Chapter 2

Importance of *Cryphonectria parasitica* intermediate phenotype isolates, stromata production, and other fungal reproductive canker characteristics in spread of CHV1-Euro7 hypoviruses on American chestnut trees at the Lesesne State Forest and the Jefferson National Forest

2.1
INTRODUCTION

In the early 1900's the introduction of the chestnut blight fungus [*Cryphonectria parasitica* (Murr.) Barr] nearly destroyed all canopy or large American chestnut trees [*Castanea dentata* (Marsh.) Borkh.] in the natural range. Fortunately the fungus does not invade the roots, so American chestnuts have been able to survive predominantly as understory shrubs. The few large American chestnut trees that survived the initial epidemic have superficial healing cankers that frequently contain pigmented hypovirulent (= reduced virulence) *C. parasitica* strains containing dsRNA hypoviruses. Low levels of host resistance, indicated by failure of virulent strains of *C. parasitica* to colonize the vascular cambium, and favorable growing sites (low altitude, mesic sites with reduced hardwood competition) have also been associated with blight control on American chestnut (Griffin 1992, Griffin *et al*. 1991). The chestnut blight epidemic was less severe in Europe where hypovirulent strains of *C. parasitica* were found to be consistently associated with healing cankers. These European hypovirulent strains contain a dsRNA hypovirus, *Cryphonectria hypovirus 1* (CHV1). Generally these strains have a white phenotype, instead of the normal orange phenotype, in laboratory culture; they also have reduced sporulation in the field (Grente 1978, Elliston 1985b).

In 1982-83 grafted American chestnut trees at Lesesne State Forest, Virginia were inoculated with one French and three Italian hypovirulent strains of *C. parasitica* (Ep-43 Ep-47, Ep-49, and Ep-51). After 20 years it was reported that CHV1 had spread throughout the grafted trees and into at least forty-five *C. parasitica* vegetative compatibility (vc) types, and to natural cankers up to 642cm outside of the hypovirulent inoculated (H-inoculated) zone (Hogan and Griffin 2002a). Based on colony morphology data of the recovered white isolates, these workers reported that the CHV1 hypoviruses from Italian *C. parasitica* isolates Ep-47, Ep-49, and Ep-51 were most likely responsible for the spread at Lesesne. Using nucleotide sequence analysis of an 844–bp region in the helicase domain, CHV1 hypovirus isolates from the white isolates recovered at the Lesesne State Forest had high identity (98.3 – 99.5%) to Italian CHV1–Euro 7 (Griffin *et*

al. 2004). This spread of CHV1 occurred in the apparent absence of appreciable quantities of *C. parasitica* reproductive structures.

Previously, Elliston (1985b), making visual estimates, indicated that Italian strains Ep-49 and Ep-51 produced few stromata on American chestnut trees following artificial inoculation. However, no quantitative data of stromata produced on cankers on American chestnut trees, based on actual stromata counts, have been reported for Italian CHV1-infected strains. Further, there have been no studies of CHV1-hypovirulent strain reproductive structures on American chestnut trees following the apparent natural spread of hypovirulence.

In 2002 single spore colonies from the isolates recovered from the grafted trees at Lesesne were classified into 4 different colony phenotypes. The most common *C. parasitica* colony phenotype recovered at Lesesne was colony morphology group 3 (CM3) (Hogan and Griffin 2002a). This phenotype is characterized by white or lightly pigmented centers with numerous, lightly pigmented yellow-orange pycnidia on margins, and is intermediate in pigmentation with typically 50% yellow-orange pigment. Intermediate isolates have also been identified in Europe. Bonifacio and Turchetti (1973) referred to intermediate isolates as those having a colony phenotype intermediate between white hypovirulent and normal virulent or pigmented types. Grente (1981) described isolates that were referred to as intermediate by Turchetti (1978) as unstable, changing to white or to pigmented types upon further transfer in the lab (Grente and Berthelay-Sauret 1978). Coskun *et al.* (1999) recovered intermediate isolates from healing or slightly sunken cankers in natural European chestnut stands in Turkey. They found that 42% of the isolates they collected were intermediate in phenotype, but only four of the isolates (white isolates) were found to contain dsRNA. The present study was conducted: (1) to determine the frequency and phenotypic diversity of CHV1-infected *C. parasitica* isolates recovered from stromata and canker tissue located on grafted American chestnut trees at the Lesesne State Forest and artificially established cankers on American chestnut at Paint Bank, Jefferson National Forest, and (2) to determine the presence or absence of CHV1-Euro7 hypovirus in intermediate (~50 +/-20% pigmented) single-spore isolates of Ep-49 and intermediate isolates recovered from American chestnut research plots.

2.2

MATERIALS AND METHODS

2.2.1 Inoculation of CHV1-containing isolates in natural cankers on grafted American chestnut trees at Lesesne State Forest and establishment of artificial cankers on natural American chestnut sprouts at Paint Bank research plots

In 1980 scion wood from large, surviving American chestnut trees was grafted to existing root-stock of naturally occurring chestnuts, established by Dietz (1978), in a reduced hardwood competition plot in the Lesesne State Forest in Nelson Co. Va. In 1982 and 1983 naturally occurring cankers, located between 0 and 187 cm on the main stems of grafted chestnuts (hypovirulent strain-inoculated zone, H-inoculated zone), were inoculated with a mixture of hypovirulent strains including white, Italian strains Ep-47, Ep-49, and Ep-51, infected with *Cryphonectria hypovirus 1* (CHV1) (Hogan and Griffin 2002a). Previous research has shown that CHV1 had spread from the inoculated cankers to newly formed naturally occurring cankers in distances of up to 642 cm from the H-inoculated zone on TH, RM, and TG grafted American chestnuts. Almost all naturally occurring cankers on these three grafts were highly superficial (Dierauf *et al.* 1997), and almost all contained some white *C. parasitica* isolates.

Between April 2002 and April 2004 artificial cankers were established by inoculating CHV1-containing isolates Ep-47, Ep-49, Ep-49(CM1), Ep-49(CM3), and Ep-51W, on natural American chestnut sprouts, approximately 6 cm in diameter, growing in a Forest clear-cut in Paint Bank, Va. All cankers were established within arms-reach on the main stem of the chestnut trees. The five Italian isolates were grown in Petri plates containing acidified (5.6 ml 25% v/v lactic acid per liter) potato dextrose agar (APDA) for 14 days and checked for cultural characteristics consistent with the isolates as described by Elliston (1985a). Discs were cut from the Petri plate cultures using a 6-mm cork borer. Using the same 6-mm cork borer, holes were cut in the bark of the trees exterior to the cambium. The mycelium-covered discs were inserted into the holes mycelium side inward and wrapped with masking tape to deter secondary infection and moisture lost. All five isolates were inoculated on five trees in a modified Latin square pattern. Maps of the tree locations were made and each tree was assigned an

identification code and labeled with an aluminum tag. Artificially established cankers were examined every October and May and assayed for canker superficiality and number of stromata present. Canker superficiality was measured by removing 2 mm bark cores from 4 canker locations and noting *C. parasitica* spread in relation to the vascular cambium. Discoloration of wood was a marker of *C. parasitica* location.

2.2.2 Isolation of *C. parasitica* from superficial cankers on Paint Bank clear-cut American chestnut sprouts and Lesesne grafted American chestnut trees

Bark-core and stromata canker isolates of *C. parasitica* were collected from main-stem and branch superficial cankers, located inside and outside of the H-inoculated zone, on TH, RM, and TG American chestnut grafts at the Lesesne State Forest, Va, and main-stem artificially established superficial cankers on American chestnut trees at Paint Bank, Va. Branch and main-stem cankers from Lesesne State Forest were identified by the measured distance from the ground to the center of the canker. When branch cankers were sampled, the distance from the canker to the origin of the branch was added to the distance from the origin of the branch to the ground. Artificially inoculated cankers at the Paint Bank research plot were identified by tree and canker number.

Bark-core samples were taken arbitrarily from each canker using a 1.7 mm diameter bark-core sampler. Samples were placed in a labeled multi-well plate, covered with masking tape for temporary storage, and placed in a cooler for transportation to the laboratory. In the laboratory, bark-core samples were removed from each well, surface disinfested in 1% NaOCl for two minutes, rinsed in sterile distilled water, and plated on APDA plates. The resulting fungal growth was monitored daily and individual colonies suspected to be *C. parasitica* were transferred to new APDA plates. Pure cultures were grown in normal room light (fluorescent) conditions for two weeks. Pure cultures of *C. parasitica* were evaluated for colony color and other cultural characteristics at 7 and 14 days, and classified as to intermediate, pigmented, or white groups, as described below. From these pure cultures, stock culture PDA slants of each isolate were made and stored at 6^{o}C.

