5-2018

# Implementing early screening methods to detect resistance to Phytophthora cinnamomi in backcross Chinese-American Chestnut hybrids

Kirsten Hein
*University of Tennessee at Chattanooga*, gvp681@mocs.utc.edu

# Implementing Early Screening Methods to Detect Resistance to *Phytophthora cinnamomi* in Backcross Chinese-American Chestnut Hybrids

Kirsten Hein

Departmental Thesis

The University of Tennessee at Chattanooga

Department of Biology, Geology, and Environmental Science

April 2018

Dr. J. Hill Craddock
UC Foundation Robert M. Davenport Professor
Biology, Geology and Environmental Science
Thesis Director

Dr. Ethan Carver
UC Foundation Collins Professor;
Assistant Dean of the Graduate School
Biology, Geology, and Environmental Science
Department Examiner

Dr. Jennifer Boyd
Associate Professor
Biology, Geology and Environmental Science
Department Examiner

## Table of Contents

# List of Figures and Tables

**Acknowledgements**

I would like to express my deepest appreciation to my thesis director, Dr. J. Hill Craddock, for his continuous support of my study and research, for his patience, motivation, enthusiasm, and knowledge. I would also like to thank the rest of my committee and co-authors, Dr. Ethan Carver, Dr. Jennifer Boyd, and Taylor Perkins, for their encouragement, insightful comments, guidance, and time spent towards my project. I am also appreciative for my friends and family who have supported me throughout this process.

I wish to thank The University of Tennessee at Chattanooga, Department of Biological and Environmental Sciences faculty and staff for their help and support.

Additionally, I thank The American Chestnut Foundation, University Honors Program, the Fortwood Street greenhouse crew, and the Tucker Foundation.

## Abstract

*Phytophthora* root rot (PRR), caused by the oomycete *Phytophthora cinnamomi* Rands., is one of the two greatest obstacles to survival of American chestnut (*Castanea dentata* Borkh.). The other is chestnut blight, caused by the ascomycete *Cryphonectria parasitica* (Murr.) Barr. Developing early and reliable PRR screening methods can facilitate the efficient introgression of PRR resistance from Chinese chestnut (*C. mollissima* Blume.) into the populations of potentially chestnut blight resistant trees currently under development by The American Chestnut Foundation (TACF). This study tests the efficacy of a method for early identification of PRR-resistant hybrid chestnuts in a greenhouse/nursery setting. The chestnut blight resistant hybrid families studied are derived from twenty years of work by the Tennessee Chapter of TACF and represent sources of PRR resistance not previously utilized by TACF. During the midsummer months of 2016 and 2017, container-grown seedlings were inoculated with *P. cinnamomi,* prepared on a clarified V8 agar medium, and rice-grain or vermiculite inoculum. Root necrotic lesions were rated using a numerical scale from 0-3, with "0" representing a plant that is completely asymptomatic, "1" representing lesions on the lateral roots only, "2" representing lesions on the lateral and on tap roots, and "3" representing plants killed by PRR (Jeffers et al., 2009). Results of the 2017 were inconclusive because of problems preparing the inoculum, and extreme environmental conditions in the early winter (freeze damage to the roots in December confounded root necrosis observations in January and February). The results of the 2016 trial show, at a 0.05 significance level, statistically significant differences in the average root rating between *C. mollissima* and *C. dentata*. Significant differences were seen in the average root rating between some hybrid families, intragenerationally and intergenerationally. Six of the backcross hybrid families; were not significantly different from the average root rating in the Chinese chestnut control families. The were no statistically significant differences between generations (B1, BB1, and B3F2). Families that were interpreted to have any degree of PRR resistance are assumed to have inherited PRR resistance alleles from *C. mollissima*. Trees identified as PRR-resistance will be transplanted into orchard settings for further observation, and represent a population of crosses that will be utilized for future breeding and restoration efforts.

# Chapter 1

# Introduction and Background

## 1.1 *Castanea dentata*: The American Chestnut

### 1.1.1 Description and Biogeography of Chestnut

*Castanea,* a genus within the family Fagaceae, is comprised of seven or so species with their native ranges dispersed throughout the northern hemisphere (Faison and Foster 2014). At least two of the species are found in North America (*C. dentata* Borkh. and *C. pumila* Mill.) (Johnson, 1988). The American Chestnut tree, *C. dentata*. is a monoecious deciduous tree that averaged 90 to 120 cm in diameter and 25 to 40 m in height (Burnham, 1988). However, during the late 19$^{th}$ and early 20$^{th}$ centuries, the introduction of the nonnative chestnut blight fungus (*Cryphonectria parasitica* (Murr.) Barr.) and root pathogen (*Phytophthora cinnamomi* Rands.) nearly eradicated the species from the North American forests. Due to its pathogenic stressors, nowadays the American chestnut typically grows as a multi-stemmed shrub that rarely exceeds a few meters (Faison and Foster, 2014).

