5-2016

Measuring Phytophthora resistance phenotypes in segregating testcross families of hybrid American chestnut trees

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Departmental Thesis

The University of Tennessee at Chattanooga

Department of Biology, Geology, and Environmental Sciences

April 2016

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Table of Contents

Page

List of Figures and Tables

Abstract

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Phytophthora root rot (PRR), caused by the oomycete *Phytophthora cinnamomi* Rands, is a formidable obstacle to the restoration of *Castanea dentata* Marsh. commonly known as the American chestnut. Genetic resistance to PRR has been observed in Asian species of chestnut including *C. mollissima* Blume, and in interspecific hybrids between *C. mollissima* and *C. dentata*. We hypothesized that root rot resistance alleles would segregate in a 1:1 ratio within progeny of crosses between PRR resistant F1 hybrids and PRR-susceptible American chestnut trees (first-backcrosses), and that PRR resistance could be successfully passed down to all families of first-backcross hybrids. To test these hypotheses, we planted seeds of 15 first-backcross (BC1) hybrid chestnut families, and seeds of *C. mollissima*, *C. dentata*, and *C. henryi* (as controls) in a randomized complete block design in five large planting containers. Some of the American chestnuts used to produce the BC1s were naturally occurring *C. dentata*; others were third- and fourth-backcross hybrids previously selected for blight resistance. Seedlings in each container were inoculated with *P. cinnamomi* cultures that we isolated from soil samples of symptomatic orchards. Percent resistance to root rot within each family was measured using a visual rating of root necrosis six months post inoculation. Results reveal that we had resistant trees in every family with resistance ratios close to expected for most families. All individuals displaying root rot resistance were planted in an experimental orchard for further evaluation, as part of the ongoing efforts of the American Chestnut Foundation to breed American chestnut hybrids with both blight and root rot resistance. We observed *C. henryi* to demonstrate 100% resistance to root rot, suggesting it may be a another valuable source for PRR resistance alleles; to our knowledge, this is the first report of PRR resistance in *C. henryi*.

Introduction

The American chestnut has been on the decline since the introduction of two terrible diseases; the chestnut blight pandemic of the early 20th century causes by the ascomycete fungus *Cryphonectria parasitica* (Murrill) Barr, and root rot caused by *Phytophthora cinnamomi* Marsh. This ink disease, also called Phytophthora root rot (PRR), of American chestnut, first reported as early as 1825, results in necrosis of the roots and quickly kills the trees (Jeffers, 2011). The search for a solution to control chestnut blight is ongoing, yet root rot stands as a formidable obstacle in the way of American chestnut restoration, especially in the Southeast United States.

Recent knowledge has suggested that the gene for resistance to root rot may be more complex than previously understood. In 2008, some chestnut scientists hypothesized that only one or two loci were involved in the expression of root rot resistance, but conclusions were unclear (Jeffers, James, Sisco, 2008). If only one or a few locus (or loci) control for resistance to root rot, resistance could be cultivated into a population of backcross chestnut hybrids over time without many complications. However, breeding resistance to root rot has been less successful than anticipated, and the presence of multiple loci controlling for root rot resistance may explain this difficulty.

Currently, Chestnut scientists are working under multiple hypotheses to explain resistance to root rot. A study done by Santos et al. (2014) suggests that at least 2 loci influence the expression of root rot, potentially more. One locus, known as linkage group E (LG_E), has been identified and appears to host an allele influencing resistance to root rot

(Zhebentyayeva et al. 2014). Future research, however, will be required to identify and locate all loci controlling for root rot.

This project had both short-term and long-term goals. The short-term goal was to screen for PRR resistance in 15 American chestnut first backcross hybrid families. Each back-cross progeny is the result of crossing a presumably PRR-susceptible American type chestnut with an apparently PRR- resistant Asian-American F1 hybrid chestnut. The Asian parent of the F1 providing root rot resistant alleles in the present study study was *C. mollissima* Blume. Once the resistant first-backcross progenies were identified, ratios of resistance to susceptibility were calculated within each family. With this information, we were able to compare data from each family to the control groups in order to determine which of our first-backcross families are likely to host the allele(s) for resistance. Another goal of this study was to evaluate *C. henryi* for root rot resistance. To our knowledge this is a novel pursuit, and no literature has been published on root rot resistance in *C. henryi*.

Results of this work will include phenotypic data that can be used in an effort to develop molecular markers for rapid selection of PRR resistant progeny and that may deepen our understanding of the genetic loci responsible for root rot resistance in American chestnut. To accomplish this, we collected and stored tissue samples for later DNA extraction and analysis in order to determine if the resistant families in our study display the same LG_E locus on their genome as the individuals in the Zhebentyayeva et al. (2014) study.

Our long term goal is to screen our PRR-resistant first-backcrosses for Chestnut Blight (*Cryphonectria* parasitica) resistance. Trees that express both PRR and Blight

resistance can then be advanced to the second-backcross, third-backcross, and fourth-backcross generations. By advancing our first-backcross hybrids through third-backcross or fourth-backcross hybrids, we hope to generate a populations of American type Chestnut trees that can survive and reproduce on their own under natural forest conditions in Southern Appalachia.

I. Literature Review

a. *Castanea dentata:* The American Chestnut

i. General description of the American chestnut. *Castanea,* a genus within the family Fagaceae, represents 6 species and 8 overall taxa. The majority of *Castanea* is distributed among the forests of the North Temperature Zone (Manos et al. 2001). *Castanea dentata* once represented a large portion of this genus. *Castanea dentata* Marsh., commonly known as the American chestnut tree, is a monoecious deciduous tree native to the eastern forests of North America. At the height of its abundance, the American chestnut was determined to comprise between 25 and 50% of the canopy throughout in some parts of North America (Russell, 1987). *C. dentata* is traditionally an impressively large tree which can surpass heights of 100 feet, with some extreme cases reaching up to 40 meters (Detwiler, 1915). Due to the symptoms of chestnut blight, today it is usually found as a multi-stemmed shrub rarely surpassing 6 meters (Burnham, 1988). However, due to the ability of *C. dentata* to regenerate from the root collar, it continues to grow despite both chestnut blight and root rot (PA-TACF, 2012).

ii. Value and Role of the American Chestnut in the Environment. Because of its prevalence and its attractive and utilitarian characteristics, the American chestnut was of great importance not only to its ecological community but also to the culture and well-being of the people of the Appalachian region. In Appalachia *Castanea dentata* was appreciated by the people for its many useful qualities. Chestnut wood is both durable and attractive, making it a choice wood for both interior and exterior design. For example, when it was readily available for timber, chestnut wood was often used for paneling, trim, and interior and exterior furniture. Stretching from New England to Georgia, chestnut wood was also the most often used wood for cabins and outhouses (Saucier, 1973). Furthermore, many rural families relied on the products of the American chestnut as a source of income and subsistence. Many local products relied on either the timber itself or even unique qualities of the bark such as tannins. The tannin of chestnut wood extract adds firmness and solidity to leather as well as making it resistant to decay (Lord, 2004). Thus, tannins of chestnut were often used to treat leather, a crucial step in the leather industry (Anagnostakis, 1987). Chestnut wood was also used as fire wood, fence posts, poles for rural telephone lines, food for farmers' hogs, and food for the wildlife (Hepting, 1974). The large variety of uses of chestnut wood made *C. dentata* a highly valued organism to the Appalachian economy.

The American chestnut is equally as important to the ecosystem as it is to the people of southeastern North America. The decline of the American chestnut dramatically disrupted the habitat and food web of its native range because it served as a foundation species of its ecosystem. A foundation species is locally abundant and creates locally stable environmental conditions required by many species within its ecological community

(Hanski, 1982). It essentially creates and defines an ecosystem. For example, the decline of *C. dentata* has affected decomposition rates, nutrient cycling, and productivity of its environment. Any disruption to the environment can have drastic consequences to the population it is supporting (Ellison et al., 2005).

