Conversion of Virulent *Cryphonectria parasitica* from the Lula Lake Land Trust to Hypovirulent Strains

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ABSTRACT:

For almost a century, the fungus, *Cryphonectria parasitica* has existed in the United States as a wound parasite to American chestnut *(Castanea dentata)* trees. However, hypovirulence, evidence of spontaneous healing in Italy and Michigan, has given the American chestnut trees hope to one day be restored into its natural range. Viruses were the cause of such spontaneous healings. My hypothesis was that a virus-containing hypovirulent strain of *C. parasitica* (in a brown background) would share alleles that control anastomoses with the local virulent strains, so a virus that causes hypovirulence could be transmitted into local virulent strains in vitro. Fifly bark samples removed from American chestnut trees at the Lula Lake Land Trust on Lookout Mountain, Georgia were explanted to an isolation medium. The *C. parasitica* isolates transferred to culture medium were paired twice with all three brown hypovinilent strains (containing either the “COLI”,”Euro7”, or “GH2” virus) for possible conversion. Indication of conversion was the presence of a third sector or the virulent strains (recipient) exhibiting similar morphology to the H strains (donor). Putative converts were transferred again for comparison with recipient and donor strains to confirm conversion. Brown *C. parasitica* did indeed share alleles for anastomoses with V strains. Strains infected with the “GH2” virus had very similar morphologies to the brown virulent strain (5-9-1B), thus no data for conversion was collected. Twenty-six out of sixty virulent isolates were successfully converted by one of the two brown virus-containing hypovirulent strains with COLT or Euro7 viruses. Local hypovirulent strains are now available for biological control experiments in the local area.

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INTRODUCTION:

The problems of the American chestnut trees are complicated by the interactions of several species across diverse environmental conditions. There are two major relationships that must be noted: (1) the chestnut tree and its fungal pathogen, *Cryphonectria parasitica* which causes chestnut blight, and (2) the fungus and its virus which infects and weakens the fungus. My project focused on the interaction between the fungus and the virus.

History of C. dentata

The American chestnut tree, *Castanea dentata,* was once an abundant tree species that had a natural range extending from Maine and Southern Ontario to Alabama and Mississippi (Figure 1) (Griffin, 2000). At its mature state, *C. dentata* could reach heights between sixty to one hundred twenty feet with straight trunks up to seven feet in diameter (Roosevelt 1902 as cited by Kuhlman, 1978). The American chestnut trees occupied about twenty-five percent of the forest population in some parts of its natural range and were considered to be forest canopy dominants in many areas. In addition to its height and abundance in the deciduous forest, the American chestnut tree is rot resistant and has “a faster rate of growth than its associated hardwood species”, which made *C. dentata* highly useful to man (Holmes, 1925 as cited by Kuhlman, 1978). The American chestnut tree was preferred because of its rot resistant lumber, which was used for furniture, fences, woodworks, houses, and for many other purposes. In addition to its fast growth and rot resistant characteristics, American chestnut trees produced an inestimable amount of sweet nuts every year, which provided food for wildlife and man (Anagnostakis, 1982). When comparing the nuts of its counterparts (European chestnut, Japanese chestnut and Chinese chestnut trees), the American chestnut trees produced sweeter nuts (J. Hill Craddock, Personal Communication, 2000). It is evident that American chestnut trees were great resources to man and wildlife.

In 1904, the fungus *Cryphonectria parasitica* was discovered at the Bronx Zoological Park in New York City on dying American chestnut trees. The fungus quickly spread throughout the American chestnut's natural range. By the 1950s, all the American chestnut trees were dead or dying in what later came known as the chestnut blight pandemic (Anagnostakis, 1987). Fortunately, the American chestnut trees still exist today, but as understory shrubs. *Cryphonectria parasitica* does not kill the roots of the trees, allowing *Castanea dentata* to sprout from the root crown collar. However, the chestnut blight fungus attacks the trees again keeping *C. dentata* from achieving its natural mature state. Thus, it can no longer maintain its status as a dominant canopy tree and a great resource to man and wildlife. However, there is hope that the American chestnut tree will be restored back into its natural range, since it is not extinct and research can be conducted with these understory shrubs and sprouts.

Introduction of <u>C. parasitica</u>

Cryphonectria parasitica, known previously as *Endothia parasitica* or *Diaporthe parasitica,* was not native to the United States (Roane et al., 1986). Early mycologists and plant pathologists thought that the chestnut blight fungus spread to the United States from China or Japan on imported nursery stock because Chinese (*Castanea mollissima*) and Japanese (*C. crenata*) chestnut trees exhibited resistance to chestnut blight. Frank Meyer, a plant explorer found *C. parasitica* in China and then later in Japan signifying that *C. parasitica* must have originated from one of these two countries (Anagnostakis, 1987). The catastrophic effects of this fungus causing the Chestnut blight pandemic in the United States provoked the government to enact the nation's first plant quarantine laws designed to protect native plants and trees (Ronderos, 2000).

In addition to infecting American chestnut trees, *Cryphonectria parasitica* also causes blight on *Castanea sativa* (European chestnut tree). First observed in Italy in the late 1930s, *C. parasitica* caused a blight epidemic in Europe that was much like the pandemic occurring in America. By comparison, "*C. sativa* was a little more resistant to blight than *C. dentata*" as noted by Berry in his 1960 publication.

Characteristics of C. parasitica in vivo and in vitro

The characteristics of chestnut blight disease in vivo are a sunken bark canker that exhibits orange fungal stromata, wilting of distal foliage, and a cream fan mycelium underneath the bark (Anagnostakis, 1987). In vitro, the normal strains exhibit the following morphology and physiology when grown on potato dextrose agar with methionine and biotin under sixteen hours of artificial light, and between 19-24°C: “abundant white to cream-colored aerial mycelium, moderately pronounced radial striations (consisting of aggregates of parallel hyphae alternating with regions of less numerous hyphae), orange, approximately hemispherical pycnidia devoid of surface ornamentation and scattered within concentric rings that correlate with photoperiod” (Elliston, 1978). In addition, a brown *C. parasitica* (apparently a mutated form of the normal orange *C. parasitica*) was found in West Virginia. This new strain has been useful in hypovirulence research as a genetic marker for the fungal background **(**Mark Double, personal communication, 2000).