In the era preceding its decimation by invasive pathogens, *C. dentata* was one of the most ecologically and economically significant trees in the eastern deciduous forests of North America (Lutts, 2004; Davis, 2006). Throughout 80 million ha of land, the American chestnut tree dominated the canopy in forests ranging from Maine down to Mississippi **(**MacDonald, 1978). Chestnut biogeography is a function of biotic factors, abiotic factors, and dispersal factors (Peterson, 2011). Since the chestnut's limited ecological amplitude

and high-water requirements, the species grew predominately on north-facing sloping topography (Faison and Foster, 2014).

The spread of chestnut was dependent on its success with seed distribution, seed establishment, and reproduction. *C. dentata* bears three nuts per spiny, green burr, and is a delectable food source to a wide variety of animals. These animals function as major dispersers of the chestnut fruit, among other environmental dispersal methods. Seedling establishment in the pre-blight forests depended on the numbers of viable seeds that survived dispersal, and on adequately moist soil conditions (Russell, 1987). Chestnut species blossom in the late spring to early summer, and they are typically wind and insect-pollinated (Fei et al., 2012). Though chestnuts are monoecious, they are self-sterile; meaning they require another tree (within ~100 m) for fertilization to occur (Forest, 1978; Cook and Forest, 1979).

### 1.1.2 The American Chestnut: A True Heritage Tree

Historically, the American chestnut tree bore an extensive ecological, economic, and cultural importance in the Appalachian region (Fei et al., 2012). As a foundational species, *C. dentata* established locally stable environmental conditions within its ecological community. The fruit of *C. dentata* was composed of the highest carbohydrate content of eastern American fruit-producing species, coupled with high quality protein and low-fat content (McCarthy and Meredith, 1988). Many game species, such as: wild turkey, squirrels, and black bears relied on chestnut as their primary food source (Davis, 2006). According to biologist James M. Hill, the decline of the American chestnut

directly contributed to the slowed recovery of wildlife populations that suffered from habitat degradation by the logging industry (Hill, 1993).

Native populations of mountain people culturally relied on chestnut as an important dietary supplement and as a marketable crop (Baxter, 2009). Families would routinely hang sacks of chestnuts on nails near their kitchens, ready to be roasted over a warm fireplace (Davis, 2006). In some areas, families would use their chestnut harvest earnings as "shoe money" to purchase their children shoes in preparation for the upcoming winter weather (Brown and Davis, 1992; Condon, 1994). Additionally, rural families depended on the products of the American chestnut as a source of income from major cities distributed across the eastern seaboard. In the early 20$^{th}$ century, records have shown that tens of thousands of pounds of chestnuts were routinely shipped to destinations along the northern train routes (Giddings, 1912; Kulman, 1978).

In 1909, the United States chestnut timber industry was valued to be more than 20 million dollars (Davis, 2006). As building material, chestnut wood was durable, highly-rot resistant and aesthetically pleasing. For example, when it was available for timber, chestnut wood was often used for roofing shingles, telephone poles, railroad ties, home paneling, and interior and exterior furniture. Furthermore, the leather industry exploited the tannins of chestnut wood and bark to treat its leather for decay resistance and solidity (Anagnostakis, 1987). The introduction and spread of the fungal blight triggered a socioeconomic disaster for both commercial operations and rural Appalachian natives. All things considered, the dying chestnut trees were exploited for their tannins by the

southern Appalachian leather industry, eventually producing over one-half of the U.S. supply of vegetable based tannins (Davis, 2006). After a decade of blight overexploitation and environmental strains, the American chestnut tree was virtually eradicated from the canopy of southern Appalachian forest.

