CHAPTER 2
LITERATURE REVIEW

For many years the American chestnut (*Castanea dentata* (Marsh.) Borkh.), was one of the most valued 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 trees provided tannins, which were used in leather processing. Perhaps the greatest loss however is the absence of these very beautiful shade trees in our landscape.

In 1904 the chestnut blight fungus *Cryphonectria parasitica* (Murrill) Barr (=*Endothia parasitica* (Murr.) P. J. and H. W. Anderson) was discovered in the United States in New York, where the first reported case of chestnut blight was in the Bronx Zoological Park (Heald 1926). It is suspected that nursery stock from Asia was imported to the U.S. and it carried the pathogen. From New York, chestnut blight spread rapidly throughout the U.S. and within 50 years had destroyed almost all American chestnut trees in the natural range (Heald 1926). Today the American chestnut is virtually absent as a competitive forest species. In a similar manner, the disease was originally quite devastating in Europe; however, it did not spread as quickly as in the U.S. This is most likely due in part to the greater level of natural blight resistance of European chestnut (*Castanea sativa* Mill.) compared to the American species (Griffin et al. 1983).

The chestnut blight fungus, *C. parasitica,* is an ascomycete that utilizes wounds in the bark of chestnut trees to gain access and cause infection. The fungus then grows in the bark tissue where it produces characteristic mycelial fans and a canker on the tree (Heald 1926; Roane, Griffin, and Elkins 1986). It colonizes the phloem and progresses to

the vascular cambium and xylem tissues, destroying tissue as it progresses. Once the fungus has invaded the vascular cambium and girdled the tree it will kill all parts of the tree present above the lethal canker.

Most cankers formed are sunken and produce numerous fruiting bodies that are present in orange stromata, which contain both the asexual pycnidia and the sexual perithecia. The pycnidia produce numerous conidia, which are exuded in a viscous mass of orange liquid. The conidia are then commonly disseminated by water, either by splashing or being washed down the tree. Conidia may also adhere to insects and birds, which 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 wind disseminated. The ostioles, or openings of the perithecial necks, can be seen as pin-head-sized black dots in the stromata. The ascospores are discharged through the ostioles and are blown by air currents, which can carry them 122 m (400 ft) or further (Heald 1926).

By 1950 it was evident that European chestnut trees in Italy were recovering from the blight (Biraghi 1953). Biraghi reported that many of the new shoots were surviving longer than the normal four or five years. It was soon discovered that not only was the spread of the disease slower in Europe, but many of the European chestnut trees were actually recovering from the blight (Bazzigher et al. 1981). Furthermore, it was revealed that many of the cankers were healing and that the fungus was limited to the outer layer of bark. In 1964 Grente (1965) isolated samples of *C. parasitica* from these cankers, reinoculated them into healthy trees and discovered that the isolates had reduced virulence. He labeled these isolates hypovirulent.

Hypovirulent strains of *C. parasitica* are usually characterized by low pathogenicity, reduced fruiting capacity, and the presence of double-stranded RNA

(dsRNA) (Ellison 1985). Moffit and Lister (1975) and Day et al. (1977) demonstrated that dsRNA was consistently associated with the hypovirulent strains examined. Hypovirulent strains of *C. parasitica* have been found in both the U.S. and Europe (Griffin 2000). In 1977, both Elliston (1978) and Griffin et al. (1977) isolated hypovirulent strains from American chestnut trees. Since then a number of dsRNA-containing hypovirulent strains have been isolated from American chestnuts. In Michigan, Day (1977) and Elliston (1985,1985) reported finding hypovirulent isolates of *C. parasitica* with dsRNA unique to the United States. Many, but not all, American hypovirulent isolates are yellow-orange and have other colony characteristics, which may make them indistinguishable from normal virulent isolates. In contrast, the hypovirulent strains from Europe have a type of dsRNA that reduces the amount of pigmentation of the isolates, when grown in culture. They usually have a white appearance.

The dsRNA in European hypovirulent strains has been designated by Hillman et al. (1995) as *Cryphonectria hypovirus 1* (CHV1). In 1992 the European dsRNA from EP-713 (an American fungal strain containing French dsRNA) was characterized (Nuss 1992). 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, and contains two open reading frames, ORF A and ORF B. ORF A contains a region that codes for the production of a protein that has been shown to suppress sporulation and pigmentation in *C. parasitica* (Nuss 1992).

CHV1 typically produces white colony morphology in *C. parasitica* when grown in culture, whereas virulent, dsRNA-free strains have yellow-orange pigmented colonies;

however, these changes in color are variable with regard to the hypovirulent fungal strain (Elliston 1985). Elliston characterized all the European dsRNA-containing strains he examined as "white for at least the first 5 days after transfer, then some developed cream, yellow or light orange centers, or light orange concentric rings". 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. 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. Robbins and Griffin (1999) utilized 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.