Physiology of C. parasitica

Considered a wound parasite, *Cryphonectria parasitica* infects the tree through wounds, which occur almost perpetually on American chestnut trees due to natural growth and shade pruning (Kuhlman, 1978). Therefore, the fungus attacks the American chestnut tree at almost all stages of the tree’s life (McCaroll et al., 1978).

The fungus produces enzymes that depolymerize the various components in the cell wall. The first enzyme to be produced is polygalacturonase, which diffuses from the mycelium of the fungus into the healthy tissue of the tree, causing the polypectate of the middle lamella (the component that 'glues' the cell walls together) to depolymerize (Albersheim, 1975). Also, the optimal pH for these enzymes is the same as the inner bark's pH of 5.5; thus these enzymes are most invasive in the inner bark. The degradation of the middle lamella causes area cell wall polysaccharides to be exposed to the other compounds like oxalic acid, which depolymerizes calcium salts of the polypectate (McCarroll et al.1978). Therefore, the fungus reabsorbs the broken down material of the American chestnut tree for its own nutrition, which creates a parasitic relationship between the tree and the fungus.

Mechanism of dissemination of C. parasitica

C. parasitica is an ascomycetous fungus that produces ascospores, a sexually produced fungal spore found within an ascus. The forcible expulsion of these ascospores from its ascus allows the wind to carry these spores to other surrounding American chestnut trees, infecting them with the chestnut blight disease (Anagnostakis, 1987). Therefore, wind is a major vector in the dissemination process of C. *parasitica* (Anagnostakis, 1987). In addition to sexual spores, *C. parasitica* produces asexual spores known as conidia. Conidia are not spread by wind, but by vectors such as insects, birds and mammals. Such disseminators pick up *C. parasitica* on their feet or body as they "move over cankers with erumpent stromata" taking conidia from an infected tree to an area of the same tree that is not infected or to a surrounding American chestnut tree (Anagnostakis, 1987).

Introduction of Hypovirulence

In the 1950's, the Italian scientist Antonio Biraghi identified "spontaneous healing" of cankers on European chestnut trees. He noticed that European chestnut trees were recovering from the chestnut blight. Jean Grente, a French mycologist sampled bark from these spontaneously healing cankers and described the cultures as a variety of unusual strains of *C. parasitica*, which he found to be associated with only healing cankers on European chestnut trees (Fulbright and MacDonald, 1991). The isolates from the "spontaneous healing" cankers of *C. parasitica* were described to be lightly pigmented, debilitated in culture, of slower growth, and produced fewer spores compared to the normal lethal strain (Fulbright, 1999). In addition, these lightly pigmented isolates seldom caused a lethal infection of European chestnut trees. Grente was first to coin the term "hypovirulence" for these new "unusual" strains that have altered morphology and pigmentation, and exhibit lower pathogenicity than the normal strains (Fulbright, 1999).

Hypovirulent strains (H) are found to have a reduced production of oxalate, which leads to their lower pathogenicity (Anagnostakis, 1978). Oxalate is a salt or ester of oxalic acid that has a direct correlation with the pathogenicity of the fungus (Anagnostakis, 1978). H strains "contain a substance that behaves like a heat-stable protein or protein complex that interferes with the activity of oxalic acid"

(Anagnostakis, 1978). Also, hypovirulent strains are considered to be diseased strains because normal lethal *C. parasitica* must have a viral infection to exhibit hypovirulence (Jaynes and Elliston, 1978).

Most fungal viruses or hypoviruses have double stranded ribonucleic acid (dsRNA) as the source of their genetic information. When the virus infects the fungus, the viral dsRNA is found in the host cell's cytoplasm (Jaynes and Elliston, 1978).

Hypovirulent strains are found to produce fewer spores than normal strains with the same nuclear genotype (Anagnostakis, 1987). This is so because of the interaction between the fungal and viral genome; the virus is interfering with the fungus's normal reproduction process. Therefore, a natural spreading of H strains for biological control has not yet been successful in the Appalachian region due to low sporulation and scattered American chestnut trees throughout the region (Fulbright, 1999).

Characteristics of hypovirulence in vivo and in vitro

The symptom of infection with hypovirulent strains is a swollen canker, due to the callusing of tissues. The growth of the disease is halted (Anagnostakis, 1978). This superficial, non-lethal canker on the bark of the American chestnut tree is known as a swollen callus. In the laboratory, the cultures of hypovirulent strains exhibit varied morphology depending upon the virus the strain contains. The Euro 7 virus-containing strain exhibits normal growth rate, lightly pigmented mycelium, and flat and few aerial hyphae (Mark Double Personal Communication, 2000). COLI virus-containing strain exhibited great debilitation, dark crusted brain-like centers, light pigmentation outside of the centers, and thin hypha (Mark Double, personal communication, 2000). GH2 virus-containing strain exhibited a dark center, aerial hyphae and fast growth (Mark Double, personal communication, 2000).

Mechanism of Conversion

Hypovirulence is determined by viral genes in the cytoplasm of *E. parasitica* and is transferred from strain to strain in the tree and on an agar medium in the laboratory (Anagnostakis, 1978). This transfer from strain to strain is made possible by a process known as hyphal anastomosis or hyphal fusion. The donor thallus (H strain) is able to transfer to the receptor thallus (V strain) the dsRNA of the virus if the two thalli are in the same vegetative compatibility (v-c) group. Vegetative compatibility (v-c) suggests that the two strains share alleles that control anastomoses. If the number of alleles in common between the two strains decreases, so does the frequency and duration of anastomoses, and the frequency of transmission of hypovirulent agents (Milgroom, 1999). If the virulent strain is converted, the morphology of the virulent strain becomes similar to that of the hypovirulent donor strain; thus it becomes hypovirulent (Fulbright, 1999).

