Chapter 3

Examination of the roles of colony age, resistance, functional mycelium units and low temperature in the survival and failure of CHV1-Euro7 hypoviruses to move throughout a vegetative compatibility type of *C. parasitica*

3.1
INTRODUCTION

In the early 20th century, the chestnut blight fungus [*Cryphonectria parasitica* (Murr.) Barr] was introduced to North America and nearly destroyed all canopy American chestnut trees [*Castanea dentata* (Marsh.) Borkh.] in its natural range. A small number of large American chestnut trees survived the initial epidemic, many of which have superficial healing cankers that frequently contain pigmented hypovirulent (= reduced virulence) *C. parasitica* strains containing dsRNA hypoviruses (Elliston 1985b; Griffin *et al.* 1983). The chestnut blight epidemic was less severe in Europe, where hypovirulent strains of *C. parasitica* were found to be consistently associated with healing cankers (Grente 1981). These European hypovirulent strains contain a dsRNA hypovirus, *Cryphonectria hypovirus 1* (CHV1) (Hillman *et al*. 1995), and generally are more successful in blight control of hypovirulence than American hypovirulent isolates (Elliston 1985a).

Unfortunately, while the European hypovirulent isolates spread naturally in Europe (Heineger and Ringling 1994) these isolates do not spread in the U.S. where most biological control experiments with hypovirulence utilize *C. parasitica* strains infected with CHV1. The overwhelmingly large number of vegetatively compatible (vc) groups of *C. parasitica* in the U.S. as compared to Europe is often cited as a major barrier to hypovirulence spread in *C. parasitica* strains via anastamosis. (Cortesi *et al.* 1998, Kuhlman and Bhattacharyya 1984, Anagnostakis 1983). However, this may not be as great a barrier as once believed as Hogan and Griffin (2002a) reported the spread of CHV1 into at least 45 new vegetative compatibility types on three grafted American chestnut trees almost 20 years after inoculation of natural cankers with a mixture of CHV1-infected hypovirulent strains.

Equally important to biological control however is the movement of CHV1 within a vc type; this can be investigated using spatial pattern analysis. In 2002 the spatial pattern of white isolates within individual superficial cankers on grafted American chestnut trees was investigated (Hogan and Griffin 2002b). Results indicated that the majority of white and pigmented isolates within a canker were in one dominant vc type and frequently these isolates were in close association with each other, indicating no

spatial separation. This finding suggests incomplete movement of CHV1 within a vc type *in vivo* can occur.

Shain and Miller (1992) also demonstrated incomplete movement of CHV1 within a vc type, but these experiments were conducted with artificially established cankers composed of only one vc type. 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 central part of the canker because the older mycelium was not functional, yet still viable. Thus, colony age may play a role in CHV1 movement throughout a vc type.

Another 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. 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. The pigmented and white colonies of this isolate were dsRNA negative and positive, respectively.

Another explanation for incomplete CHV1 movement in a vc type is that the *C. parasitica* thallus was separated into functional mycelium units (FMU's). Rayner (1991) argues that the mycelium is a "functional unit, an individual", and a fungal thallus may be composed of multiple FMU's (Olson 1999). It is possible that the FMUs 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.

Environmental factors also appear to play a role in hypovirus survival and biological control. Griffin *et al.* (1991) present evidence suggesting 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^{o}C) has a deleterious effect on hypovirus survival in the fungus (Friese *et al.* 1992). The objectives of this study are: (1) 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, (2) to examine the role of low temperatures and topographic site (elevation) on CHV1 survival within *C. parasitica* colonies *in vitro* and *in vivo*.

3.2
MATERIALS AND METHODS

3.2.1 Preparation of chestnut-bark agar (CBA)

Blight-free sections of young chestnut branches and stems were cut from Paint Bank chestnut clear-cut plots in the spring of 2002 and 2003. Chestnut sections were stripped of bark in the lab using a sharp pocket knife and long sections of stripped bark were dried at room temperature for at least 48 hr. Dried bark was then ground into a fine-granular powder using a Wiley mill, and stored at room temperature.

Chestnut-bark extract was prepared using 10g of dried, ground chestnut bark. To this was added 550 ml of distilled water and the mixture was stirred for 20 min using a magnetic stirring bar in a 1000-ml beaker. Five hundred ml of the chestnut extract solution was gravity filtered through several layers of cheese cloth to remove sediment, and then filtered stepwise using a cotton cloth filter, an AP25 glass pre-filter, and finally the extract solution was filter sterilized using a 1000-ml Nalgene autoclavable filter sterilization unit equipped with a 0.45 µm filter and AP15 prefilter (Fisher Scientific Pittsburgh PA, 15275-9952). The bark-extract was added to an equivalent volume (500 ml) of sterile, molten 4% water agar to produce a final concentration of 2% agar solution. Petri plates (8.5 cm diameter) were poured using this solution.

3.2.2 Evaluation of CHV1 movement *in vitro* using the hypovirulence conversion-plate format

CHV1 movement studies using the standard hypovirulence conversion format of Anagnostakis and Day (1979), with slight modification, were conducted using white strains TG-303 48 and RML-470 8, recovered from natural cankers on TG and RML grafted American chestnut trees at Lesesne state forest in VA in 1998. Isogenic pigmented isolates from these white isolates were obtained using the single-spore isolation technique described in pp. 17 of Chapter 2.2. Single-spore pigmented isolates were plated on acidified (5.6 ml 25% v/v lactic acid per L), Difco potato-dextrose agar (APDA) and allowed to grow for approximately 7 days. Small (6 mm) discs were cut from the advancing edge of the pigmented *C. parasitica* colony using a cork borer and placed, mycelium side down, 2 cm from the edge of a fresh plate of CBA. Each day for 7

days following inoculation, the pigmented colony growth was noted by marking the underside of each Petri plate with indelible ink at the leading edge of the pigmented colony. In addition, each day colonies were examined for the formation of conidiomata. Initial formation of was identified using a dissecting scope and florescent light source, and areas in the colony where fungal mycelium began to clump together were marked with indelible ink and the date of initial conidiomata formation was recorded.

At intervals of 3, 4, 5, or 6 days of colony growth the left half of the advancing edge of the pigmented colony was challenged with 6-mm agar discs cut from the advancing edge of the isogenic hypovirus-containing, white parent cultures of RML-470 8 and TG-303 48. The number of hypovirus-containing white discs used to challenge the pigmented colonies varied from three to six depending on the number of days' growth and subsequent size of the pigmented colony.

CHV1 movement was measured by sampling the pigmented colony using a 4-mm diameter cork borer at 7, 14, and 21 days after challenge. Sixteen samples were taken from the colony. Four agar-disk samples were taken arbitrarily from the "converted area" of the colony (identified by change in colony phenotype to white), four isolates were taken along the pigmented region just to the interior of the "conversion front" (the boundary where the pigmented *C. parasitica* colony changes to the white phenotype), four samples were taken from within the pigmented colony along a line segment oriented approximately perpendicular to the direction of growth of the pigmented colony down the Petri plate, and four samples were taken from the mycelium directly surrounding the pigmented colony inoculation point (*Figure 3.5*).

Mycelial disks were placed on APDA plates, four per plate, and evaluated for colony color following 14 days of colony growth using the criterion of Robbins and Griffin (1999) as amended on pp. 18 of Chapter 2.2. The white colony phenotype indicated presence of CHV1 and pigmented colony phenotype indicated CHV1 absence.

3.2.3 Evaluation of CHV1 movement throughout a whole colony of *C. parasitica in vitro*

CHV1 movement studies using whole-colony Petri plate cultures of *C. parasitica* were conducted using *C. parasitica* strains TG-303 48, RML-470 8, and THL-230 f. Isogenic, pigmented cultures of the tester strains were obtained as described above, and plated on

8.5-cm Petri plates containing CBA. *C. parasitica* colonies were grown for 6 days after which they were challenged just beyond the leading mycelium edge, near the edge of the Petri plate, with an isogenic CHV1-containing strain of *C. parasitica*.

Colonies were tested for CHV1 movement using a 60-cell lattice grid at 7, 14, and 21 day intervals following challenge (*Figure 3.2*). Agar disks were cut from colonies with a 4-mm cork borer and plated, four to a plate, on APDA Petri plates. Isolates were allowed to grow for 14 days and examined for colony color. White colony phenotype indicated CHV1 presence and pigmented colony phenotype indicating CHV1 absence.

3.2.4 Evaluation of CHV1 movement *in vitro* throughout 54-day-old *C. parasitica* colonies

C. parasitica strains RML-470 8, TG-303 48, and THL-230f were used to study CHV1-movement through 54-day-old colonies. Single-spore pigmented strains were plated on CBA and allowed to grow for 6 days. After 6 days the entire colony (average diameter ~5 cm) was cut from the agar using a scalpel, and placed into an empty 8.5-cm diameter Petri plate, sealed with parafilm to prevent desiccation and allowed to grow for an additional 27 days. Following this incubation period, cool but molten CBA was poured around the excised colony to the level of the excised colony and filling the plate. Colonies were allowed to grow for one day, in order for mycelium to start to grow into the new CBA, after which the colonies were challenged at the colony margin with an isogenic CHV1-containing strain of *C. parasitica,* as with the whole-colony CHV1 movement trials. Colonies were incubated for an additional 20 days and then sampled with the same lattice 60-cell lattice grid as before. Diagrams were drawn to scale to depict the position, size and shape of the excised colony relative to the entire Petri plate. This also served to determine the position of the mycelial disks (young or old region of the colony) removed from the grid for assay. The final interior colony age was 54 days for each isolate. Mycelial disks were cut from colonies with a 4-mm cork borer and plated, four to a plate, on APDA Petri plates. Isolates were allowed to grow for 14 days and examined for colony color using the criterion determined by Robbins and Griffin (1999) as amended on pp. 18 of Chapter 2.2. The white colony phenotype indicated presence of CHV1, and the pigmented colony phenotype indicated absence of CHV1.