## *1.2 Phytophthora* Root Rot

### 1.2.1 Introduction of *Phytophthora* to the Americas

Even prior to the chestnut blight pandemic, the population decline of the American Chestnut was amplified by the introduction of the oomycete pathogen, *Phytophthora cinnamomi*. It is hypothesized that *P. cinnamomi* was introduced in the late 18$^{th}$ century into the southeast region of the United States from Asia by vessel, and spread as colonial populations migrated inland (Zentmeyer, 1980). The first documented account of PRR symptoms (or ink disease) on a North American *Castanea* species dates to 1824 in Georgia (Gravatt and Crandall, 1945). In 1922, about one-hundred years after PRR was initially observed in the United States, R.D. Rands isolated *P. cinnamomi* from cinnamon trees in Southeast Asia. Consequently, in 1931, B.S. Crandall characterized *P. cinnamomi* as the causal agent of the root necrosis on *C. dentata* (Gravatt and Crandall, 1945).

### 1.2.2 *Phytophthora cinnamomi*: An Invasive Microbe

Currently, the distribution and host range of *P. cinnamomi* affects approximately 5000 species world-wide **(**Cahill et al., 2008; Jung et al., 2013). A few of the economically important plant species that demonstrate susceptibility to disease caused by the

microorganism are: chestnut, avocado, kiwi, oak, fir, pine, eucalyptus, and cinnamon. The inadvertent introduction of the pathogen into natural ecosystems, including known Global Biodiversity Hotspots, has caused devastating consequences for the biodiversity of flora and fauna **(**Hardham and Blackman, 2017). In addition, significant economic losses in agriculture and forestry caused by *P. cinnamomi* lead to its inclusion in the Key Threatening Processes in the Commonwealth Environmental Protection & Biodiversity Conservation Act 1999, and subsequently to the development of a National Threat Abatement Plan to manage and control of the impact of the disease (Australian Government, 2014). *Phytophthora* is a genus in the Class Oomycetes that acts as either a saprobic or parasitic fungus-like microorganism (Zentmeyer, 1980). In chestnut, *Phytophthora* is known to cause root rot, collar rot, defoliation, reduced vigor, and heightened mortality. *P. cinnamomi* is a soil-borne pathogen that goes through sexual and asexual phases throughout its lifecycle **(**Zentmeyer, 1980**).** The pathogen functions optimally in warm, saturated soil environments because the bi-flagellated motile zoospores are the main infective agent for plant disease. When these conditions are present, either the mycelium or chlamydospores will produce sporangia that will eventually germinate and release zoospores. The zoospores are directed through the soil by their chemical attraction to the roots of the host organism **(**Sidebottom et al., 2004). Once it reaches its host, *P. cinnamomi* penetrates the epidermal cells of the root and spreads its mycelia inside and around the host tissue. As its mycelia spreads throughout the root system, the roots begin to rot, interfering with water uptake, which results in wilting and defoliation. Sexual oospores, asexual chlamydospores, and intracellular

hyphal aggregates contribute to the long-term survival of the pathogen, making complete eradication of a species especially difficult to accomplish **(**Jung et al., 2013).

### 1.2.3 Disease Management

According to Hardham and Blackman (2017), to successfully predict, control, and manage the disease requires a thorough understanding of the pathogen's cellular biology and pathogenicity, methods for early detection and control, and knowledge of resistant plant cultivars. Since oomycetes are not phylogenetically true fungi, the application of fungicides has proven to be an ineffective method of controlling *Phytophthora* species. Two common methods used to chemically control *P. cinnamomi* are treatments with metalaxyl and phosphite. Phosphite not only inhibits the growth of the pathogen, but enhances the plant's defense mechanisms as well. However, research has shown that these treatments also inhibit the colonization of ectomycorrhizal fungi that are symbiotically beneficial for *C. dentata* and other plant species **(**Perkins, 2012**).** *P. cinnamomi*'s ability to persist in soil over an extended period, coupled with the degree of plant susceptibility, poses a challenge for identifying biological control agents for *P. cinnamomi*. Studies on the management of avocado root rot by incorporating a combination of metalaxyl, mancozeb, and silicate, with phosphite injection and organic mulches have reported reduced disease occurrence and increase of crop production by 70% and 44%, respectively (Hardham and Blackman**,** 2017). Selectively planting in well-drained sites and the incorporating ectomycorrhizal fungi on the roots of *C. dentata* have been used to manage the spread of PRR because the fungal hyphae form a mantle that encloses and protects the roots against *P. cinnamomi* (Branzanti et al., 1999). In

conclusion, there are a variety of methods used to control the spread and infection by *P. cinnamomi*; however, they have been observed to be only mildly effective at suppressing disease symptoms.