Stromata were recovered from cankers using a surgical scalpel and forceps. Bark surrounding the stroma or cluster of stromata was excised no deeper than the outer

phloem, peeled away with forceps, and placed into a multi-well plate. Each well contained one stroma or a cluster of stromata and was labeled to facilitate identification, and stromata were stored at 6°C with a damp paper towel to retain moisture.

Bark pieces containing stromata were removed from the multi-well plates and placed stromata side up on a clear microscope slide covered with double sided Scotch® tape. The bark piece containing individual stroma or cluster of stromata was held in place by the tape during dissection with a surgical scalpel. All dissection was conducted under a dissecting scope with appropriate light source. Successive cross-sections of each stroma were removed to reveal the interior of each stroma and sections were plated, two per plate, on APDA plates and monitored for colony characteristics and pigmentation as described previously. Notes were taken for each stroma including size, color, and the presence or absence of perithecia, identified by small black dots emerging or pre-emerging from the stroma body.

2.2.3 Single-spore cultures

Single-spore cultures were obtained from select stromata isolates, bark-core isolates, and laboratory hypovirulent strains of *C. parasitica*. To obtain single-spore isolates from bark-core, and laboratory-derived isolates, conidia were removed with a dissecting needle from conidiomata on 14-day-old (or older) cultures of white or intermediate isolates, and suspended in a 9-ml sterile water blank. From this blank, five 1:9 serial dilutions were made, and the contents of each dilution blank was poured onto 2% water agar plates to allow conidia to germinate. After 2 days, plates were examined under a dissecting microscope and single germinating conidia were transferred to APDA plates.

Conidia from *C. parasitica* stromata isolates were obtained by placing excised pieces of stromata in a drop of sterile water on a microscope slide and crushing the pieces with a cover slip. Conidia were observed under high magnification (400X). All pieces of stromata and water were washed from the slide and placed in a 9-ml sterile water blank. Successive serial dilutions and plating on water agar, followed by APDA plating were conducted as before.

2.2.4 Identification of cultural characteristics of *C. parasitica*

Cultural characteristics were determined by evaluating multiple singe-spore colonies and/or mass transfer APDA plate cultures from each *C. parasitica* isolate recovered from bark cores, stromata, or laboratory-derived cultures from the ATCC strains in the present research. After 14-days growth in fluorescent room light with 8-10 hr photoperiod, the cultures were evaluated for colony color, pattern, and percentage of pigmentation. Percentage of pigmentation in a colony was evaluated using "A Manual of Assessment Keys for Plant Diseases" by Clive James (Canada Department of Agriculture Publication No. 1458 1971) as a visual reference. Colony color and pattern of pigmentation were described as follows: white isolates had less than 30% pigmentation (typically yellow-orange) in the upper agar surface or mycelium of the *C. parasitica* isolate. Pigmented isolates had greater than 70% yellow-orange pigment in the agar or mycelium of the *C. parasitica* isolate. Intermediate isolates had 30% to 70% yellow-orange pigment in the upper surface agar or mycelium of the *C. parasitica* isolate. The mycelium was typically floccose (cottony) or floccose plus subtomentose.

All *C. parasitica* isolates were classified to pigment pattern type, and the intermediate isolates were given the "I" prefix designation to distinguish them from white or pigmented isolates sharing the same general colony phenotype. The mottled (M) colony phenotype had a mixture of white and pigmented mycelium present in a patchy or mottled pattern within a *C. parasitica* colony. The intermediate mottled (I-M) phenotype was best estimated using the underside of the *C. parasitica* culture, although it was also apparent on the upper surface. The concentric ring (CR) phenotype had predominantly white mycelium and numerous yellow-orange conidiomata designating the daily growth rings. The intermediate diffuse (D) phenotype had subtomentose mycelium and tan-orange pigment evenly distributed throughout the mycelium.

Digital scans of mass-transfer colonies of each *C. parasitica* isolate were taken at 14 days on a black background. Settings for the scanner remained fixed to ensure constancy of conditions for photos. Digital scans served as a permanent record for the *C. parasitica* isolates and were categorized based on the similarity of white and pigmented colony morphology of the isolates.

2.2.5 Vegetative compatibility tests on white and pigmented *C. parasitica* isolates

Vegetative compatibility (vc) tests were conducted on select pigmented bark-core isolates removed from artificially established cankers derived from inoculations with white, Italian *C. parasitica* isolates. Pigmented single-spore colonies were obtained from each white, Italian *C. parasitica* isolate using the methods described above. Pigmented, single-spore colonies were transferred to APDA plates (two per plate) and observed for 14 days to confirm stability of pigmentation. It has been found that vc tests using pigmented colonies are more reliable than those performed with white colonies (Anagnostakis 1977).

Pigmented, single-spore colonies from each white isolate were grown in pure culture on APDA plates. Disks (5 mm diameter) from isolates were taken from advancing mycelium and paired in duplicate with the pigmented bark-core isolates recovered from the cankers on APDA plates. Disks were placed (mycelium side down) 2 mm apart from opposing pigmented isolates on APDA plates. Plates were incubated in the dark and evaluated after 14 days for compatibility using ratings from Griffin and Griffin (1995). Strongly incompatible reactions produced a clear barrage zone with numerous pycnidia between isolates. Weakly incompatible reactions produced a clear barrage zone with little or no pycnidia. In compatible reactions the mycelium of the paired isolates merged.

2.2.6 dsRNA extraction assays on *C. parasitica* isolates

Extraction of dsRNA was conducted by a modification of the method of Morris and Dodds (1979) as previously described (Robbins and Griffin 1999). A total of 51 *C. parasitica* isolates recovered from bark cores and excised stromata from Lesesne and Paint Bank research plots, or from intermediate laboratory-derived isolates originating from Ep-47(’99), Ep-49(’99), and Ep-51(’99) ATCC *C. parasitica* cultures was assayed. EP-713, infected with *Cryphonectria hypovirus 1,* was used as a reference isolate for each extraction. Isolates were grown in culture on APDA plates for 14 days and evaluated for colony characteristics as previously described. Digital scans were taken to record characteristics. After 14 days the plates were sampled using a 4-mm bark-core

sampler, and five to seven mycelium covered agar disks were placed in 250-ml Erlenmyer flasks containing 90 ml of liquid glucose-yeast extract medium [10 g glucose, 2 g yeast extract, 1 g K_2HPO_4, and 0.5 g $MgSO_4.7H_2O$ per L (GYEM)]. To this medium was added 0.9 ml of an antibiotic solution containing 0.5 mg streptomycin plus 14 mg per ml chlortetracycline. Six flasks were inoculated per isolate. Mycelium was collected after 6–10 days of growth at room temperature using a Buchner funnel and a cotton filter. The mycelium was blotted dry with paper towels, wrapped in aluminum foil, weighed and stored at –20°C. Approximately 6 g fresh mycelium was collected from each isolate.

To extract dsRNA, approximately 1 g frozen mycelium was weighed and freeze dried overnight. One-hundred mg of freeze-dried mycelium was ground using a mortar and pestle and liquid nitrogen to ensure consistent and comparable extractions. The ground mycelium was placed in plastic tubes along with 10 ml of buffer containing 200 mM NaCl, 100 mM Tris, and 1 mM EDTA (=2X STE buffer ~ pH 6.8). To the tubes was added 0.5 ml of 10% sodium dodecylsulfate (SDS), 11 ml phenol containing 1% 8-hydroxyquinoline, and 5 ml of chloroform-isoamylalcohol (24:1). The tubes were placed on a rotary-arm shaker for 30 minutes to mix the contents, and centrifuged between 7,500 and 8,000 g for 30 minutes at 0 to –5°C to separate cellular nucleic acids. Eight ml of the aqueous phase containing nucleic acids was removed and mixed in 12 ml 1X STE buffer and 4 ml of 95% ethanol. This solution was filtered through 2.5 g CF-11 cellulose columns saturated with 1X STE containing 17% ethanol. These columns were rinsed with 40-50 ml of STE buffer containing 17% ethanol to remove any residual single-stranded RNA and DNA. Nine ml of STE buffer was added to each column to wash the bound dsRNA into a 30 ml tube. Two vol. cold ethanol was added to each tube which were incubated at –20°C for at least 2 hr. Following incubation the tubes were again centrifuged between 7,500 and 8,000g for 30 minutes. The resulting pellets were re-suspended using 20 µl of DNAse (Dnase I from Promega, Madison, WI) and 100 µl of 0.5 M $MgCl_2$, which digested any remaining DNA. Tubes were incubated at room temperature for 60 minutes, following which, 2 ml of ethanol was added, and tubes were again incubated at –20°C for at least 2 hr. Tubes were centrifuged at approximately 7,500 g for 40 min and pellets were re-suspended in 30 µl of RNAse-free water.

