and Grente therefore named them "hypovirulent" strains. Furthermore, it has been demonstrated that double-stranded ribonucleic acid (dsRNA) is frequently associated with the hypovirulent phenotype (19). There have been various meanings of the term "hypovirulence" over the years; throughout this thesis it will be defined as low virulence *sensu stricto* (in the strict sense) (39).

In 1979, Turchetti (66) showed that hypovirulent strains of *C. parasitica* have contributed to the high survival rate of European chestnut in Italy. Unfortunately, biological control of chestnut blight in the United States has not been as successful (62). American chestnut trees are not recovering from this disease, and now generally exist only as under-story stump sprout clusters. There are, however, a few exceptions.

In 1977, Elliston et al. (28) and Griffin et al. (39) first isolated hypovirulent strains of *C. parasitica* from American chestnut trees. Hypovirulent strains of the fungus have since been isolated from large, surviving American chestnut trees in the Eastern United States (49, 37). In

addition, Brewer (15) reported finding 24 locations in Michigan which contained American chestnut trees with abnormal cankers that appeared to be recovering. Most of these sites, however, were outside the trees' natural range.

Griffin et al. (41) conducted a study in which virulent *C. parasitica* strains were inoculated on grafted scions, seedlings, and stems of large, surviving American chestnut trees. Resulting cankers were measured, and data indicated that some surviving American chestnut trees were blight resistant. The authors concluded, therefore, that some trees may survive due to a combination of blight resistance and hypovirulence (41).

In 1980, scions obtained form large, surviving American chestnut trees were used to establish grafted American chestnut trees at the Lesesne State Forest in Virginia (24, 21). In 1982 and 1983, natural blight cankers on the stems of these trees were inoculated with a mixture of European and American hypovirulent strains of *C. parasitica* (Table 1.1). The grafted trees at Lesesne State Forest now exhibit high levels of disease control, even in the presence of a large amount of virulent inoculum from the surrounding chestnut plantation. The grafted trees, in contrast to the adjacent chestnut stump sprout clusters, contain a low number of blight-killed branches, and a high number of swollen, superficial cankers. In addition, bark cores extracted from the cankers exhibit a high ratio of healthy to necrotic tissue (21). This is an extremely rare occurrence in the eastern United States. Further information about the spread of hypovirulent strains in and around these grafted trees may help explain the unusually high level of observed disease control.

The objectives of the present study were: 1. To determine if white hypovirulent strains of *C. parasitica* have spread on grafted American chestnut trees in the Lesesne State Forest. 2. To determine if white hypovirulent strains of *C. parasitica* have spread from grafted trees to surrounding American chestnut sprout clumps. 3. To determine if white hypovirulent strains of *C. parasitica* have colonized cankers which formed after artificial inoculation of the branches on grafted trees with a virulent strain of *C. parasitica*. 4. To determine if hypovirulent yellow-orange pigmented strains, containing dsRNA, are present in the blight cankers assayed in objectives 1, 2, and 3.

Table 1.1. Hypovirulent strains of *Cryphonectria parasitica* inoculated into a zone extending from the ground to 183 cm on the main stems of grafted American chestnut trees at the Lesesne State Forest in 1982 and 1983

Strain	Pigmentation	Origin
Ep4	pigmented	France
Ep43	white/pigmented	France
Ep47	white	Italy
Ep49	white	Italy
Ep51	white	Italy
Ep60	pigmented	Michigan
Ep88	pigmented	Michigan
Ep92	pigmented	Michigan
Ep171	pigmented	Michigan
Ep172	pigmented	Virginia

CHAPTER 2
LITERATURE REVIEW

2.1 Chestnut blight epidemic in the United States and Europe

In 1904, The first case of chestnut blight in the United States was found on American chestnut trees at the Bronx Zoological Park in New York City (56). It was most likely introduced into the country on diseased nursery stock from Asia, where *C. parasitica* exists as a weak pathogen on Chinese and Japanese chestnuts (5). From New York, the disease spread rapidly via nursery stock, nuts, and natural means (62). Despite efforts to contain the pathogen, by 1945 *C. parasitica* had destroyed almost every American chestnut in the natural range, which extends along the Appalachians from New England to Mississippi (62).

Before the epidemic occurred, one-fourth of the trees native to the Appalachian region were American chestnut. These beautiful, majestic trees were important esthetically as well as economically. The wood, being rot-resistant, was used to make barns, furniture, lumber, fences, and railroad ties (36). In the lumber industry, chestnuts were very popular , as stump sprout clumps regenerate soon after trees are felled. In addition, tannins extracted from the tree bark were used in leather processing. Appalachian families gathered the sweet nuts and used them in a variety of recipes, and sometimes sold them in local markets for cash (18). Ripened chestnuts were also an important food source for wildlife. The destruction of the American chestnut trees has been labeled the worst biological disaster in history (18).

In Europe, Chestnut blight was first recorded in 1938 near Genoa, Italy (13). *Cryphonectria parasitica* spread rapidly on European chestnut, and in 30 years had spread throughout the country (62). From Italy, the disease spread into adjacent countries, although it often went unnoticed due to a decline in the chestnut population from ink disease (45). By 1967, chestnut blight was affecting most areas in Europe where chestnut grew, including France, Switzerland, Turkey, Spain, Greece, and Hungary (36). It has since been found in Austria, Slovakia, Portugal, and Germany (45).

Although chestnut blight was severe in Europe, it did not spread as quickly or cause as much damage as it did in the United States (45). Currently, spread of the disease is slow. There

are no continuos stands of chestnut trees in middle Europe (45) and European chestnut is less susceptible to blight than it's American relative (36, 41). In 1951, Biraghi (14) noticed long dark cankers in a chestnut grove, previously observed to be blight infested, that appeared healthy. This phenomenon was attributed to increased resistance of the trees, and it wasn't until 1964, when Grente (32) isolated fungal samples from these cankers, that hypovirulence was discovered.

The life cycle of chestnut blight begins when infection occurs through a wound in the bark (62). The fungus then begins to grow in the bark tissue, forming pale-colored mycelial fans. The resulting canker expands when mycelial fans penetrate areas of wound periderm, becoming lethal when the vascular cambium is invaded (36). The stem or branch on which the lethal canker occurs dies, causing blighted branches, for which this disease is named.

On the surface of these cankers, orange-yellow stromata break through the bark. Two structures can be found embedded in these stromata: perithecia, the sexual structure, and asexual pycnidia. The perithecia contain ascospores, and the tendrils of pycnidia are made of conidia (62). Ascospores are projected into the air from the ostiole, located at the end of the perithecial neck. This method of discharge allows the spores to be picked up by air currents; ascospores are therefore primarily wind disseminated (44). Expulsion predominantly occurs during the first five hours after rain, at temperatures between 20 and 27^{o}C (44). Ascospores have been retrieved 300-400 feet from stromata, and may be transported much greater distances in high winds (44). Conidia, on the other hand, are embedded in a viscous matrix, and commonly wash down trees or splash to nearby positions during rains (44, 36, 62). In addition, the mucilaginous matrix of a pycnidium allows spores to adhere onto insects and birds, which transport large numbers of conidia at a time. Surprisingly, conidia are somewhat resistant to desiccation, and have been shown to survive in dry soil for up to 2-3 months (44). Although both types of spores can initiate infection (44), ascospores are more important in the chestnut blight life cycle.

2.2 Biological control of chestnut blight with hypovirulent strains

In 1969, Grente and Sauret (35) demonstrated that the hypovirulent phenotype in *C. parasitica* was cytoplasmically transmissible through hyphal fusion. Hyphal fusion, or anastomosis, occurs between strains that belong to the same vegetative compatibility group (v-c

group). Vegetative incompatibility is controlled by five to seven vegetative incompatibility (*vic*) loci (3). Incompatible strains, when paired on acidified, potato-dextrose agar (APDA), form a barrage of pycnidia, and/or a clear zone, where their mycelia meet. When two strains of *C. parasitica* have identical alleles at all *vic* loci, their mycelia grow together, and they are vegetatively compatible. When alleles differ at one or more *vic* loci differ, strains are incompatible (3). A negative correlation has been demonstrated between the frequency of hypovirulence conversion and the number of differing *vic* genes between isolates of *C. parasitica* (54). Anagnostakis and Day (8) 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 phenomenon is known as hypovirulence conversion).

Vegetative incompatibility, however, is not always a major barrier to the spread of hypovirulence. For example, results from two separate studies (8,1) showed that pairings between incompatible strains (belonging to different v-c groups) sometimes resulted in hypovirulence conversion. Kuhlman et al. (52) confirmed this occurrence. Ninety-five percent of 118 virulent isolates, each from a different v-c group, were converted in pairings with 27 hypovirulent isolates. Anagnostakis (4) has also demonstrated that some incompatible strains of *C. parasitica* form weak barrage zones, and the hypovirulent phenotype can be transferred between most of these weakly-barraging strains.

In the forest however, conversion does not occur as easily. Kuhlman and Bhattacharyya (51) collected isolates of *C. parasitica* from cankers on American chestnut in the Appalachians. Isolates were tested for conversion capacity by hypovirulent strains from the area, and susceptibility to conversion was found to be widespread. However, hypovirulent isolates (identified based on abnormal morphology) were found in only four of the forty-one cankers sampled (51). The researchers therefore hypothesized that a factor other than vegetative incompatibility was limiting spread of the hypovirulent phenotype. In addition, Bissegger et al. (12) have suggested that vegetative incompatibility is not a major barrier for the spread of hypovirulence in Switzerland, where there are fewer v-c groups than in the USA.

In 1977, Day et al. established an association between dsRNA and the hypovirulent phenotype in *C. parasitica* (20). This dsRNA has recently been classified as *Cryphonectria hypovirus* (46), and is enclosed in pleomorphic vesicles, constructed of host-derived lipids, which are 50 - 80 nm in diameter. In addition to their association with dsRNA, many hypovirulent strains exhibit abnormal colony morphology, including reduced growth rate, pigmentation, and sporulation. There are currently three lines of evidence supporting these associations. When EP113, a white, hypovirulent, dsRNA containing strain, was paired with EP155 (a virulent, pigmented strain containing no dsRNA), the resulting strain contained dsRNA, was white in color, and hypovirulent (26). In addition, white, hypovirulent, dsRNA containing isolates can be single-spored (grown from a single conidium) to obtain a dsRNA-free isolate. When this occurs, the resulting strain is pigmented and virulent. When dsRNA is eliminated from hypovirulent strains using cycloheximide, a protein synthesis inhibitor, the same results occur (29). Finally, Choi and Nuss (17) showed that transformation of a virulent strain of *C. parasitica* with a full length cDNA copy of a viral RNA, associated with hypovirulence, conferred the complete hypovirulent phenotype.

The amount of dsRNA present in hypovirulent strains generally varies (23). Furthermore, many virulent strains have also been found to contain dsRNA (41). Latency, shown by Elliston (27) to occur as a delay in the expression of hypovirulence, may in part account for these virulent strains which contain dsRNA molecules. However, *C. parasitica* isolates have also been identified which contain no dsRNA, but demonstrate low pathogenicicty (41).

In 1978, Grente isolated hypovirulent strains in Italy which appeared white when grown in culture, due to reduced pigmentation and sporulation. These white hypovirulent strains were later found to contain high concentrations of dsRNA (23). One such strain, EP713 (a convert of EP155, an American strain) has high concentrations of dsRNA that is French in origin, and has therefore been used extensively as a reference strain. It contains a large species of dsRNA (L-dsRNA), which is 12.7 kb in length (58). It also contains two additional species, one between 8 and 10 kb (M-dsRNA), and the second between 0.6 and 1.7 kb (S-dsRNA). EP713 contains 2

open reading frames, ORF A and ORF B. ORF A has been shown by DNA mediated transformation to suppress, among other things, fungal sporulation and pigmentation (19).