## 1.3 Restoration of the American Chestnut

### 1.3.1 The American Chestnut Foundation

The mission of the American Chestnut Foundation (TACF) is to reintroduce the extirpated *Castanea* species back into its historical range, without compromising the structural and functional complexity of intact ecosystems (acf.org, 2018). Even prior to the work implemented by TACF, the U.S Department of Agriculture and the Connecticut Agricultural Experiment Station notoriously launched programs of breeding for blight resistance soon after the blight arrived in the U.S. in 1904 (acf.org, 2018). Years later, TACF was founded in 1983 by a group of plant and citizen scientists who recognized the importance in restoring the American chestnut tree to its native range within the forests of the eastern United States (acf.org, 2018). In 1989, the Wagner Research Farm in Meadowview, Virginia was established as a breeding station to implement the backcross breeding methodology developed by Philip Rutter, Dr. David French and Dr. Charles Burnham (acf.org, 2018). Their objective was to breed blight resistant Chinese-American chestnut hybrids, while maintaining the physical characteristics of the American chestnut. Today, the three research tracks TACF integrates into its restoration efforts include: breeding, biotechnology, and bio-control.

### 1.3.2 The Backcross Breeding Program

The methodology behind the backcross breeding program, employed by TACF, was proposed by Dr. Charles Burnham (acf.org, 2018). The program involves crossing the American species with the blight resistant Asiatic chestnut, including: 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. To date, most TACF hybrids descend from Chinese-American crosses. Successive crosses are made back to *C. dentata*, with each generation of backcross halving the proportion of the Chinese genome inherited (Figure 1).

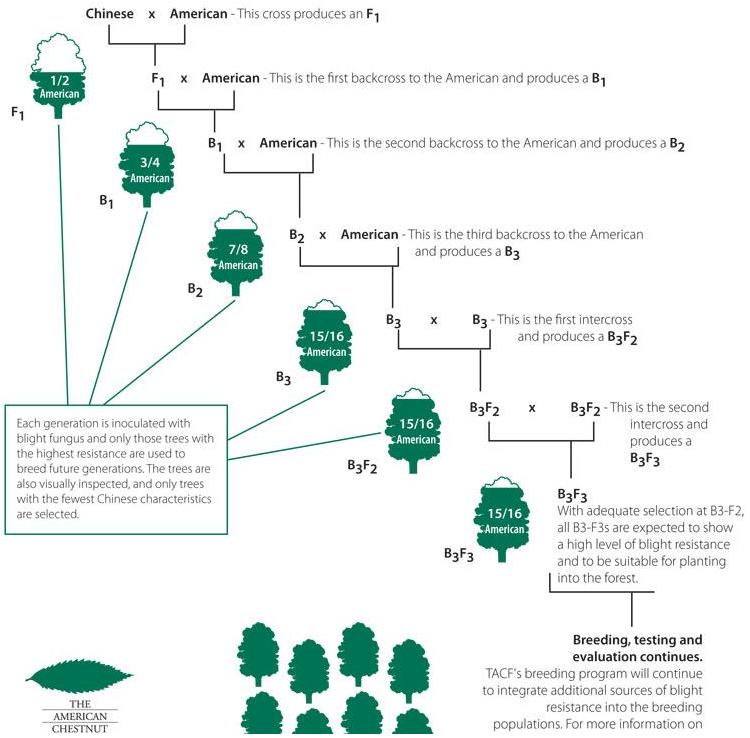


**Figure 1: A schematic of the TACF backcross breeding program for integrating disease resistance into breeding populations of chestnut.** www.acf.org

The genes that confer blight resistance in the chestnut genome are expected to be heterozygous in the backcross hybrids; meaning each offspring inherited one copy of a

resistance allele from the Chinese or hybrid parent and one copy of a susceptible allele from the American parent (Perkins et al., 2017). Burnham's hypothesis assumes that if blight resistance is an incompletely dominant trait, individuals that are heterozygous for the resistance allele will express an intermediate level of resistance. By the third-backcross generation (B3), the resulting chestnut hybrids are expected to have disease resistance and 15/16s American chestnut in their genomic composition, based on Mendelian genetics (Smith, 2012). In TACF's backcross breeding program, each generation is artificially exposed to the blight fungus, and the progeny that are identified as highly resistant are selected for future crosses. To ensure the trees are bred for local adaption, TACF is divided into state chapters that each breed separately. The provenance of the parents is important to the success of the program because the southern populations of chestnut are very diverse (Perkins, 2016). More recently, TACF has expanded its breeding program to backcross and select for PRR resistance. Integrating PRR resistance into the breeding populations of the blight resistant backcross hybrids is important in order to produce individuals that carry genes that confer multiple disease resistance at different loci.