The samples were analyzed by gel electrophoresis in a buffer containing 90 mM Tris, 90 mM boric acid, and 1mM EDTA (=1X TBE buffer ~ pH 8.0). The gel was 0.7% agarose and contained 9 µl ethedium bromide (0.6 mg per ml) for staining. Each of the isolates was analyzed in a full-strength and $1/25^{th}$ dilution for semi-quantitative dsRNA measurements. Double stranded RNA from EP 713 (12.7 kbp) in full strength and $1/25^{th}$ dilution strength was used as dsRNA intensity reference for each isolate. Twenty-nine of the *C. parasitica* isolates were extracted for dsRNA three times for confidence, while the remaining 12 *C. parasitica* isolates were extracted only once per isolate.

2.3

RESULTS

2.3.1 Recovery of *C. parasitica* isolates from stromata on superficial cankers on forest clear-cut American chestnut sprouts at Paint Bank and grafted American chestnut trees at Lesesne State Forest

Between November 2002 and June 2004, 289 stromata on bark tissues were collected from 151 locations on three grafted American chestnut trees at Lesesne State Forest, Virginia. The stromata were recovered from branch and stem cankers on three grafted American chestnut trees, TH, RM, and TG. Eighty-four of the stromata were recovered from branch cankers and 205 stromata were recovered from main-stem cankers (*Table 2.1*). The majority of the stromata were recovered from the two-stem grafted trees, RM and TH. Seventy-nine stromata were recovered from RML stem and branch cankers, 71 stromata were collected from RMR stem and branch cankers, 52 stromata were collected from THL stem and branch cankers, 61 stromata were collected from THR stem and branch cankers, and 26 stromata were collected from TG stem and branch cankers. The greatest number of stromata collected from branch isolates was 37 which was collected from the RML graft. The most stroma collected from stem cankers was 63, collected from the RMR graft.

From these 289 stromata, 245 stromata yielded *C. parasitica* isolates (84.8% recovery); they were classified as white, normal pigmented, or intermediate pigmented using the criteria previously described. Only 44 stromata did not yield any *C. parasitica* isolate, and four stromata yielded two *C. parasitica* isolates each (*Table 2.1*). From the 249 *C. parasitica* isolates recovered, 13 (5.2%) *C. parasitica* isolates were white, 23 (9.2%) isolates had intermediate pigmentation, and 213 (85.5%) *C. parasitica* isolates were normal pigmented (*Table 2.2*).

Between October 2002 and December 2004, 12 artificially inoculated American chestnut trees at Paint Bank were sampled for stromata on cankers produced by *C. parasitica*. Eleven of the twelve trees contained cankers that yielded stromata for sampling. Of the 57 total cankers on the 12 artificially inoculated trees, 38 cankers produced stromata and were sampled; however, only 31 of the 38 total cankers produced stromata that yielded *C. parasitica* isolates (*Table 2.3*). A total of 272 stromata were

Table 2.1 Total stromata and *C. parasitica* isolates collected from branches and stems of TH, RM, and TG grafted American chestnut trees at Lesesne State Forest

Graft	Stromata from branch cankers	Stromata from stem cankers	Total number stromata from cankers	Stromata yielding *C. parasitica* isolates	Number of *C. parasitica* isolates
RML	37 (44.0%)	42 (20.5%)	79 (27.3%)	70[a] (28.6%)	72
RMR	8 (9.5%)	63 (29.3%)	71 (24.6%)	62 (25.3%)	62
THL	13 (15.5%)	39 (19.0%)	52 (18.0%)	45 (18.4%)	45
THR	8 (9.5%)	53 (25.9%)	61 (21.1%)	44 (18.6%)	44
TG	18 (21.4%)	8 (3.9%)	26 (9.0%)	24[a] (9.8%)	26
Totals	**84**	**205**	**289**	**245**	**249**

[a.] Indicates where at least one stroma yielded more than one *C. parasitica* isolate.

Table 2.2 Total number of stromata sampled and white, pigmented, and intermediate *C. parasitica* isolates recovered at the Lesesne State Forest chestnut plantation

Graft	Stem/Branch	White isolates recovered[a]	Pigmented isolates recovered[b]	Intermediate isolates recovered[c]
RML	Stem	3	39	2
	Branch	3	24	1
RMR	Stem	0	49	5
	Branch	0	8	0
THL	Stem	4	26	3
	Branch	0	11	1
THR	Stem	0	28	8
	Branch	0	8	0
TG	Stem	0	5	1
	Branch	3	15	2
Totals		**13/249**	**213/249**	**23/249**
% of total isolates		**5.2%**	**85.5%**	**9.2%**

[a.] White isolates are defined as having less than 30% yellow-orange pigment in the mycelium.
[b.] Pigmented isolates are defined as having more than 70% yellow-orange pigment in the mycelium.
[c.] Intermediate isolates are defined as having 30 to 70% yellow-orange pigment in the mycelium.

Table 2.3 Total number of artificially established cankers examined for the presence of stromata, number of stroma, percentage of *C. parasitica* isolates recovered from stromata, and the number of *C. parasitica* isolates for each Italian *C. parasitica* strain recovered from excised stromata on the cankers at the Paint Bank research plot

Inoculated strain	Total no. artificial cankers examined for stromata	Cankers yielding stromata	No. stromata	Average number of stromata per canker	Cankers yielding *C. parasitica*	Stromata yielding *C. parasitica*	% total *C. parasitica* isolates
Ep-47(‘99)	9	4	16	1.8	4	13	5.4
Ep-49(’99)	10	9	121	12.1	9	111	46.9
Ep-49(CM1)	10	3	11	1.1	2	10	4.1
Ep-49(CM3)	18	17	99	5.5	12	85	35.3
Ep-51W	10	5	25	2.5	4	20	8.3
Totals	**57**	**38**	**272**	**4.58(avg.)**	**31**	**239**	**100%**

collected and 239 of these stromata yielded *C. parasitica* isolates (*Table 2.4*) with two stromata giving rise to two *C. parasitica* isolates each, for a total of 241 *C. parasitica* isolates (*Table 2.5).* Of potential biocontrol importance, white strain Ep-49(‘99) and intermediate single-spore strain Ep-49(CM3) yielded a greater average number of stromata per canker than did any of the other three strains tested (*Table 2.3*).

The nine Ep-49(‘99) and the 17 Ep-49(CM3) artificially established cankers yielded the greatest number of stromata with 121 and 99 stromata, respectively (*Table 2.4*). The 121 stromata from Ep-49(’99) artificially established cankers produced a total of 111 *C. parasitica* isolates and Ep-49(CM3) artificially established cankers produced a total of 85 *C. parasitica* isolates; this constituted 46.9% and 35.3% of the total 241 *C. parasitica* isolates, respectively. Ep-47(‘99), Ep-49(CM1), and Ep-51W artificially established cankers each produced 13, 10, and 20 *C. parasitica* isolates, respectively.

All of the 241 *C. parasitica* isolates from Paint Bank were classified as to white, pigmented, or intermediate, as described previously. The majority (160 or 66%) of the fungal isolates collected from stromata on these cankers were white. The remaining isolates were divided up almost equally between pigmented and intermediate isolates; the pigmented isolates accounted for 15% of the total and 19% of the *C. parasitica* isolates were classified to the intermediate phenotype (*Table 2.5*).

Among the groups of artificially inoculated cankers, those cankers originating from Ep-49(‘99) yielded the greatest number of white isolates (101 of 160) which accounted for 63% of the total white isolates recovered. The greatest number of pigmented isolates (13 of 35) came from the Ep-47(‘99) cankers and accounted for 37% of the total. Ep-49(‘99) and Ep-49(CM3) cankers produced 12 and 9 pigmented isolates, respectively. The greatest number of intermediate isolates originated from Ep-49(CM3) artificially established cankers. These cankers yielded 39 of 46 intermediate isolates which accounted for 85% of the total intermediate isolates collected. The only other cankers to yield intermediate *C. parasitica* isolates from stromata were Ep-51W cankers where seven intermediate isolates were recovered (*Table 2.6*).