A single spore isolate from a culture

A spore, by definition, is a single-cell reproductive body capable of growing into a new organism. Single spore isolates can be grown from *C. parasitica* cultures when a single spore is obtained. The spore contains the nuclear DNA material of the fungus and only a tiny amount of cytoplasm. The virus is usually not found in spores because of the lack of sufficient cytoplasm. When a single conidium (asexual spore) is removed from a hypovirulent strain it usually develops into a virulent culture. Single spore isolates are generally in the same v-c group as their parent strain.

Biological control efforts using hypovirulence

The first experimentation with hypovirulence in America began in 1972 with cultures sent from J. Grente (Anagnostakis, 1978). The experiment showed some trees with “fungal growth, but no wilting, even after one hundred days. The tree wound was heavily callused, and isolations of *E. parasitica* were made from this tissue, which showed hypovirulent determinants corresponding to the original French hypovirulent strain” (Anagnostakis, 1978). Because promising results were seen on the American chestnut seedlings, a field experiment was conducted in 1973. However, the hypovirulent strains used in this field experiment were the re-isolated hypovirulent strains of the in vitro experiment which lead to better disease control in the field than the initial experiment (Anagnostakis, 1978). Isolations from the healed cankers exhibited predominantly hypovirulent characteristics (Anagnostakis, 1978).

There is evidence of successful biological control in Michigan and Italy using hypovirulence (Fulbright and MacDonald, 1991). It was noted by Fulbright and MacDonald (1991) that "in both Michigan and Italy where expression of hypovirulence occurs, chestnut regeneration has continued with little or no competition from other plant species", which is not the case in the Appalachian region. Although, evidence for biological control in Italy and Michigan are positive, there is still much to be learned about hypovirulence and how it may help other areas achieve control of the chestnut blight fungus.

Purpose of research

There were three purposes to my experiment: (1) to show qualitative evidence that local virulent (virus-free) strains from the Lula Lake Land Trust on Lookout Mountain, Georgia can be converted to hypovirulent (virus-containing) strains using brown hypovirulent strains as donors, (2) to have hypovirulent strains readily available for future biological control in the Chattanooga area and for the backcross-breeding program at the University of Tennessee at Chattanooga, and (3) to sample *C. parasitica* populations from an area of the Cumberland Plateau that has never been sampled for conversion.

In doing this research, I am contributing data on a local and national level regarding the use of brown *C. parasitica* as a donor strain for biological control using viruses. The overall desire of The American Chestnut Foundation, West Virginia University, Connecticut Agricultural Experiment Station, The University of

Tennessee at Chattanooga, and many others is to restore the American chestnut trees through combinations of biological control efforts, genetic research, and production of resistant hybrids to the fungus that predominately carry genes of an American chestnut tree (Griffin,2000).

Hypothesis

My hypothesis was that the three brown H strains received from West Virginia would convert some of the fifty local orange V strains obtained to a recognizable H phenotype. Mark Double of the Plant Pathology Department of The West Virginia University suggested the three viruses “Euro 7”, “COLI”, and “GH2” because they have distinctive morphologies.

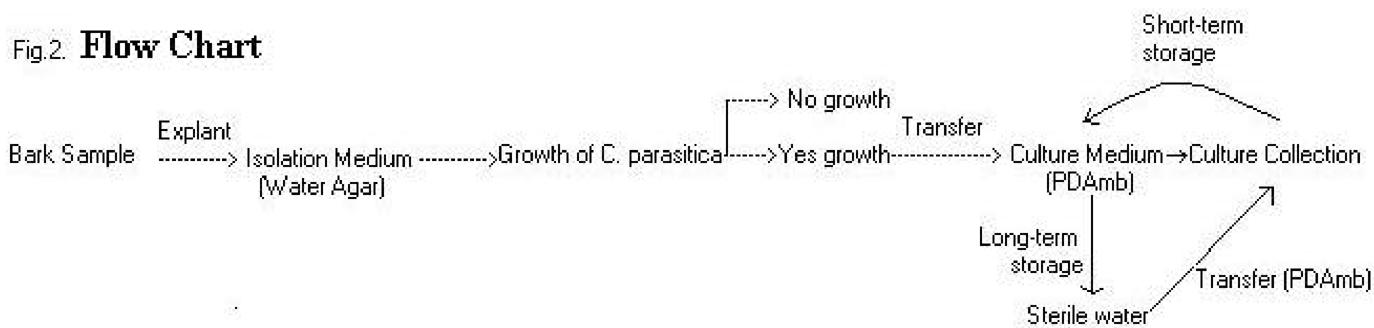
MATERIALS AND METHODS:

The summer of 2000, I visited West Virginia University’s (WVU) Plant Pathology Department to learn how to do conversions and to identify different morphologies distinctive of each of the three viruses (Euro 7, COLI, and GH2). In addition, I learned the methods for obtaining bark samples and isolating *C. parasitica*.

General Overview:

The procedures for isolating *C. parasitica* were complex, so a flow chart has been provided in figure 2 for a general overview.

Fig.2. **Flow Chart**



Media, Growth Conditions, Sterile Conditions, and Storage:

<u>Isolation medium: Water agar</u>

I mixed 10 g of agar per liter of distilled water in a large flask and placed it on a hot plate. I then turned on the heat and the magnetic stirrer and waited for it to boil. When the water agar came to a boil, the flask was taken off the hot plate and aluminum foil was wrapped over the top of the flask. The water agar was autoclaved according to the directions for liquid medium stated on the autoclave. After autoclaving, I allowed the sterilized water agar to cool before I aseptically poured the autoclaved medium into petri dishes. I allowed the poured medium to solidify in the petri dishes for approximately twenty-four hours.