3.2.5 Evaluation of resistance to hypovirus infection to the failure of CHV1 to move through a *C. parasitica* vegetative compatibility type colony *in vitro*

In 1999 a single-spore pigmented (SSP) isolate from white Lesesne research isolate THL-513b produced a white sector (WS) upon transfer to an APDA Petri plate (Hogan and Griffin 2002b). Both the pigmented colony area and the white sector were then transferred to individual plates and grown in single colonies. The pigmented colony area was dsRNA negative and virulent in field tests and the white sector was found to be dsRNA positive and intermediate hypovirulent. A stable, pigmented subculture from the original SSP THL-513b isolate was obtained, and paired with a mass transfer from THL-513b (WS) in 50 hypovirulence conversion tests using the format described above. White sector and stable SSP isolates were placed 2 mm apart from each other at the edge of an APDA plate to allow mycelial growth down the plate. None of these tests conducted in 1999 demonstrated any conversion of the pigmented single-spore strain to hypovirulence. All isolates were tested to confirm vegetative compatibility with each other. Plates were incubated in the dark and evaluated after 14 days for compatibility using ratings from Griffin and Griffin (1995). Strongly incompatible reactions were defined as those producing a clear barrage zone with numerous pycnidia between isolates. Weakly incompatible reactions were defined as those producing a clear barrage zone with little or no pycnidia. Compatible reactions were those in which the mycelium of the paired isolates merged. The THL-513b isolates were found to be vegetatively compatible with each other (Hogan and Griffin 2002b).

C. parasitica strains from the 1999 tests were stored as PDA slants at 5-6^{o}C and in March of 2000, 100 new hypovirulence conversion tests were conducted on the SSP THL-513b strain using THL-513b (WS) and white THL-513b parent as donor strains. Conversion tests were conducted using mycelial discs cut with a 6-mm cork borer, and paired together as above. Conversion tests were rated as described above and two to four 4-mm discs were cut arbitrarily from within the pigmented region of the tester strain and plated on APDA to determine if CHV1 had moved throughout the pigmented colony. Any isolates that were found to be pigmented were again subjected to conversion tests using the same two donor strains to further determine if the lack of movement was due to resistance.

Hypovirulence conversion tests were also conducted on pigmented isolates recovered from the virus movement trials using the whole-colony colony movement format. Five of these pigmented isolates were tested for resistance to hypovirus infection as they were recovered from cells that were surrounded by cells from which white isolates were recovered. Each of these pigmented isolates was tested for resistance in ten replicate hypovirulence conversion tests using an isogenic hypovirus-containing strain of *C. parasitica*, using the same method described above.

Finally, three additional pigmented *C. parasitica* isolates (THL-230f #1, THL-230f #2, and THL-230f #3), chosen because of lack of conversion in earlier tests, were subjected to the same hypovirulence conversion tests as the above isolates.

3.2.6 Examination of the role of low temperatures and forest site elevation in CHV1 survival within *C. parasitica* colonies *in vivo* and *in vitro*

Digital temperature monitors (Taylor model 1441) were placed late November 2004 at high elevations at the Virginia Tech Horton Center (975 m) and Paint Bank (975 m) research plots in Craig and Giles Co. Va, respectively. Each temperature monitor measured high and low temperatures for 5-day periods. Temperature monitors at nearby Horton Center served as temperature measurements for chestnut plots at the Center, and as a sentinel measurement for Paint Bank plots. When temperature measurements at Horton gave readings of -10^{o}C, Paint Bank temperature monitors were checked. When temperature monitors at Paint Bank indicated the mean temperature for the previous 24 hr was at least -10^{o}C, *C. parasitica*, bark-core samples were taken from artificially established superficial cankers of white strains Ep-47(’99); Ep-49(‘99); Ep-49(CM1), a white single-spore colony phenotype; and Ep-49(CM3), a colony with some pigment. Cankers were sampled using a 1.7-mm cork borer. One to four samples were taken per canker, and no more than eight samples were removed at any sampling period. Samples were then placed in a labeled multi-well plate, covered with masking tape for temporary storage, and placed in a cooler for transportation to the laboratory.

Bark-core samples were then removed from each well, surface disinfested in 1% NaOCl for 2 minutes, rinsed in distilled water, and plated on APDA. The resulting fungal growth was monitored daily and individual colonies suspected to be *C. parasitica*, were transferred to new APDA plates. Cultures were grown in normal room light

(fluorescent) conditions for 2 weeks. Colonies were rated for colony color and phenotype as describe on pp. 18 of Chapter 2.2. White colony color indicated CHV1 survival following low temperature exposure in the filed.

In vitro tests were performed with CHV1-infected *C. parasitica* strain Ep-713, according to methods established by Friese *et al*. (1992). Strain Ep-713 was grown on APDA Petri plates in multiple replicates for 4 days. Following colony growth the plates were stored upside-down in a frost-free freezer at -10^{o}C for 24 hr. After 24 hr, the plates were returned to normal room-temperature growth conditions and allowed to grow for 2 days. Following this period, the new mycelial growth was sampled and transferred to new APDA plates. Original freezer colonies and new-growth-transfer-colony plates were allowed to grow up to 14 days when they were checked for colony phenotype characteristics. White colony color indicated presence of CHV1 and pigmented colony color indicated absence of CHV1.

3.3
Results

3.3.1 Evaluation of CHV1 movement *in vitro* throughout a *C. parasitica* colony (whole-colony and hypovirulence-conversion tests)

A total of 81 whole-colony Petri plate cultures were examined for the movement of CHV1 through the *C. parasitica* colonies. The majority of the trials (36) were performed with *C. parasitica* strain TG-303 48. An analysis of covariance (ANCOVA) testing the correlation between percent hypovirus movement and the number of days after challenge, was performed using SAS v. 9.1 (SAS Institute Inc., Cary, Nc). Tests indicated that the regression analysis of all three strains (TG-303 48, RML-470 8, and THL-230f) had the same intercept and slope (P=0.13, P=0.44, respectively); therefore data from all three strains, at the three time intervals, were pooled together for analysis. On average CHV1 was able to move through approximately 27% of the lattice cell samples of the *C. parasitica* colonies 7 days after challenge with the CHV1-containing isogenic isolate. By 14 days the virus was able to move into 32% of the lattice cells of the *C. parasitica* colony (*Table 3.1*). At the end of 21 days CHV1 spread had essentially ceased, as CHV1 was only able to move into an average of 33% of the lattice cells of the *C. parasitica* colony (*Figure 3.1* and *Table 3.1*). Complete movement of CHV1 throughout the lattice cells of a colony was observed after 21 days, but only in one Petri plate replicate (TG-303 48). One replicate in the 7-day category (RML-470 8) and one replicate in the 14-day category (TG-303 48) each demonstrated almost complete movement of CHV1, moving into 97 and 98% of the cells sampled, respectively (*Figure 3.2).*

Data for isolates recovered from the lattice grid do not indicate a consistent pattern of CHV1 movement throughout a *C. parasitica* colony. The most consistent trend was that the virus almost always moved into the new growth mycelium, located peripherally, and immediately adjacent, to the challenge point (*Figure 3.3*). Furthermore, white isolates were rarely recovered from the center of the lattice grid (oldest region of *C. parasitica* colony). White isolates were more commonly found on the outer edges of the lattice grid, which was the newer growth mycelium. Typical patterns are demonstrated in *Figure 3.3.*

Table 3.1 Movement of CHV1 into virus-lacking colonies (TG-303 48, RML-470 8, and THL-230f) of *C. parasitica* 7, 14, and 21 days past colony challenge with an isogenic CHV1-containing *C. parasitica* strain

[a]Days after challenge	[b]Number of plates	[c]No. white isolates	[d]Percent white isolates
7	36	15.9	26.9
14	25	19.1	32.4
21	20	19.5	33.1
Mean	**27**	**18.2**	**30.8**

[a.] Days after challenge = the number of days past the time of colony challenge with an isogenic hypovirus-containing *C. parasitica* isolate at which time the colony was sampled to detect hypovirus movement.
[b.] The total number of plates sampled
[c.] The total number of white isolates recovered from the CBA plates using a core-sampler and the lattice plot.
[d.] The percent of lattice cell cores sampled that yielded white isolates, indicating virus movement.