Within each group of artificially established cankers, a broad distribution of *C. parasitica* phenotypes was observed. Within Ep-49(‘99) artificially established cankers,

Table 2.4 Number of stromata isolated at Paint Bank research plots from each of the artificially established cankers and the percentage of *C. parasitica* isolates recovered from stromata

Isolate	Cankers yielding stromata	No. stromata	% total stromata	No. of *C. parasitica* isolates from Stromata	% total *C. parasitica* isolates
Ep-47(’99)	4	16	5.9	13	5.4
Ep-49(‘99)	9	121	44.5	111	46.9
Ep-49(CM1)	3	11	4.0	10	4.1
Ep-49(CM3)	17	99	36.4	85	35.3
Ep-51W	5	25	9.2	20	8.3
Totals	**38**	**272**	**100%**	**239**	**100%**

Table 2.5 Isolates of *C. parasitica* recovered from stromata collected from Ep-47(‘99), Ep-49(‘99), Ep-49(CM1), Ep-49(CM3), and Ep-51W artificially established cankers on American chestnut trees at Paint Bank research plots

Tree no.	Canker no.	Inoculated strain	White isolates [a]	Pigmented isolates [b]	Intermediate isolates [c]	Total
3-3	1	Ep-49(CM3)	3	0	0	3
	2	Ep-49(CM3)	8	0	0	8
	3	Ep-49(CM3)	6	0	1	7
	4	Ep-49(CM3)	3	1	0	4
4-3	1	Ep-49(CM3)	4	0	0	4
	3	Ep-49(CM3)	4	0	0	4
	5	Ep-49(CM3)	3	0	0	3
1-4	3	Ep-49(‘99)	10	0	0	10
	4	Ep-51W	3	0	0	3
2-4	1	Ep-47(‘99)	0	6	0	6
	3	Ep-49(CM1)	8	0	0	8
	4	Ep-49(‘99)	1	0	0	1
	5	Ep-51W	1	1	2	4
3-4	1	Ep-51W	0	0	5	5
	3	Ep-49(CM3)	0	0	7	7
	5	Ep-49(‘99)	65	3	0	68
4-4	1	Ep-49(‘99)	7	0	0	7
	4	Ep-49(CM3)	1	1	28	30
5-4	2	Ep-49(’99)	4	0	0	4
	5	Ep-49(CM3)	0	7	3	10
1-5	1	Ep-47(‘99)	0	2	0	2
	2	Ep-49(’99)	4	7	0	11
	3	Ep-49(CM1)	2	0	0	2
	4	Ep-49(CM3)	4	0	0	4
2-5	2	Ep-47(‘99)	0	2	0	2
	3	Ep-49(’99)	2	0	0	2
3-5	4	Ep-49(’99)	4	0	0	4
4-5	2	Ep-49(CM3)	1	0	0	1
	3	Ep-51W	8	0	0	8
	4	Ep-47(‘99)	0	3	0	3
	5	Ep-49(‘99)	4	2	0	6
Total		**38**	**160**	**35**	**46**	**241**
% of total isolates			**66%**	**15%**	**19%**	

[a.] White isolates are defined as having less than 30% yellow-orange pigment in the mycelium.
[b.] Pigmented isolates are defined as having more than 70% yellow-orange pigment in the mycelium.
[c.] Intermediate isolates are defined as having from 30 to 70% yellow-orange pigment in the mycelium.

Table 2.6 Stromata assays: total number and overall percentage of *C. parasitica* isolates collected from stromata for Ep-47(‘99), Ep-49(‘99), Ep-49(CM1), Ep-49(CM3), and Ep-51W artificially established cankers on American chestnut trees at Paint Bank research plot, each classified as to the white, pigmented, or intermediate isolate phenotype

Original isolate Canker	White [a] isolates from stromata	% white of total	Pigmented [b] isolates from stromata	% pigmented of total	Intermediate [c] isolates from stromata	% intermediate of total
Ep-47(‘99)	0	0	13	37.1	0	0
Ep-49(’99)	101	63.1	12	34.3	0	0
Ep-49(CM1)	10	6.3	0	0	0	0
Ep-49(CM3)	37	23.1	9	25.7	39	84.8
Ep-51W	12	8.0	1	2.9	7	15.2
Total	**160**	**66.7% [d]**	**35**	**14.5% [d]**	**46**	***19.1%**

[a.] White isolates are defined as having less than 30% yellow-orange pigment in the mycelium.
[b.] Pigmented isolates are defined as having more than 70% yellow-orange pigment in the mycelium.
[c.] Intermediate isolates are defined as having from 30 to 70% yellow-orange pigment in the mycelium.
[d.] Indicates percentage of the 241 total isolates collected.

89% of 113 stromatal isolates were classified to the white phenotype while only 11% were classified to the normal pigmented phenotype (*Table 1A* Appendix). None were intermediate. For Ep-49(CM3) artificially established cankers, 44% of the isolates collected were classified as white, 46% were classified to the intermediate category, and the remaining 11% *C. parasitica* isolates were classified to the normal pigmented phenotype (*Table 1B* Appendix). Stromata collected from Ep-51W artificially established cankers produced fungal isolates classified almost exclusively to the white (60%) and intermediate (35%) groups (*Table 1C* Appendix). All stromata collected from Ep-49(CM1) and Ep-47(‘99) artificially established cankers were classified into white and normal pigmented groups, respectively (*Table 1D, 1E* Appendix). Some of the pigmented isolates from Ep-47(‘99) artificially established cankers had an atypical wavy type margin.

Single-conidium colonies were grown on APDA collected from 16 stromata recovered from the Ep-49(’99), Ep-49(CM3), and Ep-49(CM1) cankers at Paint Bank research plots. Five different artificially established cankers were sample from four separate trees. A total of 353 single-spore isolates were classified to the white, intermediate, or pigmented phenotypes. A total of 181 and 172 isolates were classified to the white and intermediate phenotypes, respectively. None of the single spore isolates from any of the stromata produced a pigmented isolate (*Table 2.7*). Eighty-five percent of 110 single-spore isolates from Ep-49(‘99) stromata were white. All of 20 single-spore isolates from Ep-49(CM1) cankers were white, and 70% of 223 single-spore isolates from Ep-49(CM3) were intermediate. Significantly, the intermediate Ep-49(CM3) isolate generated 30% of the single spore isolates having the white phenotype. All of the stromata from cankers at Paint Bank were dissected and classified to size and whether or not the stromata contained perithecia, identified by the presence of black dots (ostioles) on the stroma upper surface. The vast majority of stromata dissected did not have black dots. Two white isolates were recovered from stromata with indications of perithecia.

A small group of the normal pigmented isolates collected from cankers (stromata and bark cores) artificially established with Ep-49(‘99) were tested against a single-spore normal pigmented isolate of Ep-49(‘99) in vegetative compatibility evaluations. All six replicate pairings for each of 17 isolates yielded incompatible results (*Table 2.8*).

Table 2.7 Single-spore isolates from stromata collected from hypovirulent-strain-established cankers on American chestnut trees in the Paint Bank research plots

Tree	Canker	Stroma	Isolate	W[a]	P[b]	INT[c]
3-4	5	str 24	Ep-49(‘99)	19cm1, 1cm4	0	0
3-4	5	str 37	Ep-49(‘99)	15 cm1	0	7 cm3
3-4	5	str a	Ep-49(‘99)	20 cm1	0	3 cm3
3-4	5	str b	Ep-49(‘99)	16 cm1	0	6 cm3
4-4	1	str 5	Ep-49(‘99)	1cm1, 21cm2	0	1 cm3
3-4	5	str 15	Ep-49(CM1)	12cm1, 8cm2	0	0
3-3	2	str a	Ep-49(CM3)	0	0	24 cm3
3-3	3	str a	Ep-49(CM3)	11cm1	0	12 cm3
3-3	3	str b	Ep-49(CM3)	12 cm1	0	12 cm3
3-3	3	str c	Ep-49(CM3)	8 cm1	0	15 cm3
3-3	3	str d	Ep-49(CM3)	10 cm1	0	11 cm3
4-3	1	str a	Ep-49(CM3)	0	0	24 cm3
4-3	1	str b	Ep-49(CM3)	5 cm1	0	16 cm3
4-3	1	str c	Ep-49(CM3)	4 cm1	0	14 cm3
4-3	1	str d	Ep-49(CM3)	2 cm1	0	20 cm3
4-3	1	str c	Ep-49(CM3)	16 cm1	0	7 cm3
Total				**181**	**0**	**172**

[a.] White isolates have less than 30% yellow-orange pigment in the mycelium.
[b.] Pigmented isolates have more than 70% yellow-orange pigment in the mycelium.
[c.] Intermediate isolates have from 30 to 70% yellow-orange pigment in the mycelium.