Culture medium: Potatoe Dextrose Agar with methionine and biotin

I mixed 39 g of potato dextrose agar (Difco), 1 g of methionine and .001 g of biotin per 1 liter of distilled water in a large flask and placed it on a hot plate. I then turned on the heat and the magnetic stirrer and waited for it to boil. When the PDAmb came to a boil, the flask was taken off the hot plate and aluminum foil was wrapped over the top of the flask. The liquid PDAmb was autoclaved according to the directions for liquid medium stated on the autoclave. After autoclaving, I allowed the sterilized PDAmb to cool before I aseptically poured the autoclaved medium into petri dishes. I allowed twenty-four hours for solidification. The pH was checked before and after autoclaving. The pH was be between 5 and 6.

Long-term storage medium: Autoclaved water

Distilled water was poured into 50ml screw top vials to about ¾ full and then the tops were screwed on lightly. The vials were then placed into a rack. The screw top vials containing distilled water were autoclaved according to the directions for liquid medium stated on the autoclave. After autoclaving, I tightly screwed on the tops.

Growth Conditions

All cultures were under artificial light for 16 hours at temperatures between 19-24° C. Isolations required 4 days on water agar. Transferred cultures were evaluated after ten days on PDAmb.

Sterile Conditions

All procedures for explanting and transferring *C. parasitica* were done under sterile conditions. First, I disinfected the bench underneath a vacuumed hood with a 10% bleach solution. Second, I turned on the germicide light and the blower for about an hour prior to working under the hood. Then, I poured some ethanol into a small beaker for sterilizing the instruments. The following instruments were used: tweezers, a Bunsen burner and a scalpel**.** I lit a Bunsen burner under the hood, dipped tweezers or scalpel into the ethanol, and flamed the instrument to sterilize it each time the instrument came into contact with a sample. I sterilized the instrument(s) before and after each procedure.

Long-term storage

A 0.75 cm^3 piece of *C. parasitica* mycelium on PDAmb was cut from the outer edge of the mycelium with a scalpel. Then I unscrewed the screwtop vials containing sterile water, flamed the opening of the vial, dropped the excised piece of mycelium into the sterile water with a scalpel, and flamed the opening of the vial again before screwing the top on tightly. The vials were labeled with the name of the culture. Long-term storage was necessary in case of contamination of short-term storage. Also, there was no need to transfer long-term storage cultures unless the cultures were needed. Cultures were kept at room temperature.

Short-term storage

Cultures normally remained contamination-free for about 2 to 3 weeks depending upon the handling of cultures before another transfer was needed. Cultures were handled very carefully or the petri dishes were parafilmed.

Transfer of *C.parasitica* on culture medium:

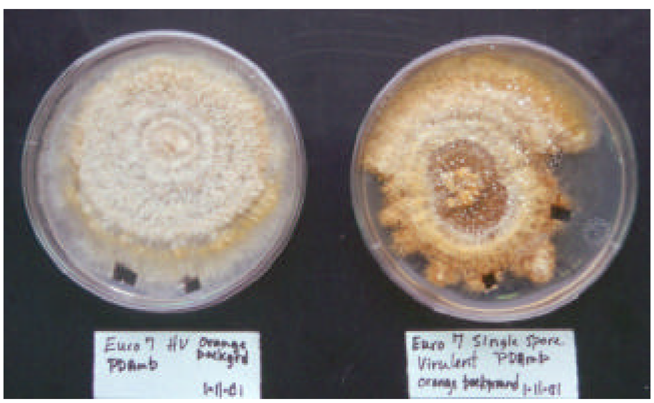
A small piece of visible *C. parasitica* mycelium (cream colored thread-like branches) was cut from the PDAmb or water agar if performing explanting procedures. An excision of 0.75 cm^3 was made from the outer edge of the mycelium with a scalpel. Using aseptic techniques, I placed the excise cube in the center of the fresh PDAmb petri dish. I made sure that the top layer of the excised PDAmb or water agar if performing explanting procedures was placed upside down to the fresh PDAmb. Plates were labeled appropriately. Extra media were stored in the refrigerator. Transferred cultures were grown under growth conditions for PDAmb as described previously.

Fungal Material:

Control

The culture collection of controls were all on PDAmb petri dishes. An orange virus-free single spore isolate (Euro 7 ss) and its parent strain, Euro 7 containing virus (Euro 7 O) were used as a positive control for conversion. Euro 7 ss was a wildtype orange strain that exhibited the following morphology: orange pigmented, and aerial hyphae in concentric rings (Figure 3). Euro 7 O, a hypovirulent strain exhibited the following morphologies: lightly pigmented and fast growth (Figure 3).

Fig 3. Euro7 O (left) and Euro 7ss (right).



Brown virus-free strain

A brown virus-free single spore isolate (5-9-1B) obtained from one of the three brown hypovirulent (virus-containing) strains used in this project was brought back from WVU, so that a comparison between a virus-free brown strain and a virus-containing brown strain could be shown in this experiment. The morphologies distinctive of 5-9-1B were concentric rings of aerial hyphae and fast growth (Figure 4).

Fig. 4. 5-9-1B (brown virus-free)

<u>Brown virus-containing strains</u>

The three viruses used were called “Euro7” from Italy, “COLI” from County Line, Michigan, and “GH2” from Grand Haven, Michigan; and all were on a brown background. The Euro7 virus-containing strain exhibited fast growth, aerial hyphae, and pale pigmentation (Figure 5). The COLI virus-containing strain exhibited great debilitation, dark crusted brain-like centers, light pigmentation outside of the centers, and thin hypha (Figure 5). The GH2 virus-containing strain exhibited a dark center, aerial hyphae and fast growth (Figure 5). All controls were transferred to long and short-term storage.