Figure 3.1 Hypovirus movement *in vitro* through a whole colony of *C. parasitica* over time. Data are the mean of three different *C. parasitica* strains (TG-303 48, RML-470 8, and THL-230f) 7, 14 and 21 days after challenge with an isogenic CHV1-infected *C. parasitica* strain. Variation expressed as standard error

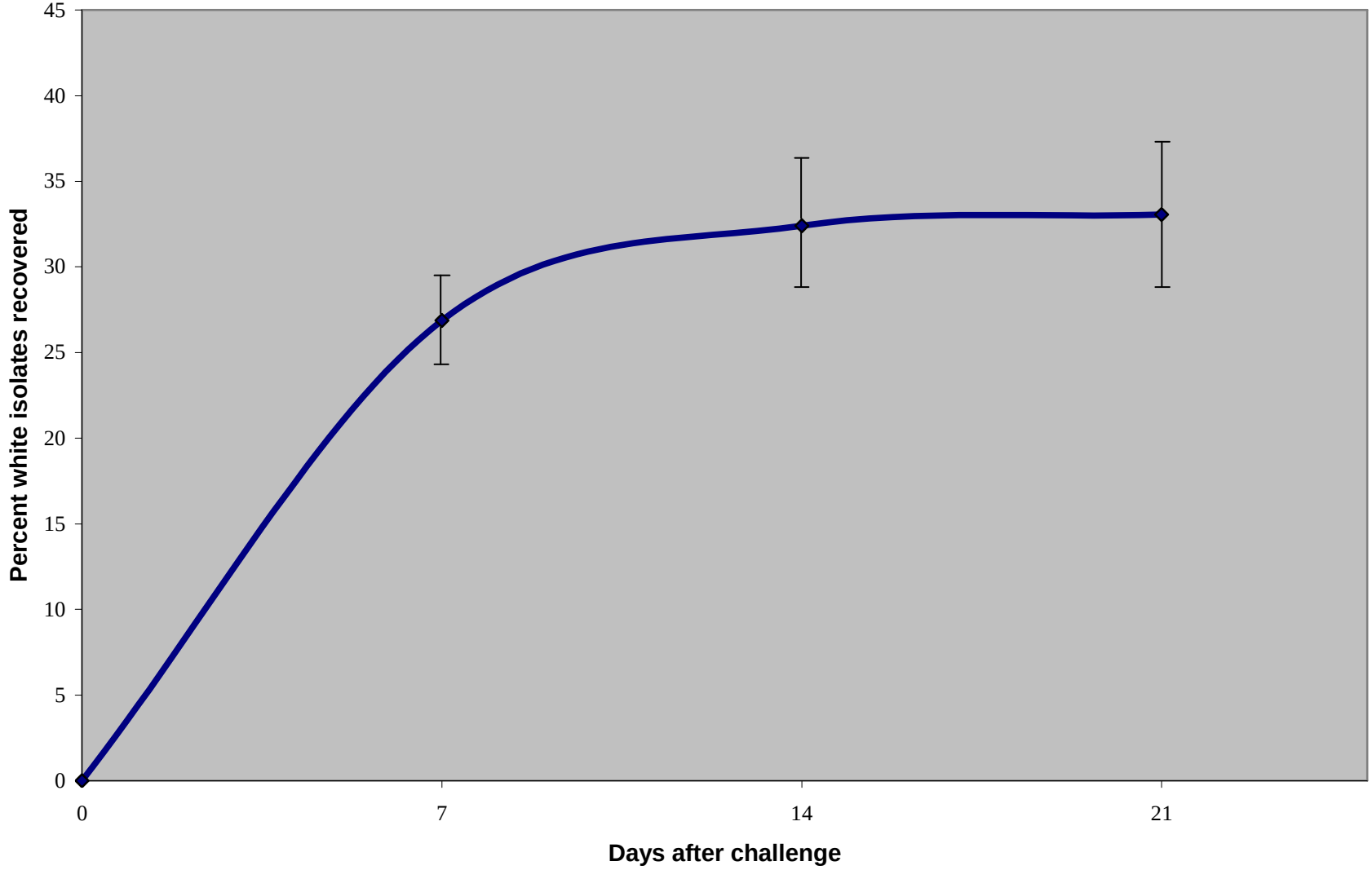


Figure 3.2 Hypovirus movement through a whole colony *C. parasitica* Petri plate colony over time represented by individual replication (◆) and mean data for *C. parasitica* strains TG-303 48, RML-470 8, and THL-230f. Line passes through the mean values for 7, 14, and 21 days

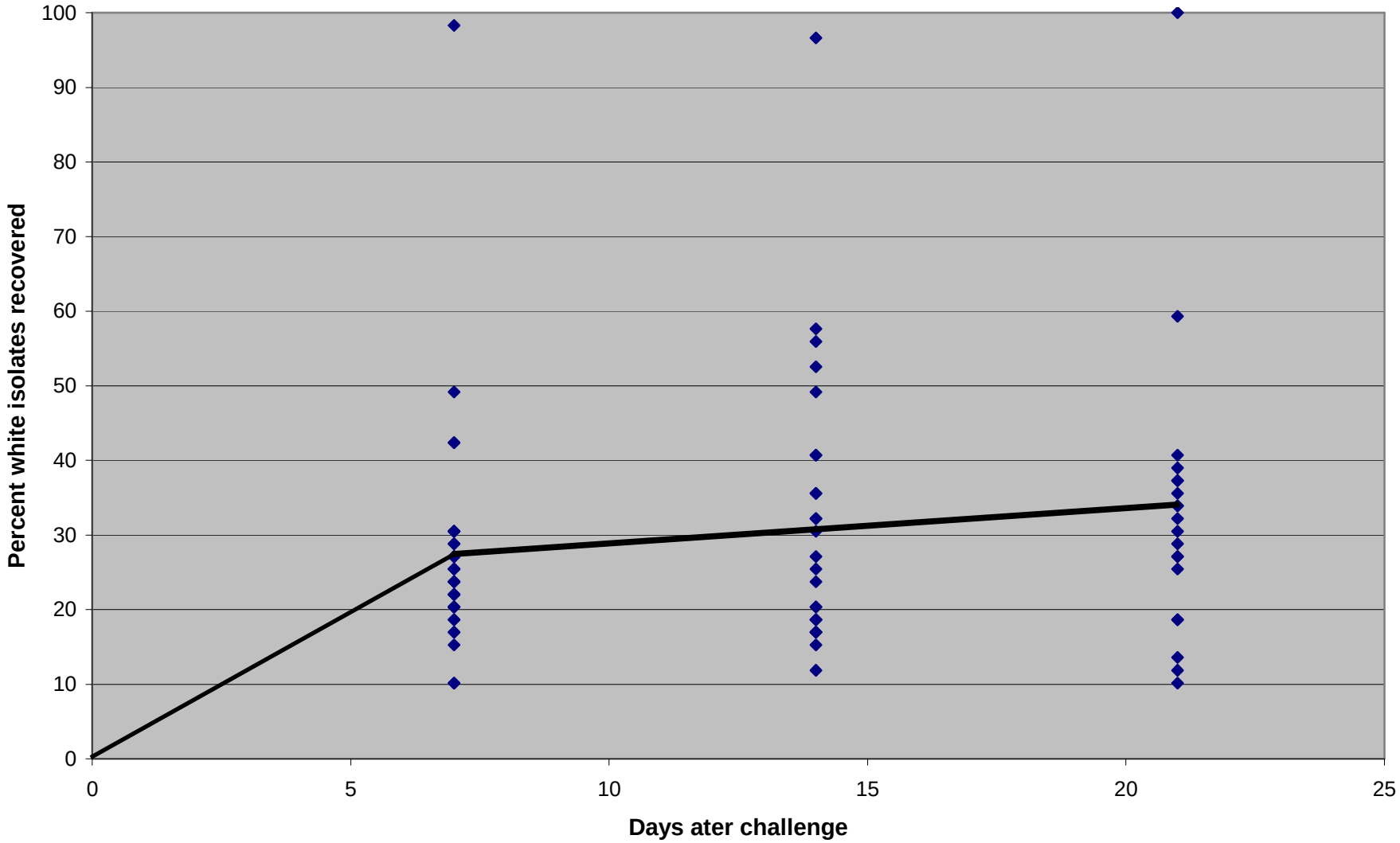
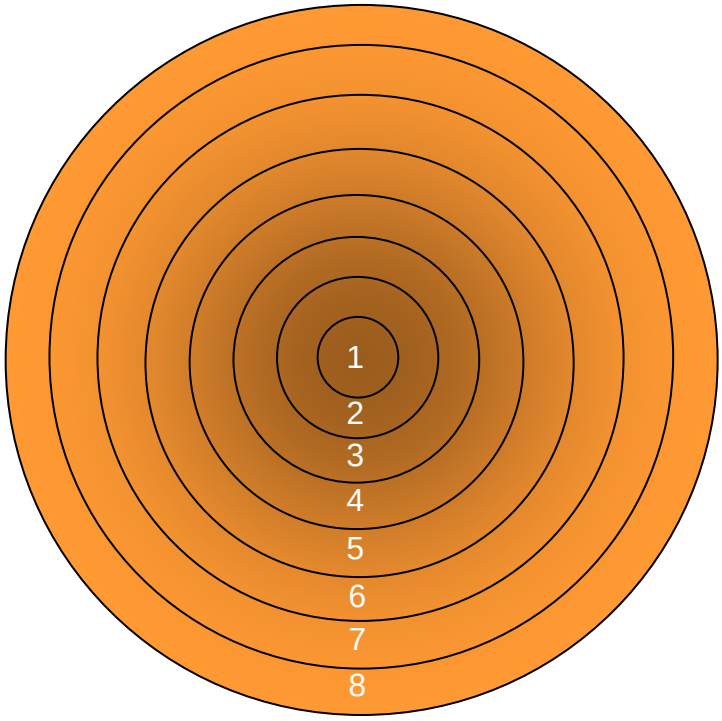
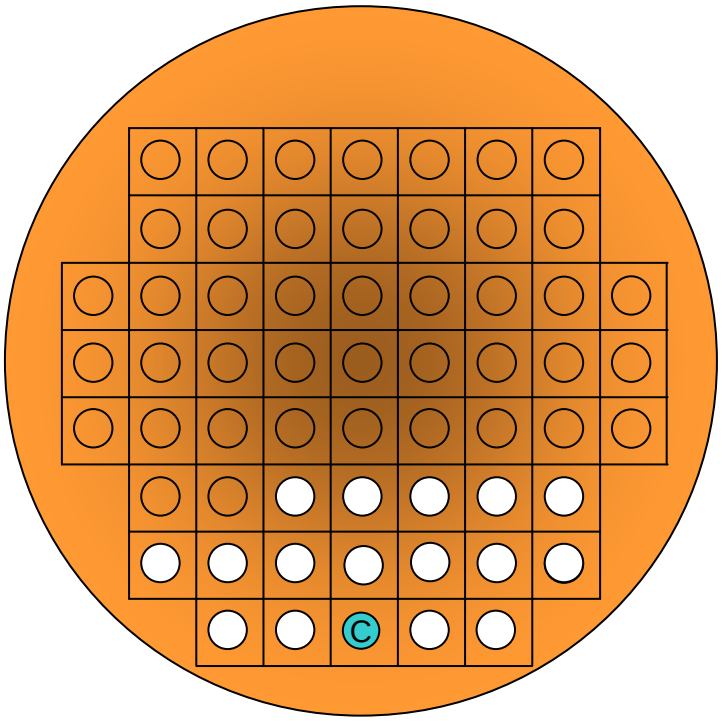


