

CHAPTER 3 MATERIALS AND METHODS

3.1 Isolation of *C. parasitica* from superficial cankers on grafted American chestnut trees

Isolates of *C. parasitica* were collected from main stem and branch superficial cankers, located outside of the hypovirulent strain-inoculated zone, on TH, RM, and TG American chestnut grafts at the Lesesne State Forest, Virginia. By 1999 the largest tree, TH, was 18.59 meters and had a dbh of 39.9 cm (Griffin 2000). Cankers were sampled throughout all parts of the three grafted chestnut trees with emphasis on main stem cankers, and 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. Six bark-core samples were taken randomly from each canker using a 1.7-mm diameter bark-core sampler. 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 two minutes, rinsed in distilled water, and plated on acidified (5.6 ml 25% v/v lactic acid per l), Difco potato-dextrose agar (APDA). The resulting fungal growth was monitored daily and individual colonies suspected to be *C. parasitica*, were transferred to new APDA plates. Cultures were then grown in normal room light (fluorescent) conditions for two weeks. Pure cultures of *C. parasitica* were evaluated for colony color at 7 and 14 days. Isolates were designated pigmented when more than 50% of the surface color was pigmented. Isolates were designated white when 50% or more of the colony

surface color was white (Robbins and Griffin 1999). From these pure cultures, stock culture PDA slants of each isolate were made and stored at 4°C.

3.2 Identification of cultural characteristics of *C. parasitica*

Cultural characteristics were determined by evaluating multiple single-spore colonies from each white isolate recovered from cankers on APDA plates. Colony morphology was also determined for the four white hypovirulent *C. parasitica* strains inoculated into the grafts in 1982 and 1983. These cultures were obtained from the American Type Culture Collection (EP-43: ATCC# 38767, EP-47: ATCC# 38760, EP-49: ATCC# 38759, EP-51: ATCC# 38758) (Jong and Edward 1991). To obtain single-spore isolates, conidia were removed with a dissecting needle from pycnidia on 14-day-old (or older) cultures of white 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 (four per plate) to six APDA plates. After 14 days growth in fluorescent room light, the 24 colonies were evaluated for colony color and pattern of pigmentation. This procedure was performed for each white isolate obtained. Colony color was estimated as described above. Pattern of pigmentation was based on the presence of white or pigmented centers in colonies, and white or pigmented outer margins of colonies. For completely white isolates, an estimation of the amount of pycnidial production was also used.

Photographs of single-spore colonies of each *C. parasitica* isolate were then taken using an incandescent light source and a camera stand with a black background. Settings for the camera, lights, and camera stand remained fixed to assure constancy of conditions

for photos. Pictures 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. The use of single-spore colonies allowed multiple replications, which enhanced reliability of cultural characteristics of colonies.

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

Vegetative compatibility (VC) tests were conducted for all white isolates recovered from cankers and the total number of VC groups was determined. These groups were then compared to the four inoculated strains (EP-43, 47, 49 and 51), which are composed of three VC groups (Jong and Edward 1991). Strains EP-47 and EP-49 were in the same VC group. Pigmented single-spore colonies were obtained from each white isolate using the methods described above. Pigmented single-spore colonies were then 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). From these pigmented single-spore isolates, a base group was established by pairing the pigmented isolates in all combinations, using the procedures of Anagnostakis (1977), as modified by Griffin and Griffin (1995).

Pigmented single-spore colonies from each white isolate were grown in pure culture on APDA plates. Disks (5-mm diameter) from isolates were then taken from advancing mycelium and paired in duplicate with other pigmented single-spore isolates on APDA plates. Disks were placed (mycelium side down) 2-mm apart from opposing pigmented, single-spore isolates, on APDA plates. Plates were then 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. Once a base of VC groups was established, all other isolates were tested against this group.