Table 2.8 Vegetative compatibility tests for pigmented bark-core and stromata *C. parasitica* isolates recovered from artificially established cankers at the Paint Bank research plots, using tester strains Ep-47(‘99), Ep-49(‘99), Ep-51W, and Ep-49(CM1)

Tree no.	Canker no.	Pigmented isolate tested	Tester strain[a]	Vegetative compatibility reactions
Tree 2-4	1	a	Ep-47(‘99)	6/6 incompatible
Tree 2-4	1	b	Ep-47(‘99)	6/6 incompatible
Tree 2-4	1	c	Ep-47(‘99)	6/6 incompatible
Tree 2-4	1	d	Ep-47(‘99)	6/6 incompatible
Tree 2-4	3	a	Ep-49(‘99)	6/6 incompatible
Tree 2-4	3	b	Ep-49(‘99)	6/6 incompatible
Tree 2-4	3	c	Ep-49(‘99)	6/6 incompatible
Tree 2-4	3	d	Ep-49(‘99)	6/6 incompatible
Tree 2-4	5	b	Ep-51W	6/6 incompatible
Tree 3-4	1	d	Ep-51W	6/6 incompatible
Tree 3-4	2	a	Ep-47(‘99)	6/6 incompatible
Tree 4-4	3	d	Ep-47(‘99)	6/6 incompatible
Tree 4-4	5	a	Ep-49(CM1)	6/6 incompatible
Tree 5-4	1	d	Ep-49(CM1)	6/6 incompatible
Tree 3-4	5	Str 58	Ep-49(‘99)	6/6 incompatible
Tree 3-4	5	Str 59	Ep-49(‘99)	6/6 incompatible
Tree 3-4	5	Str 16b	Ep-49(‘99)	6/6 incompatible
Totals	**9**		**17**	**102/102 incompatible**

[a.] Pigmented single-spore isolates of tester strains were used in vc tests. Tester strains were identical to the strains inoculated at the Paint Bank research plots

2.3.2 Recovery of *C. parasitica* isolates from bark cores on superficial cankers on forest clear-cut American chestnut sprouts at Paint Bank and grafted American chestnut trees at Lesesne State Forest

Between October 2001 and November 2003, 277 bark-core isolates were taken from Ep-47(‘99), Ep-49(‘99), Ep-49(CM1), Ep-49(CM3), and Ep-51W artificially established cankers on the trees at the Paint Bank research plots. The greatest number of bark cores (116) was taken from the 27 Ep-49(CM3) cankers. Each group of artificially established cankers originating from Ep-47(‘99), Ep-49(‘99), Ep-49(CM1), and Ep-51W *C. parasitica* strains yielded between 36 and 45 cores. From these 277 cores, 238 *C. parasitica* isolates were recovered. The greatest number of *C parasitica* isolates was recovered from Ep-49(CM3) cankers (101), wheras the fewest *C. parasitica* isolates was recovered from Ep-47(‘99) cankers (*Table 2.9*).

All the isolates were classified as white, pigmented, and intermediate using the criteria previously described. Among all groups of artificially established cankers, the majority of *C. parasitica* isolates were classified to the white category (63%), while only a small percentage of isolates were assigned to the pigmented category (16%). Twenty one percent of the isolates were classified as intermediate (*Table 2.10*). The number of white *C. parasitica* isolates collected was composed primarily from isolates recovered from Ep-49(‘99), Ep-49(CM3), and Ep-51W artificially established cankers. The majority of pigmented isolates (24 or 63%) were collected from Ep-47(‘99) cankers, and the vast majority of intermediate isolates (47 or 93%) were collected from Ep-49(CM3) (*Table 2.11*). Significantly, however, the intermediate Ep-49(CM3) isolate had the ability to generate a large number (50 or 33%) of white isolates (*Table 2.11)*

Within the groups of artificially established cankers, 80% of the *C. parasitica* isolates collected from Ep-47(‘99) cankers were classified to the pigmented phenotype. Only 16% where classified to the white phenotype and the remaining 3% had intermediate pigmentation. *C. parasitica* isolates from Ep-49(‘99) artificially established cankers were classified exclusively to the white phenotype. Isolates from Ep-49(CM1) artificially established cankers were classified primarily as white (70%), with the majority of the remaining isolates classified as pigmented (24%). Ep-49(CM3) artificially established cankers, from which the majority of *C. parasitica* isolates were

Table 2.9 Number of bark cores isolated at Paint Bank research plots from each of the artificially established cankers and the percentage of *C. parasitica* isolates recovered

Inoculated strain	Cankers sampled	No. cores	% total cores	Cores yielding *C. parasitica*	% total *C. parasitica* isolates
Ep-47(‘99)	9	36	13.0	30	12.6
Ep-49(‘99)	10	45	16.2	36	15.1
Ep-49(CM1)	9	40	14.4	34	14.3
Ep-49(CM3)	27	116	41.9	101	42.4
Ep-51W	10	40	14.4	37	15.5
Totals	**65**	**277**	**100%**	**238**	**100%**

Table 2.10 Isolates of *C. parasitica* recovered from bark cores collected from Ep-47(‘99), Ep-49(‘99), Ep-49(CM1), Ep-49(CM3), and Ep-51W artificially established cankers on American chestnut trees at Paint Bank research plots

Tree no.	Canker no.	Inoculated strain	White [a] isolates	Pigmented [a] isolates	Intermediate [a] isolates	Total
3-3	1	Ep-49(CM3)	2	0	4	7
	2	Ep-49(CM3)	2	0	5	7
	3	Ep-49(CM3)	5	0	5	10
	4	Ep-49(CM3)	2	0	4	6
4-3	1	Ep-49(CM3)	9	2	0	11
	2	Ep-49(CM3)	2	2	0	4
	3	Ep-49(CM3)	4	0	0	4
	5	Ep-49(CM3)	8	0	0	8
1-4	1	Ep-49(CM3)	0	0	4	4
	2	Ep-49(CM1)	3	0	0	3
	3	Ep-49(‘99)	4	0	0	4
	4	Ep-51W	4	0	0	4
	5	Ep-47(‘99)	2	2	0	4
2-4	1	Ep-47(‘99)	0	4	0	4
	2	Ep-49(CM3)	1	0	7	7
	3	Ep-49(CM1)	1	4	1	8
	4	Ep-49(‘99)	2	0	0	2
	5	Ep-51W	1	1	0	2
3-4	1	Ep-51W	3	1	0	4
	2	Ep-47(‘99)	0	4	0	4
	3	Ep-49(CM3)	2	0	7	9
	4	Ep-49(CM1)	4	0	0	4
	5	Ep-49(‘99)	4	0	0	4
4-4	1	Ep-49(‘99)	4	0	0	4
	2	Ep-51W	4	0	0	4
	3	Ep-47(‘99)	1	1	0	2
	4	Ep-49(CM3)	0	0	7	7
	5	Ep-49(CM1)	1	1	0	2
5-4	1	Ep-49(CM1)	2	1	0	3
	2	Ep-49(‘99)	4	0	0	4
	3	Ep-51W	4	0	0	4
	4	Ep-47(‘99)	2	0	1	3
	5	Ep-49(CM3)	2	0	4	6
1-5	1	Ep-47(‘99)	0	4	0	4
	2	Ep-49(‘99)	4	0	0	2
	3	Ep-49(CM1)	4	0	0	4
	4	Ep-49(CM3)	4	0	0	4
	5	Ep-51W	4	0	0	4
2-5	1	Ep-51W	3	0	0	3
	2	Ep-47(‘99)	0	4	0	4

	3	Ep-49(‘99)	4	0	0	4
	4	Ep-49(CM1)	3	0	1	4
3-5	1	Ep-49(CM3)	3	0	0	3
	2	Ep-51W	4	0	0	4
	3	Ep-47(‘99)	0	3	0	3
	4	Ep-49(‘99)	4	0	0	4
	5	Ep-49(CM1)	3	1	0	4
4-5	1	Ep-49(CM1)	3	1	0	4
	2	Ep-49(CM3)	4	0	0	4
	3	Ep-51W	4	0	0	4
	4	Ep-47(‘99)	0	2	0	2
	5	Ep-49(‘99)	3	0	0	3
5-5	1	Ep-49(‘99)	3	0	0	3
	4	Ep-51W	4	0	0	4
Total		**54**	**150**	**38**	**50**	**238**
% of total isolates			**63%**	**16%**	**21%**	

[a.] White isolates are defined as having less than 30% yellow-orange pigment in the mycelium.
[b.] Pigmented isolates are defined as having more than 70% yellow-orange pigment in the mycelium.
[c.] Intermediate isolates are defined as having between 30 and 70% yellow-orange pigment in the mycelium.