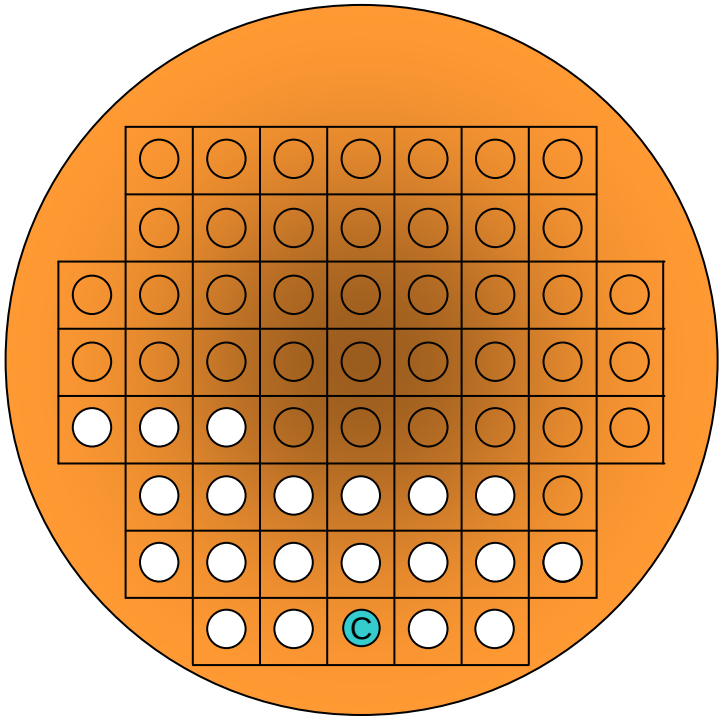
Figure 3.3 Pattern and extent of CHV1 movement throughout *C. parasitica* colonies, indicated by white lattice cells, at 7, 14, and 21 days after being challenged (C) with an isogenic CHV1-containing *C. parasitica* isolate



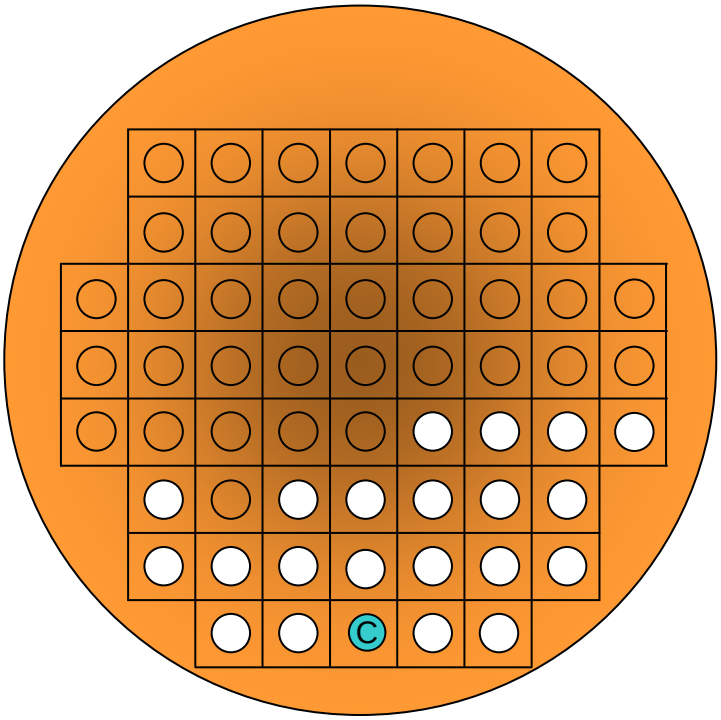
Typical growth rings



7 day representative colony



14 day representative colony



21 day representative colony

CHV1 movement using the hypovirulence-conversion method demonstrated similar results as the whole-colony method. The majority of CHV1 movement into the virus-free colony took place in the first week after challenge (*Table 3.2*). All F-tests indicated there were no significant differences in white isolate recovery (P=0.05) for assays done 7, 14, or 21 days after challenge for both CHV1-infected strains; therefore, all data for these assay times were pooled. However, there was a significant association (P<0.05) between the age of the pigmented tester colony and the percentage of white isolates recovered based on ANOVA tests comparing all treatments (*Table 3.2*). The greatest number and percentage of white isolates recovered was from the 3-day-old colonies (95%) and the smallest number and percentage of white isolates recovered was from the 6-day-old colonies (50%) (*Figure 3.4, Table 3.2).*

There was also a distinct trend of hypovirus movement into the pigmented *C. parasitica* colony behind the conversion front of new growth mycelium (conversion front). Just beyond this front region is located the converted colony, indicated by a change in colony morphology to white phenotype, and random cores sampled from this region all yielded white isolates (*Figure 3.5).* For the "mean plate" shown in *Figure 3.5* the percent of white isolates recovered from the challenged colony decreased as the distance from the CHV1 challenge points, shown in blue, increased (*Figure 3.5*). White isolates were less frequently recovered from the oldest part of the *C. parasitica* colony (colony origin or assay points 13 to 16 in the mean plate) where the mean percent of white isolates recovered ranged from 48% to 40% (*Figure 3.5* and *Table 3.3).*

3.3.2 Evaluation of CHV1 movement *in vitro* throughout 54-day-old *C. parasitica* colonies

CHV1 was not able to move throughout 54-day-old mycelium. On average CHV1 was only able to move through 7.8 to 14.4 lattice cells (*Figure 3.6*) or 17.3% (*Table 3.4*) of the entire *C. parasitica* colony, consisting of young and old mycelium. This is roughly one half of the average number of lattice cells that CHV1 was able to move into for the whole-colony *C. parasitica* colonies, 21 days post challenge with CHV1-containing isogenic isolates. Typically only one to three of these lattice cells yielded white isolates from the older (54-day-old) mycelium located towards the interior of the colony (*Figure*

Table 3.2 Percentage of white isolates collected from *C. parasitica* colonies challenged with an isogenic CHV1-infected *C. parasitica* strain using the hypovirulence-conversion format method

[b]Assay time (days) after CHV1 challenge	**[a,c]Age of colony at time of CHV1 challenge and percentage of white isolates**				
	3-day colonies	4-day colonies	5-day colonies	6-day colonies	**Mean**
	%	%	%	%	**%**
7	91.7	79.2	53.3	45.0	**67.5[d]**
14	96.7	79.2	64.2	53.3	**73.3[d]**
21	97.5	65.0	57.5	52.5	**68.3[d]**
Mean	**95.0%[e]**	**75.8%[e]**	**58.3%[e]**	**50.0%[e]**	**69.8%**

[a.] Number of days of virus-free colony growth previous to challenge with isogenic CHV1-infected *C. parasitica* strain.
[b.] Number of days after colony challenge with an isogenic CHV1-infected containing *C. parasitica* strain, and before sampling with a bark-core sampler for presence of CHV1, indicated by recovery of white isolates.
[c.] Strains TG-303 48 and RML-470 8 were used for the trials.
[d.] F tests indicate there were no significant differences in white isolate recovery (P=0.05) for assays done at 7, 14 and 21 days after CHV1 challenge.
[e.] ANOVA tests indicate means for all #-day colony treatments were significantly different from each other (P=0.05).

