Chapter 1

LITERATURE REVIEW

For many years the American chestnut [*Castanea dentata* (Marsh.) Borkh.] was one of the most prized trees in the eastern United States. It not only provided food and shelter for wildlife, but was highly valued by people. The American chestnut was a relatively fast growing, rot-resistant hardwood. The lumber was highly valued for building, and it provided a plentiful supply of sweet chestnuts that were used in various foods. Furthermore, chestnut tree bark provided tannins, which were used in leather processing. Perhaps the greatest value however was the majestic presence of these very beautiful shade trees in our landscape, and timber trees in our hardwood forests.

Unfortunately in the early part of the 20th century the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr [=*Endothia parasitica* (Murr.) P.J. and H.W. Anderson], was introduced into Bronx, New York on nursery stock from Asia. Within 50 years the fungus had spread throughout the natural range of American chestnut, destroying nearly all American chestnut trees. Today the once magnificent American chestnut tree survives predominantly as an understory tree species.

Cryphonectria parasitica is an ascomycete that utilizes wounds in the bark of chestnut trees to gain access and cause infection. The fungus grows in the bark of the tree, in characteristic mycelial fans, producing a canker on the tree (Heald 1926; Roane *et al.* 1986). As the fungus grows, it colonizes the phloem and moves towards the vascular cambium and xylem tissues, destroying tissue as it progresses. Research indicates that *C. parasitica* can utilize chestnut bark tannins as a nutrient source and that hamamelitannin, which is found in high concentrations in American chestnuts but not in blight resistant Chinese chestnut, can be used by virulent strains of *C. parasitica* as the sole nutrient source (Elkins *et al.* 1979, Elkins 1981). A tannase isolated and purified from *C. parasitica* is able to hydrolyze hamamelitannin and produce gallic acid (Farias *et al*. 1994, Roane *et al.* 1986). It is believed that the increase in hydrogen ion concentration can break down pectin in cell walls and kill parenchyma cells (Hebard *et al.* 1984). Other factors, such as phytotoxins, are also believed to precede the advancing mycelial fans and destroy host cells, thus allowing uptake of nutrients by the fungus (Roane *et al.* 1986).

After the fungus has invaded the vascular cambium and girdled (surrounded) the stem, it will kill all parts of the tree above the lethal canker.

Most cankers are sunken and produce numerous fruiting bodies that are present in orange stromata which contain both the asexual conidiomata (pycnidia) and the sexual perithecia. The conidiomata produce numerous conidia, and are exuded in a viscous orange mass of liquid. Commonly, the conidia are disseminated by water, either by splashing or being washed down the tree. Conidia may also adhere to insects and birds that are able to distribute spores over great distances (Heald 1926). The primary method of spread, however, is believed to be the sexual ascospores, which are ejected from the openings of the perithecial necks (ostioles) and are blown by air currents. These currents can carry ascospores 122 m or further (Heald 1926). The ostioles can be seen as pin-head-sized black dots in the stromata.

The situation is less severe in Europe, where the first signs of chestnut blight were observed somewhat later than in the U.S. By 1950 researchers noted that European chestnut trees (*Castanea sativa*, Mill.) were recovering from the blight (Biraghi 1953). European chestnut was demonstrated to contain slightly higher levels of resistance to *C. parasitica* than American chestnut (Griffin 1983), but much of the recovery has been attributed to naturally occurring hypovirulent strains of *C. parasitica* (Grente 1965). These strains were characterized as having lower virulence, reduced fruiting capacity, and the isolates from Europe have a white colony morphology when grown in culture, and are often referred to as white isolates. These characteristics were later attributed to the presence of double stranded RNA (dsRNA) viruses (Moffit and Lister 1975). Hypovirulent strains of *C. parasitica* containing dsRNA have been identified in the United States, but are rare and have not spread to any extent (Griffin *et al.* 1983, Elliston 1985b). Furthermore, the hypovirulent strains in the U.S. are yellow-orange pigmented and are difficult to distinguish from naturally occurring virulent strains.