American hypovirulent strains of *C. parasitica* are pigmented, making many of them visibly indistinguishable from virulent strains. Most have been shown to contain dsRNA, but at much lower concentrations than the white European strains (23). Dodds (23) lists the dsRNA content of EP713 as 40 μg per 2.0 g of mycelium, and the dsRNA content of most American isolates as 1 μg per 2.0 g of mycelium. American isolates of dsRNA native to Michigan contain slightly higher concentrations of dsRNA, about 4.0μg per 2g of mycelium (23). Pigmented hypovirulent strains which contain dsRNA have been found in the grafted American chestnut trees, which were inoculated with the strains indicated in Table 1.1, at Lesesne State Forest (Griffin, unpublished). There are three types of dsRNA based on molecular weight. Type 1, which is European and characterized by EP113 (from which EP 713 is derived), has estimated molecular weights of 6.2×10^6, 5.9×10^6, 5.0×10^6, and 4.6×10^6. Type 2 is also European, and characterized by EP 4, 47, 49, and 51. It contains dsRNA of approximately 6.0×10^6 and 5.5×10^6, with 4 minor bands. Type 3, native to the United States, has dsRNA with a molecular weight of 5.5×10^6; except in EP 60, native to Michigan, where the molecular weight of the dsRNA is 4.8 and 4.3×10^6.

In attempt to achieve disease control of chestnut blight, researchers have used hypovirulent strains to inoculate cankers on American chestnut trees. Jaynes and Elliston (48) inoculated cankers caused by normal virulent strains with a mixture of ten hypovirulent strains, each with a varying level of pathogenicicty. The mixture used included French-derived American, American, and Italian strains. The treatments were found to be significantly effective in limiting canker growth. In addition, mixtures of four hypovirulent strains, selected for their relatively high pathogenicities, produced cankers that expanded more slowly than those caused by only one of the hypovirulent strains (48). According to these results, combinations of hypovirulent strains seem to be effective in controlling individual cankers.

Despite extensive effort, high levels and long term control of chestnut blight with hypovirulence has not been successful in the natural range of the American chestnut (21). In

Connecticut, Jaynes and Depalma (47) located four chestnut plots , and in 1978 treated cankers on stump sprouts with mixtures of white and pigmented hypovirulent strains over a period of four years. In each plot, inoculations were made out to 25 m from a central point. After four years, Jaynes and Depalma made measurements in the treated area, as well as in the surrounding non-treated area. Data indicated that a higher percentage of trees were surviving within the treated area, and that the average stem diameter was higher in the treated area than in the surrounding non-treated area. Although there was no statistical analysis of this data, trends were the same in all four plots. In 1990, Anagnostakis (7) did a follow-up study of this work. After establishing two comparison plots as controls, she measured stems and average diameter at breast height (dbh) in two of the plots that had been treated by Jaynes and Depalma. Measurements were taken in three areas, basically corresponding to the original treated inner circle, to the non-treated outer circle, and out to 100 m from the central starting point. Data showed that was a difference in the number of large (>2.5 cm diameter) stems and their average dbh, in the measurements taken out to 100m, between the treated plots and the control plots. A conclusion cannot be made from these data, however, as no statistical analysis was done. In addition, dsRNA was extracted from *C. parasitica* isolated from abnormal cankers, and was found in strains with vegetative compatibility types different from those of the original treatment strains. Anagnostakis suggested that dsRNA had spread to other strains and had been maintained in the population. Anagnostakis also suggested that hypovirulent strains may have spread from the treated areas into the surrounding non-treated areas. There is, however, no proof that strains have spread. White strains, which were inoculated at the beginning of the study, were not monitored.

Recently, dsRNAs were recovered from *C. parasitica* isolates obtained in research plots in West Virginia where hypoviruses were released for biological control in the late 1970's (53). Although the hypovirus from Michigan (CHV3) seems to persist in the population of *C. parasitica*, the European hypovirus (CHV1) did not. No biological control of the trees was observed (53).

In contrast to the situation in the United States, trees in Europe are recovering from chestnut blight. Successful disease control has been attributed to the natural occurrence and spread of hypovirulent strains in the *C. parasitica* population (45). Other factors may also be contributing to this success. First, there is a difference in susceptibility between *C. dentata* and *C. sativa* (30, 11). In addition, there are less vegetative compatibility groups among the fungal population (62). Furthermore, most American chestnut trees in the central and southern section of the natural range grow at relatively high altitudes. Griffin et al. (42, 37) suggest 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 to some hypovirulent strains of *C. parasitica*. Severe cankers have surprisingly been found on Chinese chestnut, which is normally highly blight resistant, growing in high altitude locations that have low temperatures (50).

Bissegger et al. (12) studied the Switzerland *C. parasitica* population in two 6-year old European chestnut coppices over a period of four years. The authors propose that hypovirulence plays an important role in the decline of disease severity. In the first year of sampling, 59% and 40% of the *C. parasitica* isolates obtained from cankers occurring in the two plots where white. Data show that cankers containing white isolates killed fewer sprouts, and expanded more slowly, than cankers with orange isolates. In addition, most cankers in the study yielded white isolates of *C. parasitica* at some time during the 4-year period. Results obtained from hypovirulence conversion studies, along with the effective transmission of the hypovirus into rare VCGs, suggest that vegetative incompatibility is not a major barrier for the spread of dsRNA in Switzerland (12). Furthermore, cankers were found in both research plots that contained only orange isolates, yet did not kill their host. This suggests that hypovirulence is not the only factor responsible for disease control (12). The authors concurred with the suggestion that blight resistance in European chestnut may be an important factor (12, 36). The mortality of European chestnut may be delayed, giving hypovirulent strains the opportunity to establish themselves and spread within the canker and throughout the tree (15, 36).

Remarkably, the grafted American chestnut trees at Lesesne State Forest in the United States are exhibiting a high level of chestnut blight control (21). The white strains inoculated into these trees in 1982 and 1983 (Table 1.1) may provide a good marker for the spread of hypovirulence. Previous work, in Virginia and surrounding areas, shows that no white strains found have been virulent, and that none of over 500 *C. parasitica* isolates recovered from American chestnut in the eastern U.S. have been white (40, 35, Griffin, unpublished).

There are several studies which provide insight into possible vectors of *C. parasitica*. Russin and Shain (63) reported finding *C. parasitica* associated with 75 insect species, most belonging to the order Coleoptera. Some fungal isolates recovered from these insects contained dsRNA. Insects carrying the fungus were found up to 32 m from the nearest source of inoculum. In a later study, Russin and Shain (64) found that other fungi, which commonly colonize blight cankers, attract insects to the cankers, which may then spread strains of *C. parasitica*. Mites have also been found in and around chestnut blight cankers, and were shown to carry propagules of the fungus (68), including some hypovirulent strains (43). In addition, carpenter ants have been found feeding on *C. parasitica* in nature, and may help disseminate the fungus (2). In one study, Garrod et al. (31) placed stem agar cultures of hypovirulent and virulent strains on American chestnut trees. Results indicated that both hypovirulent and virulent strains spread equally from the artificial cankers, and were found up to 100 cm from source of inoculum.

In 1992, Choi and Nuss (17) constructed a full-length complementary DNA copy of the L-dsRNA from EP713. When this cDNA was introduced into virulent strains *of C. parasitica* through DNA-mediated transformation, the complete hypovirulent phenotype was conferred. This significant advance presented many new opportunities, including the engineering of strains with enhanced ability to transmit the hypovirulent phenotype. Chen et al. (16) demonstrated that the introduced cDNA is transferred through asexual sporulation, and is effectively transmitted to ascospores; an event that has never occured with natural hypovirus dsRNA (6). According to Nuss (59), this meiotic transmission of RNA to ascospores represents a method of hypovirulence transmission that is expected to bypass existing barriers, including vegetative

incompatibility. Currently, studies are in progress which are designed to test the efficacy of engineered hypovirulent strains in the field.

CHAPTER 3
MATERIALS AND METHODS

3.1 Isolation of *C. parasitica* from superficial cankers on grafted American chestnut trees and American chestnut stump sprouts

At the Lesesne State Forest in Virginia, natural blight cankers of the superficial and swollen type (38) were identified and labeled on branches and main stems of the three largest grafted American chestnut trees (TH, TG, and RM, which were inoculated with hypovirulent strains of *C. parasitica* in 1982 and 1983). The distances from the ground to each branch, the ground to each main stem canker, and the main stem to each branch canker were measured. Using a small bark-core sampler (1.7 mm diameter), three bark cores were collected from each canker, and rated for superficiality. Cores with white tissue at the phloem side of the cambium were designated superficial; non-superficial cores showed necrosis at the cambium. Samples were placed in a pre-labeled, multi-well ELISA plate, and wells containing bark cores were covered with masking tape. Plates were transported to the laboratory in a cooler, where cores were removed from the ELISA plate, surface disinfested in 1% NaOCl for 2 min, and plated on acidified potato-dextrose agar (APDA). Cores were monitored daily for mycelial growth, and subcultures of *C. parasitica* isolates were transferred onto APDA plates. Colony pigmentation was evaluated after 7 and 14 days of growth in room light. White isolates appeared to be at least 50% white over the colony surface after 7 and 14 days of growth. When 50% or more of the colony surface contained a yellow-orange pigment, isolates were designated pigmented. Each isolate was evaluated in this manner at least once. When results were unclear, additional transfers and evaluations were made. Stock cultures were made of each isolate, and maintained on PDA (potato-dextrose agar) slants at 4^{o}C.

Superficial and swollen blight cankers on American chestnut stump sprout clusters, which grow in the area adjacent to the grafted trees (Fig. 4.4), were identified and labeled. The distance from each sprout cluster to the nearest grafted tree was measured. In the spring and summer of 1996, three bark cores were collected from each canker. Using the small bark core sampler, cores were rated for superficiality and temporarily stored in a multi-well ELISA plate. In the

laboratory, cores were surface disinfested in 1% NaOCl for 2 min, plated on APDA, evaluated, and maintained as described above.

3.2 Isolation of *C. parasitica* from cankers which formed after artificial inoculation with the pigmented virulent strain WK

In May 1992, inoculations were made on two of the three grafted trees with a standard virulent strain of *C. parasitica* (WK) (G. Griffin, unpublished). Resulting blight cankers were sampled in July 1996: three from the TH graft and three from the TG graft. Two of the cankers sampled from the TG graft were located on a branch, stored at 4° C, that was removed from the tree in 1994 to prevent growth of the WK canker into the main stem. All other cankers sampled were located in the field. After measuring the distance from each canker to the main stem of the tree, ten cores were collected along the periphery of each canker. Bark cores were assayed for superficiality, transported to the laboratory, and assayed as described in 3.1.

In May, 1996, a standard virulent strain of *C. parasitica* (WK) was used to inoculate branches on the TG, TH, and RM grafted trees. Branches, with diameters of at least 3.9 cm and adequate asymptomatic bark space for canker growth, were selected and marked. Three inoculations were performed on each grafted tree using the agar-disk, cork-borer method described by Griffin et al. (40). A bark plug (4 mm diameter) was removed from the branch using a cork borer. A disk of mycelium and agar, removed from a WK colony growing on APDA, was inserted mycelium side down into the site where the bark core was removed. Inoculation sites were then covered with masking tape. To determine if white strains were present in resulting cankers, they were sampled at 5 and 11 months after inoculation. Four bark cores were extracted from the margin of each canker (one from each of four quadrants or sectors). Cores were assayed for superficiality, transported to the laboratory, plated, evaluated, and maintained as described in 3.1.

3.3 Vegetative compatibility among pigmented strains of *C. parasitica*

Pigmented strains recovered from cankers which formed after inoculation with WK were paired with the standard virulent strain (WK) in vegetative compatibility tests using a method

similar to that developed by Anagnostakis (1). Agar disks (5 mm diameter), cut from pigmented colonies, were placed 2 mm apart from WK agar disks near the center of an APDA plate. Duplicate pairings were done on each plate. Test plates were placed in the dark for 14 days, then rated for compatibility as described by Griffin et al. (37). Strongly incompatible reactions produced barrage zones with pycnidia between the two colonies. Weakly incompatible reactions were characterized by barrage zones with little or no pycnidia, and compatible reactions occurred when the two colonies merged.