### 1.3.3 Biotechnology

The biotechnology program, developed in part by researchers from the State University of New York, College of Environmental Science and Forestry (SUNY-ESF) and the New York chapter of TACF, integrates the current backcross breeding program with types of transgenic and possibly soon CRISPR-based trees (Zhang et al., 2013). Genetic engineering and molecular biology has provided the tools to test individual genes in the

American chestnut that might correlate to pathogen resistance. Researchers at SUNY-ESF have identified a gene that has been found to enhance the tree's blight resistance ability by producing an oxalate detoxifying enzyme (Zhang et al., 2013). The trees selected for restoration purposes will be evaluated by the following: effective blight resistance, safety to humans and the environment, and traditional American chestnut growth characteristics. To effectively reintroduce the American chestnut tree to its historical native range, the trees will need to confer PRR resistance as well. Identifying PRR resistant trees, and subsequently crossing them with the blight resistant transgenic trees, will allow stacking of genes that confer multiple disease resistance in the resulting progeny with a higher proportion of the American chestnut genome. (Zhang et al., 2013).

## 1.4 Screening for Disease Resistance

### 1.4.1 Overview PRR Screening Methods

Disease screening through phenotypic evaluations can be used to determine how a host plant physiologically responds to disease pressures and to measure symptom severity of a disease in a host plant (Russell, 1978). Arbitrary symptom severity scales, usually dependent on pathological system being studied, are developed to easily assess how the host plant responds to the pathogenic pressures that are visible to the naked eye. To effectively implement this methodology in an experimental design, the scale must clearly define the difference between each rating value and the scale must account for the full range of symptoms exhibited by the host organism (Russell, 1978). To date, TACF has been developing a symptom severity scale to reliably assess the expression of PRR in

chestnut by phenotypic evaluation. The hybrids identified to have PRR-resistance represent a population of crosses that will be utilized for future breeding and restoration efforts. PRR disease screening occurs in the late winter to ensure that the inoculated seedlings are dormant in order to minimize the effects attributed by root exposure. The most common scale, utilized by TACF, to evaluate PRR-resistance is a numerical scale from 0-3, with "0" representing a plant that is completely asymptomatic, "1" representing lesions on the lateral roots only, "2" representing lesions on the lateral and on tap roots, and "3" representing plants killed by PRR (Jeffers et al., 2009). More recently, Suzy Sharpe and Dr. Steven Jeffers used a 0 to 5 numerical scale to score the trees based on phenotypic observations: where 0 = healthy—no evidence of wilt, 1 = 1-10% of foliage with symptoms, 2 = 11-50% of foliage with symptoms, 3 = 51-90% of foliage with symptoms, 4 = 91-99% of foliage with symptoms, and 5 =100% of foliage with symptoms—plant dead (Sharpe, 2017). In addition to the foliage assessments, the roots were given a symptom severity score of 0 to 5 based on the levels of root necrosis caused by *P*. *cinnamomi*: where 0 = 0%, roots healthy, 1 = 1-10%, 2 = 11-50%, 3 = 51-90%, 4 = 91-99%, and 5 = 100%, all roots dead (Sharpe, 2017). Additional research should be considered to test efficacy and consistency of the results generated between the two PRR symptom severity scales.

### 1.4.2 Importance of Early Screening

To effectively breed and manage the chestnut backcross orchards, a considerable amount of time, labor, and money is required to sustain the efforts that go into germplasm conservation and species restoration. Eliminating the individuals that are symptomatic

before orchard establishment and planting only the individuals that exhibit PRR-resistance and chestnut blight resistance as seedlings will expedite field studies and selection. In addition, developing early and reliable screening methods can facilitate better ways to study the inheritance of multiple disease resistance genes simultaneously (Ousmane et al., 2005). Finally, by selecting crosses that demonstrate high disease resistance through early screening, researchers can selectively determine which crosses they should utilize for molecular analyses.

# Chapter 2

## Materials and Methods

In this experiment, different methodologies were implemented in the trials conducted in the 2016-2017 growing season and the 2017-2018 growing season. However, the methods and results will only be presented for the 2016-2017 season due to various complications that are reviewed in Chapter 4.