The dsRNA viruses associated with *C. parasitica* have been extensively characterized and have been classified in the family *Hypoviridae* (Hillman *et al.* 1995). The most highly studied dsRNA hypovirus, which is found naturally in European hypovirulent strains, has been designated as *Cryphonectria hypovirus 1* (CHV1). CHV1 is composed of three separate dsRNA segments. The two shorter segments are the M and

S-dsRNA, which are 8-10 kb, and 0.6-1.7 kb, respectively. No function has been associated with these segments. They appear to be related to the third segment, but have internal deletions. The last segment, L-dsRNA, is 12,712 bp in size, has no protein coat and contains two open reading frames, ORF A and ORF B. Sequence homology exists in both ORF A and ORF B with ssRNA potyviruses (Koonin *et al.* 1991), and ORF A contains a region that codes for the production of a protein (p29) that has been associated with the suppression of sporulation and pigmentation in *C. parasitica* (Nuss 1992). However, other research (Griffin *et al.* 2004) indicates no association of p29 amino acid sequences to the level of pigmentation in *C. parasitica*. Two types of CHV1 are presently recognized (CHV1-Euro 7 in Italy and CHV1-EP713 in France), which have 88-89% sequence identity (Chen and Nuss 1999). Newhouse *et al.* (1983) demonstrated through freeze-substitution, and phase-contrast microscopy that virus particles (latter determined to be CHV1) tend to accumulate in the apex of growing mycelium of *C. parasitica.*

Three other hypoviruses associated with *C. parasitica* have been identified and characterized to a lesser extent: CHV2 (Hillman *et al.* 1994), CHV3 (Smart *et al.* 1999) and CHV4 (Hillman 2000), which does not appear to be associated with hypovirulence. CHV2 was first isolated from *C. parasitica* strain NB58, found in New Jersey, and bears similarity to a hypovirus isolated from China. CHV3 occurs naturally in Michigan and Ontario and has been partially attributed to limited blight control in both of those areas (Fulbright *et al.* 1983). CHV4 appears to have no effect on virulence or phenotype. Recently, a new hypovirus from the family *Reoviridae* was isolated from *C. parasitica* (Hillman *et al.* 2004). The virus is a double stranded RNA virus with 11 segments of dsRNA and a protein coat. Using Koch's Postulates, the reovirus substantially altered the phenotype of the *C. parasitica* isolates and was associated with reduced virulence *in vitro*; however, virulence associated with chestnut I *in vivo* is still to be determined.

In 1980 a reduced hardwood competition chestnut research plot was established in an American chestnut plantation at the Lesesne State Forest, VA on a low altitude (1,350 ft) mesic site. Scion wood from large surviving American chestnut trees were grafted into existing rootstocks in the chestnut plantation (Dierauf *et al.* 1997). After two years (1982-83) a mixture of 10 hypovirulent strains of *C. parasitica*, including one French and

three Italian white hypovirulent strains (Ep-47, Ep-49 and Ep-51), were inoculated into natural cankers in a zone ranging from the ground to 187 cm, termed the "hypovirulent strain inoculated zone" (H-inoculated zone). The four European hypovirulent strains contained CHV1. Eighteen years later the three trees were thriving despite the presence of numerous blight cankers. These cankers are highly superficial with no necrosis or colonization of the inner phloem or vascular cambium (Dierauf *et al.* 1997). It was found that the white hypovirulent strains had spread at high frequency outside of the H-inoculated zone into naturally occurring cankers (Robbins and Griffin 1999). Furthermore, no pigmented isolate collected from outside the H-inoculated zone was found to contain dsRNA, indicating that American hypovirulent strains did not spread on the trees (Robbins and Griffin 1999; Hogan and Griffin 2002a). Based on nucleotide sequence analysis of a 844-bp region in the helicase domain, CHV1 hypovirus isolates from the white isolates recovered at the Lesesne State Forest had high identity (98.3 – 99.5%) to CHV1–Euro 7 (Griffin *et al.* 2004).

Spread of CHV1-containing strains occurs naturally in Europe (Heineger and Rigling 1994), but with exception of the situation at Lesesne indicated above, spread of white, European, hypovirulent strains of *C. parasitica* has not persisted to any extent in the U.S. (Peever *et al.* 1997). This may be due in part to the vast number of vegetative compatibility (vc) types of *C. parasitica* in the U.S. relative to Europe (Anagnostakis 1982). The phenomenon of vegetative compatibility is widespread in fungi. This mechanism is believed to restrict the passage of deleterious nuclear or cytoplasmic determinants from one fungal strain to another through programmed cell death. Vegetative compatibility in *C. parasitica* is believed to be controlled by 5 to 7 loci with two alleles at each locus, and when any one of the alleles at these loci are different the two strains are incompatible (Anagnostakis 1982). Compatible strains of *C. parasitica* allow for the transfer of hypoviruses from one strain to another through hyphal fusion or anastomosis (horizontal transmission) (Anagnostakis and Day 1979). Typically, transfer of hypoviruses does not occur rapidly between individuals of different vc types *in vitro*, however, this sometimes depends on the number of loci with differing alleles and on the specific loci that differ (Liu and Milgroom 1996, Huber and Fulbright 1994).