The approximate number of vegetative compatibility (VC) groups was determined in a population of 65 pigmented *C. parasitica* isolates from the chestnut graft area. Twenty-five pigmented (20 from branch cankers and five from stump sprout cankers) *C. parasitica* isolates were used to create a base group of tester isolates. Isolates were paired in all possible combinations. Agar disks from these VC tester groups were then paired in duplicate with 34 pigmented isolates of *C. parasitica* from branch cankers, and six isolates from stump sprout cankers. Reactions between test isolates were rated using the method described above.

3.4 Hypovirulence conversion of pigmented *C. parasitica* strains by white *C. parasitica* strains

Hypovirulence conversion assays were done to determine if white strains, collected from cankers which formed after artificial inoculation with WK, were capable of converting WK to the white hypovirulent phenotype *in vitro*. White strains were paired with the standard virulent strain WK in the following manner. Using a cork borer (5 mm diameter), disks of WK and disks of white isolates were obtained from cultures growing on APDA. Agar disks were removed and placed, mycelium side down, approximately 10 mm apart at the top portion of an APDA plate (52). Each test was done in duplicate. After 14 days of growth in room light, plates were examined for any change in the WK pigmented colony morphology as it grew down the plate. Four cores were then removed from the pigmented side of the pairing, close to the colony intersection line, and transferred to new plates. Resulting colony color was evaluated after 7 days of growth. When a change in the colony morphology of WK from pigmented to white was

observed after 14 days, an agar disk was removed from the new sector and paired with a disk of WK. Conversion was confirmed if WK was converted by the new sector in this second pairing.

To determine if hypovirulence conversion capability was widespread among white isolates on the grafts, 35 pigmented isolates, recovered from branch cankers, were assayed for hypovirulence conversion. Each pigmented isolate was paired against six different white strains (two of which were collected from the RM graft in a previous study), also isolated from branch cankers, using the method described above. Each conversion test was done in duplicate.

3.5 Assay of dsRNA in *C. parasitica*

A total of 60 *C. parasitica* isolates were assayed for the presence of dsRNA. This group consisted of 39 pigmented branch isolates, 11 white branch isolates, and 10 isolates recovered from stump sprout cankers. Four to six disks of mycelium, cut from colonies growing on APDA, were grown in liquid glucose-yeast extract medium containing 10 g glucose, 2 g yeast extract, 1 g K_2HPO_4, and 0.5g $MgSO_4.7H_2O$ per L (GYEM). Four flasks, each containing 90-100 ml of GYEM with 0.9 ml of an antibiotic mixture containing 0.5 mg streptomycin and 43 mg per ml chlortetracycline, were prepared for each isolate. After 14 days of growth in room temperature, mycelium was harvested through a cotton filter using a buchner funnel and a vacuum pump. Fungal mycelium was dried, weighed, and stored at -20^0C. To begin the extraction process, mycelium patties were ground in liquid nitrogen using glass beads (0.17 mm diameter) and a chilled mortar and pestle. DsRNA was extracted and analyzed from the *C. parasitica* samples using the method described by Morris and Dodds (57). Crushed, frozen mycelium (>1g) was placed into tubes with 10 ml of a buffer that contained 200 mM NaCl, 100 mM Tris, , and 1 mM EDTA (=2X STE buffer). Next, 0.5 ml of 10% sodium dodecylsulfate (SDS), 11 ml phenol, containing 1% 8-hydroxyquinoline, and 5 ml of chloroform- isoamylalcohol (24:1) were added. The contents in tube were mixed on a rotary-arm shaker for 30 min, and cellular nucleic acids were separated by centrifugation at 7,649g for 30 min at 0 to -5^{o}C. The aqueous phase was removed, and mixed with 10-15 ml 1X STE buffer and 4 ml 95% ethanol. This solution was purified through columns consisting of 2.5 g CF-11 cellulose powder saturated with 1X STE

buffer that contained 17% ethanol. This ethanol concentration has previously been shown to provide optimum conditions for the binding of dsRNA to the cellulose particles (57). To remove any unbound single-stranded RNA, or DNA, columns were washed with 40-50 ml of STE buffer that contained 17% ethanol. STE buffer (9 ml) was added to elute the bound dsRNA from the column into a tube. Cold ethanol (18 ml) was added, and tubes were incubated at -20°C for at least 2 h. The solution was centrifuged at 7, 649g for 30 min, pellets were resuspended, and 20 µl DNAse (Dnase I from Promega, Madison, WI) plus 100 µl of 0.5 M $MgCl_2$ were added to remove any traces of DNA. After a 60 min incubation, 2 ml of ethanol were added, and tubes were placed at -20°C for at least 2 h. After centrifugation at 7,649g for 40 min, pellets were resuspended, and purified dsRNA was analyzed by 0.7% agarose gel electrophoresis in a buffer that contained 90 mM Tris, 90 mM boric acid, and 1 mM EDTA (=1X TBE buffer). Gels were stained in 1X TBE buffer, containing 40 µl of ethidium bromide (0.6 mg per ml), for 10 minutes. Gels were then de-stained in distilled water for 15-20 min, and photographed on an ultraviolet light source (302 nm) using Polaroid 57 film. Three concentrations of the 12.7 kb dsRNA in EP713, along with a dsDNA ladder were used as markers. Each isolate was assayed and analyzed at least two times. All isolates showing bands on the agarose gel were subjected to RNAse treatment to confirm the presence of dsRNA (57). Gels were placed in a 0.3 M solution of NaCl, with RNAse (Rnase-A from Sigma, St. Louis, MO). Denatured dsRNA extracted from EP713 was used as a control in gels treated with RNAse.

3.6 Virulence trials of selected of *C. parasitica* strains

Virulence trials were performed with 24 isolates of *C. parasitica* (13 isolates from branch cankers, nine isolates from stump sprout cankers, WK, and EP713) in the Jefferson National Forest, using the method described by Griffin et al. (40). In the first week of May 1997, blight-free stump sprouts with sufficient diameter (>3.7 cm) were selected and marked. Using a cork borer (6 mm diameter), a bark core was removed from the stem of a stump sprout. Agar disks (6 mm diameter), cut from 7 to 10-day-old cultures of *C. parasitica* growing on APDA, were used as inoculum. The disk was placed, mycelium side toward cambium, in the inoculation site. Each

isolate was inoculated into five different stump sprouts using a disconnected Latin square design. Five inoculations were done on each stem, and all inoculation sites were covered with masking tape.

On October 6, 1997, canker length and superficiality were measured using the procedure described by Griffin et al. (42). Bark samples were then removed from each of four quadrants of the canker, with the inoculation site at the center, using the small bark core sampler. Non-superficial cores showed necrosis at the cambium; cores with white tissue at the cambium were designated superficial. Canker severity index for each isolate tested was then calculated by multiplying the average canker length by the percentage of necrotic cores obtained for each isolate (41).

CHAPTER 4
RESULTS

4.1 *C. parasitica* isolates recovered from superficial cankers on grafted American chestnut trees and American chestnut stump sprouts

A summary of the number of pigmented and white isolates recovered from the TH, TG, and RM grafted American chestnut trees at the Lesesne State Forest is presented in Tables 4.1, 4.2, and 4.3. Sixty-two natural cankers were sampled, yielding 156 isolates of *C. parasitica*. Fifty-three of these isolates were white, and 103 isolates were pigmented. Bark cores from 10 of the cankers sampled yielded *Trichoderma spp.* only; no *C. parasitica* was recovered. Forty-two (23 white and 19 pigmented) isolates were recovered from 18 cankers on the TH graft (Table 4.1). Seventeen cankers from the TG graft yielded 40 (10 white and 30 pigmented) *C. parasitica* isolates (Table 4.2). Twenty-seven cankers on the RM graft were sampled, yielding 74 isolates (20 white and 54 pigmented) of *C. parasitica* (Table 4.3). The farthest canker containing a white strain was located 564 cm from the H-inoculated zone, on the main stem of the TH graft. The location, position, and pigmentation of all isolates recovered from the TH, TG, and RM trees in this study can be seen in Figures 4.1, 4.2, and 4.3. All bark cores extracted were superficial.

Nineteen superficial cankers located in stump sprout clusters adjacent to the grafts were sampled. Fifty-one isolates of *C. parasitica* (50 pigmented and 1 white) were recovered (Table 4.4). The presence of the white strain could not be confirmed; 11 additional bark cores extracted from the same canker yielded only pigmented colonies. The position of the stump sprout clusters in relation to the grafted trees is presented in Figure 4.4.

The three bark cores extracted from each main stem, branch, or stump sprout canker were evaluated for growth of *C. parasitica* separately. When three pigmented colonies grew from three bark cores, each pigmented colony was counted as one isolate and assigned an isolate code, consisting of the canker number from which it was extracted, and a letter. Occasionally, two or more pigmented or white colonies with distinct morphologies grew from one bark core. In this case, each distinct colony was considered to be one isolate.

The TH graft was the tallest of the three trees studied at 17.1 m when measured in 1996 (21). The RM grafted tree was 14.6m tall, and the TG grafted tree was 12.1 m high. The portion of each grafted tree sampled, relative to its height, was found by dividing the distance of the farthest canker sampled on each tree by tree height. The results were similar for each tree: TH, 0.44; RM, 0.42, and TG, 0.40.

The size of the largest and smallest branches sampled on the grafted trees is summarized in Table 4.5. The smallest branch sampled in the study was located on the RM tree, with a diameter of 4.9 cm at the middle of the branch. This measurement is smaller than the diameter at breast height (DBH) of the largest live stems found on the stump sprout clusters (Table 4.6). All American chestnuts were planted at the same time at the Lesesne State forest, and the sprout cluster age is therefore equivalent to the age of the root stocks on the grafted trees (28 years).

A summary of stem size and survival of stems on American chestnut stump sprout clusters sampled in this study in 1996 and 1997 is presented in Table 4.6. Size and survival determinations were made at breast height in October, 1997. The largest live stem in the stump sprout clusters included in this study was located in sprout cluster F, and measured 8.2 cm at breast height (Table 4.6).

The TH tree had a DBH of 33.8 cm when measured in 1996 (21), more than four times greater than the DBH of the largest stump sprout stem. The contrast between the success of the grafted American chestnut trees, inoculated with hypovirulent strains, and the stump sprout clusters can be seen more strikingly in Figure 4.5. The observer is standing next to dead stems in a stump sprout cluster, with the TH grafted tree (17.1m tall) in the background. In Figure 4.6, the observer is pointing to the top of the zone inoculated with hypovirulent strains (H-inoculated zone) on the RM tree (DBH =33.5cm), with a marked branch, which was sampled in this study, in the background.

The distance from the H-inoculated zone to the farthest canker sampled on each tree was divided by 2 to find the half-way point of the sampling distance (0.5 maximum sampling distance). For cankers yielding *C. parasitica*, the number of white strains per canker on the grafted trees near the H-inoculated zone (<0.5 maximum sampling distance) and on the grafted

trees far from the H-inoculated zone (>0.5 maximum sampling distance) was calculated. The results for cankers in the area near the H-inoculated zone were: RM, 1.23; TG, 0.86; and TH, 2.13. For cankers located far from the H-inoculated zone, the following values were obtained: RM, 0.36; TH, 1.20; and TG, 0.50. For each tree, the number of white strains per canker found near the H-inoculated zone was divided by the number of white strains per canker found far from the H-inoculated zone. The computed ratios were: RM, 3.42; TH, 1.66; and TG, 1.72; with a mean ratio of 2.27.

To determine if a significant difference existed between the number of white strains per canker on the grafted trees near the H-inoculated zone (<0.5 maximum sampling distance) versus the number of white strains per canker far from the H-inoculated zone (>0.5 maximum sampling distance), the data were pooled for all three trees. Using the SAS system and a non-linear Poisson regression (65), the number of white strains per canker near the H-inoculated zone was found to be significantly greater (P=0.0039) than the number of white strains per canker far from the H-inoculated zone. When assessing the goodness of fit for the frequency of white strains per canker on all trees to the Poisson distribution, the Pearson chi-square value obtained was 63.999. This value was compared to the 0.95 percentile value (67.505) of the chi-square distribution, and the fit to the Poisson was not rejected (P=0.0882). The Lloyds index of patchiness (m*/m, where m*=m + [v/m - 1], m= mean, and v= variance) value for these data was found to be 1.36. This value is slightly greater than 1.0, the value indicative of a random pattern (61).