Figure 3.4 Average movement of CHV1 through 3, 4, 5, and 6 day-old hypovirus-free TG-303 48 and RML-470 8 *C. parasitica* colonies (following challenge with an isogenic CHV1-infected *C. parasitica* strain) using the hypovirulence-conversion format test. Line indicates combined means of 7, 14, and 21 day sampling times

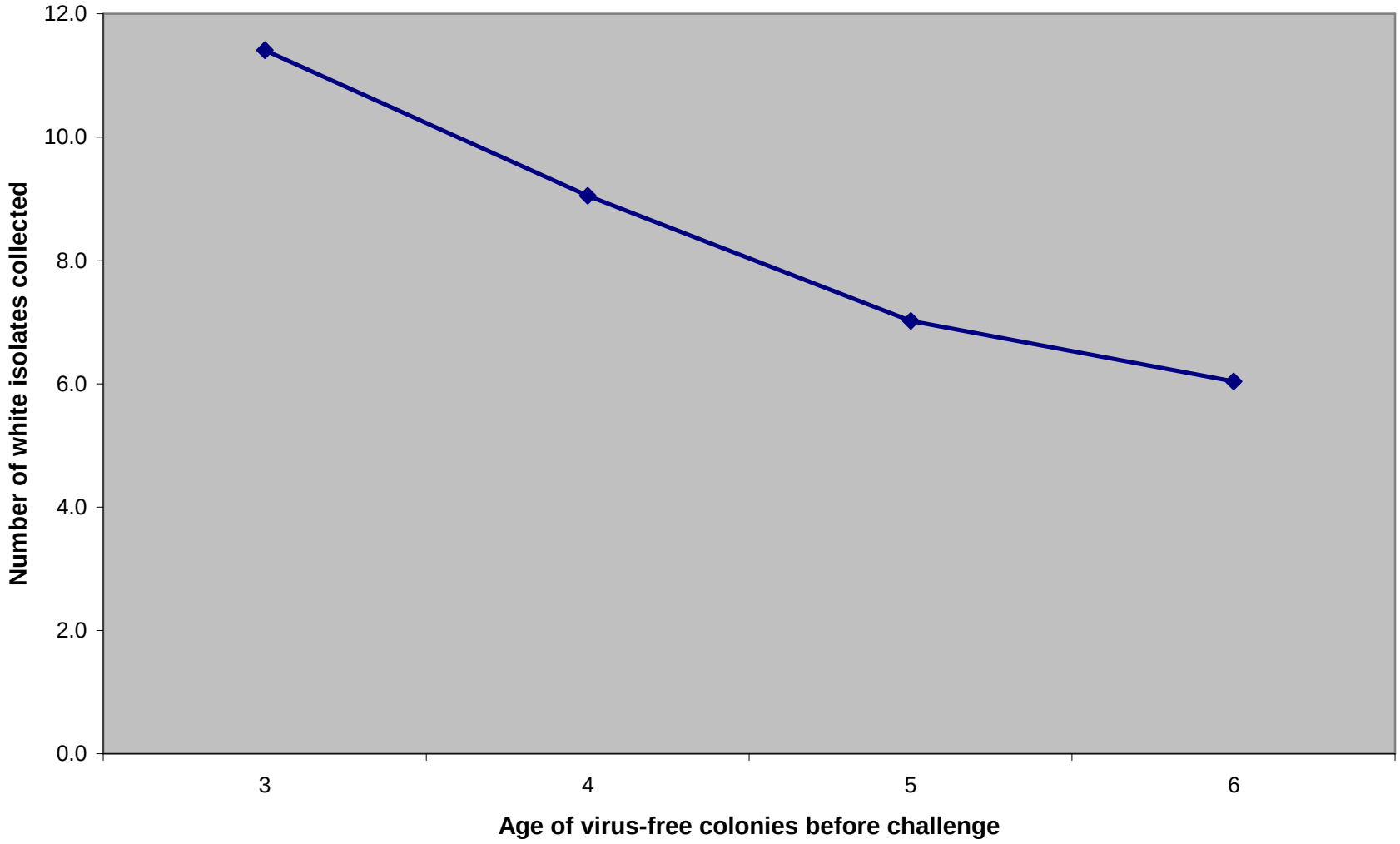


Figure 3.5 Hypovirus movement using hypovirulence-conversion plate cultures. Results for various challenge times (CHV1 challenge after 3-6 days of colony growth) are indicated below. Sampling point numbers are indicated on the Mean plate below. Sampling points 1-4 were taken arbitrarily from the white region below the conversion front as a control (not indicated in figure)

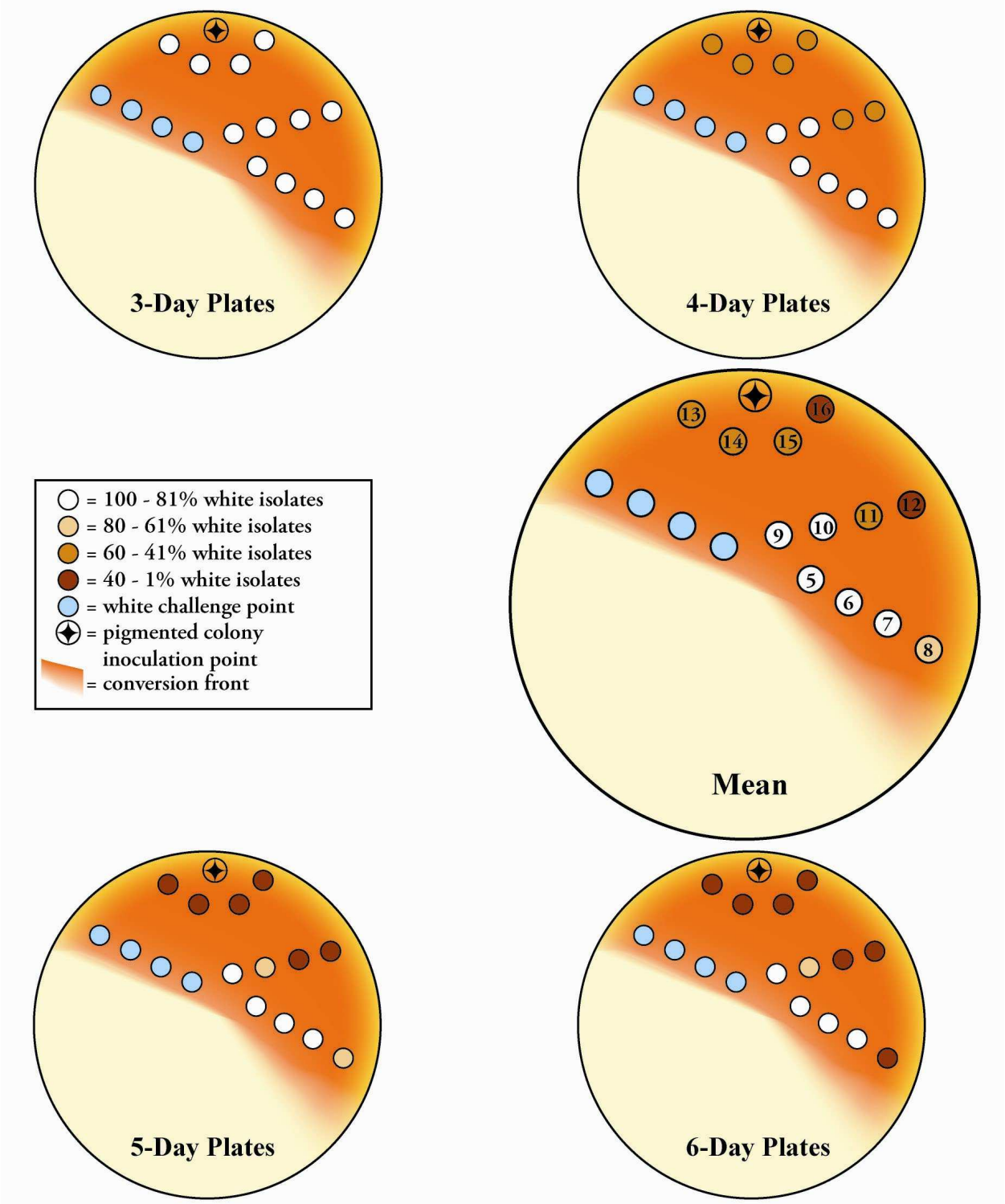


Table 3.3 Percentage of white isolates collected from different sampling points (see *Fig 3.5*) in hypovirulence-conversion-plate tests with TG-303 48 and RML-470 8 *C. parasitica* cultures

[b]Colony age	[a]Sampling point and percentage white isolates											
	5	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
	%	%	%	%	%	%	%	%	%	%	%	%
3	100	100	100	98	100	100	98	81	83	90	93	85
4	100	100	100	90	100	94	54	48	54	54	48	44
5	100	100	100	79	100	72	26	17	28	28	32	21
6	100	100	98	39	100	63	17	13	23	15	17	11
[c]Mean %	**100**	**100**	**100**	**77**	**100**	**82**	**49**	**40**	**47**	**47**	**48**	**40**

[a.] Position of the sampling points was relative to the size of the colony produced before challenge with an isogenic CHV1-containing strain of *C. parasitica*, and the conversion front, where a noticeable change in colony morphology was evident in the pigmented colony.

[b.] The age of the colony, in days, before challenge with an isogenic CHV1-containing strain of *C. parasitica*. Each plate was sampled 7, 14, and 21 days after challenge

[c.] Mean percentage of white isolates collected from sampling points from all colony age conversion plates

Figure 3.6 Hypovirus movement into young and 54-day-old mycelium of TG-303 48, RML-470 8, and THL-230f *C. parasitica* Petri plate colonies. Young mycelium is defined as new colony growth occurring just prior to and after the time of challenge. Plates were sampled 21 days after challenge. Variation for young plus old mycelium and old mycelium is expressed as standard error