When hypoviruses are transmitted to virulent strains, the virulent strains can be converted to hypovirulent strains, but this hypovirulence conversion generally occurs more slowly, or does not occur *in vitro* when isolates are in different vc types. Anagnostakis (1983) demonstrated that weekly incompatible interactions could result in hypovirulence conversion *in vitro*. Biella *et al.* (2002) indicated that this may be the result of delayed cell death. Transmission of hypoviruses is inversely related to the rate of programmed cell death. When cell death occurs rapidly, very little hypovirus transmission occurs; however, if the rate of cell death is slowed, hypoviruses can sometimes be transmitted between incompatible strains. Lui and Milgroom (1996) have identified a direct correlation between the number of vc genes that are different and a lack of hypovirulence conversion *in vitro*, and Huber and Fulbright (1994) indicated that changes at specific *vic* loci have differing effects on hypovirus transmission. Currently, most workers favor the belief that vegetative incompatibility is a major barrier to successful biocontrol of chestnut blight due to the failure of hypovirulent strain inoculation to control blight on American chestnut trees in several trials. For example, Hobbins *et al.* (1994) found that vegetative incompatibility was restrictive to CHV1 transmission in *C. parasitica* on artificially inoculated forest American chestnut trees, and no blight control was obtained after 52 weeks. Other data suggests that vegetative incompatibility can be overcome to some degree in cankers, although blight control was still not obtained in these studies (Grente 1981). Shain and Miller (1992) have provided evidence that on American chestnut trees in a forest situation, artificial inoculation of CHV1- infected strains can partially overcome vegetative incompatibility and convert virulent strains *in vivo* if left in contact for a long period of time (65 weeks); however, CHV1 did not persist at this location (Peever *et al.* 1997). Hogan and Griffin (2002a) have provided evidence that CHV1 has spread into at least 45 different vc types on grafted American chestnut trees in the Lesesne plantation, where high levels of blight control have been observed for more than 20 years. The grafted American chestnut trees in this study were derived from large survivors, and earlier tests indicated that low levels of blight resistance in the grafted trees may have provided the extended time needed for CHV1 spread, hypovirulence conversion and blight control (Robbins and Griffin 1999).

In 1998-2001, 110 white isolates were collected from stem and branch cankers from outside the H-inoculated zone of the grafted American chestnut trees at the Lesesne

State Forest. These isolates were classified to vc type and colony morphology (CM) groups using single-spore isolates. A total of 48 different vc types were identified from the 110 white isolates collected. Vegetative compatibility tests revealed that none of the four inoculated white strains were compatible with isolates from the twenty-five major vc types (those consisting of two or more isolates) identified in this study. It appears therefore that the CHV1 hypoviruses, instead of the inoculated white isolates, have spread and 45 vc types represent the minimum number of new vc types into which CHV1 had moved. This finding contradicts the belief that vc diversity poses a major barrier to the spread of hypovirulence (Hogan and Griffin 2002a).

Elliston (1985a) identified a correlation between the specific hypovirulence agent (dsRNA strain) and the colony morphology of European white hypovirulent isolates, while Chen and Nuss (1999) demonstrated that the genotype of *C. parasitica* may also contribute to the colony phenotype of CHV1-infected isolates. Additional studies have used different criteria for classifying the European hypovirulent strains, such as amount of sporulation, presence of orange concentric rings and color of mycelium. Bonifacio and Turchetti (1973) were the first to note the presence of "intermediate" isolates, and described them as having "small pycnidia distributed uniformly on the whole colony". More recently, Coskun *et al.* (1999) described normal virulent strains as those that had "cream colored mycelium, abundant orange pycnidia scattered within concentric rings and spore tendril production". Hypovirulent isolates had "white mycelium and few and large pycnidia" and intermediate isolates were classified as "whitish-cream mycelium with pycnidia uniformly distributed over the entire colony". These descriptions of Coskun *et al.* (1999) assume that no pigmented strain can be hypovirulent and allow for a great deal of subjectivity in rating virulence since pathogenicity trials have not been used by most European researchers. Furthermore, the number of intermediate isolates collected from this and other studies is significant; however often these isolates are classified by most workers to the pigmented, virulent category. Robbins and Griffin (1999) classified *C. parasitica* isolates using a system whereby isolates were classified as pigmented when more than 50% of the colony is pigmented after 7 and 14 days and they designated isolates white when the colony surface is 50% or more white. This system helps with classification; however, it allows for only two categories and a vast amount of morphological variation between and among both pigmented and white groups.

