CHAPTER 5
DISCUSSION

In 1999 it was reported that the white hypovirulent strains of *C. parasitica* had spread throughout the grafts in Lesesne State Forest (Robbins and Griffin 1999). This spread and subsequent biological control is very unusual for the United States and is the most successful form of chestnut blight control on American chestnut in the United States to date. The size of these trees alone can attest to the remarkable control. As of 1999 the largest graft (TH) was over 18.5 meters tall.

In 1982 and 1983 there was a total of four white strains, consisting of three VC groups, inoculated into natural cankers on the grafted trees. Vegetative compatibility tests performed in this study revealed that none of these original VC groups were found among 25 major white VC groups (those consisting of two or more isolates) recovered from the trees. Forty-five VC groups therefore represent the minimum number of “new” VC groups into which one or more of the original European hypoviruses (CHV1) had spread. It is also possible that some spread has occurred since 1996 because five of the 11 major pigmented VC groups (those with two or more isolates) established by Robbins and Griffin (1999) were common to the 25 major white VC groups reported here. The VC ratio (0.43) found here is identical to that found by Robbins and Griffin (1999) and close to results obtained by Anagnostakis for pigmented isolates in Connecticut research plots (0.41) (1986). Furthermore, the Shannon diversity index value found here (3.64) is similar to data from other studies (Anagnostakis 1986). Current belief is that vegetative incompatibility in *C. parasitica* poses a major barrier to the spread of hypovirulence and blight control (Hobbins et al. 1982, Anagnostakis 1983, Kuhlman and Bhattacharyya 1984, Liu and Milgroom 1996). It is apparent from this study, however, that vegetative
incompatibility among *C. parasitica* isolates in the 48 white VC groups collected was not a significant enough factor to limit the spread of CHV1 in the trees at Lesesne.

The presumed dominating role that vegetative incompatibility plays in limiting spread of hypovirulence also comes into question for the cankers sampled with the lattice plot method. The RML-470 and TG-303 cankers are of particular interest because the majority of both pigmented and white isolates within each canker were in the same VC group. Tests with pigmented isolates from canker RML-470 indicated that these isolates are dsRNA negative. Previous studies have demonstrated that when artificially inoculated cankers are challenged with hypovirulent strains of the same VC group, pigmented strains common to that VC group are readily converted to hypovirulence (Shain and Miller 1991, Hobbins et al. 1994). In the present study, pigmented strains, recovered from lattice cells that were adjacent to lattice cells containing white strains in the same VC group, were not converted to hypovirulence *in vivo*. This finding is likely to have far-reaching significance to understanding the factors that limit the spread of CHV1 in blight cankers on both American and European chestnut trees.

In order to explain these results, it is possible that 1) the white and pigmented isolates in the same VC groups are physically separated by host tissue, 2) there was incomplete movement of CHV1 throughout the thallus of the vegetatively compatible pigmented strain (Shain and Miller 1992), or 3) the pigmented isolates are resistant to infection by CHV1. In support of the first hypothesis is the separation of the sampling holes in the grid. The sampling holes are located in the middle of the 2.54 cm x 2.54 cm lattice cells; therefore, there is a maximum of 2.54 cm of tissue that could separate isolates sampled from adjacent cells. Furthermore, isolates could be present at different depths in the tree, and separated by healthy tissue, as demonstrated by Griffin (1999). Periodically
some cores in this study were observed that had a thin layer of healthy tissue between two necrotic regions. The problem with this first hypothesis is that the pigmented and white isolates in the same VC group would have to become established by at least two separate invasions, both of which produced by strains in the same VC group. Both strains would then form separate networks in the canker. However, there was no evidence of healthy tissue between the lattice holes; therefore, this tissue is probably colonized completely by C. parasitica. Furthermore, it is unlikely that two isolates of the same VC group, but differing in pigmentation, could become established in the same canker, in close proximity, without making contact with each other at some place in the canker. Support of this hypothesis is even less since the phenomenon was observed in both the RML-470 and TG-303 cankers.

The data of Shain and Miller (1992) support the second hypothesis. They found that most (58-81%) of the isolates removed from artificially established virulent cankers challenged with hypovirulent isolates, were converted to hypovirulence after 65 weeks. However, these data are especially of interest because of the appreciable number of isolates collected that were not converted to hypovirulence, despite being in the same VC group. Further data from the same study indicated that new growth of the virulent thallus, at the canker margin, was more susceptible to conversion than the older growth at the center of the canker. After 9 weeks the entire circumference of the cankers yielded white isolates, while the center continually gave virulent pigmented isolates.