The height of each tree was plotted against the average number of white isolates per canker from the entire tree length sampled (Figure 4.7), for the average number of white isolates per canker near the H-inoculated zone (Figure 4.8), and for the average number of white isolates per canker far from the H-inoculated zone (Figure 4.9). A correlation analysis could not be performed on these graphs, as three observations give too few degrees of freedom to carry out the test. All three figures demonstrate that the tallest tree in the study (TH) contained the highest number of white isolates per canker.

Table 4.1. Number of pigmented and white *Cryphonectria parasitica* isolates recovered from natural cankers in 1996-1997 on the TH grafted American chestnut tree at various distances from the main stem zone inoculated with a mixture of hypovirulent strains in 1982-1983

	Distance from H-inoculated[2] zone (cm)	Isolate Code	Number of *C. parasitica* isolates white	pigmented
Branches	25	32A, 32B, 32C, 32D	2	2
	68	33A, 33B, 33C, 33D, 33E, 33F ,33G	3	4
	80	38B	1	0
	110	34A, 34B, 34C	1	2
	143	35A, 35B, 35C, 35D, 35E	1	4
	169	36A, 36B, 36C, 36D, 36E	5	0
	220	37A, 37B, 37C	3	0
	232[1]	57	0	0
	303	39A	0	1
Main stems	227[1]	50	0	0
	263	58	1	2
	317	59	0	1
	321[1]	51	0	0
	341[1]	52	0	0
	377[1]	53	0	0
	437	THln1, THln2, THln3	1	2
	515	60	4	0
	564	THhn1, THhn2	1	1

[1] *Trichoderma* spp. only were recovered from these cankers.

[2] Hypovirulent(H)-inoculated zone extends 183 cm from ground on main stem. The 0.5 maximum sampling distance =282 cm.

Table 4.2. Number of pigmented and white *Cryphonectria parasitica* isolates recovered from natural cankers in 1996-1997 on the TG grafted American chestnut tree at various distances from the main stem zone inoculated with a mixture of hypovirulent strains in 1982-1983

	Distance from H-inoculated[2] zone (cm)	Isolate code	Number of *C. parasitica* isolates	
			white	pigmented
Branches	70	20A, 20B, 20C	1	2
	132	26A, 26B, 26C, 26D, 26E, 26F	1	5
	134	21A, 21B, 21C, 21D	3	1
	157	22A, 22B	0	2
	174	23A, 23B, 23C, 23D	1	3
	208	24A, 24B, 24C	0	3
	217	27A, 27B	0	2
	302	47A	1	0
	338	25A, 25B, 25C	1	2
	339	48A, 48B	0	2
Main stem	83	61	0	1
	124	62	0	1
	154	63	1	2
	220	64	1	2
	257[1]	45	0	0
	272	65	0	2
	297[1]	46	0	0

[1] *Trichoderma* spp. only were recovered from these cankers

[2] Hypovirulent(H)-inoculated zone extends 183 cm from ground on main stem. The 0.5 maximum sampling distance =170 cm.

Table 4.3 . Number of pigmented and white *Cryphonectria parasitica* isolates recovered from natural cankers in 1996-1997 on the RM grafted American chestnut tree at various distances from the main stem zone inoculated with a mixture of hypovirulent strains in 1982-1983

	Distance from H-inoculated[2] zone (cm)	Isolate code	Number of *C. parasitica* isolates white	pigmented
Branches	52	1A	0	1
	57[1]	16	0	0
	72	12A, 12B	0	2
	88	2A, 2B, 2C, 2D	2	2
	92	17A, 17B, 17C	3	0
	109	3A, 3B, 3C, 3D, 3E	3	2
	127	4A, 4B, 4C, 4D, 4E	0	5
	137	13A	1	0
	160	5A, 5B, 5C	2	3
	182	18A, 18B, 18C	1	2
	189	6A, 6B, 6C, 6D	0	4
	199	14A, 14B	2	0
	213[1]	57	0	0
	272	7A, 7B, 7C	0	3
	279	15A	0	1
	312	19	1	0
	318	8A, 8B, 8C	0	3
	438	9A, 9B, 9C	0	3
	471	10A, 10B, 10C	0	3
	498	11A, 11B, 11C	0	3
Main stem	97	66	1	3
	185	67	1	3
	251	68	0	2
	281[1]	55	0	0
	299	69	1	2
	338	56	0	4
	427	70	2	3

[1] *Trichoderma* spp. only were recovered from these cankers.

[2] Hypovirulent(H)-inoculated zone extends 183 cm from ground on main stem. The 0.5 maximum sampling distance =249 cm.

Table 4.4. Number of pigmented and white isolates of *Cryphonectria parasitica* recovered from natural superficial cankers in 1996-1997 on American chestnut stump sprout clusters at various distances from the nearest grafted American chestnut tree inoculated with a mixture of hypovirulent strains in 1982-1983

Sprout cluster	Number of cankers sampled	Distance from nearest grafted tree (cm)	*C. parasitica* isolates	
			white	pigmented
A	3	395	0	5
B	3	275	0	8
C	2	325[1]	0	7
D	4	885	0	11
E	4	290	0	11
F	4	626	1[2]	8

[1] The grafted tree from which this distance was measured was not included in this study.
[2] Finding was not confirmed; 11 additional bark cores yielded only pigmented strains.

Table 4.5. Size of largest and smallest branches on grafted American chestnut trees sampled for *Cryphonectria parasitica*

	Branch diameter (cm)[1]	
Grafted tree	**Largest**	**Smallest**
RM	8.5	4.9
TG	14.3	6.4
TH	11.3	5.4

[1] Determined at branch midpoint.

Table 4.6. Size and survival of stems on American chestnut stump sprout clusters sampled for *Cryphonectria parasitica*

Sprout cluster code	DBH[1] largest live stem (cm)	DBH[1] largest blight-killed stem (cm)	Number of live stems[2]	Number of blight-killed stems
A	3.1	7.7	4	10
B	5.7	5.6	5	10
C	4.2	5.6	3	7
D	4.9	7.4	5	10
E	4.1	4.9	7	11
F	8.2	5.6	3	4

[1] DBH =diameter at breast height.
[2] Stems greater than 0.3 cm DBH.

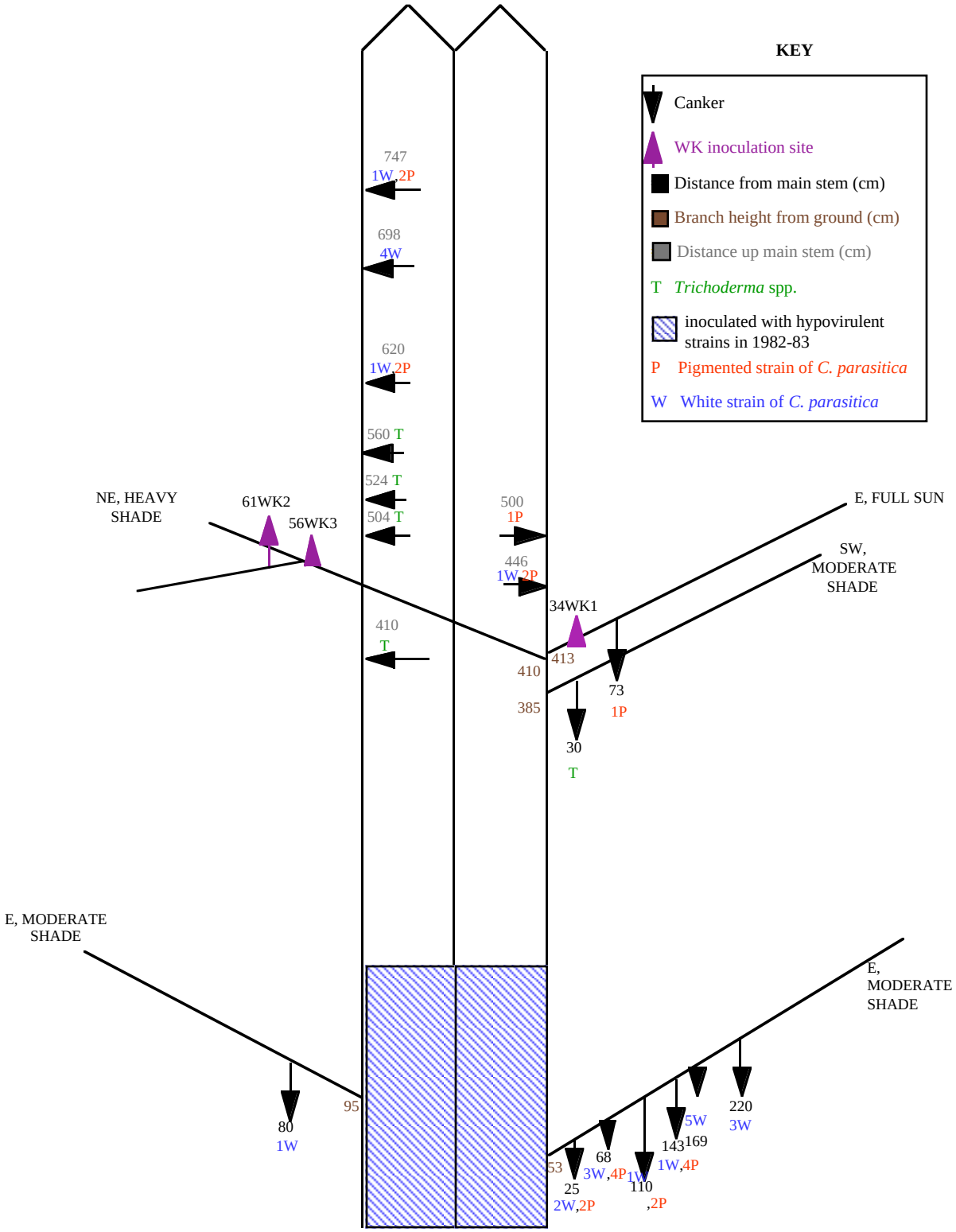

Figure 4.1. Location and position of branches and cankers on the TH grafted tree, with type, number, and pigmentation of fungal strains isolated.