C. dentata served as a foundational species because of the many important resources it provided to other wildlife. Notably, its fruit has characteristics which differentiate it from most other common trees of the southeastern forests of America. For example, its fruit has one of the highest carbohydrate contents of eastern American fruit-producing species and has high quality protein and low fat content (McCarthy and Meredith, 1988). Its nut is more available than many other tree nuts because it avoids destruction by frost. *C. dentata* blooms in late June, unlike many other species such as oak trees which flower up to a month earlier and may lose many of their fruits due to frost (PA-TACF, 2012). Its leaves have a low carbon to nitrogen ratio and thus decompose quickly to release nutrients to the environment (Smock and MacGregor, 1988). Furthermore, a healthy American chestnut provides a canopy of over 100 feet, thus providing shade and shelter for all organisms living below (Ellison et al., 2005).

To conservationists, ecologists, and others, the decline of the American chestnut has been a problem of great concern for many years because of the important role it plays ecologically, economically, and culturally. (Seiler et al. 2015). Unfortunately, in the early 20th century, a fungal blight was introduced and spread at rates so rapid that the tree was all but immediately eliminated. This rapid population decline resulted in both widespread socioeconomic and ecological strain for the Appalachian region of eastern North America (Roane et al. 1986).

iii. Decline of Population by Chestnut Blight. In 1904, Hermen Merkel, a forester at the New York Zoological Gardens, made note of a phenomenon occurring to the American chestnut within the Bronx Zoo. He observed many chestnuts which had large portions of rotting bark. He also noticed larger tumor-like formations of the infected trees (Hepting, 1974). He recognized this as a blight to the chestnuts. Merkel's observation was not the only report made around this time; in 1903, a report very similar to Merkel's was made on Long Island, and by 1907, reports of occurrences of the disease had been made from Poughkeepsie, New York to Trenton, New Jersey (Harrisburg, 1912).

The causal agent for Chestnut Blight, discovered by William Murrill, was originally known as *Endothia parasitica,* but today is known as *Cryphonectria parasitica* (Roane et al. 1986). *Cryphonectria* infects the bark by sending fine threads of mycelia into the inner bark, destroying the cambium layer. This results in cankers, which can expand enough to be fatal (Kuhlman, 1964). *C. parasitica* was introduced to America as a consequence of an effort to breed a chestnut which boasted the size of the Asian variety of chestnut while maintaining the sweetness of the American chestnut (Anagnostakis, 1987). The nursery stock brought to New York was an Asian species of the chestnut that carries genetic resistance to *C. parasitica.* It is now known that while Asian species are resistant, the American species are known to be fairly susceptible to the disease (Jaynes, 1975). Thus, by 1940, less than 40 years after the introduction of chestnut blight, few Chestnuts were alive or without symptoms of the blight. (Davis, 2000). Seemingly every large stem of *C. dentata* has now been affected (Hebard, 2006). Today, most American chestnuts are reduced to multi-stemmed shrubs, with few examples reaching the fruiting stage. The

fruiting stage is necessary for naturally occurring reproduction, thus only a very small percentage of the remaining population is capable of continuing the species. (Paille, 2003).

b. *Phytophthora* Root Rot

i. Introduction of Root Rot to the Southeast. There is in fact another pathogen playing a role in the extreme population decline of *C. dentata.* This pathogen, *Phytophthora cinnamomi* Rands, was most likely introduced from the South Pacific to the United States by boat at a southern port around the 1700s (Crandall and Gravatt, 1967). In 1922, *P. cinnamomi* was isolated by R.D. Rands from cinnamon trees in Southeast Asia where it was found to cause stripe cankers. The species name originates from its association with this disease (Zentmeyer, 1977). The first report of Chestnut fatality from root rot occurred in 1825, and by 1840 the frequency of deaths had become noticeable (Clinton 1912; Butterick 1913; Crandall et al. 1945). However, it was not until 1931 that Crandall identified *P. cinnamomi* to be the causal agent of the root rot affecting the American chestnut tree (Gravatt and Crandall, 1945).

ii. Genus: *Phytophthora.* *Phytophthora* is infamous for its ability to infect a wide range of hosts in a wide range of ecosystems. For example, *Phytophthora* is known to cause disease in chestnut, oak, fir, pine, eucalyptus, cinnamon, and more. It has been found in nurseries, Christmas tree plantations, landscapes, and forest soils**.** As a pathogen, it acts on its hosts by causing rot of fine and thick roots, collar rot, stripe cankers, wilting of woody hosts, trunk cankers, and stem and root lesion (Erwin and Ribiero 1996, Shearer et al. 1989, Sinclair and Lyon, 2005). All species of *Phytophthora* are oomycetes. They are fungus-like microorganisms which can act as either pathogens or saprobes (Zentmeyer,

1980). They are notorious for causing destructive disease of plants. Most species of *Phytophthora* function best in moist soils because of their production of zoospores that swim through soils saturated in water. Phytophthora produces both oospores and chlamydospores, which both contribute to long-term survival, making this collection of a species markedly difficult to control.

iii. Root Rot Symptoms and Method of Infection. *Phytophthora cinnamomi* is one of the most well-known species of *Phytophthora*. More than 5,000 hosts are known to be affected by *P. cinnamomi*, and these hosts are found in most temperate and tropical regions of the world (Erwin, 1996). The symptoms of disease from infection by *P. cinnamomi* are known as "root rot" or "ink disease". (Anagnostakis, 2001). The first symptoms of root rot are yellowing and wilting of the leaves. Upon examining the roots, necrosis will be noticeable. (Brosi, 2001). *P. cinnamoni* is also known to cause collar rot, branch dieback, defoliation, reduced vigor, and increased mortality. In the case of root rot of chestnut, it is a soil-borne pathogen that causes fatality to *C. dentata* by reducing water and nutrient uptake and forming lesions on roots (Maurel et al. 2001).

P. cinnamomi can infect its host in two different ways and can remain dormant in the soil as mycelium or as chlamydospores (Sidebottom et al. 2004). When soil is moist and warm, either the mycelium or the chlamydospores produce sporangia which can germinate and directly colonize roots or release zoospores. The zoospores first direct their movement towards the roots of is host based on chemical attraction (Sidebottom et al. 2004). Once *P. cinnamomi* reaches its host, it spreads its mycelia inside and around the tissue of the host plant. In the American chestnut, it can either penetrate epidermal cells or enter through pre-existing host wounds (Gow et al. 1999). It spreads its mycelia both

intra- and inter-cellularly through the plant tissue of the *C. dentata*. *P. cinnamomi* then feeds on the inner root cortex (Zentmeyer 1980; Day 1938).

iv. Controlling Root Rot. *P. cinnamomi* is both intense and quick to spread from one host to another; it spreads itself naturally by sending zoospores through near-saturated soils and is also spread mechanically through means such as unwashed agricultural equipment used at a site of infection, physically relocated infected soils, or flowing water from an infected area to a previously uninfected area (Reeves and Jackson, 1974; Sidebottom, 1998). Like all members of *Phytophthora*, it is both saprophobic and pathogenic; in the case of root rot, *Phytophthora* acts as a parasitic plant pathogen (Zentmeyer, 1980). The ability to act as a saprophyte results in widespread survival and persistence for the organism because it can persist in the soil in the absence of a host (Erwin et al. 1983). Furthermore, *P. cinnamomi* has a pronounced ability to thrive in soil with poor living conditions. It functions best in shallow, infertile, degraded, eroded, poorly drained, or poorly managed soils (Campbell and Copeland, 1954). It also prefers poorly aerated soil high in moisture and clay because these conditions promote the formation of sporangia and of zoospore release (Wilcox and Mircetich, 1985). Thus, when planting to re-introduce hybrid American chestnuts, it is important to avoid planting sites with these soil characteristics.

The production of the asexual resting structures known as chlamydospores is considered to be the primary means of long-term survival in northeastern American soils (Zentmyer, 1980). *P. cinnamomi,* unlike most members of *Phytophthora,* does not commonly produce oospores; however, it does produce chlamydospores which allow the organism to function as a saprobe in soil or on another organism for long periods of time.