The third hypothesis, indicated above, stems from a phenomenon observed in our laboratory and the findings of Polashock et al. (1994). We have observed “sectoring” of C. parasitica strain EP-713 in culture whereby a pigmented sector arose from a growing white colony. When these pigmented sectors were then tested for the presence of dsRNA;
none was recovered (unpublished data). In addition, sectoring of a pigmented single spore isolate from white isolate THL-513b has been observed. The pigmented single spore colony was transferred to an APDA plate and a white sector arose from the pigmented colony. Both the white and pigmented colonies were transferred again. The pigmented colony sectored again to give another white colony; the white colony remained stable (Fig 5.1). Both colonies were tested for presence of dsRNA. The white colony had dsRNA but the pigmented colony tested negative for dsRNA. Twenty-nine single-spore cultures from the pigmented colony were paired with the white parent in *in vitro* hypovirulence conversion tests. No hypovirulence conversion was found for any of the pairings.

A similar phenomenon was described by Polashock et al. (1994). It was found that a dsRNA (CHV2)-containing hypovirulent strain (NB58) of *C. parasitica*, recovered from American chestnuts in New Jersey, produced a “phenotypically-distinct sector” in culture, which was dsRNA negative. Attempts to infect this sector, by pairing with the parent strain, were unsuccessful despite findings that the sector and parent strain were isogenic. It appears that some defense mechanism may prevent transmission of dsRNA. The authors indicate they observed a similar sectoring in EP-713. It is proposed that a mechanism similar to this may occur within some of the cankers on the grafts at Lesesne. However, further work must be done to test all the hypotheses mentioned above.

Cultural morphology groups recovered from the three grafts suggest that hypoviruses from *C. parasitica* strains EP-49 and EP-51W have spread the most. These two isolates were the only inoculated strains to be categorized in the two most common CM groups (CM 1 and CM3) and have the fastest growth rate *in vitro* of the inoculated strains. Since none of the recovered isolates were found to be compatible with either of the two inoculated strains, the spread appears to be a result of the hypoviruses rather than
the fungal strains. The discrepancy in cultural morphology between ATCC# 38758 EP-51 and Elliston’s (1985) original photo and description for EP-51 suggests virus competition among virus mixtures or virus mutation may be occurring in EP-51. The single-spore EP-51W colonies closely resemble the photo and description of the EP-51 colony shown by Elliston (1985). Therefore, it may be possible that a viral mutation produced the abnormal colony in the ATCC culture, and the original virus was recovered in EP-51W. Another possible explanation is that there are multiple viruses present in EP-51 (ATCC# 38758). Elliston (1982, 1985) indicated that multiple hypoviruses (agents) may exist in hypovirulent strains from Italy and have varying effects on colony morphology. When single-spore colonies of strains containing only one hypovirus are isolated, the morphologies of each colony were distinct. In this study, EP-49 (ATCC# 38759) also had two types of colony morphologies among the single-spore progeny. It is possible that both EP-49 and EP-51 from ATCC have a mixture of different hypoviruses, or a mixture of viruses resulting from mutation. Because of this confusion, further experiments using single-spore and single-colony mass transfer plates should be conducted under controlled light and temperature conditions to confirm colony morphology stability for the three strains. Elliston (1985) has indicated that serial transfers of dsRNA-containing strains may result in changes, including cultural characteristics in these strains.

The cankers sampled using the grid method were quite varied with respect to the number of white isolates collected. The percent of white isolates varied from 0 to 60%; however, the two cankers for which isolates of *C. parasitica* were identified to VC groups, (TG-303 and RML-470) had percentages of white isolates close to the mean (47%) observed for isolates collected from all three trees. It is reasonable that collecting a large number of isolates from single cankers might produce varying results since the area of
sampling is substantially smaller than collecting from all over branches and stems of the three trees. Furthermore, when collecting isolates (six per canker) outside of the H-inoculated zone, the individual cankers sampled also had variable percentages of white isolates recovered: 0 – 66% (0/6 – 4/6).

A total of eleven pigmented isolates from two cankers sampled by the lattice plot method (THL-660 and RML-470) were tested for dsRNA. All of the isolates tested were negative for dsRNA. The majority of pigmented isolates recovered from the American chestnuts grafts were virulent and did not contain dsRNA (Robbins and Griffin 1999); however, pigmented hypovirulent isolates containing dsRNA have been isolated from the inoculated zone of these grafts in the past (Griffin 1999). Isolates recovered from the THL-660 canker were exclusively pigmented, and examination of the bark cores removed from the canker identified the canker as superficial. This would suggest that one or more blight control factors, including host resistance, might be preventing the canker from developing in a nonsuperficial manner and girdling the tree.

The random distribution of white isolates, and the aggregation of VC groups among the cankers in the trees at Lesesne are probably both favorable to biocontrol. A random spatial pattern of white isolates allows equal chance for virulent pigmented strains to come in contact with hypovirulent white strains and an increased occasion for hypovirulence conversion. The aggregation of VC groups suggests that if a pigmented, virulent isolate is converted to hypovirulence by CHV1, then all neighboring or contiguous isolates in the same VC group have a greater chance to be converted to hypovirulence. In this situation, the apparent barrier of vegetative incompatibility is no longer a factor. These events could then lead to aggregation of white VC groups among cankers as found in this study. The random distribution of white isolates and aggregation
of VC groups may therefore serve as factors contributing to the high level of blight control on the Lesesne trees.