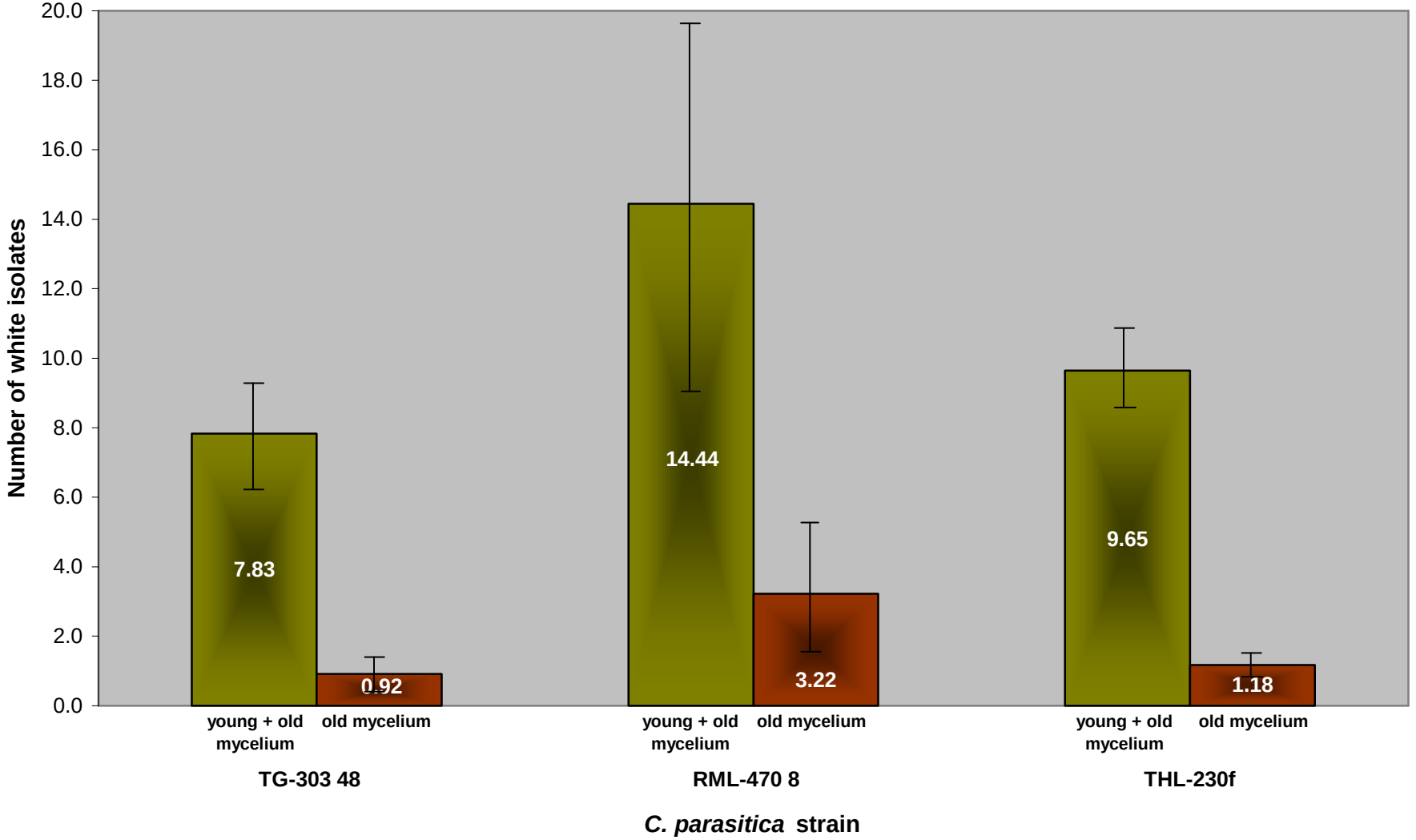


Table 3.4 Movement of CHV1 into 54-day-old hypovirus-free colonies following challenge with an isogenic CHV1-containing *C. parasitica* strain

Strain	No. plates assayed[a]	White isolates recovered			
		Young + old mycelium[b]		Old mycelium	
		%	No.	%	No.
TG-303 b	12	13.3%	7.8 +/- 1.5	1.5%	0.9 +/- 0.5
RML-470 8	9	24.5%	14.4[c] +/- 5.2[d]	5.4%	3.2[e] +/- 1.4[d]
THL-230f	17	16.4%	9.7 +/- 1.1	2.0%	1.2 +/- 0.4
Total/Avg.	**38**	**17.3%**	**10.21**	**1.6%**	**1.58**

[a.] Total number of 54-day-old colonies sampled for each *C. parasitica* strain.
[b.] Old mycelium consists of the 33-day-old colony challenged and young mycelium is the newly emerging mycelium from the colony just prior to and after challenge.
[c.] Mean number of total white isolates recovered from 54-day-old colonies plus young growth.
[d.] Variation expressed as standard error. No significant difference in white isolate recovery (P=0.05) was found for assays done with any of the *C. parasitica* strains.
[e.] Mean number of total white isolates recovered from 54-day-old colonies only.

3.6 and *Table 3.4*). The largest difference of hypovirus movement was between RML-470 8 and TG-303 48, which had CHV1 movement into an average of 14.4 lattice cells and 7.8 lattice cells, respectively; however, all analysis of variance (ANOVA) tests indicated there were no significant differences in white isolate recovery (P=0.05) for assays done with any of the *C. parasitica* strains (*Table 3.4).*

The pattern of CHV1 movement was similar to the whole-colony plate *C. parasitica* movement trials conducted at 7, 14, and 21 days. CHV1 did not move into the central or oldest part of the colony; rather, the lattice cells into which CHV1 did move, were located towards the exterior of the colony, just beyond the challenge point, in the new growth mycelium.

3.3.3 Evaluation of resistance in *C. parasitica* to CHV1 transmission *in vitro*

The original 50 conversion tests in 1999, using a single-spore pigmented (SSP) isolate from THL-513b (parent) and THL-513b (WS), were all negative for hypovirus transmission. In 2000, all 100 conversion tests for *C. parasitica* strain SSP THL-513b were positive for hypovirus transmission, regardless of whether SSP THL-513b tester strain was paired with hypovirus-containing THL-513b (parent) or THL-513b (WS) donor strains. Four cores were removed from the pigmented area behind the conversion front in each colony and grown out for confirmation of hypovirus movement. Most of these cores yielded white isolates, but a small number (19) of those cores produced pigmented isolates; these isolates were subjected to further hypovirulence-conversion tests. An additional 74 conversion tests were conducted on the 19 pigmented isolates from the above trials for a total of 86 conversion tests between SSP THL-513b and the parent strain and 88 conversion tests between SSP THL-513b and THL-513b (WS) (*Table 3.5*). Five of these tests demonstrated partial conversion, indicated by only partial movement of the hypovirus into the pigmented tester strain colony, based on number of mycelial disks sampled from behind the conversion front of the colony yielding white isolates. The other 69 tests were fast conversions (conversions within one to three days after challenge) with apparent complete movement of virus throughout the tester colony, since all colony disks yielded white isolates.

Table 3.5 Hypovirulence conversion tests on *C. parasitica* isolates with possible resistance

Donor strain[a]	Tester strain[b]	No. tests	No. conversions
	Strain 513b tests		
THL-513b (parent) '99	SSP THL-513b sector '99	50	0
THL-513b (parent)	SSP THL-513b sector	86	[c]84
THL-513b (WS)	SSP THL-513b sector	88	[c]85
	Isolates from CHV1 movement trials		
THL-230f (parent)	14 DPC THL-230f plate #2 isolate #36	10	10
THL-230f (parent)	21 DPC THL-230f plate #1 isolate #36	10	10
THL-230f (parent)	21 DPC THL-230f plate #4 isolate #36	10	10
THL-230f (parent)	21 DPC THL-230f plate #4 isolate #36	10	10
THL-230f (parent)	21 DPC THL-230f plate #3 isolate #37	10	10
THL-230f (parent)	21 DPC THL-230f plate #2 isolate B	10	10
	THL-230f "possible resistant" isolates		
THL-230f (parent)	THL-230f #1	10	10
THL-230f (parent)	THL-230f #2	10	10
THL-230f (parent)	THL-230f #3	10	10
Total	**11**	**314**	**259**

[a.] Donor strain is the isogenic *C. parasitica* strain containing CHV1 and having typical white colony phenotype.

[b.] Tester strain is the isogenic *C. parasitica* strain lacking CHV1 and having typical pigmented or normal colony phenotype.

[c.] A small number (5) of isolates were found to have partial conversions indicated by a noticeable change in colony phenotype as the tester strain moved down the plate; however some of the isolates removed from behind the conversion front, inside the pigmented region of the tester strain, were pigmented upon transfer.

Sixty hypovirulence conversion tests conducted on individual isolates, recovered from the whole-colony CHV1 movement trials, yielded fast or very fast conversions, and apparent complete movement of the hypovirus throughout the tester strain based on colony disk transfer. The additional 30 conversion tests with additional "possible resistant" THL-230f isolates demonstrated the same fast or very fast conversion results with complete movement of CHV1 throughout the pigmented colony (*Table 3.5*).

Field tests using SSP THL-513b and THL-513b (parent) yielded similar results as the *in vitro* tests noted here. An artificially established canker on a 10-12 cm diameter American chestnut tree at the Paint Bank research plot was established using SSP THL-513b in May of 1999, and challenged with THL-513b (ws) strain in May 2000. The 12.6 cm long canker was a typical sunken canker in May of 2000 and was sampled in May 2001. All the isolates recovered from the canker were white hypovirulent isolates of *C. parasitica* that were vegetatively compatible with THL-513b.

3.3.4 Effect of low temperature on CHV1 survival in a *C. parasitica* canker colony

There were five bark-core collection dates where the preceding days temperature at the Paint Bank research plots met or exceeded -10^{o}C (*Table 3.6).* The mean temperature on the collection dates was -13.4^{o}C and generally the Paint Bank research plot was colder than the Horton Center research plot. A total of 31 isolates were recovered from bark cores taken from superficial cankers established with hypovirulent strains of *C. parasitica* at the Paint Bank Research plots on the collection dates indicated. All but two of the *C. parasitica* isolates recovered were white, indicating CHV1 survival at -10^{o}C. The two pigmented isolates were both collected from the same canker, and were subjected to vegetative compatibility tests. Both isolates were found to be incompatible with the original *C. parasitica* strain inoculated on the tree (*Table 3.6*).