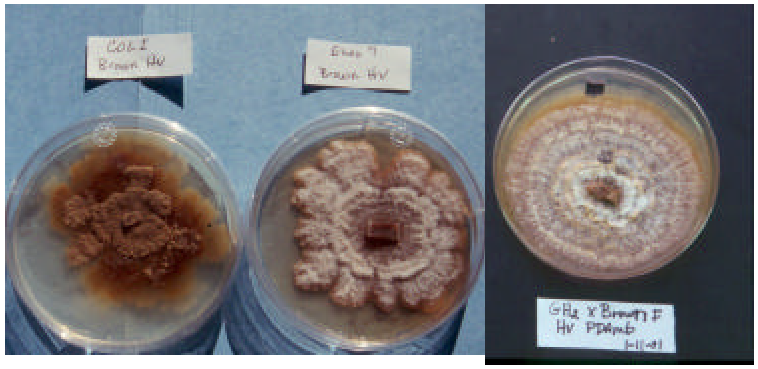


Fig. 5. "COLI" (left), Euro 7 (middle) and "GH2"(right). COLI exhibited crusted brain-like dark centers; Euro7 was lightly pigmented and had fast growth; and GH2 had a dark center, aerial hyphae, and fast growth.

Lula Lake Land Trust virus-free strains (orange *C. parasitica*)

Field sampling procedures:

Samples of *Cryphonectria parasitica* were obtained at the Lula Lake Land Trust on Lookout Mountain, Georgia using a pocketknife or a bone marrow extractor to sample from the edge of a sunken canker. Twelve American chestnut trees were examined for cankers. Three of the twelve trees did not have symptoms of the blight and were healthy. There were twelve samples taken from the edge of each canker. The bark samples were either placed into a microtiter plate with an index card indicating which tree corresponds to which row on the microtiter plate or into labeled Ziplock bags. Tape was then placed over each filled row of the microtiter plate. The samples were all labeled by the following notation: LL-1-2 meaning Lula Lake, tree 1, and isolate number 2, and etc. LL-13 has two cankers on the tree, one was a swollen canker and the other one was a sunken canker, thus LL-13 Swollen (LL-13 Swo) and LL-13 Normal (LL-13Norm) were their assigned tree and canker names.

All American chestnut trees sampled from have been labeled in previous years. Samples were stored in a refrigerator until the isolation process could be performed.

Preparation of bark samples for the explanting of *C.parasitica*:

If samples were in microtiter plates then the following procedures were conducted to wash the samples with bleach. The tape was carefully removed from each row on the microtiter plate. I made certain that the bark samples remained in the proper wells. I took a wire screen and laid it over the microtiter plate. Using five rubberbands, the screen was rubberbanded to the microtiter plate. 900 ml of distilled water was mixed with 100 ml of bleach, then I poured the mix solution into a pan. Afterwards, I submerged the microtiter plate(s) for twelve minutes in the pan with the mixed solution. I carefully poured off the bleach solution by tilting one corner of the plate. I lightly pounded the microtiter plate(s) several times on a hard surface to remove excess bleach solution. Carefully, I removed all rubber bands. I kept the bark samples in the wells until explanting process. I prepared the bark samples the same day I performed the explanting procedures.

If samples were in Ziplock bags then the samples were cut into little pieces so that they could fit into the microtiter plate(s). Then I followed the above methods.

Explant procedures for isolation of *C. parasitica*:

A washed bark sample was placed on one end of the water agar plate, approximately 0.5 inch from the edge of the plate. A second bark sample from the same tree was placed on the other side of the same petri dish. I sterilized the tweezers before and after contact with a bark sample. The petri dish was labeled with tree identification and the isolate number with a marker or wax pencil. Unused media were kept in the refrigerator for storage. Explants were place under the isolation conditions as described above.

First transfer of *C.parasitica* on culture medium:

The procedures of this section were the same as the transfer of *C. parasitica* for short- term storage.

Second transfer of *C.parasitica* on culture medium:

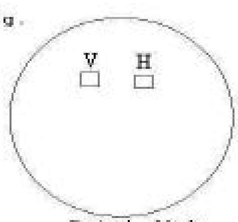
C. parasitica appeared orange with cream aerial hyphae as described in the Introduction. Only successful initial transfers of *C. parasitica* proceeded through this second transfer on fresh PDAmb. The purpose of this step was to separate contaminates from *C. parasitica*. I performed the same procedures for short- term storage on culture medium.

Initiating Conversions:

Pairing H and V strains in petri dishes

I simultaneously paired successful isolations of normal virulent strains from Lula Lake with each of the three brown hypovirulent strains from West Virginia twice (example LL-38-2 X COLIA and LL-38-2 X COLI B). The excised pieces were of the same size ($0.75cm^3$) and 0.5 cm apart (figure 6). Each sample was taken from the outer edge of the culture. LL-5-4 and LL-13-normal-5 had an extra isolate that was plated out for conversion because of possible contaminations (example LL-5-4a and LL-5-4b). Thirty pairings were done for each brown hypovirulent strain (A and B). The plates were labeled with tree number, canker number and virus name (LL-1-2 X COLI). Also, hypovirulent controls (Euro 7, COLI, and GH2) on brown background were all paired with the virulent 5-9-1B. Euro 7 O single spore isolate was also paired with the Euro 7ss parent strain. These plates were labeled with names of the donor and the recipient strains (COLI X 5-9-1B). Conversions occurred under the artificial light conditions as previously described.

Fig.



Petri dish

Transfer controls and Lula Lake virulent (normal) strains

I also transferred the individual controls and the normal strains each time pairings were done so that all conversions would have controls and normal strains from the same day for comparison purposes. I performed the same procedures for short- term storage on culture medium.

Identify the outcome of the pairings:

All three plates (the pairing, the H strain, and the V strain used in that particular pairing) were the same age so that proper comparison could be done.

Criteria for no conversion

1. No change in the morphology of the V strain (Figure 7)
2. No third sector present (Figure 7)
3. A distinct line between H and V strains (Figure 7)

Fig. 7. Note that in the middle petri dish there was no change in the V strain morphology, no third sector present and a distinct line between H and V strains, therefore this pairing was categorized as no conversion.

Criteria for possible conversions

1. A change in the morphology of the V strain to similar to that of the H strain or donor strain (Figure 8 right)

   or

2. A third sector was present (Figure 9).

Fig.8

Fig. 8. "Pairings of virulent (right) and hypovirulent (left) isolates of C. parasitica. Conversion has occurred in the virulent strain (arrow), and the pattern of mycelial growth as changed" (Fulbright and MacDonald, 1991).