These spores germinate when the preferred amount of moisture and nutrients is available. The walls of its chlamydospores thicken over time and are found to have a higher lipid content than most other *Phytophthora* species. (McCarren, et al. 2005; Zentmyer, 1980). Though *P. cinnamomi* will not survive for long in temperatures below 0 degrees Celsius, chlamydospores have been found to endure these low temperatures for up to 2 weeks (Zentmyer, 1980).

Many methods of control have been utilized in efforts to control *P. cinnamomi*. For example, the implementation of well-drained sites and the incorporation of ectomycorrhizal fungi on the roots of *C. dentata* have been used to prevent the spread of root rot because the fungi form fungal mantles around the root system and thus provide a protective barrier against *P. cinnamomi* (Branzanti et al. 1999). Some methods of chemical control of *P. cinnamomi* such as trunk injection, soil fumigation or aerial spraying have been utilized, but various environmental, financial, logistical, and public relations concerns with these efforts limit the success of these methods (Gravatt and Crandall 1945; Colquhoun et al. 2000; Benson and Grand, 2000; Tynan et al. 2001). For example, mono- and di- potassium salts of phosphorous acid have been used to treat root rot, but research has shown that the treatment inhibits the colonization of ectomycorrhizal fungi which provide important benefits for *C. dentata* and other trees. (Perkins, 2012). In the case of root rot, the ectomycorrhizal fungi are important because the fungal hyphae form the mantle which encloses and protects the root from root rot. (Kendrick, 2000). Damaging the mycorrhizas increases the chances of infection by *P. cinnamomi* because of the protective nature of the mycorrhizal formation. Thus, though many of these methods have been mildly effective, none have proven to be worth further attention.

v. Regional Effects on Root Rot. The temperate climate of the native range of the American chestnut is another contributing factor to its susceptibility to root rot. Very high temperatures are known to be fatal to the organism, and very low temperatures result in a decrease or even a cessation in pathogenic activity; *P. cinnamomi* rarely causes infection below temperatures of 15ºC **(**Shew and Benson, 1983**;** Erwin et al. 1983**).** Studies show that the optimal temperature for pathogenic activity is 19-27ºC (Zentmyer, 1980). The effects of different climates on the pathogenic activity of *P. cinnamomi* are apparent from studying the different behavior of *P. cinnamomi* in its eastern North America range vs. its behavior in slightly less temperate ranges. For example, as well as being found in the Appalachian forests of North America, *P. cinnamomi* is found in southwest France where it causes root rot in *Castanea sativa* Mill., the sweet chestnut, *Quercus rubra* L., the northern red oak, and *Quercus robur*, the indigenous pedunculate oak (Foex, 1941). Root rot is especially a concern for *Q. robur* because the tree is very economically and ecologically important to its range. Currently, there is concern that root rot in pendunculate oak is spreading in nursery stock (Maugard, 1997). *P. cinnamomi* produces different symptoms in pendunculate oak than it does in the American chestnut. In root rot in pendunculate oak, *P. cinnamomi* attacks the host via roots and causes the formation of a canker of the lower trunk of the tree that severely decreases the value of the timber. Unlike root rot in American chestnut, the disease does not affect the overall health of the tree. (Robin et al., 1992) This is believed to be because *P. cinnamomi* attacks the roots of chestnut species much more severely than in oak species (Brasier et al. 1993; Crandall et al. 1945; Tainter et al. 2000).

Furthermore, root rot in red oak and pendunculate oak is not spreading at rates like root rot in American chestnut. The disease is believed to have originated in the 1950s and is still today only present in southwest France and a small region in the Pyrenean Peidmont area in Spain (Levy 2000; Marçais et al. 1996). Root rot in chestnuts of the region was first reported in 1848 in the Basque country of France and, like root rot in American chestnut, has spread to most ranges of the chestnut in Europe (Grente, 1961; Morel et al. 2003). The range of *P. cinnamomi* in Europe is more limited than in North America. A study performed in southwest France explored the cause of this limited range and found the cause to be a susceptibility to frost. It was found that *P. cinnamomi* performs very poorly with temperatures below freezing, and as a result, canker development is severely diminished (Marçais et al. 1996). However, the impact of frost is not as severe when *P. cinnamomi* attacks chestnuts because the disease mostly attacks the roots. The soil of its North American range rarely freezes below 10 cm, and most of the roots of the chestnut are found in this layer of the soil. (Marçais et al. 1996) Thus, the vulnerability of American chestnut to *P. cinnamomi* is partially a result of the tendency of the soil of its range to remain above freezing for all, if not most, of the year.

c. Restoration of the American Chestnut

Today, reintroduction biology and restoration ecology are two approaches that are being practiced with the restoration of the American chestnut tree. Reintroduction biology introduces one species into its historical range, (Seddon et al. 2007) and restoration ecology is used to bring back community and ecology health (Young, 2000). The single species *Castanea dentata* is being reintroduced into its historical range, the southern Appalachians, and this reintroduction is projected to lead to the restoration of a healthy

functioning ecosystem in this region. Currently, a series of backcrosses to breed chestnut hybrids is being used to reintroduce both blight-resistant and root rot resistant American chestnuts into their native range (Smith, 2012).

i. The American Chestnut Foundation. From the 1920s to the 1960s, chestnut scientists were breeding American chestnut with resistant Asian species (Smith, 2012). Their efforts were shut down after 40 years of efforts failed to produce resistant Chestnut hybrids that still had American characteristics. However, 20 years later, Dr. Charles Burnham suggested that second, third, and fourth generation hybrids should be crossed back with American chestnut trees in efforts to maintain American characteristics. In 1983, it was on this premise that a non-profit organization known as The American Chestnut Foundation (TACF) was founded with a mission to "restore the American chestnut tree to its native range within the woodlands of the eastern United States" (Jeffers et al., 2008). Their current focus is to breed both Chestnut blight and root rot resistance into the American chestnut by backcrossing American chestnuts with resistant Asian species. The American Chestnut Foundation's attempt to breed resistance to *Cryphonectria parasitic* is threatened by both *P. cinnamomi* Rands and *P. ramorum.* The latter species is mostly a threat to American chestnuts in Europe, but it has recently begun to establish itself in the eastern United States. However, its specific effects on the American chestnut are unknown. Currently, the main focus of the American Chestnut Foundation's efforts are on the eradication of the threat of *P. cinnamomi*, but the threat of *P. ramorum* should be monitored (Bowles, 2006).

ii. Backcrossing for Resistance to Chestnut Blight. The American Chestnut Foundation is practicing an inter-species breeding strategy (primarily between *C. dentata*

and *C. mollissima*) using backcross breeding in which chestnut blight resistant species are crossed with American chestnuts to generate resistant hybrids. Highly resistant species include four Asiatic species: the Japanese chestnut, *C. crenata* Sieb. and Zucc.; the Chinese chestnut, *C. mollissima* Blume.; the Senguin chestnut, *C. senguinii* Dode.; and the Henry chinquapin, *C. henryi* Skan. Rehd. and Wils. (Milburn and Gravatt 1932; Crandall et al. 1945; Gravatt and Crandall 1945). Most commonly, *C. dentata* is crossed with *C. mollissima*, the Chinese chestnut and *C. crenata*, the Japanese chestnut in efforts to produce blight resistant hybrids (Burnham et al. 1986). In the backcross breeding method employed by TACF, three backcross generations are produced, and the progeny are all selected for blight resistance. The selected BC3F1 trees are intercrossed to produce BC3F2 populations that provide the seeds used to plant in the American chestnut's historical range. In this process, because it promotes local adaptation, it is important to use parent trees that originated in the region the planting will occur (Hebard, 2006).