Hogan and Griffin (2002a) classified 110 white, hypovirulent *C. parasitica* isolates recovered from natural cankers into four distinct colony morphology (CM) groups. CM group 1 colonies had white centers with white, fast-advancing margins; CM group 2 colonies had white centers with light to dark brown, wavy margins; CM group 3 colonies had white to moderately pigmented centers with numerous pigmented yellow-orange pycnidia on the margins and sometimes the colony was greater than 50% pigmented; lastly, CM group 4 colonies had lightly yellow-orange pigmented centers with white, fast-advancing margins. Of the four major CM groups, CM1 and CM3 were the most predominant and many of them could be considered intermediate in pigmentation. Furthermore, these two CM groups were found among the single-spore colonies in three of the four original Italian white inoculated strains. It appears therefore that some CHV1 strains from the original *C. parasitica* hypovirulent isolates, inoculated into the grafted trees at Lesesne, contain CHV1 that have greater fitness for spread than others. The work of Hogan and Griffin (2002a), Coskun *et al.* (1999) and Bonifacio and Turchetti (1973) demonstrate (1) the prevalence of intermediate-pigmented isolates in the U.S. and Europe; (2) there is inadequate information on the presence of dsRNA hypoviruses in intermediate isolates; and (3) the importance of intermediate isolates as part of biological control of chestnut blight using hypovirulence. Therefore, for all these reasons, intermediate isolates must be studied more extensively.

This vc and CM typing provides limited information about the population structure of the *C. parasitica* isolates on the grafted trees at Lesesne State Forest. Further knowledge of the *C. parasitica* reproductive capabilities in the cankers at Lesesne could provide information regarding the source of inoculum or method of fungal spread. Vc typing has given some insight, but the spread of vc groups could be due to clonal propagation, either by conidia or hyphal fragments, or by the airborne ascospores produced by sexual mating. Abundant infection by ascospores should result in high genetic diversity of *C. parasitica* on the grafted trees. Currently, ascospores appear to be the primary method of spread; however ascospores do not carry the CHV1 virus and the vast majority of cankers at Lesesne contain white isolates (Hogan and Griffin 2002a, Robbins and Griffin 1999). Therefore, cankers could be formed by either sexual or asexual inoculum, but the spread of CHV1 is most likely due to subsequent spread of

CHV1-containing asexual inoculum (vertical transmission) or by vectors such as mites (Wendt *et al.* 1983).

Experimentation in biocontrol of chestnut blight using hypovirulence necessitates the identification of strains of *C. parasitica*, which have greater ecological fitness or superior traits for CHV1 spread. In the previous study (Hogan and Griffin 2002a), a number of CHV1-infected *C. parasitica* isolates in the same vc type were recovered from different cankers, and even different grafted trees. Furthermore, the cankers at Lesesne were composed of multiple vc types; however, one vc type in each canker appeared to dominate the others.

The movement of CHV1 within a vc type is also of importance for biological control, and can be investigated using spatial pattern studies. The spatial pattern of white isolates within individual cankers at Lesesne was investigated using a 7x7 lattice grid (Hogan and Griffin 2002b). One branch and one main-stem canker was sampled on each of the three trees yielding a total of forty-nine bark-core samples. The isolates were identified as to white or pigmented for each canker and the vc type was identified for all isolates in two of the cankers. All but one canker containing white isolates had a random pattern of white isolates, and both of the cankers identified to vc type indicated that the majority of white and pigmented isolates were in one dominant vc type. Frequently, lattice cells containing white isolates were adjoined to lattice cells containing pigmented isolates in the same vc type, indicating no spatial separation. This finding suggests there is incomplete movement of CHV1 within a vc type *in vivo*.

One possible explanation for the lack of virus movement within a vc type is that the fungus developed resistance to virus infection. Polashock *et al.* (1994) identified a virus-resistant mutant of *C. parasitica in vitro*. A hypovirulent, dsRNA (CHV2)-containing strain of *C. parasitica*, NB58, produced a "phenotypically-distinct sector" in culture, which was found to be free of dsRNA. The virus-free sector was found to be isogenic with the parent, and multiple conversion pairings between the parent and other vegetatively compatible virus-containing strains were unsuccessful. This lack of conversion was consistent regardless of viral strain. A similar sectoring phenomenon was observed with white CHV1 containing isolate, THL-513b (Hogan and Griffin

2002b). Single spores from this field isolate were pigmented upon isolation, yet gave rise to a white sector on three separate mass transfers of the pigmented colony.