3.3.5 *In vitro* low temperature tests with *C. parasitica* hypovirulent strain Ep-713

Approximately 25 *in vitro* low temperature tests were conducted with colonies of strain Ep-713. After placing each APDA Petri plate in the freezer at -10^{o}C for 24hr. the new mycelial growth at room temperature was slightly pigmented and suppressed in the agar. There was very little subtomentose type mycelium in the new growth. Two mass-transfer

mycelial samples were taken from the new growth of each colony and plated separately on APDA plates. All resulting colonies, from the 25 original colonies, had white mycelial growth and colony characteristics consistent with white strain Ep-713.

Table 3.6 Phenotype of *C. parasitica* isolates collected at Paint Bank research plots following at least 24 hr. of -10°C or lower temperature

Date(s)	Average temp. (°C)[a]	Collection date	No. total isolates recovered	No. white isolates[b]
12/20/04	-11.0	12/21/04	4	4
1/05/05 – 1/07/05	-13.3	1/09/05	8	7[c]
1/10/05	-15.5	1/12/05	8	7[c]
1/20/05	-12.6	1/21/05	8	8
1/29/05 – 1/31/05	-14.7	1/31/05	3	3
Total	**-13.42**		**31**	**29**

[a.] Average daily temperature calculated from daily maximum and minimum temperatures
[b.] Number of white isolates recovered from bark cores collected
[c.] Subsequent vegetative compatibility tests revealed that the pigmented isolates recovered were incompatible with the inoculated strain; thus the pigmented isolates were likely the result of secondary colonization.

3.4
DISCUSSION

In this study both the whole-colony studies and the long-term 54-day colony studies indicate that the age of the *C. parasitica* colony limits the movement of CHV1 throughout the colony after hypovirulence challenge. In the whole-colony studies many of the cells into which CHV1 moved were occupied by the younger or new mycelial growth of the *C. parasitica* colony. In the vast majority of the trials CHV1 was able to move into the lattice cells of mycelium located just to the left and right of the challenge point and around the perimeter of the colony. These cells were located in parts of the *C. parasitica* colony that emerged after the CHV1 challenge. This pattern of CHV1 movement may be due to (1) the inability of CHV1 to move into the young or new growth mycelium which is located distant to the original challenge point (distance effect), and/or, (2) the inability of CHV1 to move into older mycelial growth (age effect). It seems that the second hypothesis is the most important. The mycelium most distant to challenge point may also be too old after 7 days, as CHV1 moved rapidly in the first 7 days of the 21-day test period.

In support of the mycelium-age hypothesis, in the whole-colony studies, CHV1 was often unable to move into the central regions of the colony located only one or two lattice cells away from the challenge point, but was able to move into the perimeter areas of the colony in areas located up to nine lattice cells distant to the challenge point. This is evidenced in *Figure 3.3* where many of the lattice cells in the 7-to 8-day growth rings contain white isolates while other lattice cells located in 2-to 3-day growth rings remain free of CHV1 despite their closer proximity to the challenge point.

One explanation why CHV1 moves more easily into new growth mycelium could be due to the numerous hyphal anastamoses and nutrient translocation that exists in the hyphal tips of new-growth mycelium. Olson and Gray (1998) found tangential movement of nutrients along the hyphal front of various agar grown basidiomycete cultures indicating anastamosis along the periphery of the colony. Using hypovirulent cultures of *C. parasitica*, Newhouse *et al.* (1983) found that the hypovirus concentration was greatest in the hyphal tip of the new growth mycelium. It seems likely then that as

the nutrients are translocated along the hyphal front of *C. parasitica* in new growth, CHV1 is also translocated along the front, presumably through numerous anastamoses. In addition, Newhouse *et al.* (1990) identified woronin bodies in *C. parasitica,* which occluded the septal pore. Woronin bodies are microscopic organelles present in the cytoplasm of fillamentous fungi which are consistently associated with the septal pore. Some research (Trinci and Collinge 1974; Markham and Collinge 1987) has proposed that woronin bodies can explain why some nutrients, or cytoplasmic entities are restricted to areas of the fungal thallus. Although woronin bodies have been identified with *C. parasitica,* they have not been suggested to be responsible for restricting hypovirus movement. The presence of or multiplication of woronin bodies in *C. parasitica* could contribute to the formation of functional mycelium units (FMUs) (Rayner 1991), which are sections of the mycelium that act independently of the entire mycelium. Conversely, woronin bodies may act in conjunction with the age of the *C. parasitica* thallus to restrict hypovirus movement.

Results from 54-day colony experiments also indicate that colony age plays a role in CHV1 movement. Very little CHV1 movement was observed in any part of the 54-day-old colonies tested and movement into the center (oldest) part of the colony was very rare. More than five times as many white isolates were recovered from lattice cells located in the newest mycelial growth (perimeter) than in the oldest growth (center). This would appear to be further evidence that older *C. parasitica* mycelium provides a barrier for CHV1 movement. Interestingly, the overall number of lattice cells into which CHV1 moved was fewer than in the whole-colony tests and movement throughout, even in the new growth mycelium, was less. This may be due in part to the non-uniform growth emerging from the colony center. As per the methods mentioned, the ~5-cm diameter colony center was grown for 27 days before it was transferred to a new Petri plate. Mycelial growth was revived in the new agar medium, but rather than mycelial growth from a central point, there were many mycelial fan outgrowths coming from the perimeter of the 5-cm diameter plated colony center. The emerging growth was a patchwork of mycelial fans originating from various locations of the 5-cm colony center. Thus the new growth mycelium was not uniform, like in the whole-colony experiments. All the separate origins of mycelial growth may be in fact separate FMU's.

Functional mycelial units may act independently of each other, or in association through hyphal anastomoses. When acting in association, the FMUs can communicate with each other through nutrient translocation, or signaling. In natural situations, FMUs can be beneficial to the fungus when available nutrients are scarce. It has been suggested long-distance electrical signals can be produced when nitrogen reserves change and that through signaling, parts of the mycelium die-back while other parts closest to the nutrients may thrive (Watkinson 1999). It is possible therefore that hypovirus moves more abundantly through new-growth mycelium and not old growth because nutrient translocation is favored in new growth mycelium and the old-growth mycelium "area of nutrient deficiency" is allowed to "die-back in order to support the entire colony" (Watkinson 1999). Usually associated with old-growth mycelium is a high degree of vacularzation in cells and loss of cytoplasm (Ainsworth and Sussman 1965). In this study it was noted that older growth mycelium had substantially larger vacuoles versus new growth mycelium, when examined microscopically.

Alternatively, it is possible that the same electrical or chemical signaling that exists to support a mycelium during periods of nutrient duress may also serve to protect a fungus from an invading hypovirus in the cytoplasm. Timonen *et al.* (1996), and Olsson and Gray (1998), have used a radioactive nutrient labeling technique to identify the reallocation patterns of nutrients in intact mycorrhizal systems, and agar-grown fungal cultures following formation of the fungal thallus. Both studies identified special patterns of movements in the mycelium, indicating that formation of FMUs can take place after the formation of the intact mycelium. In order to limit the hypovirus infection to part of the mycelium, electrical or chemical signals may trigger a type of hypersensitive response leading to containment of hypovirus within an FMU.

It is possible that colony mycelium age plays a role in biological control of chestnut blight *in vivo.* Hogan and Griffin (2002b) reported finding large natural cankers on the Lesesne trees with both white and pigmented isolates of the same vc type coexisting in the same canker. In addition, the white and pigmented isolates appeared to be in contact with each other based on the lattice-cell locations in a 7x7 lattice sampling grid. Despite the apparent connections, CHV1 did not convert the entire bark colony of the same vc type to hypovirulence *in vivo*. It is possible that the CHV1-containing

isolates recovered from these cankers were recovered from the younger mycelium in the canker, and the CHV1-lacking isolates were from the older regions of the underlying *C. parasitica* colony.

The natural cankers at Lesesne sampled were approximately 30 x 40 cm in size, yet exhibited many signs of superficial cankers containing hypovirulent isolates, including bark cores showing asymptomatic phloem tissue at the cambium, swollen and black appearance, and very few *C. parasitica* reproductive structures. It is reasonable that a virulent *C. parastica* strain established and expanded the canker for many months, or years, before a hypovirulent strain carrying CHV1 colonized the canker and/or CHV1 was transmitted to the *C. parasitica* strain in the canker (Hogan and Griffin 2002a). The grafted trees at Lesesne have a small amount of natural resistance (Robbins and Griffin 1999), which may in the short term prevent *C. parasitica* from killing the tree, and provide time for CHV1 to spread to the canker and convert the underlying colony to hypovirulence. If a long period of time exists before hypovirulence conversion, the age of the underlying colony in the canker could be a barrier to CHV1 spread. In addition, the mycelial growth of the original strain is three-dimensional within the canker, so the youngest and oldest regions of the colony may not be oriented in a radial pattern such as in a Petri plate. This could account for the random patterns of white isolates within the lattice grid of the canker (Hogan and Griffin 2002b). Furthermore, some white isolates recovered could be the result of post–conversion new growth mycelium, growing in a random pattern.