When hypovirulent strains are inoculated into healthy chestnut trees they commonly produce small, superficial, swollen cankers, which have fewer stromata (Griffin et al. 1983). Occasionally, a hypovirulent strain will not behave in the predicted manner and will produce much larger, but superficial cankers. Elliston (1978) found that fungal isolates taken from the margin of large cankers, resulting from artificial inoculation with white European strains, might still be hypovirulent if reinoculated into a healthy tree. This suggests that latency of hypovirulence expression may occur with some isolates.

The spread of European hypovirulent isolates occurs naturally in Europe; however the same situation does not exist in the U.S. (Heineger and Ringling 1994). This may be due in part to the overwhelming number of vegetatively compatible (VC) groups in the United States as compared to Europe (Cortesi et al. 1998; Kuhlman and Bhattacharyya 1984; Anagnostakis 1983). Vegetative compatibility is controlled by at least seven loci, and when any one of the alleles at these loci is different between fungal strains, they are vegetatively incompatible (Anagnostakis 1982). When isolates are vegetatively compatible it is possible for the hypovirulence viruses (hypoviruses) to be transferred from one fungal strain to another through hyphal fusion, or anastamosis (Anagnostakis and Day 1979).

Anagnostakis (1983) demonstrated that through anastamosis, hypovirulent isolates could "convert" virulent isolates in the same VC group to the hypovirulent state. This "hypovirulence conversion" generally does not occur when isolates are in different VC groups. There is evidence however, that transmission of CHV1 and other hypoviruses can overcome vegetative incompatibility and to some extent convert normal strains to hypovirulent strains *in vitro*. Anagnostakis (1983) demonstrated that "weekly" incompatible interactions can result in hypovirulence conversion, and Liu and Milgroom (1996) identified a correlation between number of vegetative compatibility loci that were different between strains and hypovirulence transmission. It was found that when two isolates differed by one *vic* gene, transmission frequency was 0.50. The frequency dropped to 0.13 when two genes were different, and 0.03 - 0.04 when isolates differed by more than two *vic* genes. Bazzigher et al (1981) identified a "bridging" mechanism, whereby two VC groups, not converting each other to hypovirulence, are "linked" by a third VC group which can convert *in vitro* the other two VC groups to hypovirulence.

Furthermore, Kulman and Bhattacharyya (1984) identified a group of isolates, called broad converters, which can convert many VC groups to hypovirulence *in vitro*, and additional isolates that are susceptible to conversion *in vitro* by many VC groups.

The barrier that vegetative incompatibility poses is reduced significantly in Europe where the number of VC groups is significantly lower than in the U.S. In 1998 Cortesi et al. (1998) surveyed the number of VC groups from 11 Italian and five Swiss subpopulations of *C. parasitica* and found a total of 31 different VC groups, 15 of which were common to both countries.

It appears that for *in vivo* forest situations, time may play a large role in conversion. Robbins and Griffin (1999) artificially established cankers on American chestnut trees with virulent strain WK. The cankers were known to also be infected with "white" hypovirulent strains inoculated into the tree at an earlier date. It was found that after 5 months WK could not be converted to the white phenotype *in vitro* by any white isolate taken from the artificially established canker. After 50 months however, 6 of 13 isolates retrieved from the canker were able to convert WK to the hypovirulent phenotype *in vitro*. This suggests that over time the WK strain in the cankers was converted to hypovirulence *in vivo.* One factor that may allow the time needed for hypovirulence conversion is low levels of natural host resistance to *C. parasitica* (Robbins and Griffin 1999; Bizzegger et al. 1996; Griffin et al. 1983). Low levels of host resistance may prevent "quick kills" and allow time for hypovirulence conversion to take place *in vivo.*

The phenomenon of hypovirulence conversion has allowed researchers to study the effects of different hypovirulence agents present in various strains of *C. parasitica*. Elliston (1985) found that generally colony morphology of the isolates was dependent on the hypovirulence agents present in each strain. Furthermore, variability of colony

morphology was observed in white *C. parasitica* cultures from Italy containing the hypovirulence agent labeled HI_1 (Elliston 1982). It was suspected that HI_1 was actually a mixture of at least two different hypovirulence agents.

Further barriers preventing the spread of hypovirulent strains may be due in part to the limited dissemination of the asexual conidia. It has been shown that the dsRNA hypoviruses are found in asexual conidia, but are not found in the wind-disseminated, sexual ascospores (Anagnostakis 1977). However, other methods of dissemination, including insects and mites, have been reported. Anagnostakis (1982) identified a species of carpenter ant that commonly feeds on *C. parasitica*, and Wendt et al. (1983) found mites that could carry propagules of *C. parasitica,* associated with blight cankers. Hypovirulent strains were also associated with mites recovered from American chestnut trees (Griffin et al. 1984). In 1998, Nannelli et al. (1998) discovered that fragments of *C. parasitica* remained viable after passing through the digestive system of corticolous mites. Furthermore, Robbins and Griffin (1999) found natural dissemination of hypovirulent strains of *C. parasitica* among artificially inoculated trees; but almost no hypovirulent strains were recovered from surrounding chestnut stump sprouts, thus suggesting any vector would most likely be wingless.