3.4 Spatial pattern determinations of white *C. parasitica* isolates in superficial cankers on grafted American chestnut trees

The spatial patterns of the white isolates within and among main stem and branch cankers were determined. In addition, the spatial pattern of VC groups was examined for one main stem (RML-470) and one branch (TG-303) canker. For spatial pattern within a canker, a 17.8 x 17.8 cm sheet metal lattice grid with 49 equally-spaced sampling holes, each located at the center of a lattice cell, was placed on the cankers to be sampled. The grid was rounded to conform to the shape of the main stem and branch cankers. Once the grid was held in place, small bark-core samples (1.7-mm diameter) were obtained from the canker using the sampling holes as a guide. Bark-core samples were removed, placed in a multi-well plate, transported to the laboratory, surface disinfested, and plated on APDA using the same techniques mentioned above. Once isolates were recovered they were categorized as to white or pigmented using the criteria described above. For the two cankers used in VC tests, described above, pigmented single-spore isolates were recovered from the white isolates and tested for VC as previously described. Pigmented isolates recovered from the cankers were then tested for VC in a similar manner (no single spores were needed). Once the base of VC groups were established for the white and pigmented

isolates these bases were compared to each other to determine the VC groups common to white and pigmented isolates.

The presence of white isolates and VC groups were then mapped for each canker assayed. The pattern of the characters on these maps was also tested for randomness, using the join-count statistics described by Pielou (1977). Lloyd's index of patchiness (Pielou 1977) was used to evaluate the overall spatial pattern of white isolates among cankers on the grafted chestnut trees. The overall spatial pattern of white VC groups among cankers on the grafted American chestnut trees was evaluated using the double matrix test of Harvey et al. (1988), as described by Bisseger et al. (1996) and Milgroom et al. (1990). The data from objectives 1 and 2 were used in these analyses.

3.5 dsRNA extraction assays on *C. parasitica* isolates

Extraction of dsRNA was conducted on 11 randomly selected pigmented isolates from two spatial-pattern grid cankers (five isolates from canker RML-470 and six isolates from canker THR-660) using the techniques described by Robbins and Griffin (1999). EP-713, infected with *Cryphonectria hypovirus 1*, was used as a reference isolate. Isolates were grown in culture on APDA and five to seven disks were taken from advancing mycelium. These disks were placed in 250-ml Erlenmyer flasks containing 90-100 ml of liquid glucose-yeast extract medium [10 g glucose, 2 g yeast extract, 1 g K_2HPO_4 , and 0.5 g $MgSO_4 \cdot 7H_2O$ per L (GYEM)]. To this medium was added 0.9 ml of an antibiotic solution containing 0.5 mg streptomycin plus 43 mg per ml chlortetracycline. Four to seven flasks were inoculated per isolate. Mycelium was collected after 14 days of growth at room temperature, using a Buchner funnel. The mycelium was then dried at room temperature, wrapped in aluminum foil, and stored at -20 C .

To extract dsRNA, mycelium was ground up using a mortar and pestle, liquid nitrogen and glass beads (0.17-mm diameter). The ground mycelium was then placed in plastic tubes along with 10 ml of buffer containing 200 mM NaCl, 100 mM Tris, and 1 mM EDTA (=2X STE buffer). 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 then centrifuged at 7,649 g for 30 minutes at 0 to -5 C to separate cellular nucleic acids. The aqueous phase containing nucleic acids was removed and mixed in 10-15 ml 1X STE buffer and 4 ml of 95% ethanol. This solution was then filtered through 2.5 g CF-11 cellulose columns saturated with 1X STE containing 17% ethanol. These columns were then 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 then added to wash the bound dsRNA from the column into a tube. Cold ethanol was then added to the tubes, which were incubated at -20 C for at least 2 h. Following incubation the tubes were again centrifuged at 7,649 g for 30 minutes. The resulting pellets were resuspended using 20 µl of DNase (Dnase I from Promega, Madison, WI) and 100 µl of 0.5 M MgCl₂, which removed 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 h. Tubes were then centrifuged at 7,649 g for 40 min and pellets were resuspended 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). The gel was 0.7% agarose and contained 1µl ethidium bromide (0.6 mg per ml) for staining. A dsDNA ladder was

used as a marker for band sizes. Double stranded RNA from EP 713 (12.7 kbp) and two white isolates recovered from the grafts were used as references.