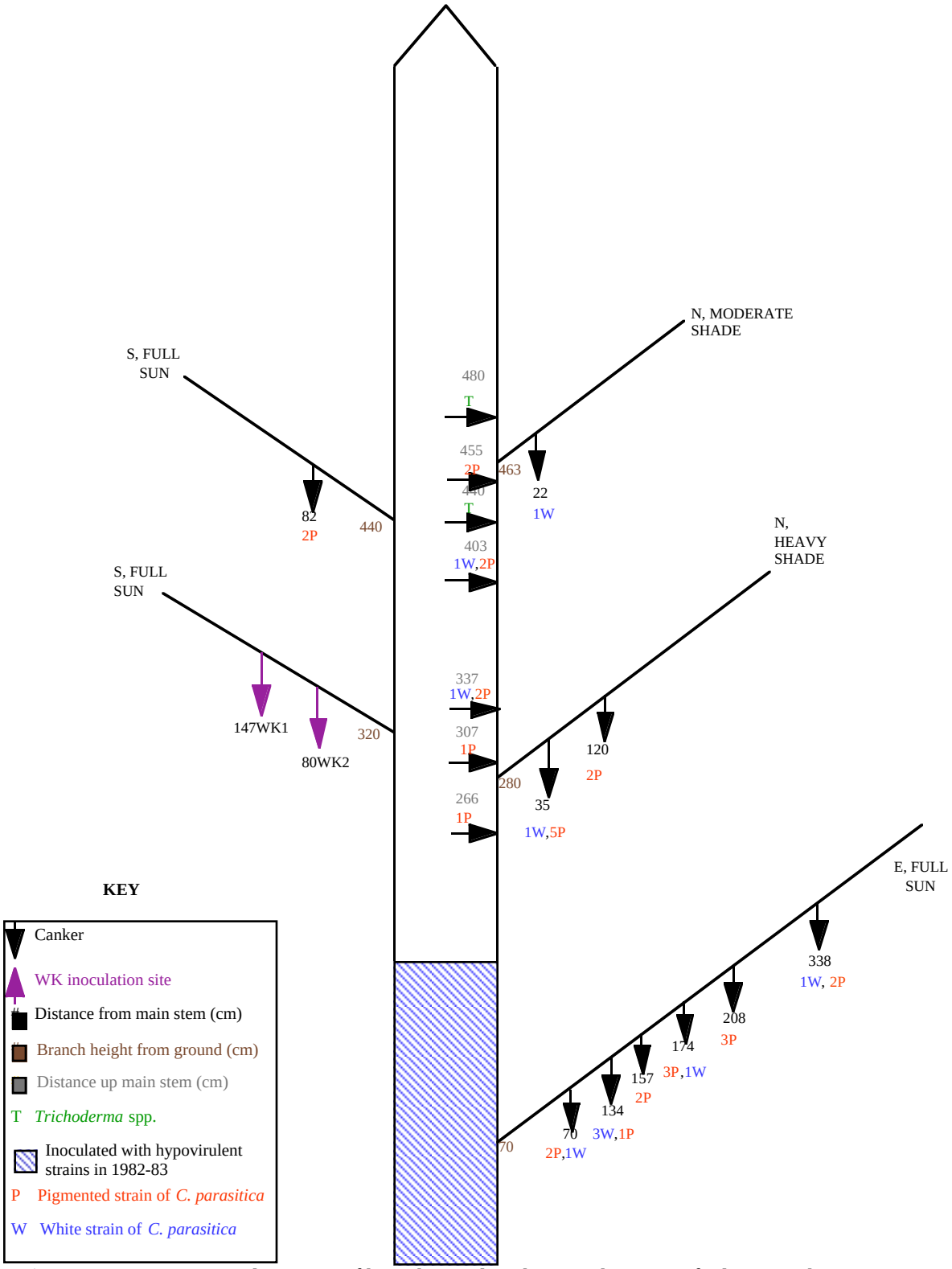

Figure 4.2. Location and position of branches and cankers on the TG grafted tree, with type, number, and pigmentation of fungal strains isolated.

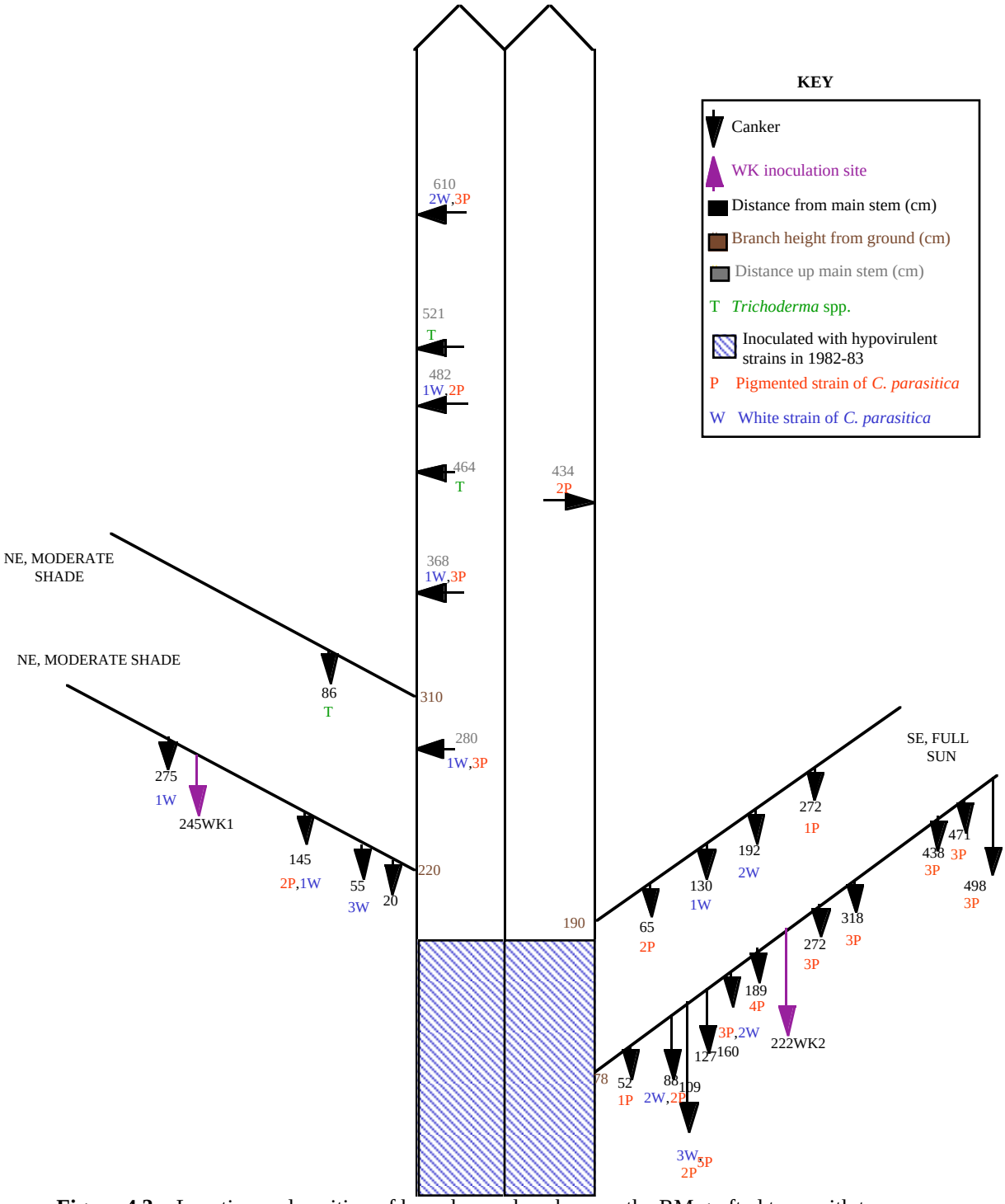

Figure 4.3. Location and position of branches and cankers on the RM grafted tree, with type, number, and pigmentation of fungal strains isolated.

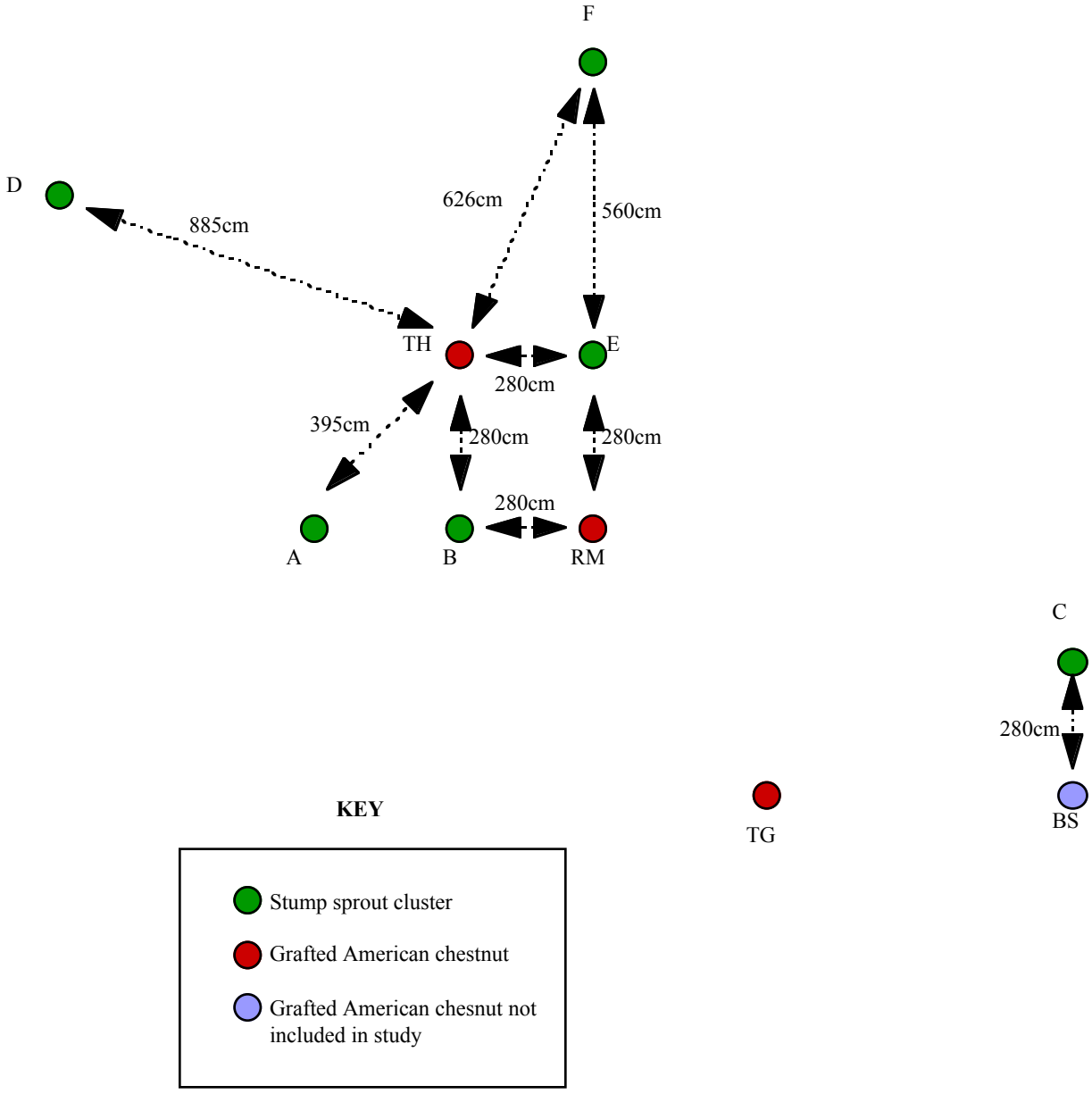

Figure 4.4. Map of American chestnut stump sprout clusters sampled in relation to the position of grafted American chestnut trees.

Figure 4.5. Observer is standing next to blight-killed American chestnut stump sprouts, with the TH grafted American chestnut tree in the background.

Figure 4.6. Observer is pointing to a superficial main stem canker on the RM grafted tree, near the approximate location of the H-inoculated zone upper boundry. A low branch sampled in this study is visible in the background.

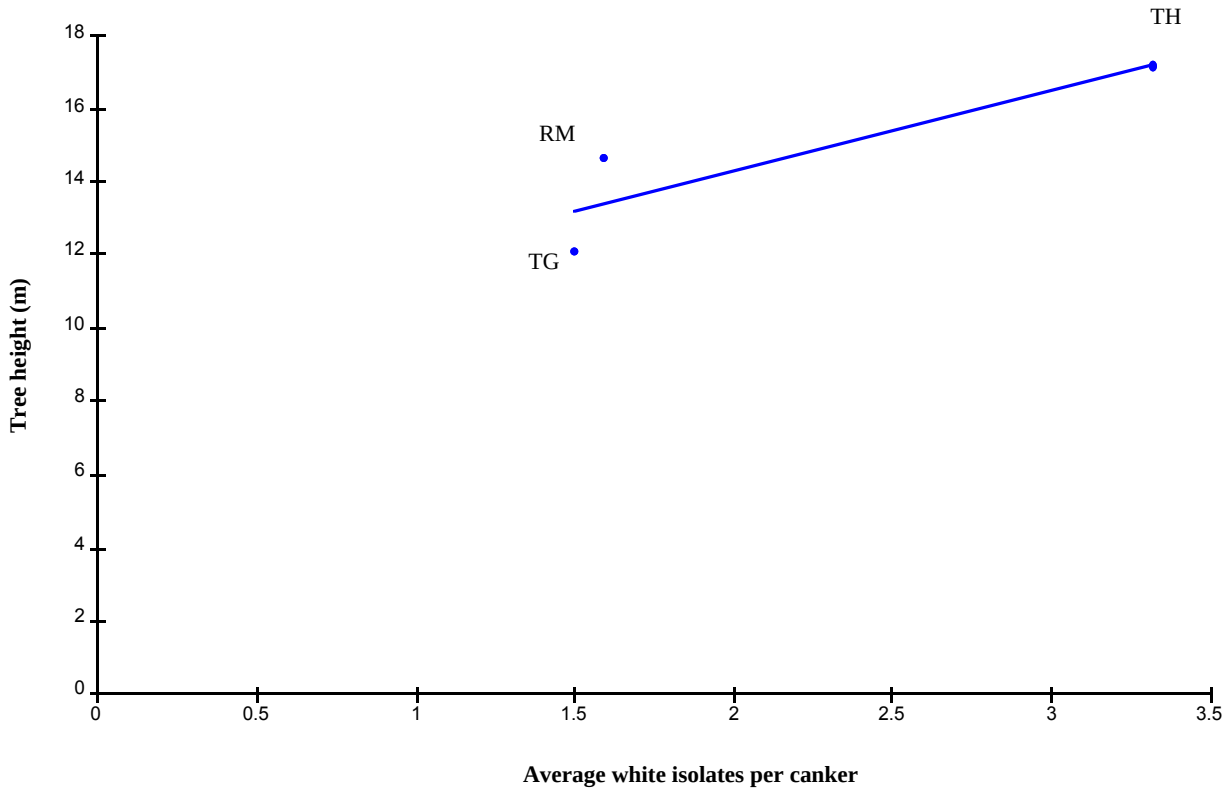

Figure 4.7. Average number of white isolates per canker, combined from the area on the grafted trees near and far from the H-inoculated zone, plotted against tree height.