Results of the present study also indicate that the extent of hypovirus movement through an entire colony does not change very much over a long period of time. In this study the majority of the CHV1 movement through a *C. parasitica* colony occurred between 0 and 7 days following challenge with CHV1, and the amount of hypovirus movement through a *C. parasitica* colony was statistically the same after 7, 14, and 21 days using the whole-colony method. *Figure 3.1* shows a trend in hypovirus movement over time. There was very little increase in the number of the mycelial lattice cells occupied by CHV1 between 7 and 14 days (gain of 3 cells or 5.5% of the entire lattice grid) or between 14 and 21 days after challenge (gain of 0.5 cells or 0.7% of the entire lattice grid). At the day of challenge, none of the mycelium in the lattice cells contained

CHV1. Thus, over the span of 7 days CHV1 moved into an average of 16 lattice cells and 19.5 lattice cells by 21 days.

Shain and Miller (1992) described incomplete movement of hypovirus into an artificially established canker 9 weeks after challenge with a hypovirus-containing *C. parasitica* strain. After 9 weeks, the interior of the canker (oldest region) was essentially free of hypovirus, but the periphery (location of hypovirus challenge) was converted to hypovirulence. In this study, the cankers sampled were only a total of 21 weeks old, and much younger than the natural cankers at Lesesne. According to Shain and Miller, CHV1 was unable to spread throughout the entire canker after more than 2 months of contact. In fact, they report an almost equal amount of hypovirus movement throughout a canker colony at 6 and 9 weeks after challenge (68% and 70% white isolates, respectively, based only on bark cores). In comparison the percentage of white isolates recovered *in vivo* by Shain and Miller at 21 days after challenge (30%) is almost equal to the percentage of white isolates recovered at 21 days *in vitro* (33%) noted here.

Shain and Miller also reported hypovirus spread of 56% to 81% *in vivo* following long-term incubation periods of 65 weeks. Furthermore, Hobbins *et al.* (1994) reported *in vivo* hypovirus spread of up to 94% within one vc type, although 2/3 of the isolates were recovered from the new-growth mycelium periphery of the canker where one would most expect to recover hypovirulent isolates. These are some of the longest-term studies on virus movement in a canker, and the percentage of recovered isolates infected with hypovirus is among the highest reported to date. Despite this length of time, hypovirus in these studies was still unable to move throughout the entire canker even though the cankers were artificially established and presumably the entire mycelial thallus inside the canker was of one genotype (Shain and Miller 1992). Furthermore, the experimental method of these studies allows for maximal contact between the virulent and hypovirulent strains as they both expand to produce the canker. In nature, cankers may grow for years before coming into contact with hypovirulent strains, at which point the canker is 1.) composed of multiple vc types and 2.) densely composed of large mycelial fans such that new hypovirulent growth and anastomoses with virulent mycelium are severely limited by both the size of the existing canker, and the available healthy phloem tissue contained within. Hogan and Griffin (2002) reported limited hypovirus movement

in naturally formed cankers composed of multiple vc types. Using a sampling grid, the vc composition of the canker was identified and mapped. The cankers were many years old and had been classified as "healing cankers" for several years, yet hypovirus had only moved into 35% to 60% of the recovered isolates in the major vc group of each canker. Thus, although the present study has the shortest experimental time, and the lowest percentage of hypovirus movement into a single vc type colony, the above *in vivo* studies indicate that even in the longest term studies, complete hypovirus movement will not occur.

An examination of the hypovirulence conversion-plate test means in *Figure 3.5* and *Table 3.3* indicates a combined effect of distance and white-pigmented mycelium contact time on hypovirus movement through a colony. Similar to the whole-colony tests, the sampling points 13-16, located in the oldest region of the *C. parasitica* cultures (mycelium surrounding the inoculation point at the top of the plate), typically yielded the lowest percentage of white isolates. As the isolation point moves further from the hypovirus challenge point, the percentage of white isolates collected decreases, indicating that distance from the nearest challenge point may be a factor in CHV1 movement, although not as important as age of mycelium. It is possible that the time it takes for CHV1 to reach the more distant isolation points (eg. points 11 and 12) may cause the colony to mature past the age of unrestricted hypovirus movement.

It appears that low temperature (-10^{o}C) has no effect on hypovirus maintenance or survival, although some phenotypic changes in the fungus were associated with the low temperature stress. All bark cores recovered from the artificially established cankers in field experiments at Paint Bank yielded white *C. parasitica* isolates, indicating successful hypovirus maintenance after exposure to temperatures below -10^{o}C on 1 to 3 successive days, and for five separate periods from December, 2001 to January, 2002. Two isolates recovered were normal pigmented isolates; however, they were not the same vc type as the inoculated strain. These isolates were most likely secondary colonizers, which is very common (Griffin and Griffin 1995). The *in vitro* experiments gave similar results. CHV1 remained stable and produced a white Ep713 colony upon transfer to new APDA plates, following exposure to -10^{o}C for 24hr., indicating the survival of CHV1, despite some temporary phenotypical changes in the new mycelial growth on the frozen *C.*

parasitica colony plates. Furthermore, on a number of occasions, white *C. parasitica* colonies were revived from mycelium stored at -20^{o}C (personal observation). Storing CHV1-infected mycelial patties in the freezer is a standard procedure for dsRNA extraction, and often patties are stored for more than a year, yet they still yield viable, CHV1-containing *C. parasitica* colonies upon transfer.

The phenotype changes in the *C. parasitica* colonies subjected to low temperature may be the result of stress on the colony. The agar in the Petri plates lost 3-4 ml of water due to evaporation and condensation of water on the Petri-plate when they were in the freezer. Once removed from the freezer, the water was decanted. The resulting agar was denser than before. This desiccation may account for the slower growth of the colony after freezing. In addition, some intracellular nutrients may have been lost through cellular injury; thus, the mycelium may have lacked some nutritional or physiological requirements for rapid colony growth after freezing. Low temperature injury to cell membranes is common in fungi (Roth *et al.* 1979). Therefore, the temporary change in phenotype may be due mostly to low temperature effects on the host.

Friese *et al.* (1992) reported that storing *C. parasitica* at temperatures of -10^{o}C for 24 hr resulted in the loss of dsRNA from the new growth mycelium. The loss of dsRNA was measured by the return of normal pigmentation to the new growth mycelium after returning the isolates to normal growth conditions, and sampling conidia from the new-growth conidiomata. Although the present study confirmed a temporary change in colony color in new growth mycelium after exposure to freezing temperatures, we could not confirm the loss of CHV1, as all transfers from mycelial growth emerging after freezing yielded white isolates. Mass transfers of mycelium were used in this study rather than conidia because mycelium has been found to be more reliable for detecting hypovirus as often individual conidia are hypovirus free. Furthermore, from a biological control perspective, the fungal thallus is responsible for stem and branch injury, and thus presence of hypovirus in the thallus should be primary. These results, in combination with the survival of CHV1 in cankers over the winter indicate that freezing temperatures (of up to -20^{o}C) are not detrimental to hypovirus maintenance.

No long-term *C. parasitica* resistance to CHV1 infection or movement was identified in this study. It appears that if any resistance exists, it is temporary. The same *C. parasitica* strains that were identified to have resistance to hypovirulence conversion in the fall of 1999 were found to have no resistance 1 year later, after storage at 5°C. It is possible that the short-term exposure at 5°C had an effect on the resistance that was observed in the previous year, or that the resistance was lost due to propagation through mass transfers. It has been noted that dsRNA (CHV1) can be regained after temporary loss during multiple mass transfers (Anagnostakis 1981); this may be a type of temporary resistance.

The original sectoring of isolate THL-513b and lack of hypovirulence conversion of the pigmented colonies was similar to sectoring of other *C. parasitica* strains believed to have hypovirus resistance. 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 or mutant was found to be isogenic with the parent, and multiple conversion pairings between the parent and other vegetatively compatible virus-containing strains, were unsuccessful. These workers indicated the mutation involved virus maintenance. In addition, Hogan and Griffin (2002b) observed sectoring of *C. parasitica* strain Ep-713 in culture in which a pigmented sector arose from a growing white colony. When these pigmented sectors were then tested for the presence of dsRNA, none was recovered (Hogan and Griffin 2002b). Polashock *et al.* (1994) reported similar sectoring *in vitro* for Ep-713. When inoculated in the field at Paint Bank the THL-513b pigmented sector produced a sunken, killing type canker, 12.6 cm in length, on an American chestnut stem. One year later, the THL-513 white sector was inoculated in the tree at the periphery of the killing canker. Within the year, the canker developed healthy callus tissue, and 1 year later white hypovirulent isolates were removed from the canker, indicating hypovirus spread, and loss of resistance (Hogan and Griffin, unpublished).

One can only hypothesize about the nature of the temporary resistance in isolate THL-513b. A possible explanation for the loss of resistance to hypovirus infection may be due to a system of virus-induced gene-silencing as observed in plants (Napoli *et al.*

1990) or quelling as observed in fungi (Romano *et al.* 1992). There are a number of models of gene-silencing of viral pathogens in plants whereby short dsRNA agents in the host can recognize the invading RNA and degrade the virus using nucleases (Matzke 2002; Voinnet 2001). These systems may be inducible and transient and the virus can respond with viral suppressors (Matzke *et al.* 2002). CHV1-encoded protein p29 (Choi *et al.* 1992) has been demonstrated to have similarities to HC-Pro, the potyvirus-encoded suppresser of RNA silencing in plants, and has therefore been suggested to serve a similar function. Very recently suppression of RNA silencing by p29 has been demonstrated in both *Nicotiana benthamiana* and *C. parasitica* (unpublished, in Nuss 2005). A system of gene silencing and gene silencing suppression could possibly explain the development of resistance as well as the loss of hypovirus resistance and the subsequent ability of hypovirus to infect *C. parasitca* strains. Much more research would need to be conducted however to determine if gene silencing actually acts as an anti-hypoviral defense in *C. parasitica*.