Fig. 9. Note a third sector between the H and the V strain in the middle petri dish. This was the possible conversion.

Transferring possible conversions:

An excision of $0.75 cm^3$ was made in the area where possible conversion could have occurred for each pairing that met the possible conversion criteria. The area sampled was closest to the side of the V strain, furthest from the H strain, and the outer edge of the mycelium being questioned for conversion to assure that the area sampled was not the donor strain (H strain). Then, I continued the steps for transferring *C. parasitica*. All petri dishes were marked with the date and the proper name of the virus and tree.

Confirmation of Conversions:

The virulent strain (virus-free), the hypovirulent strain (virus-containing) and the possibly converted strain were placed side by side with the possibly converted strain in the middle so that the three strains could be compared (Figure 10 and 11). If the possibly converted strain exhibited similar morphologies to the virus-containing strain with the exception that the possibly converted strain was on an orange background (only for Lula Lake strains not on controls) then the possibly converted strain was indeed converted to a hypovirulent strain.

Fig 10. LL-32-2 (recipient)(Left), LL-32-2XCOLI (Middle), and COLI (donor) (Right). LL-32-2 exhibited a crusted brain-like center representative of the "COLI" virus and was on an orange background, which means conversion had occurred.

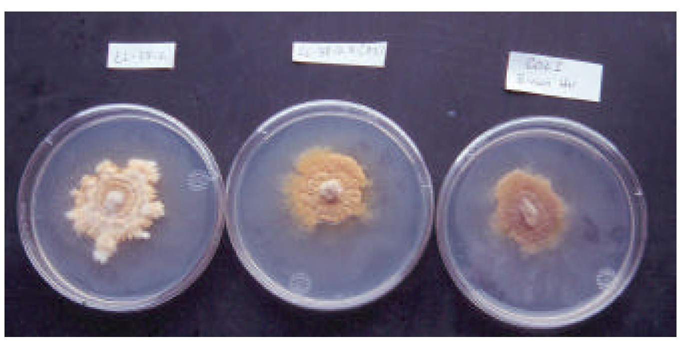
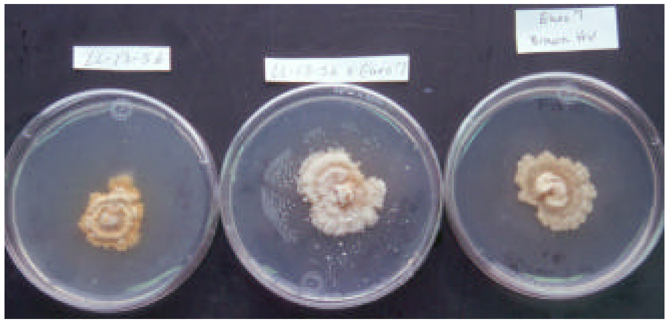


Fig.11. LL-13-5b (recipient)(Left), LL-13-5bXEuro7 (Middle), and (right) (donor) (Left). LL-13-5b exhibited a fast growth, and pale pigmentation representative of the “Euro7” virus and was on an orange background, which means conversion had occurred.



Storage of Successful conversions for biological control:

Successful conversions were kept in long and short-term storage. After ten days in the proper growth environment, the short-term stored materials were placed into a refrigerator to avoid contamination.

RESULTS:

Isolation of <u>C. parasitica</u>

Out of the fifty bark explants, I had success isolating *C. parasitica* from thirteen of them (Table 1). The other thirty-seven were contaminated with other fungi found on American chestnut trees (29 transfers) or no *C. parasitica* was isolated from the explants (8 explants).

Table 1

Isolation of *Cryphonectria parasitica*

Tree	isolate	Explant	1st Transfer	2nd Transfer
LL-2	1	no isolatation	N/A	N/A
LL-2	2	no isolatation	N/A	N/A
LL-2	3	no isolatation	N/A	N/A
LL-2	4	no isolatation	N/A	N/A
LL-2	5	no isolatation	N/A	N/A
LL-3	1	no isolatation	N/A	N/A
LL-3	2		Contamination	N/A
LL-3	3		Contamination	N/A
LL-3	4		Contamination	N/A
LL-3	5		Contamination	N/A
LL-4	1		Isolated *C.parasitica*	transferred
LL-4	2		Isolated *C.parasitica* w/contamination	transferred
LL-4	3		Isolated *C. parasitica*	transferred
LL-4	4		Isolated *C. parasitica*	transferred
LL-4	5		Contamination	N/A
LL-5	1		Isolated *C. parasitica* w/contamination	transferred
LL-5	2		Isolated *C. parasitica*	transferred
LL-5	3		Contamination	N/A
LL-5	4		Isolated *C.parasitica*	transferred
LL-5	5		Isolated *C. parasitica* w/contamination	transferred
LL-13Swo	1		Contamination	N/A
LL-13Swo	2		Contamination	N/A
LL-13Swo	3		Contamination	N/A
LL-13Swo	4		Contamination	N/A
LL-13Swo	5		Contamination	N/A
LL-13Norm	1		Contamination	N/A
LL-13Norm	2		Contamination	N/A
LL-13Norm	3		Isolated *C.parasitica*	transferred
LL-13Norm	4		Isolated *C.parasitica*	transferred
LL-13Norm	5		Isolated *C.parasitica*	transferred
LL-15	1		Contamination	N/A
LL-15	2	no isolatation	N/A	N/A
LL-15	3		Contamination	N/A
LL-15	4		Contamination	N/A
LL-15	5	no isolatation	N/A	N/A
LL-17	1		Contamination	N/A
LL-17	2		Contamination	N/A
LL-17	3		Contamination	N/A
LL-17	4		Contamination	N/A
LL-17	5		Contamination	N/A
LL-38	1		Contamination	N/A
LL-38	2		Isolated *C.parasitica*	transferred
LL-38	3		Contamination	N/A
LL-38	4		Contamination	N/A
LL-38	5		Contamination	N/A
Lake Brow	1		Contamination	N/A
Lake Brow	2		Contamination	N/A
Lake Brow	3		Contamination	N/A
Lake Brow	4		Contamination	N/A
Lake Brow	5		Isolated *C. parasitica*	transferred

Conversion

<u>Controls</u>

The Euro 7 ss and its parent (Euro 7 O) served as the positive control of the experiment. When the two strains were paired it revealed a positive conversion as expected (Figure 12) (Table 2).