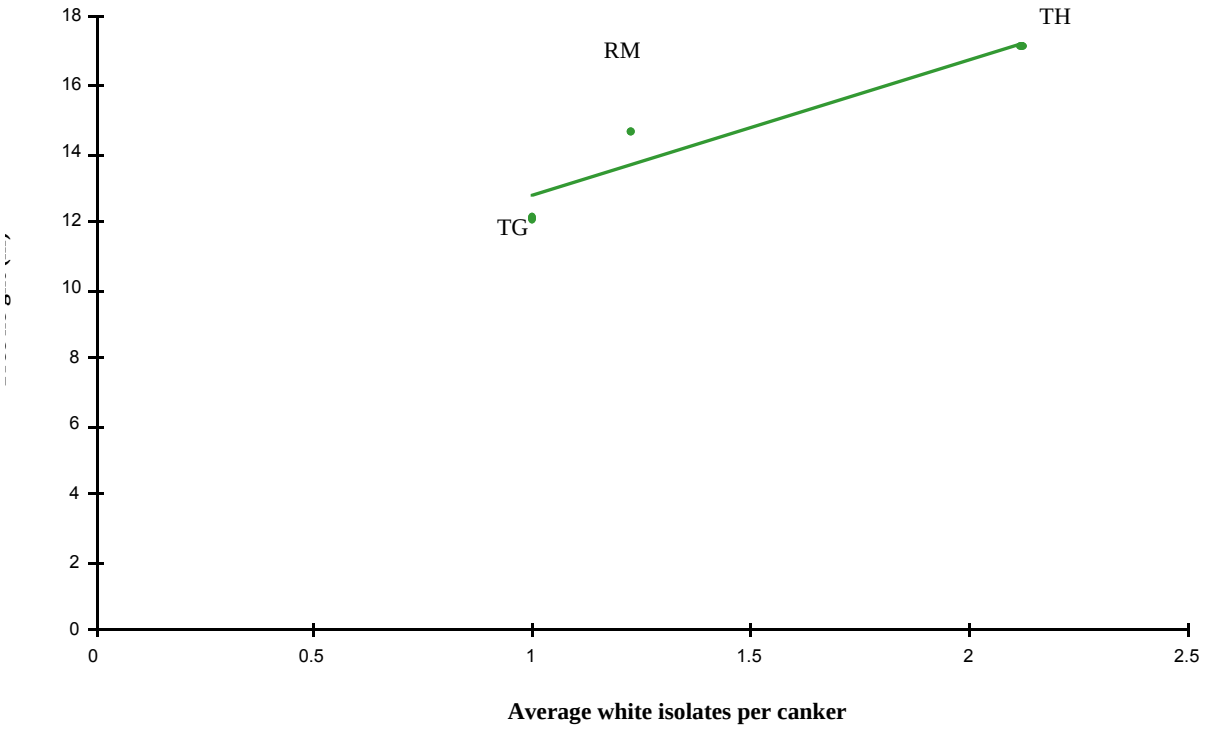

Figure 4.8. Average number of white isolates per canker from the area on the grafted trees near the H-inoculated zone, plotted against tree height.

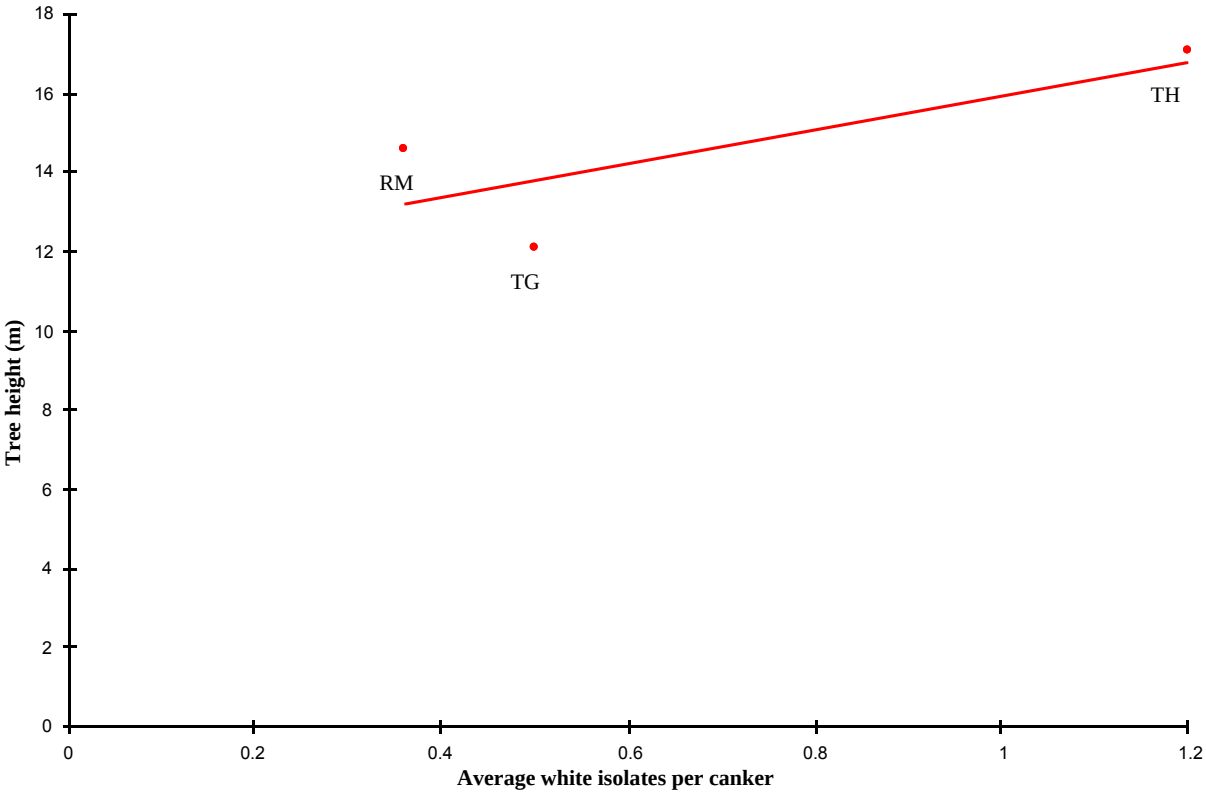

Figure 4.9. Average number of white strains per canker from the area on the grafted trees far from the H-inoculated zone, plotted against tree height.

4.2 *C. parasitica* isolates recovered from cankers formed on grafted American chestnut trees after artificial inoculation with virulent strain WK

A summary of the bark core superficiality and pigmentation of *C. parasitica* isolates recovered 5, 11, and 50 months after artificial inoculation of the three grafted trees with the virulent strain WK is presented in Table 4.7. After 5 months, two cankers resulting from these inoculations had naturally occurring blight cankers adjacent to the WK cankers that were not apparent at the time of inoculation, and the WK cankers were therefore not sampled. All bark cores extracted from WK cankers after 5 months showed superficial necrosis (Table 4.7). Two of the eight *C. parasitica* isolates collected from WK cankers formed on the RM tree after five months were white. None of the eight isolates collected from cankers on the TG tree were white after five months, and four of the eight isolates collected from 5 month cankers on the TH tree were white. Eleven months after artificial inoculation, five of the seven isolates recovered from WK cankers on the RM tree were white. Cankers on the TG tree yielded no white isolates from the 11 month sampling, and three of the eight isolates collected from WK cankers on the TH tree were white (Tables 4.7, 4.8). All bark cores collected from the 11 month sampling showed superficial necrosis. WK canker length and distance from the main stem on the three grafts, along with pigmentation *C. parasitica* isolates recovered from each of these cankers 5 and 11 months after artificial inoculation with WK, are presented in Table 4.8.

Six cankers resulting from inoculation with the standard virulent strain WK in 1992 were sampled. Three cankers from the TH graft yielded 10 pigmented and 16 white isolates of *C. parasitica*. All 30 bark cores removed from these three cankers showed superficial necrosis. Thirty bark cores were removed from the TG graft, 70% of which showed superficial necrosis. Eight pigmented and 13 white isolates were recovered from these three cankers on the TG graft; two of these cankers were located on a branch removed in 1994 and stored at 4^{o} C. This branch, and the branch on the RM tree containing artificial WK inoculations done in 1991, were removed in cooperation with the Virginia Department of Forestry to prevent movement of the virulent strain into the main stem of the graft. Because the branch removed from the RM tree was unavailable at the time of this study, *C. parasitica* isolation data were not collected from this graft.

Table 4.7. Canker superficiality and percentage of *Cryphonectria parasitica* isolates, recovered from cankers, that were pigmented or white 5, 11, and 50 months after inoculation of three grafted American chestnut trees (TH, TG, and RM) with pigmented virulent strain WK

	5 months			11 months			50 months[6]		
Character	**RM**	**TG**	**TH**	**RM**	**TG**	**TH**	**RM**	**TG**	**TH**
Percent canker bark cores superficial	100(8)[1]	100(8)[1]	100(12)[1]	100(8)[1]	100(8)[1]	100(12)[1]	ND[7]	100(30)[1,8]	70(30)[1]
Percent isolates pigmented	75(6/8)[2]	100(8/8)[2]	50(4/8)[2]	29(2/7)[2]	100(9/9)[2]	63(5/8)[2]	ND	38(8/21)[2]	38(10/26)[2]
Percent pigmented isolates compatible with WK *in vitro*[3]	83(5/6)[3]	100(4/4)[3]	100(4/4)[3]	100(2/2)[3]	78(7/9)[3]	80(4/5)[3]	ND	100(4/4)[3]	50(3/6)[3]
Percent isolates white	25(2/8)[4]	0(0/8)[4]	50(4/8)[4]	71(5/7)[4]	0(0/9)[4]	38(3/8)[4]	ND	62(13/21)[4]	62(16/26)[4]
Percent white isolates that convert WK *in vitro*[4]	0(0/2)[5]	-	0(0/2)[5]	0(0/5)[5]	-	67(2/3)[5]	ND	43(3/7)[5]	50(3/6)[5]

[1] Percentage of bark cores with superficial necrosis; numbers in parenthesis indicate number of bark cores that were assayed for superficiality.
[2] Numbers in parenthesis indicate number of pigmented *C. parasitica* isolates over total number of isolates recovered from bark cores.
[3] Numbers in parenthesis indicate number of pigmented isolates vegetatively compatible with WK, over number of isolates assayed.
[4] Numbers in parenthesis indicate number of white *C. parasitica* isolates over total number of isolates recovered from bark cores.
[5] Numbers in parenthesis indicate number of white isolates recovered from cankers that converted WK to the white hypovirulent phenotype over number of isolates assayed.
[6] These data were collected from WK inoculations made in 1992.
[7] No determination.
[8] Two cankers sampled were from a branch removed in April, 1994 and stored at 4^{o} C.