Table 2.11 Bark-core assays: total number and overall percentage of *C. parasitica* isolates collected from bark cores for Ep-47(‘99), Ep-49(‘99), Ep-49(CM1), Ep-49(CM3), and Ep-51W artificially established cankers on American chestnut trees at Paint Bank research plot, each classified as to the white, pigmented or intermediate phenotype

Inoculated strain	White [a] isolates from cores	% of total white	Pigmented [b] isolates from bark cores	% of total pigmented	Intermediate [c] isolates from bark cores	% of total intermediate	Total *C. parasitica* isolates	% of total isolates
Ep-47(’99)	5	3.3	24	63.2	1	2.0	30	12.6
Ep-49(’99)	36	24	0	0	0	0	36	15.1
Ep-49(CM1)	24	16	8	21.1	2	4.0	34	14.3
Ep-49(CM3)	50	33.3	4	10.6	47	93.0	101	42.4
Ep-51W	35	23.3	2	5.3	0	0	37	15.5
Total	**150**	**63%[d]**	**38**	**16%[d]**	**50**	**21%[d]**	**238**	**100%**

[a.] White isolates are defined as having less than 30% yellow-orange pigment in the mycelium.
[b.] Pigmented isolates are defined as having more than 70% yellow-orange pigment in the mycelium.
[c.] Intermediate isolates are defined as having between 30 and 70% yellow-orange pigment in the mycelium.
[d.] Indicates percentage in relation to total number of *C. parasitica* isolates.

collected, yielded almost equal numbers of white and intermediate isolates with 50 and 47 isolates, respectively. Ep-51W artificially established cankers yielded almost exclusively white isolates (95%), with the remaining isolates classified to the pigmented phenotype. (*Tables 1F -1J Appendix*)

2.3.3 Extraction trials for dsRNA performed on white, intermediate, and pigmented *C. parasitica* isolates collected from stromata, bark-core or laboratory strains

A collection of 51 *C. parasitica* isolates was tested for the presence of a 12.7 kb dsRNA band (*Table 2.12*). The collection was composed of *C. parasitica* isolates originating from 18 stromata collected from the Lesesne research plot, seven stromata and eight bark cores collected from the Paint Bank research plot, and 18 laboratory cultures derived from the Ep-47(‘99), Ep-49 (’99), and Ep-51 (’99) ATCC *C. parasitica* strains. All of the isolates and strains in the collection were classified to the white, pigmented or intermediate phenotype, and when applicable, isolates were further classified into descriptive colony phenotypes (*Table 2.13*). Digital image scans were taken after 14 days growth on APDA for permanent records. Eleven of the isolates were classified as white, seven isolates were classified as pigmented, and 33 isolates were classified as intermediate (*Table 2.12*).

Twenty-nine of the isolates were tested with three replications of extraction and electrophoresis for confirmation of findings (*Table 2.14*). Twenty of these 29 isolates tested positive for the presence of dsRNA. The test results indicated that a high level of confirmation was obtained in the dsRNA assays. For example, 88% of these assays (53/60) with three replications yielded the same dsRNA positive result for the 20 isolates tested. Furthermore, for the isolates yielding high-intensity bands of dsRNA, 100% of the 39 assays gave the same dsRNA positive result. All seven of the negative replications were observed from four intermediate isolates that otherwise produced faint or very faint bands in the agarose gel. Seven intermediate isolates each had three identical extractions, yielding either strong or faint banding patterns. Seven of the eight white isolates tested positive for dsRNA (*Table 2.14*). The one white isolate that tested negative was a CM3-type isolate classified with 15% pigmentation. Two of the four pigmented isolates tested positive. One pigmented isolate (Ep-49 #5 ss2) had only faint gel bands, but tested positive in all three replications. The other pigmented isolate was classified as 85% pigmented and each replication produced a

Table 2.12 Total *C. parasitica* isolates collected from field or laboratory and tested for the presence of dsRNA

Source	Colony phenotype	Field L	Field PB	Lab	Total
Stromata	White	3	1	0	4
	Intermediate	12	5	0	17
	Pigmented	3	1	0	4
Bark cores	White	0	1	0	1
	Intermediate	0	6	0	6
	Pigmented	0	1	0	1
ATCC	White	0	0	6	6
	Intermediate	0	0	10	10
	Pigmented	0	0	2	2
Total		**18 (35.3%)**	**15 (29.4%)**	**18 (35.3%)**	**51**

Table 2.13 Evaluation of dsRNA in white, pigmented and intermediate isolates of *C. parasitica* isolates from the field or laboratory

	Isolate code	Colony type [a]	% pigmentation [b]	Origin [c]	dsRNA evaluation [d]
1.	RM. Str 54a	P-N	99	Field L	++ [i]
2.	T 4-5. C 4. Str d	P-N	85	Field PB	++++ [e]
3.	Ep-49(’99)M	P-M	85	Lab	- [e]
4.	TH. Str 124	P-N	85	Field L	- [e]
5.	T 3-4. C 3. Cor a	P-M	75	Field PB	- [i]
6.	TH. Str 125	P-CR	75	Field L	++ [i]
7.	Ep-49 #5 ss2	P-CM3	75	Lab	++ [e]
8.	TH. Str 70	I-CR	70	Field L	- [i]
9.	RM. Str 130a	I-CR	70	Field L	++
10.	T 4-4. C 4. Cor III	I-M	70	Field PB	+ [f]
11.	T 3-3. C 1. Cor I	I-D	70	Field PB	++++ [e]
12.	T 4-4. C 4. Str t	I-CM3[g]	70	Field PB	- [e]
13.	T 2-4. C 1. Str c	I-CR	70	Field PB	- [e]
14.	T 3-4. C 1. Str d	I-CR	65	Field PB	++ [e]
15.	RM. Str 62a	I-CR	65	Field L	+ [i]
16.	Ep-49 #4 ss1	I-CM3	65	Lab	- [e]
17.	RM. Str 61a	I-CM3[a]	60	Field L	++ [i]
18.	TH Str 165a	I-CR	60	Field L	+ [i]
19.	RM. Str 61b	I-CR	60	Field L	++ [i]
20.	RM. Str 34a	I-CR	60	Field L	+ [i]
21.	Ep-49(’99) ss1	I-CM3	60	Lab	+ [f]
22.	T 5-4. C 5. Str d	I-M	60	Field PB	- [e]
23.	T 4-4. C 4. Cor a	I-M	55	Field PB	+++ [i]
24.	T 4-4. C 4. Str 2	I-M	55	Field PB	- [i]
25.	RM. Str 135	I-CR	55	Field L	- [i]
26.	Ep-49 #6 ss1	I-CM3	55	Lab	+ [h]
27.	Ep-49 #7	I-M	55	Lab	+ [f]
28.	Ep-49 #7 ss1	I-CM3	55	Lab	- [e]

29.	Ep-49 #7 ss2	I-CM3	50	Lab	- [e]
30.	TH. Str 162a	I-CM3[a]	50	Field L	++ [i]
31.	RM. Str 35a	I-CR	50	Field L	+++ [i]
32.	T 5-4. C 5. Cor a	I-M	50	Field PB	- [i]
33.	TH. Str 108	I-CR	45	Field L	++ [i]
34.	Ep-49 #5 ss1	I-CM3	45	Lab	++++ [e]
35.	T 5-4. C 5. Cor d	I-M	45	Field PB	- [i]
36.	Ep-49 #4 ss2	I-CM3	45	Lab	++++ [e]
37.	Ep-49 #6	I-CR	40	Lab	++++ [e]
38.	Ep-49 #6 ss2	I-CM3	35	Lab	++++ [e]
39.	TH. Str 119	I-CR	30	Field L	- [i]
40.	T 3-3. C 2. Cor I	I-D	30	Field PB	++++ [e]
41.	Ep-49 #4	W-CR	20	Lab	++++ [i]
42.	Ep-49 #5	W-M	20	Lab	++++ [e]
43.	T 3-3. C 2. Str d	W-CM3[a]	15	Field PB	- [e]
44.	Ep-49 #6 ss3	W	10	Lab	++++ [e]
45.	T 4-3. C 1. Cor II	W-CR	10	Field PB	++++ [e]
46.	TG. Str 48a	W	7	Field L	++++ [i]
47.	TH. Str 164a	W	2	Field L	++++ [i]
48.	RM. Str 86	W	1	Field L	++++ [i]
49.	Ep-49 #11	W	0	Lab	++++ [e]
50.	Ep-49 #11 ss1	W	0	Lab	++++ [e]
51.	Ep-49 #11 ss2	W	0	Lab	++++ [e]