Fig. 12. Euro7 ss (left petri dish), Euo7ssXEuro7O (middle petri dish), and Euro 7 O (right petri dish). In the middle petri dish, the V strain (left in middle petri dish) exhibited a change in morphology at the edge of the normal *C. parasitica*. Light pigmented, aerial hyphae were starting to form on the side of the V strain, which indicates conversion of the V strain (Euro 7ss).

Grand Haven 2 virus on brown background had similar morphologies to virulent 5-9-1B on brown background (Figure 13). Therefore, it was difficult to distinguish conversion when 5-9-1B (virus-free) strain was paired with the "GH2" virus–containing strain. This pairing was categorized as no data collected because of the ambiguity (Table 2).

Fig.13. 5-9-1B(left) and "GH2"virus-containing (right) showed similar morphologies, in that both exhibited similar rate of growth, contained concentric rings, and had aerial hyphae.

"Euro 7" and "COLI" virus-containing brown strains were both able to convert 5-9-1B to a hypovirulent strain (Table 2). When converted, 5-9-1B showed similar morphologies to the donor strain it was paired with in the petri dish (Figure 14 top row). "Euro 7" had fast growth and lightly pigmented, whereas "COLI" had crusted brain-like centers and lighter pigmentation outside of its centers. (Note: successful conversions of LL-5-4a x COLI and LL-5-4aXEuro7 on the bottom row of Figure14).

Fig. 14. Left to right top row (5-9-1B, 5-9-1BXEuro7, 5-9-1BXCOLI). Left to right bottom row (LL-5-4a, LL-5-4aXEuro7, LL-5-4aXCOLI). Notice 5-9-1BXEuro 7 and LL-5-4aXEuro7 both exhibited fast growth and were lightly pigmented. 5-9-1BXCOLI and LL-5-4aXCOLI were both debilitated in growth and had the crusted brain-like dark centers. All showed successful conversions.

Table 2. This table showed that "Euro7" and "COLI" containing strains were able to convert their parent strain, 5-9-1B to a H strain. And, the Euro7 O was able to convert Euro 77ss as was expected.

Table 2 Conversion Table for Controls

	Virus-free Strains	
	5-9-1B Brown bckgrd	Euro 7ss Orange bckgrd
Euro 7Brown bkgrd	converted	N/A
COLI Brown bkgrd	converted	N/A
GH2 Brown bkgrd	N/A	N/A
Euro 7Orange bkgrd	converted	converted

Confirmed conversion of virulent Lula Lake strains to hypovirulent strains

It was difficult to distinguish if conversion had occurred between the pairing of “GH2” virus-containing strain and Lula Lake (virus-free) strains because “GH2” did not have clear distinct morphologies like “COLI” or “Euro 7”. Therefore, no data was collected for “GH2” virus-containing strain.

“COLI” virus-containing brown strain had more successful conversions with the Lula Lake Land Trust local V strains (17/30) than the “Euro 7” virus-containing brown strain did (9/30) (Table 3).

LL-38-2 (virus-free) strain (Figure 15, left) was clearly converted by the hypovirulent strains containing the “Euro7” (Figure 15 middle) and the “COLI” (Figure 15, right) virus. The morphologies were clearly distinctive with LL-38-2 X Euro7 exhibiting pale pigmentation and fast growth, and LL-38-2 X COLI exhibiting a crusted brain-like center with a lighter pigment surrounding the center (Figure 15). In Figure 16, more successful conversions occurred with the exception of LL-13-3 X Euro 7 (Second row, middle petri dish) being a no conversion [Note: the morphology changes of the virus-free strains (first column on the right) when paired with brown virus-containing strain (first row) were very distinctive (all petri dishes in second and third columns and second through fourth rows with the exception of LL-13-3 X Euro 7 had successful conversions) in this matrix].

Fig. 15. LL-38-2(Left), LL-38-2XEuro7, and LL-38-2XCOLI. Note the distinctive morphologies of "Euro 7" virus in LL-38-2X Euro7 and of "COLI" virus in LL-38-2XCOLI.