The spatial pattern findings of the present study are similar to Robbins and Griffin (1999) and Bissegger et al. (1996). Robbins and Griffin (1999) found a nearly random to slightly aggregated pattern of white isolates in the cankers on the grafts at Lesesne, using the Lloyd’s index of patchiness (1.36). Only 37% of the cankers sampled were main stem cankers in their study, whereas 88% of the cankers sampled were main stem cankers in the present study. Both the Lloyd’s index of patchiness for Robbins and Griffin and the value obtained here (0.91) are close to 1.0, which would indicate a random pattern. Using the double matrix test of Harvey et al. (1988) as described by Bissiger et al. (1996) and Milgroom et al. (1990), the spatial patterns of white isolates and white VC groups found here are generally comparable to results from Bissegger et al. (1996) for one of two locations, using the same statistical test. Their study found both a random distribution of C. parasitica isolates (pigmented and white isolates combined P=0.102) and an aggregated distribution of pigmented and white VC groups (P=0.004) in the Lumino C. parasitica population in Switzerland.

In 1999 Robbins and Griffin (1999) found that 34% of the C. parasitica isolates collected outside the H-inoculated zone of the three grafts were white. This number is lower than the 47% observed in this study, possibly suggesting that the white hypovirulent isolates have since spread to a greater degree outside of the H- inoculated zone. In the present study the percent white isolate recovered from the TH, RM and TG grafts were 50, 49 and 41% respectively. Since all three trees had percentages of white isolates higher than the 34% observed previously, it is likely that the 47% white observed here is not a product of bias sampling of one tree. A possible explanation could be that the high
number of main stem cankers, sampled in the present study, skewed the results. In the Robbins and Griffin 1999 study, 37% of the 62 natural cankers sampled were main stem cankers. In this study, 88% of the 49 cankers sampled were from the main stem. It is possible that the branch cankers were more accessible to invasion of airborne, pigmented, virulent inoculum (ascospores) than the more vertical main stem cankers. Furthermore, stem cankers may have a greater possibility of acquiring hypovirulent strains through mites that may feed on a hypovirulent thallus and carry pieces of this inoculum to neighboring cankers on the stem (Wendt et al. 1983, Nannelli et al. 1998). Either of these two factors may skew the frequency of white and pigmented strains in cankers.

The presence of specific VC groups of white isolates in the different grafts is also of interest. There does not appear to be a dominant hypovirulent VC group among the trees. Although VC group I was the largest group sampled, it was present only on the TH and RM grafts. In comparison, all three grafts possessed VC group XVII, which contained only half the number of isolates (5) of VC group I. Furthermore, even though there are noticeable differences in the percent live crown of the three grafts, there does not seem to be any correlation between the relative frequency of white isolates to number of white VC groups (VC ratio), total number of VC groups, or specific VC groups present in the grafted trees and the percent live crown of the trees. The TH graft is currently the largest and healthiest (95% live crown) graft, followed by the RM and TG grafts, respectively. Although the TH graft had the lowest VC ratio, it had more total white VC groups than the TG and shared three white VC groups with the RM and two VC groups with the TG. Similarly, the RM graft is noticeably healthier than the TG graft and it had nine more white VC groups and a higher VC ratio than the TG graft. Furthermore, the RM graft shared two common white VC groups with the TG graft. It appears therefore
that the differences in height and percent of live crown are not highly related to the fewest numbers of VC groups or the presence of specific VC groups in the healthier grafts. These findings further support the possibility that factors other than hypovirulence and number of VC groups are contributing to the observed blight control.

One suggested factor associated with blight control on the grafts is a low level of host resistance (Robbins and Griffin 1999; Dierauf et al. 1997). Unequal levels of host resistance could account for the differences in overall health of the grafts. In addition, low levels of resistance may account for the large amount of hypovirulence spread and numerous “new” hypovirulent VC groups present in the grafts at Lesesne. It has been previously demonstrated that hypovirulence conversion between unlike VC groups is more likely to occur in vivo than in vitro (Grente 1981, Double 1982). Robbins and Griffin (1999) found that white isolates taken from a superficial canker, resulting from inoculation with a virulent strain of C. parasitica, initially could not convert the virulent strain to hypovirulence in vitro. However, after 23-50 months, 62% of the isolates recovered were white and almost half of these isolates (46%) were able to convert the virulent strain in vitro to hypovirulence (Robbins and Griffin 1999). It is possible therefore that the low level of resistance has allowed the time necessary for hypovirulence conversion of isolates in incompatible VC groups.
Fig 5.1. *Cryphonectria parasitica* white isolate THL-513b single-spores yielded white and pigmented colonies (top). The pigmented colony was transferred, before colonies came in contact with each other, to test the stability of pigmentation. Upon transfer, the pigmented colony yielded a dsRNA-containing white “sector” (bottom left). The completely pigmented colony was transferred from stability plates three consecutive times, each time producing a white sector before it became a stable, pigmented colony. The white parent (THL-513b) was paired with the stable pigmented single spore colony for hypovirulence conversion tests. After 29 tests, no conversion was observed.