In 2014, it was reported that 10 to 30% of TACF's Restoration Chestnut 1.0 trees are as resistant as *C. mollissima*, depending on the year (Hebard, 2014). Restoration Chestnut 1.0 trees are 94% American and 6% Chinese chestnut. Since 2002, TACF has planted 62,343 seeds in their orchards. The US Forest Service has been evaluating the performance of the Chestnut 1.0 trees since 2009 and has reported that many of the hybrids are the fastest growing trees on site and that they are similar in growth and appearance to the American chestnut controls. Furthermore, computer simulations have also indicated that these progeny have adequate genetic diversity (Hebard, 2014).

iii. Backcrossing for Resistance to *Phytophthora* Root Rot: Experimentation suggests that backcrossing resistance into *C. dentata* is the most effective mode of

controlling root rot. This process takes many years, but is expected to result in Chestnut hybrids that are resistant to root rot are 15/16 American and only 1/16 Chinese (Smith, 2012). Since 2004, the backcross breeding program of TACF has been applied to *C. mollisima* with *C. dentata* with a focus on root rot resistance. In 2011, a fourth backcross generation was evaluated for root rot resistance at the Chestnut Return Farm in Seneca, SC (Jeffers, 2011). Jeffers and his colleagues evaluated 242 hybrid families and found 50 of them to be resistant to root rot. Over five years of work, 208 hybrid survivors from 35 different families have been cultivated and replanted in Joe James's Oconee County, S.C., farm, representing a 3% survival rate (James, 2009). Steve Jeffers and Joe James (2008) claim that using controlled pollinations from two of their resistant individuals will boost the survival rate from 3% to nearly 50%. They hope to incorporate the root rot resistance into breeding populations of backcrossed American chestnuts.

d. Quantitative Trait Locus Mapping

i. Description of QTL. A quantitative trait locus is a section of a genome that correlates to the specific phenotype being studied. The section of DNA is known as the locus, and the correlating phenotype is known as the quantitative trait. Evolutionary scientists developed the science of Quantitative Trait Locus (QTL) mapping in response to many questions of genetic variation. (Shrimpton & Robertson 1988; Mackay 1995). This method, developed by Gelderman in 1975, uses molecular markers and genetic maps to answer questions of genetic variation. (Barton and Turelli 1989). QTL mapping is used to determine the correlation between genotypic and phenotypic information. Molecular markers define the genotype information while trait measurements are used to define the phenotypic information.

ii. General Uses of QTL. The goal of QTL mapping is to pinpoint the location(s) on a chromosome that code(s) for the expression of the trait being studied and understand how the location(s) acts. (Kendrick, 2000). In QTL mapping, phenotypic data of organisms are collected and used to create a mapping population. Genetic markers on an organism's genome are compared against the genetic map of the population to search for physical linkages between a genetic marker and a QTL. QTL can be used to study patterns of segregation and is also used to study whether multiple regions interact to produce the correlated phenotype. Thus QTL can help determine whether one or a few loci are creating a large effect or if many loci are working together to create the effect (Bernatzky and Mulcahy, 1991). In relation to this study, QTL mapping can be used to attempt to determine the number and location of loci which determine resistance to root rot. This information can then be used to perform selection by assistance of genetic markers.

iii. Identification of the LG_E locus using QTL mapping. Zhebentyayeva et al. (2014) identified a locus on the American chestnut genome which appears to influence the expression of root rot resistance. They named this locus LG_E. They identified this locus using QTL mapping. After extracting DNA from both the parent trees and the hybrid progeny, they used two types of molecular markers to genotype each individual: single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs). Genetic linkage maps of progeny from the same cross were then generated by using centimorgan distance between each locus. The centimorgan distance was determined by calculating the frequency of recombination between target loci. Loci which had high frequency of recombination were determined to be farther apart, whereas loci with low frequency of recombination were determined to be closer together.

After Zhebentyayeva et al. (2014) generated genetic linkage maps for the progeny of all crosses, they used maximum likelihood and Kruskal-Wallis tests to correlate low disease severity scores (indicating expression of root rot resistance) and sequence variation at each locus being studied. The LG_E locus was determined to host a particular allele which was observed in the individuals expressing resistance to root rot. The progeny that had low resistance scores contained a different allele at this LG_E locus. This linkage group is believed to be on chromosome 5.

e. Disease Screening

i. Overview of Disease Screening Methods. Many different methods of disease screening have been used to measure severity of disease in host plants. Disease screening is helpful to determine resistance when a disease trait ranges on a gradient which is able to be criticized by the naked eye (Russell, 1978). Disease severity scales correspond to the intensity of the expression of a disease and have often been scales of 0-3, 0-5, or 1-9. The scale used depends on the pathosystem being studied. Illustrations and keys associated with the different ratings are often provided (James, 1974; Strange et al., 2004). These tools help illustrate the judgements used to rate disease severity. Because humans are intrinsically subjective and the human eye can only accurately assess obvious levels of disease, disease screening is difficult to standardize (James, 1974). In order to aim for the highest accuracy possible, the difference between each rating must be clear, the full range of disease symptoms must be provided for, the data must be easily recorded, and experiment conditions should be as similar to environmental conditions as possible (Porta-Puglia and Aragona, 1997).

ii. The Method of Root Rot Screening Developed by TACF. The American Chestnut Foundation has run several experiments in which the expression of root rot in different hybrids is measured by a rating of necrosis ranging from healthy and without symptoms to dead (Sisco, 2004). The goal in these studies is to identify the hybrid families expressing high levels of resistance. These resistance hybrids are then used to create a resistant population for future breeding and restoration. After all hybrid seeds have matured, they are inoculated with *P. cinnamomi.* The inoculation occurs around 3 months after planting, and the seeds begin to die within about 3 weeks after inoculating. The screening of the hybrids occurs around 5 months after inoculation.

Usually in late winter, each hybrid is removed from its growing plot for evaluation. Each plant is rated on a scale of 0-3 for necrosis. A rating of 0 signifies a lack of lesions of roots, 1 signifies lesions on lateral roots, 2 signifies lesions on tap roots, and 3 signifies plant fatality. The plants given a rating of 0 or 1 are considered highly resistant and can used to help create a resistant family in future breeding efforts, to locate the resistant genes in the genome by QTL, and more (Jeffers, 2011).

II. Methods

In my study, I screened hybrid testcross families for root rot resistance in order to evaluate the percent of resistant individuals within each family. As seen in **Table 1,** 15 different first-backcross (BC1) hybrid families and 3 control families were evaluated. Most of these 15 first-backcross families are the result of controlled backcrosses using hand selected individuals of *C. dentata* as the pistillate individual and an F1 progeny screened for root rot resistance as the male individual. For four of the 15 first-back cross individuals, the male individual was either a third-backcross or fourth-backcross individual selected for resistance to chestnut blight. These 4 backcrosses are known as "better backcrosses" because the screening for chestnut blight had already been performed. The 3 controls included one family each of *Castanea dentata* (American chestnut), *C. mollissima* (Chinese chestnut), and *C. henryi* (Chinese Chinquapin). The 15 BC1 seedling families all derive from 2014 hand pollinations and/or open pollinated seeds of selected *Phytophthora*-resistant F1 hybrids (provided by Dr. Hill Craddock and The American Chestnut Foundation). The sources of PRR-resistance alleles in the BC1s are six different *C. mollissima* cultivars: 'Amy', 'Byron', 'Gideon', 'Lindstrom '99', 'Nanking', and 'Payne'. The pedigrees and notes about the parents of the BC1 families are listed in **Table 1.** In total, 435 first-backcross individuals were planted and 165 were sent to Allatoona.

Table 1: Pedigrees and Notes on the 2015 Phytophthora Screening Families.