Adequate environment may also play a role in the biological control of *C. parasitica*. Griffin et al. (1991) found that the highest level of biological control on American chestnuts was observed on forest clear-cut mesic sites. In addition, both temperature and altitude have been identified as stresses, which may increase the susceptibility of American chestnuts. Griffin (2000) identified a correlation between electrolyte leakage, a measure of plant stress, and superficial canker breakdown over the colder winter months at high altitudes (1187 m). Furthermore, high altitude stress may

increase susceptibility to invasion by virulent strains (Griffin and Griffin 1995). The presence of either high altitude or low temperature, or a combination of the two, may provide enough stress to allow even weak pathogens, such as the hypovirulent strains of *C. parasitica*, to produce killing cankers.

All of these barriers have contributed to the absence of natural and artificially established biological control of chestnut blight in the United States (Peever et al. 1997). In 1975, Van Alfen and Jaynes inoculated American chestnut sprouts with virulent and white hypovirulent strains of *C. parasitica* with European dsRNA and facilitated disease control. A similar experiment was conducted five years later where more than 250 trees were inoculated with hypovirulent strains around the circumference of cankers (Jaynes and Elliston 1980). After one year, 86% of these cankers were still healthy, but in the following years subsequent natural cankers proved lethal to the inoculated trees. Limited disease control has been observed in Connecticut where trees in an American chestnut clear-cut were inoculated with hypovirulent strains (Anagnostakis 1990).

A novel situation exists in Lesesne State Forest, where an experimental plot was established in 1980 by grafting scions from large surviving American chestnut trees on the rootstocks of American chestnuts growing in a plantation. In 1982-1983, natural blight cankers on these trees were inoculated with a mixture of six pigmented, dsRNA-containing hypovirulent strains of *C. parasitica* and four white (CHV1) European hypovirulent strains (Dierauf et al. 1997). It was found that after 14 years, the white strains had spread throughout the stems and branches of the inoculated trees (Robbins and Griffin 1999). The grafting site at Lesesne is a favorable one as a low altitude (411 m) mesic site with no hardwood competition. These factors, in combination with the inoculated hypovirulent strains and low levels of host resistance, provide an integrated

management system, which is currently the most successful known biological control situation in the United States (Griffin 2000).

The spatial pattern of white isolates in the cankers at Lesesne may be critical to biological control of chestnut blight. A random pattern favors biological control because it increases the chances that virulent strains may come in contact with hypovirulent strains, provided the frequency of hypovirulent strains is not too low (Griffin 1999). This increased chance of contact also increases the possibility of hypovirulence conversion and subsequent spread of hypoviruses. Aggregated patterns of white isolates are less favorable in biological control. These patterns are selective in distribution, and therefore provide less possibility of contact between virulent and hypovirulent *C. parasitica* strains in a canker. In 1999 Griffin examined the spatial pattern of white isolates in natural cankers on the grafted American chestnuts in Lesesne, using a 49-hole lattice grid. All cankers were superficial and located within a zone from the ground to 183 cm on the stem of the grafted trees. A join-count statistical test for random mingling of lattice cells designed by Krishna Iyer (Pielou 1977) was used. All cankers had patterns in which white isolates were randomly distributed throughout the canker. This random distribution may contribute to the high level of blight control on the grafts at Lesesne.

A random pattern of white, hypovirulent *C. parasitica* isolates throughout the trees is also favorable to biological control. In 1999 Robbins and Griffin (1999) examined the spatial pattern of recovered white isolates from cankers throughout the three grafted American chestnuts at Lesesne. The pattern of white isolates in cankers was nearly random to slightly aggregated. This finding is based on a marginal p-value obtained from a goodness of fit test to the randomized Poisson distribution, and a low value for Lloyd's index of patchiness, which is density independent. This pattern allows all cankers on the

trees equal chance of receiving white strains, thus favoring biological control. Milgroom et al. (1990) conducted a similar study in which pigmented *C. parasitica* samples were taken from cankers in eight clear-cut plots and each classified to VC group. Spatial pattern of the VC groups was analyzed using a double matrix comparison technique (Harvey *et al*. 1986). It was found that nonrandom patterns were found at least once in each plot; however, all but one of these patterns were due to multiple occurrences of VC groups on the same tree. When these occurrences were eliminated, most patterns were random. Bisseger et al. (1996) used the statistical methods of Milgroom to determine the spatial pattern of white isolates and VC groups in cankers present in two clear-cut situations in southern Switzerland. Two different situations were found to exist in these plots. The first plot had a random distribution of white isolates and nonrandom distribution of VC groups, while the second plot had a nonrandom distribution of white isolates and a random distribution of VC groups (Bisseger et al. 1996).

Lastly, the diversity of vegetative compatibility groups and presence of white isolates has been measured for individual chestnut plots using the ratio of the number of VC groups to the sample size, and by the Shannon diversity index (Anagnostakis 1986, Bissigger 1996). The VC ratio, or S/N number is simply the number of phenotypes or groups (VC), divided by the total number of individuals in the sample. Because this measure is a ratio, it is independent of sample size. The Shannon diversity index takes into account the fraction of the total sample size that each group represents. When all the individuals are in the same group, the value is 0. When they are all different, the Shannon index is at its maximum for that population.