Table 4.8. Canker length, location, and pigmentation of *Cryphonectria parasitica* strains recovered 5 and 11 months after artificial inoculation of the TH, TG, and RM trees with virulent strain WK

Canker code	Canker length 5 months after inoculation (cm)	Canker distance from main stem of grafted tree (cm)	White and pigmented strains recovered 5 and 11 months after inoculation[1]
TGWK1	12.7	147	4P(5); 4P(11)
TGWK2	10.3	80	4P(5); 5P(11)
THWK1	5.0	34	1W(11)[2]
THWK2	10.7	61	4W(5); 2W, 1P(11)
THWK3	0.4	56	4P(5); 4P(11)
RMWK1	9.7	245	2W,2P(5); 3W,1P(11)
RMWK2	8.3	222	4P(5); 2W, 1P(11)

[1]P= pigmented, W= white; numbers in parenthesis represent months after inoculation.

[2] No *C. parasitica* was recovered from this canker at the 5-month sampling.

4.3 Vegetative compatibility among pigmented strains of *C. parasitica*

Vegetative compatibility of pigmented strains, recovered from cankers 5 and 11 months after inoculation with WK, are summarized in Table 4.7. Five months after inoculation, all four pigmented isolates from the TG tree tested were compatible with WK. Four of four pigmented isolates recovered from 5 month cankers on the TH tree were also all compatible with WK. Five of six pigmented isolates of *C. parasitica* collected from cankers on the RM tree were compatible with WK (Table 4.7). Eleven months after inoculation, four of five pigmented isolates tested from cankers on the TH tree were compatible with WK. Seven of nine pigmented isolates collected from the TG tree after 11 months were compatible with WK, and both pigmented RM isolates were compatible with WK after 11 months.

A vegetative compatibility assay was also done with pigmented isolates recovered from cankers formed after artificial inoculation with WK in 1992. All four isolates tested from the TG tree were compatible with WK, and three of six isolates tested from the TH tree were compatible with WK.

Sixty-five pigmented isolates, 54 from branch cankers and 11 from stump sprout cankers, were paired in all possible combinations to determine the approximate number of VC groups present among the isolates tested. Twenty-eight VC groups were found among the 65 isolates tested. Table 4.9 summarizes the number of branch and stump sprout isolates of *C. parasitica* belonging to each VC group. Ten isolates of *C. parasitica* were found in VC group 3, the largest of the 28 groups. Five VC groups contained more than 3 isolates, and 16 of the 28 VC groups contained only one isolate.

Table 4.9. Number of branch and stump sprout isolates of *Cryphonectria parasitica* belonging to each vegetative compatibility (VC) group

VC group	Branch isolates	Stump sprout isolates	Total isolates
1	6	0	6
2	1	0	1
3	9	1	10
4	1	0	1
5	4	0	4
6	4	0	4
7	2	0	2
8	2	1	3
9	2	0	2
10	1	0	1
11	2	0	2
12	0	1	1
13	0	1	1
14	0	1	1
15	0	3	3
16	1	0	1
17	1	0	1
18	1	0	1
19	1	0	1
20	0	1	1
21	0	1	1
22	0	1	1
23	1	0	1
24	1	0	1
25	8	0	8
26	2	0	2
27	3	0	3
28	1	0	1

4.4 Hypovirulence conversion of pigmented *C. parasitica* by white strains

Four white isolates recovered from cankers five months after artificial inoculation with WK (two from the RM tree and two from the TG tree), were paired with WK in a hypovirulence conversion assay. No conversion occured in any of these pairings. Eleven months after artificial inoculation, none of 5 white isolates recovered from cankers on the RM tree converted WK, while two of the three white isolates from cankers on the TH tree converted WK to the white hypovirulent phenotype (Table 4.7).

Hypovirulence conversion assays were also done with white isolates recovered from cankers formed after artificial inoculation with WK in 1992. Three of the six white isolates from cankers on the TH tree, and three of the seven white isolates from cankers on the TG tree, converted WK to the white hypovirulent phenotype (Table 4.7).

Thirty-five pigmented isolates recovered from branch cankers were each paired against six different white isolates in an additional hypovirulence conversion assays. A list of isolates included in the test is presented in Table 4.10. None of the white strains converted any of the pigmented strains to the white hypovirulent phenotype.

Table 4.10. Pigmented and white isolates of *Cryphonectria parasitica*, recovered from branches and main stems of the TH, TG, and RM grafted American chestnut trees, used in conversion assays

Isolate code	Pigmentation
11C	pigmented
18C	pigmented
15A	pigmented
32C	pigmented
20A	pigmented
33G	pigmented
2D	pigmented
35D	pigmented
28B	pigmented
23C	pigmented
2C	pigmented
3C	pigmented
5B	pigmented
27D	pigmented
6A	pigmented
10B	pigmented
20B	pigmented
21C	pigmented
24A	pigmented
26F	pigmented
31A	pigmented
30C	pigmented
34A	pigmented
4A	pigmented
32A	pigmented
22A	pigmented
23B	pigmented
25C	pigmented
33F	pigmented
29A	pigmented
35A	pigmented
33B	pigmented
Thhn3	pigmented
Thln2	pigmented
27D	pigmented
28A	white
21B	white
36C	white
3B	white
RM1MW[1]	white
RM1MSW [1]	white

[1] These isolates were collected from the RM tree in a previous study.

4.5 Assay of dsRNA in *C. parasitica*

Fifty isolates from branch cankers on the grafted trees, and 10 isolates from stump sprout cankers were assayed for the presence of dsRNA (Table 4.11). All pigmented isolates tested were negative for dsRNA (Table 4.11). All white isolates included in the assay contained an approximately 12.7 kbp dsRNA segment, with a band intensity equivalent to that of undiluted EP713 (Figure 4.10). These bands were resistant to digestion by RNAse A at high ionic strength (0.3 M NaCl), confirming that dsRNA was present.

Table 4.11. Strains of *Cryphonectria parasitica* collected from branches on grafted American chestnut trees and surrounding American chestnut stump sprouts that were assayed for the presence of dsRNA

Isolate code and pigmentation	Location	dsRNA[1]	Isolate code and pigmentation	Location	dsRNA[1]
39A(P)	branch	neg	48A(P)	branch	neg
2D(P)	branch	neg	26F(P)	branch	neg
33B(P)	branch	neg	30C(P)	branch	neg
3C(P)	branch	neg	22A(P)	branch	neg
4A(P)	branch	neg	24C(P)	branch	neg
5A(P)	branch	neg	33F(P)	branch	neg
6A(P)	branch	neg	THln3(P)	branch	neg
7B(P)	branch	neg	49A(P)	branch	neg
8A(P)	branch	neg	18C(P)	branch	neg
9B(P)	branch	neg	2A(W)	branch	pos
10B(P)	branch	neg	3B(W)	branch	pos
11C(P)	branch	neg	21D(W)	branch	pos
15A(P)	branch	neg	17A(W)	branch	pos
31B(P)	branch	neg	17B(W)	branch	pos
18A(P)	branch	neg	17C(W)	branch	pos
20A(P)	branch	neg	34C(W)	branch	pos
20B(P)	branch	neg	19A(W)	branch	pos
31A(P)	branch	neg	33E(W)	branch	pos
21C(P)	branch	neg	20C(W)	branch	pos
31C(P)	branch	neg	37A(W)	branch	pos
23C(P)	branch	neg	GG6,2b(P)	stump sprout	neg
24A(P)	branch	neg	GG9,2(P)	stump sprout	neg
25C(P)	branch	neg	SS3B,b(P)	stump sprout	neg
26D(P)	branch	neg	GG6,1(P)	stump sprout	neg
27D(P)	branch	neg	SS4B(P)	stump sprout	neg
28B(P)	branch	neg	SS3A,b(P)	stump sprout	neg
28E(P)	branch	neg	SS7B(P)	stump sprout	neg
29B(P)	branch	neg	GG10,1a(P)	stump sprout	neg
33G(P)	branch	neg	GG6,2a(W)[2]	stump sprout	pos
35D(P)	branch	neg	SS1B(P)	stump sprout	neg

[1] neg= contained no dsRNA; pos= contained a band of dsRNA approximately the same size (kbp) and intensity of undiluted EP713.

[2] see text.

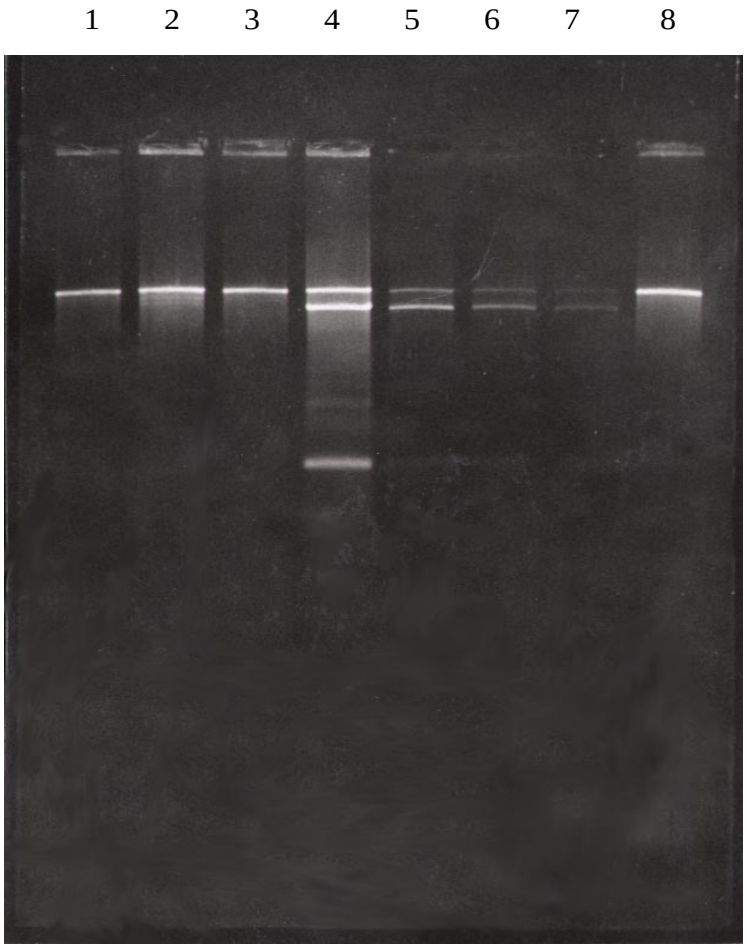

Figure 4.10. Double stranded (ds) RNA banding patterns from four white hypovirulent isolates of *Cryphonectria parasitica* on a 0.7% agarose gel stained with ethidium bromide. Lanes 1, 2, 3, and 8 contain dsRNA from white hypovirulent islolates (3B, 17B, 18B, 17A) collected from branch cankers. Lane 4 contains undiluted dsRNA from EP713 (1.3 -12.7 kbp). Lane 5 contains a 1/10 dilution of dsRNA from EP713; lane6 contains a 1/25 dilution; lane 7 contains a 1/50 dilution.

4.6 Virulence trials of selected strains of *C. parasitica*

A summary of virulence, dsRNA presence, VC group, and distance from main stems of the 24 isolates of *C. parasitica* included in the virulence trials is presented in Table 4.12. Previous cluster analyses, using a large population of *C. parasitica* isolates, yielded four virulence groups with canker disease-severity index (CSI) rating breaks occurring at 25.1, 50.8, and 74.3% of the CSI for virulent reference strain WK (42). Using these percentages and the WK CSI index found in the Lesesne study (1,190), four virulence groups were established among the 24 isolates. Six branch canker, and two stump sprout canker isolates were virulent (V). One branch canker and three stump sprout canker isolates were placed in the intermediate virulent (IV) category. Three branch canker and two stump sprout canker isolates were intermediate hypovirulent (IH). All isolates in the V, IV, and IH categories were pigmented. The four white isolates included in the virulence trials, in addition to one pigmented isolate, were hypovirulent (H). The pigmented isolate in the hypovirulent category came from a stump sprout, and its CSI was based on three variable readings, as two of the cankers could not be evaluated due to interference by natural cankers. The white hypovirulent isolate GG6,2a was found on a stump sprout. As mentioned in section 4.1, this finding could not be confirmed.