Pedigree	Notes	Planted Per Family	Sent to Allatoona
UTC1 TTU-A-4 x ALA Frames 1	TTU-A4 = 2004 TNCLA1 x Gideon, ALA Frames 1 = *C. dentata* from Alabama	27	15
UTC2 Mcinturff FF-1 x OP	McInturff FF-1 = 2006 TNMON8 x Nanking; Pollen cloud = select Clapper BC3s/ BC4s	80	28
UTC3 TTU-A-4 x ALA Frames 4	TTU-A4 = 2004 TNCLA1 x Gideon, ALA Frames 4 = *C. dentata* from Alabama	29	5
UTC4 TTU-A-4 x OP	TTU-A4 = TNCLA1 x Gideon; Pollen cloud = selected Clapper BC3s	116	46
UTC5 TN-SUM1 x Neel 6-193	TNSUM1 = *C. dentata* from Sumner Co., TN Neel 6-193 = 2003 TNLIN-1 x Payne	15	9
UTC6 TN-MAC1 x Neel 4-195	TNMAC1 = *C. dentata* from Macon Co., TN Neel 4-195 = 2004 Bendabout E10 x Amy	40	8
UTC7 TN-MAC1 x Neel 6-268	TNMAC1 = *C. dentata* from Macon Co., TN Neel 6-268 = Bendabout L10 x Bryon (Lindstrom-67)	7	0
UTC8 TN-MAC1 x Neel 2-127	TNMAC1 = *C. dentata* from Macon Co., TN Neel 2-127 = 2004 TNRUT1 x Lindstrom-99	27	6
UTC9 TN-MAC1 x Sam's 2 II-1	TNMAC1 = *C. dentata* from Macon Co., TN Sam's 2 II-1 = 2006 TNMON8 x Nanking	6	0
UTC 10 Mcinturff DD-1 x OP	McInturff DD-1 = 2006 TNMON8 x Nanking Pollen cloud = selected Clapper BC3s/BC4s	1	1
UTC11 Mcinturff II-1 x OP	McInturff II-1 = 2006 TNMON8 x Nanking Pollen cloud = selected Clapper BC3s/BC4s	1	0
UTC12 TTU-A-4 x ALA Frames 5	TTU-A4 = 2004 TNCLA1 x Gideon, ALA Frames 5 = *C. dentata* from Alabama	12	9
UTC13 Neel 5-238 x ALA Frames 1	Neel 5-238 = 2004 Bendabout L10 x Byron (Lindstrom-67) ALA Frames 1 = *C. dentata* from Alabama	23	11
UTC14 Neel 3-262 x TN Carroll County #1	Neel 3-262=2003 TNRUT1 x unknown Chinese Carroll County #1 (TNCAR-1) = *C. dentata* from TN	49	23
UTC15 Neel 6-268 x ALT-3	Neel 6-268 =2004 Bendabout L10 x Lindstrom-67 ALT-3 = *C. dentata* from the Talladega National Forest in Alabama	4	4

Female parent is listed first for every cross, followed by staminate (pollen, or male) parent. OP = open pollinated. Pollen cloud = the mixture of pollens of selected BC3 and BC4 trees present in the experimental orchards.

Isolation of *Phytophthora* from Orchard Soils. On April 22, 2015, soil samples believed to contain *Phytophthora cinnamomi* were collected by Taylor Perkins, Dr. Hill Craddock, and me from Sam McInturff's farms in Blount County, Tennessee. We used protocol established by Jeffers and Sisco working with The American Chestnut Foundation (Jeffers et al. 2008). The two orchards signified as Sam's I and II are backcross orchards that were planted in the late 1990s and early 2000s, and are maintained by volunteers of the Tennessee Chapter of the American Chestnut Foundation. Soil samples were also collected from Bendabout Farm in Bradley County, Tennessee by Taylor and Cameron Perkins in April 2015. The Bendabout Farm is managed as a habitat conservation and game preserve and also contains several backcross orchards of TACF material. The soil samples collected from this farm were brought by Taylor Perkins to Clemson University for the detection of *P. cinnamomi.* Taylor Perkins was operating under Dr. Steven N. Jeffer's APHIS Permit. Suzy Sharpe and Dr. Steven N. Jeffers confirmed the presence of *P. cinnamomi* in 3 different locations of Sam McInturff: Sam's I K-15, Sam's II DD-20, and Sam's II DD-5. Two samples of *P. cinnamomi* were also detected in Bendabout Farm Orchard 3. Once the organism was detected, *P. cinnamomi* was isolated and stored. Taylor Perkins transferred the isolates to vials for permanent storage in Dr. Steven N Jeffer's lab and for transport back to UTC. Protocol for preparation of PAR(PH) medium and cV8A basal medium of the *Phytophthora* inoculum followed that used by the Jeffer's lab at Clemson can be found in the appendix.

Experimental Tub Setup. The *Phytophthora* root rot tubs were set up and planted on May 3rd 2015. This setup was a randomized block design. The design was created in an effort to account environmental effects so that all variation is due to genetics. For the

experiment, 5 Rubbermaid 568 liter stock tank (Model 4245) tubs were set up to represent 5 different replicates. The pots were set up in a row along the north end of the Fortwood Street Greenhouse. Each tub was placed on top of a wooden palette which was set inside 22 gallon plastic kiddie pools. These pools were used to prevent contamination of the surrounding soil: after inoculation, they collect water containing *P. cinnamomi* zoospores as the tubs are watered. The pools were treated with a disinfecting solution or StorOx and drained as they filled up with water. StorOx is a fungicide that extends shelf life and reduced spoilage of stored crops (StorOx 2.0, 2016). It is used against many pathogens including early and late blight, bacterial ring rot, bacterial soft rot, silver scurf, and fusarium tuber rot. After the tubs were placed in a row, each tub was filled with around 560 liters with Sun Gro Metro-mix 360 growing soil, 79 liter bags. The tubs were then heavily saturated with water in order to settle the soil. More soil was then added to each tub to level off each tub.

Planting of Chestnut Seeds. The planting of the seedlings began after the tubs were allowed to sit for 3 days. Before planting the seeds, 10 rows were set up in each tub. Chicken wire was inserted around the circumference of the tub, and strings were tied to the wire at regular intervals in order to create a grid providing 11 linear rows for planting. After the rows were set up for each tub, the seedlings were planted. A randomized number calculator was used to determine where each family would be planted in each tub. The same number of seedlings from each family would be planted in all five tubs.

In each tub, eleven rows were dug with a trowel. The rows were approximately twice as deep as a chestnut seed. Seeds were placed in the rows two inches apart from each other. Before planting, each chestnut seedling was visually screened for presence of

molding. Seedlings which showed signs of molding were not planted and were discarded, as they could have resulted in skewed results. Tubs were covered with chicken wire mesh to prevent seed predation by squirrels, jays, etc. After planting was completed, the seedlings were watered every few days and the tubs were monitored for any signs of growth. **Figure 1** and **Figure 2** show the tub directly after set up and 2 months after setup.

Figure 1: Tub 1 after tub was set up and seeds were planted and families labeled. (May 2016)

Figure 2: All tubs are pictured and illustrate mid-summer growth progress. Trees appear healthy and are growing as expected. Inoculum had not yet been applied. (July 2016)

Tagging of Hybrid Individuals and Inoculation of Tubs. On August 25-27, each plant was tagged by Taylor Perkins, Hill Craddock, and Paola Zannini with the following format: UTC 1-1 represents the first individual of the family UTC1 (progeny of the cross TTU A4 X ALA Frames 1), and UTC 1-2 represents the second individual of this family. Once the seedlings had sufficient time to become established in their new environment, the first-backcross hybrid families were inoculated with *Phytophthora cinnamomi*. This timeline allowed for the seedlings to grow enough to have a reasonable chance at expressing resistance against infection. On September 2, a 5 gallon bucket was used to mix with three liters of vermiculite and 2 liters of potting medium. This inoculum was poured into furrows that were dug in between each row of plants. Each tub served as a sort of "death chamber" for the seedlings, in which the tubs were kept in conditions favorable for the expression of PRR symptoms. They were well watered so that the soil would be saturated enough for the movement of zoospores for the spread of infection.