[a.] The colony type designation indicates the overall colony designation: white (W= having less than 30% yellow-orange pigment in the mycelium), pigmented (P = having more than 70% yellow-orange pigment in the mycelium), or intermediate (I = having from 30 to 70% yellow-orange pigment in the mycelium), and colony characteristics: mottled (M), concentric ring (CR), diffuse pigmented (D), or single-spore colony morphology group 3 (CM3). Dotted lines indicate delineation of white, pigmented, and intermediate groups.

[b.] Each isolate was rated for the percent of pigmentation +/- 5%.

[c.] All isolates were obtained from either Paint Bank research plots (Field PB), Lesesne research plots (Field L), the American Type Culture Collection or Virginia Tech laboratory-produced collection (Lab).

[d.] The dsRNA electrophoresis gel band intensity is rated with a series of (+)'s. Very faint bands are +, faint bands are ++, moderate intensity bands are +++, bright intensity bands are ++++. Isolates that produced no gel band are indicated with a minus (-).

[e.] Three dsRNA extraction replications all with the same result.

[f.] One positive dsRNA extraction and two negative dsRNA extractions.

[g.] CM3 "type" isolate. Isolate is not from a single spore, but has CM3 phenotype.

[h.] Two positive dsRNA extractions and one negative extraction.

[i.] Results from one dsRNA extraction

Table 2.14 Total white, pigmented and intermediate *C. parasitica* isolates of different colony types tested for the presence of dsRNA

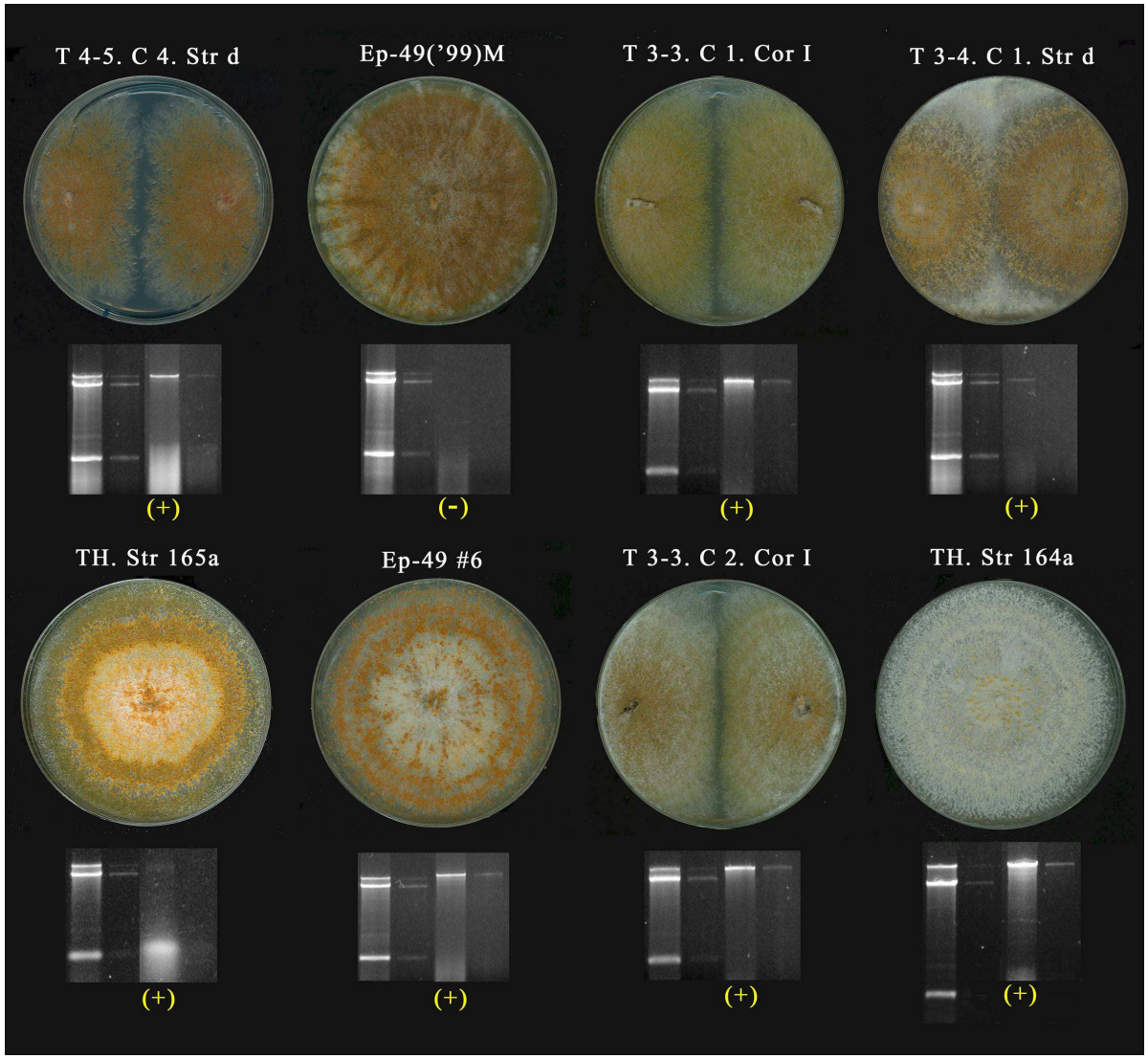
Colony type	Three replications			One replication			Total
	W[a]	**P**[b]	**I**[c]	**W**	**P**	**I**	
CM3	1	1	9	0	0	2	13
Mottled	1	1	3	0	1	4	10
Concentric Ring	1	0	3	0	1	10	16
Diffuse	0	0	2	0	0	0	2
Typical	4	2	0	3	1	0	10
Total	**8 (15.7%)**	**4 (7.8%)**	**17 (33.3%)**	**3 (5.9%)**	**3 (5.9%)**	**16 (31.4%)**	**51**
Positive dsRNA	7	2	11	3	2	10	35
Negative dsRNA	1	2	6	0	1	6	16

[a.] White isolates are defined as having less than 30% yellow-orange pigment in the mycelium.
[b.] Pigmented isolates are defined as having more than 70% yellow-orange pigment in the mycelium.
[c.] Intermediate isolates are defined as having from 30 to 70% yellow-orange pigment in the mycelium.

very strong gel band (*Table 2.13*). Representative cultures and gel photographs are shown in *Figure 2.1*.

The remaining 32 isolates were tested once for the presence of dsRNA. Results from this group of isolates were very similar to the results from the isolates with three replications (*Table 2.14*). Ten of the 16 intermediate isolates tested positive for the presence of dsRNA (63% positive dsRNA results vs 65% positive for the isolates with three replications), all of which produce gel bands of faint to medium intensity. One of the pigmented isolates produced a faint band and all of the white isolates produced gel bands of high intensity (*Table 1K* Appendix). There was no consistent relationship between phenotype and the presence of dsRNA.