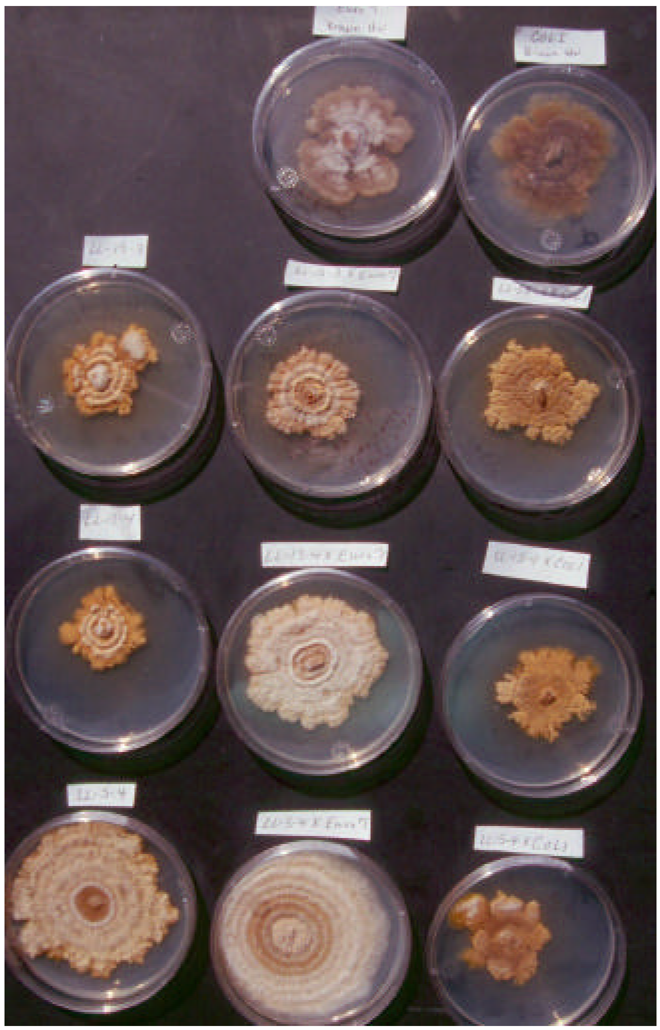


Fig 16. Left to right and top to bottom: Euro7, COLI, LL-13-3, LL-13-3XEuro7, LL-13-3XCOLI, LL-13-4, LL-13-4XEuro7, LL-13-4XCOLI, LL-5-4, LL-5-4XEuro7, and LL-5-4XCOLI. LL-13-3XEuro7 was a no conversion. All others were converted.

Table 3 Conversion Table of Virulent Lula Lake Strains

		Virus-Containing Strains			
Tree	Isolate	Euro 7A	Euro7B	COLI A	COLI B
LL-4	1	no	no	no	no
LL-4	2	no	no	no	no
LL-4	3	no	no	no	no
LL-4	4	no	no	no	no
LL-5	1	no	no	no	no
LL-5	2	no	no	converted	converted
LL-5	4a	converted	converted	converted	converted
LL-5	4b	no	no	no	no
LL-5	5	no	no	no	converted
LL-13 Norm	3	no	no	converted	converted
LL-13 Norm	4	converted	No	converted	converted
LL-13 Norm	5a	no	no	converted	converted
LL-13 Norm	5b	converted	converted	converted	converted
LL-38	2	converted	converted	converted	converted
LL-Lake Brow	5	converted	converted	converted	converted

DISCUSSION:

The results of this experiment have provided a better understanding of whether the brown strain should be used to help transmit viruses like “COLI” and “Euro7” to local virulent orange strain. The results did meet the expectation of this project in that brown hypovirulent strains were able to convert a number of the local virulent strains (26 converted out of 60) I was hoping for greater compatibility between the brown background and the orange local V strains, so that more conversion would have occurred. A modification of this experiment for future experiments in the local area would be to use a local hypovirulent strain like the ones converted in this experiment as the donor strain for the conversion of local virulent strains. By using fungal backgrounds from the same area, a possibility of having better compatibility in alleles for anastomoses is greater, thus yielding a greater percentage of hyphae anastomoses, which will increase the chances of virus transmission.

The amount of contamination observed was definitely an unexpected result, since all proper precaution was practiced in hope of fewer contaminations. The contaminants were normal fungal inhabitants of American chestnut trees and it could be that the contaminants were more successful at competing for nutrients on the PDAmb. Mark Double confirmed that contaminants are seen all the time when isolating *C. parasitica*. For future improvement of this section, more bark samples could be explanted.

The "GH2" virus-containing strain had morphologies that were too similar to the 5-9-1B virus-free brown strain. It was difficult for Dr. J. Hill Craddock and myself to distinguish between the two strains, thus there was no data collected for "GH2". As an amateur identifying morphologies, a future suggestion would be for amateurs like myself to use viruses with extremely distinctive characteristics like "COLI" and "Euro 7" in their experiment.

"COLI" virus-containing brown strain had more successful conversion than "Euro7" virus-containing brown strain was purely due to chance, since both fungal background was the same (brown background), which means that the fungal genome was the same for both brown hypovirulent ("Euro7" and "COLI" virus-containing) strains. The donor (H strain) thallus was able to transfer to the receptor (V strain) thallus the dsRNA of the virus if the two fungal thalli were in the same vegetative compatibility group, which means that the virus's transmission abilities were dependent upon the background's genes for vegetative compatibilities and not the virus's genome.

For future research in the local area, I suggest that v-c groups be identified, so that the local fungal population can be analyzed for other biological control efforts. V-C typing can help answer many questions about the different types of fungal strain here in the local area. The more V-C groups in the area, the greater the difficulty in establishing biological control (Fulbright and MacDonald, 1991). In addition, there are other ways to restore the American chestnut trees besides biological control like the backcross-breeding program currently underway at different locations. Dr. J. Hill Craddock is crossing Chinese onto American chestnut trees here at the University of Tennessee at Chattanooga in hope of producing a hybrid with resistant traits to the blight fungus. Genetic research on the virus, the fungus, the American chestnut trees and its counterparts are being conducted for a better understanding of genes involved in resistance, sporulation, pigmentation, and other traits. There are many factors that are keeping the American chestnut trees from thriving, but with constant research and collaboration, we will have American chestnut trees as resources once again.

GLOSSARY:

Alleles-Two or more alternative forms of a gene at a particular locus that confer alternative characters.

Anastomosis- The fusion of two haploid hyphae regulated by vegetative compatibility genes.

Background- The expression of the fungal genome's phenotype only, which is determined by fungal chromosomal genes without the effects of dsRNA. Background is the wildtype of *C. parasitica*, the virus's host, and without their host these viruses cannot survive.

Brown *Cryphonectria parasitica* strain- It was first discovered in West Virginia. Brown *C. parasitica* is a good genetic marker of the fungal background (brown phenotype) and a mutated form of the normal orange *C. parasitica*.

Genetic marker- A known heritable trait associated with a particular allele that is used to indicate the presence of that gene.

Genotype-The particular alleles at specified loci present in an organism.

Locus- The specified chromosomal location of a gene.

Mycelium- The vegetative thallus of a fungus consisting of a mass of branching threadlike hyphae.

Phenotype-The expression of a specific trait based on genetic and environmental influences.

Pycnidia- A flask-shaped structure that produces conidia, asexual spores.

Thallus- Any simple vegetative plant body that lacks roots, stems, and leaves.

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