Table 4.12. Virulence, dsRNA presence, VC group, and location of *Cryphonectria parasitica* isolates recovered from natural superficial cankers on branches of TH, TG, and RM grafted American chestnut trees and American chestnut stump sprouts

Isolate code and pigmentation[1]	Location	Distance from main stem of graft(cm)	VC group	Relative canker severity index[2]	Virulence[3]	dsRNA[4]
WK(P)	reference	-	-	100	V	neg
18C(P)	branch	143	16	107.2	V	neg
11C(P)	branch	498	3	98.9	V	neg
32C(P)	branch	25	17	89.7	V	neg
15A(P)	branch	272	6	87.4	V	neg
20A(P)	branch	70	5	86.3	V	neg
33G(P)	branch	68	18	82.5	V	neg
GG10,1a(P)	stump sprout	885	21	77.6	V	neg
SS3A,b(P)	stump sprout	290	12	76.0	V	neg
23C	branch	174	1	65.8	IV	neg
SS1B(P)	stump sprout	395	22	65.3	IV	neg
GG9,2(P)	stump sprout	290	20	58.1	IV	neg
SS7B(P)	stump sprout	275	3	54.8	IV	neg
28B(P)	branch	150	19	49.5	IH	neg
GG6,1(P)	stump sprout	626	8	48.1	IH	neg
35D(P)	branch	143	5	32.1	IH	neg
2D(P)	branch	88	7	31.9	IH	neg
SS4B(P)	stump sprout	275	15	31.4	IH	neg
SS3B,b(P)	stump sprout	290	14	11.8	H	neg
17B(W)	branch	55	-	4.2	H	pos
34C(W)	branch	110	-	1.2	H	pos
3B(W)	branch	109	-	0.1	H	pos
GG6,2a(W)[5]	stump sprout	626	-	0.1	H	pos
EP713(W)	reference	-	-	0.1	H	pos

[1] Letters in parenthesis indicate pigmentation of isolate; P= pigmented; W= white.
[2] Based on canker severity index (CSI) of virulent reference strain WK (CSI= 1,190).
[3] V= virulent; IV= intermediate virulent; IH= intermediate hypovirulent; H= hypovirulent.
[4] Neg= contained no dsRNA; pos= contained a band of dsRNA approximately the same size (kbp) and intensity of undiluted EP713.
[5] See text.

CHAPTER 5
DISCUSSION

In 1982 and 1983, the TH, TG, and RM grafted American chestnut trees at the Lesesne State Forest were inoculated with hypovirulent strains of *C. parasitica*. Fourteen years later, the results indicate that white hypovirulent strains have spread to cankers on the main stems and branches throughout the grafted trees. This spread of white hypovirulent strains may be responsible, in part, for the high level of blight control exhibited by the TH, TG, and RM grafts (21). All American chestnuts at the research site in the Lesesne State Forest were planted at the same time. The stump sprout clusters and the root stocks of the grafted trees are both 28 years old (22). There is a striking contrast between the large size and good condition of the stems of the grafted trees (21) and the small size and poor condition of stems in the surrounding stump sprout clusters (Table 4.6, Figure 4.6). Both grafts and sprout clusters have been exposed to the same virulent inoculum, originating from the plantation and the surrounding forest.

The number of white strains on the grafted trees near the H-inoculated zone was significantly greater than the number of white strains on the grafted trees far from the H-inoculated zone. In the future, to maximize spread of white hypovirulent strains on grafted American chestnut trees, it may be beneficial to re-inoculate the trees with hypovirulent strains at a greater distance up the main stem after approximately 10 years of growth. This may result in more rapid spread of white and other hypovirulent isolates, and less branch death on the grafts. As of 1996, a few branches on the grafted trees had died from blight (21). Twelve of 18 pigmented isolates (eight branch and four stump sprout) were found to be virulent or intermediate virulent on American chestnut stems in virulence trials; both are capable of killing chestnut stems. Of these 12 isolates, eight were collected from cankers located far from the three H-inoculated zones. Ascospore showers from pigmented virulent strains, carried by wind currents, may be landing on these branches and stump sprouts. If hypovirulence is to be established in cankers at substantial

distances from the H-inoculated zones, a second inoculation of hypovirulent strains farther up the tree, and perhaps on the larger branches, may be beneficial.

The RM graft had a low average number of white strains per canker (0.36), and a high number of pigmented strains, located far form the H-inoculated zone. This tree has long, low branches that extend past the dense upper canopy of the RM tree. In the early spring, when leaves are down, and rainfall and temperatures are optimal (>20^{o}C) (44), these branches may be in the path of ascospore showers which originate from perithecia of pigmented virulent strains of *C. parasitica* in the chestnut plantation (21). The long, roughly horizontal branches of the RM graft may be more likely to receive ascospore deposits than the vertical main stem. The TH tree, on the other hand, had a high average number of white strains per canker (1.20), and a low number of pigmented strains, in the area far from the H-inoculated zone. The branches on the TH tree are not horizontally oriented.

A high number of white hypovirulent strains of *C. parasitica* in cankers on branches and main stems may be associated with tree vigor. The TH graft, which was the tallest tree at 17.1 m and had 95% of its tree crown alive in 1996 (21), contained the highest average number of white strains per canker (1.67) overall. Further research is needed to confirm or reject this hypothesis.

The results indicate that the pattern of white strains in the cankers on the three grafted trees is nearly random to slightly aggregated, based on the marginal P value obtained for the goodness of fit test to the Poisson distribution, and the low LIP value. This pattern of white isolates found in cankers sampled suggests that the vector or vectors responsible for dissemination of these strains have a chance traveling to all cankers almost equally. The LIP value is density-independent, and all cankers in the vicinity of the vector or vectors have nearly the same likelihood of receiving white strains.. In addition, the size of the canker did not appear to affect the occurrence of white strains in cankers. Five months after WK was inoculated into branches, two white strains were found in one canker on the RM graft. This 9.7 cm long canker, located 245 cm from the main stem of the tree, was much smaller than the natural cankers sampled.

Results from VC assays demonstrated that after 5 and 11 months, 26 of the 30 pigmented isolates recovered from WK cankers were compatible with WK *in vitro*. These compatible strains,

therefore, may be WK. The superficial cankers from which these strains were collected may contain a high population density or biomass of this virulent strain, as 71% of the isolates recovered from the WK cankers were pigmented. Twenty-eight VC groups were found among 65 pigmented natural canker isolates tested, with 16 groups containing one isolate each. These results are similar to VC data found in other studies (9, 51, 55), and indicate that there is considerable diversity in the *C. parasitica* population at this site.

WK was not converted to the white hypovirulent phenotype by any of the white isolates collected from cankers 5 months after artificial inoculation with WK. Two of nine white isolates collected 11 months after artificial inoculation, and six of 13 white isolates collected after 50 months, converted WK to the white hypovirulent phenotype. It is therefore possible that some of these white strains were WK that have been converted to the white hypovirulent phenotype. White isolates from WK cankers on the TH tree, with the most vigor of the three grafts, had the most success converting virulent, pigmented WK to the white hypovirulent phenotype *in vitro*. None of 35 pigmented *C. parasitica* isolates, collected from natural branch cankers, were converted to the white hypovirulent phenotype when paired with six white natural branch canker isolates in conversion assays. Hypovirulence conversion capability is often found to be widespread *in vitro* among some hypovirulent isolates (52), but blight control with hypovirulence generally has not been successful in the eastern United States (21, 62, 45). If hypovirulence conversion is occurring in the TH, TG, and RM trees it was not widespread among the limited number of isolates tested in this study.

A dsRNA molecule of 12.7 kbp, present at high concentrations, was found in all white isolates tested. European white strains containing this type of dsRNA (EP47, EP49, EP51) were included in the mixture of strains inoculated into the grafted trees in 1982 and 1983 (Table 1.1). None of over 500 *C. parasitica* isolates collected from American chestnuts in Virginia and neighboring states were white (41, 42, Griffin, unpublished). Thus, it is likely that the white hypovirulent phenotype of the isolates recovered in this study is due to the dsRNA present in the white isolates inoculated into the grafts in 1982 and 1983. Previous work at Lesesne has demonstrated the presence of pigmented hypovirulent isolates, which contained low or high

concentrations of dsRNA, among isolates collected from a limited sampling near the H-inoculated zone on the grafted trees (Griffin, unpublished). However, none were found in the present study. Further research is needed to confirm the type of dsRNA present among these white and pigmented *C. parasitica* isolates. Samples have been sent to collaborators in Italy, where the genome of dsRNA from white isolates in the grafted trees will be compared to the genome of European dsRNA using molecular hybridization techniques. Recently, Liu et al (53) recovered *C. parasitica* isolates from forest sites were dsRNA had been released in the late 1970's, but biocontrol was unsuccessful (Y.-C. Liu, personal communication). They found that the European type dsRNA (CHV1) did not persist in the population of dsRNAs present at the site.

During the winter of 1996-1997, a branch located 97 cm above the H-inoculated zone on the TG tree was killed. This north-facing branch was in heavy shade for most of the day. The same winter, a low, north-facing branch on the TH tree, inoculated with WK in 1992, was also killed. Branch death due to combined blight and shade-stress has been reported for blight-resistant Japanese chestnut (67), and shade stress may be a factor in the death of branches on the grafted trees.

If white hypovirulent strains have spread from the grafted trees to the surrounding American chestnut stump sprout clusters, it does not appear to be ecologically significant. One white strain was found in a canker located on stump sprout D, 626 cm from the TH graft. In attempt to confirm this finding, 11 additional bark cores were extracted from this small canker. All 11 yielded only pigmented strains. If the white strain were present, it probably was not a significant component of the canker. Four of the nine stump sprout isolates (44%) used in virulence trials were either intermediate hypovirulent or hypovirulent. This value is similar to the 49% intermediate hypovirulent plus hypovirulent isolates found in superficial stump sprout cankers in an American chestnut plantation in a previous study (42). The CSIs of the virulent reference strain WK were also similar in the Lesesne and previous study in a plantation (1,190 and 1,152, respectively). This level of hypovirulence may account, in part, for the presence of superficial cankers on these stump sprouts.

Little or no dissemination of white strains of *C. parasitica* to the stump sprout clusters suggests that a wingless vector is most likely spreading the white hypovirulent strains. Air dissemination of ascospores from the white strains is unlikely, since white strains have not been found to produce ascospores (62). The few stromata that were found on the grafted trees yielded only pigmented strains (Griffin, unpublished).

Ants and mites have been found associated with virulent or hypovirulent strains of the chestnut blight fungus in previous studies (2, 43, 68), and may be partially responsible for the spread of white strains. Little information exists about spread of white strains of *C. parasitica* following artificial inoculation in nature. In France, Grente (33) noted that cankers occurring close to healing cankers, which contained white strains, were more likely to become healing cankers themselves. Grente stated that white hypovirulent strains had spread approximately 5 m around the artificially inoculated cankers after 5 years (34). Spread of white strains from the H-inoculated zone at Lesesne was found out to 5.6 m, a distance similar to that found by Grente, although the time periods are different. Samples at Lesesne were taken 14-15 years after inoculation with white strains; at 5 years natural cankers may not have been present at a distance of 5 m as grafts were probably not very large. During the course of this study, two different insects were found associated with cankers on American chestnut trees. At the Lesesne State Forest, an insect identified as *Ellychnia corrusca* (E. Day, personal communication) was found in the crevice of a canker. It is a member of the lightning bug family, *Lampyridae*, and is a predatory insect often found on tree trunks in the spring. In the Jefferson National forest, a small tube-tailed thrip was found on the bark of an American chestnut stem near a blight canker. It was identified as a member of the *Phelothripidae* family, which includes many insects that feed on fungus spores (E. Day, personal communication). Although further research is required, mites and ants, along with both of the insects collected in this study (in particular the flightless thrip), should be investigated as vectors of *C. parasitica* in nature.

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