On November 3, two leaf samples from each plant were collected. Each sample was cut into two 30-40 mg aliquots and a 100 mg aliquot. For each plant, one 30-40 mg aliquot was added to a plate to be used for DNA extraction, and the other 30-40 mg aliquot was added to a plate to serve as a backup. In total, 96 plates were sent to Clemson. For each plant, a 100 mg aliquot was also wrapped in aluminum foil for DNA extraction with the CTAB method in case of failure of the first DNA extractions.

Disease Screening for Root Rot in First-Backcross Test Hybrids. On January 19, 2016, Dr. Hill Craddock, Taylor Perkins, and I began phenotyping the F1 backcross hybrids inoculated with *P. cinnamomi.* We met at the greenhouse every Tuesday and Thursday until the phenotyping was complete. Due to weather, we often had to postpone phenotyping to

allow the soil to thaw. Commonly assisting us was Paola Zanini and a group of undergraduate volunteers from the UTC biology department.

To begin phenotyping, we carefully dug up each plant from the tub in the order in which they were planted (starting with the first individual planted in row 1 of tub 1). We pulled plants one row at a time and analyzed each row separately from the next. After the plants were pulled, we cleaned the roots of each plant by submerging the plant in a bucket of water. We then laid the plants back into the tray and rated them one-by-one in the order they were planted. To rate the plants, we used a scale used by Jeffers and Sisco (2011). If there were no symptoms of root rot, we gave the plant a "0". If there was necrosis seen on any of the lateral roots, we have the plant a "1". We modified this aspect of the scale and decided to give all plants lacking of lateral roots a 1 as well. A study done by Cahill et al. (1989) observed that root rot stopped root growth of chestnut across different groups of plant species within 24-48 hours. We concluded that a lack of lateral roots indicated that root rot had stopped the growth of the lateral roots or that the roots had rotted from root rot necrosis and fallen off. If the plant had necrosis on the tap root, we gave it a "2". Finally, if the plant was dead from root rot, we gave it a "3". If the plant was only dead at the top, we determined the plant died from other causes and gave it a "0". We rated each plant in all 5 tubs and collected data in printed Excel sheets.

Statistical Analysis. I used SPSS to analyze my data. I decided to transform all the scores of 1, 2, and 3 to 1, indicating "symptomatic" as Jeffers and colleagues did in their root rot screening (Jeffers 2011). I concluded that the scale of disease expression was not necessary in the evaluation of the data, though it may be valuable information to refer to in

the future. Simplifying the dependent variable into two responses made evaluating the data a clearer and more approachable process.

To evaluate my data, I ran a cross tabulation and 36 separate Chi-square tests in order to display the root rot resistant to symptomatic ratios within each family. I referenced course materials from the course Ecological and Evolutionary Statistics taught by Dr. Hope Klug. I originally included all 15 first backcross hybrid families and the 3 controls; however, I ultimately decided to not include any of the families with less than 5 individuals, as I concluded that the data for these families have no statistical credibility. I ran 36 separate Chi-square tests as post-hoc analyses to look for significant differences of rot root resistance between the first backcross hybrid families and the 3 controls.

My null hypothesis is there would be no significant difference between the percent resistance of the American control and the first backcross hybrid families. This would reveal that no PRR resistance alleles would be passed down from the original Chinese parent *C. mollissima* to the first backcross hybrid progeny. In this scenario, all first backcross families would show no sign of root rot resistance, and all individuals would show symptoms of root rot. My alternative hypothesis is that all families would display intermediate resistance to root rot. In this situation, a 50/50 ratio of resistant to symptomatic trees would be observed. In my analyses, I accepted P values less than 0.05 to be significant (i.e. $\alpha = 0.05$).

III. Results

The proportion of root rot within families varied, from no symptoms in the two Asian species (*C. mollissima and C. henryi*), to 75% symptomatic in the American species (*C. dentata*). The BC1 hybrid backcross families, although they averaged about 58.02% symptomatic (41.98% resistant), also varied in their response to the pathogen. Overall, 246 of the 424 backcross hybrids were symptomatic. Results of the root necrosis screening are presented in a cross tabulation table **(Table 2)** and in a histogram **(Figure 3)**. Some of the families had higher percent resistance than other families. The most resistant families were **UTC 1** (85.2% resistant), **UTC 12** (83.3% resistant) and **UTC14** (73.5% resistant). These families deviated from the expected value of 50% resistance. Both the Chinese control and *C. henryi* were 100% resistant. The most susceptible families were **UTC2** (52.5% susceptible) and **UTC 3** (51.7% susceptible). They only slightly deviated from the expected value of 50% susceptibility.

Table 2: Cross Tabulation of Pedigree vs Resistance: A score of 0 represents resistant plants, and a score of 1 represents plants symptomatic of root rot. Root rot scores of "1", "2", and "3" were compiled to represent plants symptomatic of root rot.

Pedigree		Resistant	Symptomatic	Sample Size
American Control (UTC-17)	Count	3	9	12
	% within Pedigree	25.0%	75.0%	
Chinese Control (UTC-16)	Count	11	0	11
	% within Pedigree	100.0%	0.0%	
Henry's Chinkapin (UTC-18)	Count	17	0	17
	% within Pedigree	100.0%	0.0%	
Mcinturff FF-1 x OP (UTC-2)	Count	38	42	80
	% within Pedigree	47.5%	52.5%	
Neel 3-262 x TN Carroll County #1 (UTC-14)	Count	36	13	49
	% within Pedigree	73.5%	26.5%	
Neel 5-238 x ALA Frames 1 (UTC-13)	Count	13	10	23
	% within Pedigree	56.5%	43.5%	
TN MAC1 x Neel 2-127 (UTC-8)	Count	15	12	27
	% within Pedigree	55.6%	44.4%	
TN MAC1 x Neel 4-195 (UTC-6)	Count	24	16	40
	% within Pedigree	60.0%	40.0%	
TN MAC1 x SAMS ? (UTC-9)	Count	3	3	6
	% within Pedigree	50.0%	50.0%	
TTU A-4 x ALA Frames 1 (UTC-1)	Count	23	4	27
	% within Pedigree	85.2%	14.8%	
TTU A-4 x ALA Frames 4 (UTC-3)	Count	14	15	29
	% within Pedigree	48.3%	51.7%	
TTU A-4 x ALA Frames 5 (UTC-12)	Count	10	2	12
	% within Pedigree	83.3%	16.7%	
TTU A-4 x OP (UTC-4)	Count	61	55	116
	% within Pedigree	52.7%	47.3%	
TTU SUM1 x Neel 6-193 (UTC-5)	Count	9	6	15
	% within Pedigree	60.0%	40.0%	
Total	Count	(all families) 274 (BC1 families) 243 (all families)	(all families) 184 (BC1 families) 175 (all families)	458
	% within Pedigree	59.8% (BC1 families) 58.13%	40.2% (BC1 families) 41.87%	

Each subscript letter denotes a subset of 0 categories whose column proportions do not differ significantly from each other at the .05 level.