Another explanation for incomplete CHV1 movement in a vc type is that the *C. parasitica* thallus was separated into functional units. Rayner (1991) argues that the mycelium is a "functional unit, an individual". Olson (1999) has adapted this idea to form a model whereby the entire mycelium of a fungal individual is one genetic mycelium unit (GMU), which is composed of a number of functional mycelium units (FMU). According to this model the FMUs, which make up the GMU, may act independently of each other, or in association through hyphal anastomoses. When acting in association, the FMUs could communicate with each other through nutrient translocation, or signaling. Olson (1999) has hypothesized that signaling may form as a result of variable nutrient supplies in the environment of the thallus. It has been suggested that intracellular nitrogen reserve changes may stimulate long-distance electrical signals within the fungal mycelium (Watkinson 1999). Through signaling, parts of the fungal thallus may then die-back and parts of the thallus closest to nutrients may thrive, thus producing FMUs. This hypothesis is also supported by Davidson *et al.* (1996), who demonstrated that "shifts in mycelial pattern can be explained by purely contextual, rather than genetic changes."

Because a FMU shares nutrients, identification of FMUs is observable through the use of radiolabeled nutrients, followed by autoradiography. Timonen *et al.* (1996), and Olsson and Gray (1998), have used this technique to identify the reallocation patterns of nutrients in intact mycorrhizal systems, and agar-grown cultures. Both studies identified special patterns of movements in the mycelium. Olson and Gray (1998) found tangential movement of nutrients along the hyphal front of agar grown cultures, indicating anastomosis along the periphery of the colony.

Another possible explanation for incomplete hypovirus movement in a vc type is that the age of mycelium plays a role in virus movement throughout a thallus. Shain and Miller (1992) demonstrated incomplete movement of CHV1 throughout an artificially established canker composed of one vc type. Artificially established cankers were challenged at the base with a vegetatively compatible CHV1 containing strain of *C. parasitica*, and fungal isolates were taken from all parts of the canker over a period of 65

weeks. Fungal isolates taken from the periphery of the canker contained hypovirus; however, most isolates taken from the center of the canker were found to be free of hypovirus. It is possible therefore that the hypovirus was unable to spread to the oldest part of the canker, the center, because the older mycelium was not functional, yet still viable.

Trinci and Collinge 1974, and Markham and Collinge 1987 proposed that woronin bodies can explain why some nutrients or cytoplasmic entities are restricted to areas of the fungal thallus. Woronin bodies are microscopic organelles present in the cytoplasm of filamentous fungi which are consistently associated with the septal pore. Newhouse *et al.* (1990) identified woronin bodies in *C. parasitica* which occluded the septal pore; however, these workers did not consider the possibility that woronin bodies were restricting hypovirus movement. The presence of woronin bodies in *C. parasitica* could contribute to the formation of FMUs or act in conjunction with the age of the *C. parasitica* thallus to restrict hypovirus movement.

Environmental factors also appear to play a role in biological control. Griffin (1992) and Griffin *et al.* (1991) suggested that low altitude, mesic sites with low hardwood competition are the most favorable to chestnut blight biological control with hypovirulence. Furthermore, an increase in electrolyte leakage from American chestnut bark tissue, a measure of plant stress, and breakdown of superficial cankers derived from hypovirulent strain inoculation, have been demonstrated at high altitudes (1067 – 1158 m) versus low altitude (180 m) (Griffin 2000). The effects of high altitude and the associated low temperatures on CHV1-infected strains of *C. parasitica* have not been examined extensively; however, some evidence has shown that freezing temperatures (-10^0C) has a deleterious effect on hypovirus survival in the fungus (Friese *et al.* 1992). Knowledge of the effects of environmental factors such as low temperature on CHV1-infected *C. parasitica* is imperative in a biocontrol system. The overall objectives of this study are: (1) to determine the frequency and phenotypic diversity of CHV1-infected *C. parasitica* isolates recovered from stromata and canker tissue located on grafted American chestnut trees at the Lesesne State Forest and artificially established cankers on American chestnut at Paint Bank, Jefferson National Forest; (2) to determine the presence or absence of CHV1-Euro7 hypovirus in intermediate (30% to 70% pigmentation) single-

spore isolates of Ep-49(’99) and intermediate isolates recovered from American chestnut research plots; (3) to investigate the roles of colony age, resistance to hypovirus infection, and functional mycelial units in the failure of CHV1 to move throughout a vegetative compatibility type of *C. parasitica in vitro*; and (4) to examine the role of low temperatures and topographic site (elevation) on CHV1 survival within *C. parasitica* colonies *in vitro* and *in vivo*.