Figure 2.1 Colony phenotype and presence or absence of dsRNA for eight representative isolates of *C. parasitica* recovered from Lesesne and Paint Bank research plots or laboratory cultures. Top left to bottom right show colony phenotypes of designated isolates ranging from pigmented to white. For each isolate, representative dsRNA assay gels are shown for reference strains Ep713 at full strength and 1/25 dilution (lane 1 and 2), and for the unknown isolates at full strength and 1/25 dilution (lanes 3 and 4). For each unknown (+) indicates the presence of a 12.7 kb dsRNA band (uppermost band in Ep-713 gel) and (-) indicates the absence of this band



2.4
DISCUSSION

The results indicate that there was no direct correlation between colony phenotype or amount of pigmentation and the presence or relative concentration of dsRNA. Previous research on the isolates inoculated at the Paint Bank research plots and all dsRNA-containing *C. parasitica* isolates recovered from the grafted trees at Lesesne indicated that the hypovirus present in those *C. parasitica* isolates is identical to CHV1-Euro 7 when comparing an 844-bp region in helicase domain of ORF B, and an 894-bp region in ORF A, using sequencing data (Griffin *et al.* 2004). Therefore there is high confidence that the dsRNA hypovirus contained in the isolates tested is CHV1-Euro 7. Within each of the pigmented, intermediate and white categories various *C. parasitica* isolates tested positive for the presence of CHV1. Furthermore, within the intermediate, CM3, CR, M, and D colony phenotype groups there were *C. parasitica* isolates that tested positive for the presence of CHV1 and isolates that tested negative for CHV1. In general, however, the pigmented isolates were found to be CHV1 negative and the white isolates were CHV1 positive, and the intermediate isolates with more pigmentation were typically CHV1 negative, while the isolates with less pigmentation were typically CHV1 positive. Although the amount of pigmentation cannot be used as an absolute indicator of hypovirus presence or absence, it can be used as general guide.

Only one white isolate was found to be CHV1 negative (T 3-3. C 2. Cor I). This isolate was a bark core isolate recovered from an Ep-49(CM3) canker at the Paint Bank research plots. Results from this study indicate that intermediate, CM3 cultures are highly variable in regards to presence or absence of dsRNA content regardless of amount of pigmentation. Although this isolate was classified as a white isolate (<30% pigmentation), the CM3 characteristics may have contributed to the variability. Our lab has found that *C. parasitica* isolates with the CM3 phenotype can also change phenotype upon transfer in the lab, possibly due to CHV1 presence or absence. It is possible therefore that upon transfer to the liquid medium flasks the white phenotype was lost and the CHV1 contained within was also lost.

The two most highly pigmented isolates (RM. Str 54a and T4-5. C 4. Str d) were found to have dsRNA. Both of these colonies were of the pigmented normal phenotype. RM. Str 54a is a stroma isolate from Lesesne and T4-5. C 4. Str d is a Paint Bank stroma isolate from an Ep-47(‘99) canker. Ep-47(‘99) has been found by our lab to be somewhat variable in colony color. It has the ability to look very white and also pigmented. The mycelium in the agar is suppressed and has little subtomentose hyphae. Despite this “flat” pigmented appearance, Ep-47(‘99) has consistently been found to contain CHV1 in high concentration. Ep-43 is also a pigmented isolate that has been shown to contain CHV1 in high concentrations. Previous research has demonstrated that pigmented isolates are capable of containing CHV1 as three dsRNA isolates sequenced for CHV1 identity were from *C. parasitica* isolates (Ep-43, STR1 and RM1M), and were classified as pigmented (Griffin, *et al.* 2004).

White isolates appear to consistently have a much higher relative concentration of CHV1 than non-white isolates which tested positive for CHV1. All of the white isolates had a high relative concentration of CHV1 and those white isolates that were tested multiple times, consistently had high intensity CHV1 bands with each replication. In contrast, the intermediate isolates that tested positive for the presence of CHV1 had isolates of varying degrees of band intensity yet the intensity was generally consistent with each extraction replication. Among the different intermediate phenotypes, the I-D phenotype appears to be the most likely intermediate phenotype to contain CHV1. Both of the I-D isolates tested had high band intensity and gave consistent results through replication. The I-M phenotype appeared to be the least likely phenotype to contain CHV1 as only three of the seven total I-M isolates gave a positive result, and two of the isolates with multiple replicates each only yielded one positive replication.

It appears that intermediate isolates may perform an important function in biological control of chestnut blight. In this study stromata collected from cankers at Lesesne and Paint Bank gave rise to 23 and 46 intermediate *C. parasitica* isolates, respectively. These stromata have the potential to disseminate CHV1 in conidia as single conidia were recovered from 16 of the stromata from Paint Bank and gave rise to 181 white isolates and 172 intermediate isolates. Other research here indicates that intermediate isolates have the potential to carry CHV1. Thirty-three intermediate isolates

were tested for the presence of CHV1 and 21 isolates tested positive for CHV1. Of these isolates, 12 of them had multiple positives, confirming further that the intermediate isolates tested here were infected with CHV1. The importance of these isolates is even more noteworthy as 9.2% of the stromata isolates collected from the grafted American chestnut trees at Lesesne were classified to the intermediate phenotype, as compared to the 5.2% white *C. parasitica* isolates collected. Results from this study therefore indicate that a possible 14.4% (9.2% + 5.2%) of the *C parasitica* isolates collected from stromata could be CHV1-containing hypovirulent isolates. Furthermore, previous research has indicated that as high as 47% of the isolates from bark-core samples, collected from cankers on the grafted trees at Lesesne, were white, CHV1-containing isolates (Hogan and Griffin 2002a). During that study, the importance of intermediate isolates was not considered, and it is possible that the number of CHV1 containing hypovirulent *C. parasitica* isolates would actually surpass 50% if intermediate isolates had been tested.

Very little other research documents the importance of intermediate isolates; however, intermediate isolates are not uncommon in scientific literature. Coskun *et al*. (1999) document the finding of intermediate isolates collected from healing or intermediate cankers in natural European chestnut stands in Turkey. They found that 42% of the isolates they collected were intermediate in phenotype, and none of the 18 intermediate isolates tested contained dsRNA. Only four of the isolates (white isolates) were found to contain dsRNA. These researchers postulated that the relative health of the trees was due to either a combination of host resistance and unfavorable local climate for the pathogen, or an unexplained loss of virulence by the pathogen.

A large number of stromata were collected from the three trees at Lesesne; however, in general the stromata were infrequent and approximately 10-12 stromata were found in 0.25 m^2. In comparison, a normal canker incited by a virulent canker will commonly have around 30-35 stromata per cm^2 on smooth bark (unpublished data). Although sexual reproduction is taking place on the healing cankers containing hypovirulent isolates at Lesesne, it is far less frequent than in a natural chestnut population. The cankers initiated by hypovirulent strains at Paint Bank exhibit the same trend. For the cankers established using Ep-47(‘99), Ep-49(‘99), Ep-49(CM1), Ep-49(CM3), and Ep-51W the average number of stromata collected per canker was 4.58.

The overwhelming majority of the stromata came from isolate Ep-49(‘99) which had an average of 12.1 stromata per canker. Measurements taken at Paint Bank for Ep-49(’99) hypovirulent-strain-established cankers in past years indicates a mean density of 0.06 stromata/cm^2 with a range of 0.0 – 0.25, and an average canker length of 5.4 cm (unpublished data). A WK virulent comparison strain tested during the same growing seasons had a mean density of 33.8 stromata/cm^2, with an average canker length of 10.6 cm.

It is likely that the stromata yielding white isolates at Lesesne are produced from new growth white mycelium inside the canker, rather than a virulent strain thallus converted to white hypovirulence by CHV1. Shain and Miller (1992) demonstrated incomplete movement of CHV1 through one vc type of *C. parasitica in vivo*, including the stromata. Even after challenge, while CHV1 converted the underlying thallus to white hypovirulence, the existing stromata remained pigmented. In this study, it was found that only 5.2% of the stromata yielded white *C. parasitica* isolates. In light of the measured stromata density of the hypovirulent isolates, the amount of competing virulent strains in the cankers, and the apparent failure of CHV1 to move into stromata, one would expect a very low number of white isolates recovered from stromata.

At Paint Bank the number of intermediate isolates recovered was much greater than at Lesesne. There were a total of 46 intermediate *C. parasitica* isolates or 19% recovered from the stromata collected at Paint Bank. It appears that Ep-49(CM3) and Ep-51W are the most important strains for yielding intermediate isolates. In the Paint Bank Research plot, Ep-49(CM3) and Ep-51W artificially established cankers contained all of the intermediate isolates, with 84.8% coming from Ep-49(CM3). Cankers originating from Ep-49(CM3) also produced 22% of the white isolates recovered. This, coupled with the findings of Hogan and Griffin (2000a) that intermediate CM3 single spore colonies were the most common phenotype of *C. parasitica* isolates recovered at Lesesne, indicates that intermediate isolates recovered from bark cores or stromata at Lesesne are likely the result of the CHV1 strains from either Ep-49 or Ep-51 which were the original *C. parasitica* strains that were inoculated in the trees in 1982-83.