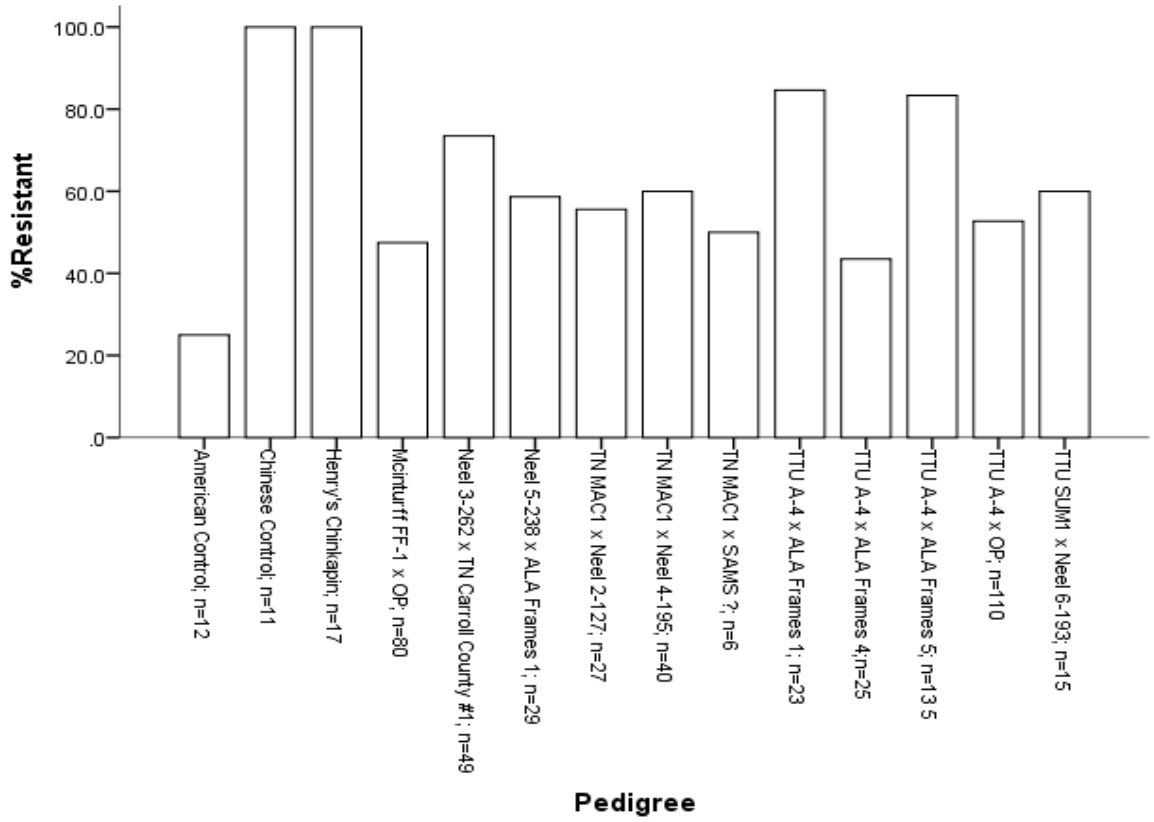

Figure 3: Percent of Resistant Individuals by Family: Bars represent families and are defined by the percentage of individuals within each family that were rated as "0" (asymptomatic). Error bars cannot be included when measuring percentages within predictor variables. Root rot scores of "1", "2", and "3" were grouped together as symptomatic. Sample sizes of families are represented as "*n* = ".

The Chi-square analysis in **Table 3** shows a statistical difference between families. (χ^2 = 48.16; df= 13; p = 0.00).The results from pairwise comparisons of all backcross hybrid families to the three controls are presented in **Table 4. UTC1** (χ^2 =13.542; df= 1; p = 0.00), **UTC6** (χ^2 =4.530; df= 1; p = 0.033) **UTC12** (χ^2 = 8.224; df= 1; p = 0.004), and **UTC14** (χ^2 = 4.821; df= 1; p = 0.002) differ statistically from the American control. **UTC1** (χ^2 = 1.821; df= 1; p = 0.177), **UTC12** (χ^2 = 2.008; df= 1; p = 0.156), and **UTC 14** (χ^2 = 3.726; df= 1; p = 0.540) differ statistically from the Chinese control. Only **UTC1** (χ^2 =

2.770;df=1; p = 0.096)and **UTC12** (χ^2 = 3.043; df=1; p = 0.81) are statistically different from *C. henryi*.

Table 3: Chi-Square Evaluation for Differences Among Families

	χ^2 Value	Degrees of freedom	P-value
Pearson Chi-Square	4.160	13	p = 0.000

This chi-square analysis measures for a significant difference of resistance among the all families tested. This is including the 12 different first backcross hybrid families and the 3 control groups.

Table 4: Pairwise Chi-Square Analyses

Family	American Control	Chinese Control	*C. henryi*
UTC1 TTU A-4 x ALA Frames 1	χ^2 = 13.542; p= 0.00	χ^2 = 1.821; p = 0.177	χ^2 = 2.770; p = 0.096
UTC2 Mcinturff FF-1 x OP	χ^2 = 2.138; p = 0.144	χ^2 = 10.725; p = 0.001	χ^2 = 15.740; p = 0.000
UTC3 TTU A-4 x ALA Frames 4	χ^2 = 1.895; p = 0.169	χ^2 = 9.103; p = 0.003	χ^2 = 13.048; p = 0.000
UTC4 TTU A-4 x OP	χ^2 = 3.310; p = 0.69	χ^2 = 9.529; p = 0.002	χ^2 = 13.744; p = 0.000
UTC5 TTU SUM1 x Neel 6-193	χ^2 = 3.308; p = 0.69	χ^2 = 5.720; p = 0.017	χ^2 = 8.369; p = 0.004
UTC6 TN MAC1 x Neel 4-195	χ^2 = 4.530; p 0.033	χ^2 = 6.411; p = 0.011	χ^2 = 9.454; p = 0.002
UTC8 TN MAC1 x Neel 2-127	χ^2 = 3.121; p = 0.077	χ^2 = 7.145; p = 0.008	χ^2 = 10.389; p = 0.001
UTC9 TN MAC1 x SAMS ?	χ^2 = 1.125; p = 0.289	χ^2 = 6.679; p =0.010	χ^2 = 9.775; p = 0.002
UTC12 TTU A-4 x ALA Frames 5	χ^2 = 8.224; p = 0.004	χ^2 = 2.008; p = 0.156	χ^2 = 3.043; p = 0.81
UTC13 Neel 5-238 x ALA Frames 1	χ^2 = 3.157; p = 0.076	χ^2 = 6.775; p = 0.009	χ^2 = 9.855; p = 0.002
UTC14 Neel 3-262 x TN Carroll County #1	χ^2 = 4.821; p = 0.002	χ^2 = 3.726; p = 0.540	χ^2 = 5.616; p = 0.018

Chi-square analyses were performed between each first backcross hybrid family and each control group. χ^2 = Chi-square value. For each analysis, the degrees of freedom = 1. Chi-square analyses against the American control detect families that are statistically more resistant than the control for resistance, and chi-square analyses between the Chinese control and *C. henryi* detect families who are not statistically less resistant than the resistant control(s).

Table 5 shows the Chi-Square tests done for each tub to evaluate for significant differences across the tubs. **Tub 1** was significantly different from the other tubs (χ^2 = 31.623; df= 13; p = 0.03). **Tub 4** was also significantly different from the other tubs (χ^2 = 33.894;df = 13; p = 0.001). Tubs 2, 3, and 5 were not determined to be significantly different from the other tubs. Thus, a significant tub effect was present, meaning either environmental variation was not eliminated or improper procedure was performed.

Table 5: Chi-Square Evaluation of Tubs

Tub	Pearson Chi-Square	Degrees of freedom	P value
1.0	χ^2 = 31.643 n= 78	13	p = 0.003
2.0	χ^2 = 21.046 n= 98	13	p = 0.072
3.0	χ^2 = 13.901 n= 95	12	p = 0.307
4.0	χ^2 = 33.894 n=100	13	p = 0.001
5.0	χ^2 = 14.370 n=86	12	p = 0.278
Total	χ^2 = 48.408 n=457	13	p=0.000

This table contains results from a chi-square analysis measuring for significant differences across the tubs. Each tub represents a block in the randomized block design.

IV. **Discussion**

a. Overview

The aim of this study was to screen 15 first backcross (BC1) hybrid chestnut families for resistance to Phytophthora root rot (PRR) as part of the Tennessee Chapter of The American Chestnut Foundation's effort to breed PRR-resistant American type hybrids for chestnut restoration in the Southeast. We expected the BC1 families to demonstrate approximately 50% resistance, and we expected all families to be significantly different from the American control group, Chinese control group, and *C. henryi*. The families we determined to vary the most from these expected ratios were UTC-1 [TTU A-4 x ALA Frames], UTC-12 [TTU A-4 x ALA Frames 5], and UTC-14 [Neel 3-262 x TN Carroll County #1]. These families were determined to be significantly less susceptible than the American chestnut control group but not significantly less resistant than the Chinese control group (*C. mollissima*). Thus, they displayed a higher ratio of resistance than expected. This variance from expected values may be a result of chance or a result of improper procedure. However, it is also possible that the American parents involved in the backcross contributed alleles for resistance, thus increasing the resistance ratio.

Though many of the families were significantly less resistant than both the Chinese control and *C. henryi*, all families displayed some level of resistance. Most families were approximately 50% resistant. Due to the nature of this study, a family does not need to display 100% resistance to *Phytophthora cinnamomi* to be valuable in our study. Any family that displays a degree of resistance illustrates that the root rot resistance allele was passed down from *C. mollissima* during the first cross generating the F1 generation.

Testcross families with 50% resistance suggest that traits for root rot resistance were simply inherited from the Chinese source as dominant alleles. The F1s must have been all heterozygous, which led to the approximately 1:1 segregation in the testcross (1st backcross) to American.

b. *C. henryi*

To our knowledge, no information on *Phytophthora* root rot resistance in *C. henryi* (Henry Chinkapin of China) has been published up to this point. Our study suggests that *C. henryi* carries alleles for resistance to root rot. According to our study, 100% of individuals of *C. henryi* displayed resistance to root rot. It is thus possible that *C. henryi* is homozygous resistant at the locus (or multiple loci) for root rot resistance. This information suggests that *C. henryi* may be a valuable player in the pursuit of root rot resistance in hybrid American chestnuts. The root rot resistance alleles have the potential to be moved from *C. henryi* to backcross progeny.

c. Critique of Methods

The Chi-Square analysis evaluating for significant differences among the 5 tubs reveals that tubs 2 and 4 were significantly different from the other 3 tubs. This discrepancy has many possible explanations. It may be a result of tub effect, imperfect procedure, or environmental variation. It is possible that the inoculum was improperly distributed in these 2 tubs at the time of inoculation. The inoculum may have also not spread well from the points original points of inoculation. It is also possible that the tubs received unequal amounts of sunlight or precipitation.

The tub effect observed (**Table 5)** suggests there are various aspects of this study design which could be improved. A future repetition of this study should better control for environmental variation. Though the tubs were painted white to avoid edge effect, it is still possible that edge effect occurred in our experimental design. To better avoid the confounding variable of uneven heating within the tub, the tubs should be shaded or the tub design could be set up inside a greenhouse with more consistent climate conditions. The timing of procedures of this study was approximately one month postponed. In the future, the seeds should be planted about a month earlier (early April). Inoculation should occur in late July or early August. We suspect that our timing of root rot disease screening was sufficient, and that disease screening should occur shortly after the soil within the tubs begins to thaw.

It is likely that the inoculation procedure may have been the greatest limitation of our study. The tub effect observed in our study suggests that all plants may not have been properly inoculated. Furthermore, we expected to see 100% susceptibility in American controls, but only 25% were given disease ratings of "0". The most probable explanation is that the inoculum did not take well where these specific American controls were located in the tub. To avoid this, the tubs should receive multiple inoculums and/or receive inoculums at a greater volume.

Another limitation of our study is the sample sizes across families. Unfortunately, we had little control over this variable. We collected as many seeds as possible from each first backcross hybrid family, but many families had only few seeds. Furthermore, some of these seeds did not develop into trees. An ideal version of this experimental setup would

include at least five individuals per family per tub and would have more space between each seed in the tub to avoid competition between individuals.

d. Future Directions.

i. Stem Assay. Alternative to our experiment design, a stem assay could be used to evaluate expression of *Phytophthora* root rot. Excised stem segments of first backcross hybrid families could be collected and then inoculated with *P. cinnamomi*. The stem segment would be evaluated for the expression of root rot. This would be a quicker method used to distinguish between susceptible and resistant individuals. Unlike our experimental setup, this method would be nondestructive to the first backcross hybrid test subjects and would allow for the test subjects to be utilized in future research. Specifically, it would allow us to screen for first backcross progeny for root rot resistance and then screen the same progeny for Chestnut blight resistance, fulfilling the ultimate goal of breeding American chestnut hybrids with resistance to both Chestnut blight and *Phytophthora* root rot.

ii. LG_E locus

The data from this study have the potential to be screened for the presence of molecular marker on the the LG_E linkage group which is believed to control for root rot resistance. In the future, tissue samples from resistant individuals from our first-backcross progeny could be used in QTL mapping to determine if their alleles match the resistant plants evaluated in Zhebentyayeva's (2014) study. However, identical crosses would need to be performed and phenotyped and genotyped for resistance over multiple years in order to control for environmental variation, and funding would need to be acquired (Zhebentyayeva 2014).

iii. Allatoona. In total, 165 individuals from this study were sent to Allatoona Lake Chestnut Restoration Project. We plan to inoculate these root rot survivors with chestnut blight in approximately five years and perform a second backcross. Based on prior work at the first backcross level, we expect 1/8 to have adequate blight resistance. The goal of this project is to breed an American chestnut hybrid that has nearly identical characteristics as *C. dentata* as well as both Chestnut blight and root rot resistance.

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Appendix

V-8 Vermiculite Inoculum for *Phytophthora* Root Rot Experiments Protocol for preparation of V8 vermiculite of the *Phytophthora* inoculum followed that used by the Jeffer's lab at Clemson:

1. Recover isolates of *Phytophthora* sp. on plates of PAR(PH) medium at 25 degrees Celsius, transfer to 10% clarified V8 agar (cV8a) to get actively growing-colonies on a non-amended medium; check for purity; bacteria can lurk among hyphae growing on selective medium.
2. Use a standard ratio of V8B to vermiculite (1 part V8 broth: 2 parts fine textured vermiculite).
3. Prepare enough 10% V8 broth for the volume of vermiculite to be moistened; recipe for 1.0 liter: 100 ml of V8 juice + 1.0g $CaCO_3$ + 900 ml distilled water; do not sterilize before use.
4. Use fine textured, horticultural-grade vermiculite; store in a dry place.
5. Pyrex bottles work best for growing inoculum as these can be autoclaved repeatedly; usually we use 300-500 ml of vermiculite per bottles.
    a. We used 9 pyrex bottles with 300ml of vermiculite in each bottle. We used 1350mL of V8 broth
6. Place bulk vermiculite in an aluminum pan and covered with foil.
7. To each bottle, add 300 ml of vermiculite + 150 ml non-sterile V8 broth; place lids loosely on bottles to allow for ventilation during autoclaving – do *not* tighten or pressure will break bottles. Cover tops and necks of bottles with aluminum foil.

8. Autoclave bottles for 45 min; remove bottles from autoclave soon after autoclaving to prevent evaporation of V8 broth and subsequent desiccation of vermiculite.
9. The following day, autoclave bottles for another 45 min, following same protocols.
10. When bottles have completely cooled – aseptically seed each bottles with three 5-mm plugs of an isolate and replace lids and foil on bottles – lids should be tightened and then opened ¼-turn; be sure isolates are clean by growing on a non-selective medium (cV8a)
11. Incubate cultures in bottles at 25 degrees Celsius (dark) for 10 days.
12. Every other day, tighten lids on bottles and carefully shake bottles to evenly distribute mycelium and encourage uniform colonization of vermiculite; be careful to not get vermiculite on the lids or near the lip of the bottles; open lids ¼ turn and return bottles to incubator.
13. Before using inoculum, check each bottles for purity
    a. Aseptically in a laminar-flow hood – remove foil and lid from each bottles – one at a time
    b. With a sterile spatula, remove a small amount of vermiculite and sprinkle it on a plate of non-amended cV8a – without antibiotics
    c. Place these plates at 25 degrees Celsius for 24-72 hr – check for growth of *Phytophthora* from each and every piece of vermiculite and for any evidence of contamination