### 2.1 Isolation of *Phytophthora* from Orchard Soils

On April 22, 2015, soil samples from *P. cinnamomi* symptomatic orchards were collected by Taylor Perkins, Hill Craddock, and Anna Claire Robinson from Sam McInturff's farms in Blount County, Tennessee. The two orchards (Sam's I and II) are backcross orchards that have been maintained by volunteers of the Tennessee Chapter of the American Chestnut Foundation since the late 1990s and early 2000s. A second set of soil samples was collected from Bendabout Farm in Bradley County, Tennessee by Taylor and Cameron Perkins in April 2015. The farm is managed as a habitat conservation and game preserve, and is composed of several TACF backcross orchards. Dr. Steven N. Jeffers and Suzy Sharpe confirmed the presence of *P. cinnamomi* in 3 different locations in Sam McInturff's farm (Sam McInturff: Sam's I K-15, Sam's II DD-20, and Sam's II DD-5) and in two samples collected from Bendabout Farm Orchard 3. Taylor Perkins and Anna Claire Robinson isolated *P. cinnamomi* from soil samples where the microorganism was detected, and transferred the isolates to vials for permanent storage in Dr. Steven N Jeffers' lab. Reference Jeffers et al. (2008) for a thorough review of the methods used for soil collection and *Phytophthora* isolation and appendix I for the protocol for preparation of PAR(PH) medium and cV8A basal media of the *Phytophthora* inoculum.

## 2.2 Seed Planting and Experimental Setup

In February 2016, hybrid chestnut seeds of known and open-pollinated crosses from *C. dentata* and *C. mollissima* were planted into 656 mL D40 (Stuewe & Sons) containers and were organized into a completely randomized design. The experimental controls included one family of *Castanea dentata* (American chestnut) and one family of *C. mollissima* (Chinese chestnut). The source(s) of PRR-resistance alleles in each hybrid family is derived from novel *C. mollissima* cultivars not previously utilized by TACF to date, as well as *C. mollissima* cultivars that are currently utilized in the backcross breeding program. Information about the pedigrees, sources of resistance, and codes for each cross is listed in Table 1.

**Table 1: Crosses, Seed Types, and Identification Codes of the chestnut families inoculated with *P. cinnamomi* in 2016.** The crosses are depicted by the following orientation: female parent x male parent. In the greenhouse, each individual was tagged with a number that denotes which genetic family it belongs to, followed by a number that identifies the individual within the family. All of the backcross hybrid chestnuts were sourced by The American Chestnut Foundation.

| Cross | Seed Type | *Family Code | Source of Resistance | Pedigree of Mother | Pedigree of Father |
|---|---|---|---|---|---|
| *C. dentata* x opAm | Am | 1 | None | *C. dentata* x opAm | *C. dentata* x opAm |
| *C. mollissima* x opCh | Ch | 2 | *C. mollissima* x opCh (from Smith Farm Mix) | *C. mollissima* x opCh | *C. mollissima* x opCh |
| Cataloochee 2007 Tree 273 x TN-TTU-A29 | B1 | 3 | 'Gideon' | *C. dentata* x opAm | TNCLA1 x Gideon |
| Cataloochee 2007 Tree 33 x Neel 2-127 | B1 | 4 | 'Lindstrom-99' | *C. dentata* x opAm | 2004 TNRUT1 x Lindstrom99 |
| Cataloochee 2007 Tree 80 x Neel 4-195 | B1 | 5 | 'Amy' | *C. dentata* x opAm | 2004 TN-BF1-E10 x Amy |
| Cataloochee 2007 Tree 80 x Neel 8-192 | B1 | 6 | *C. mollissima* x opCh | *C. dentata* x opAm | 2004 TNLIN1 x opChinese |
| Cataloochee 2007 Tree 80 x Pryor Seed Orchard Tree 3-50 | B1 | 7 | *C. mollissima* x opCh | *C. dentata* x opAm | Old NC10 x opChinese |
| Cataloochee 2007 Tree 80 x TN-TTU-A30 | B1 | 8 | 'Gideon' | *C. dentata* x opAm | TNCLA1 x Gideon |
| GABE001-165 x GAHA14 | B1 | 9 | 'Lindstrom-67' | 2006 GAWA1 x Lindstrom67 | *C. dentata* x opAm |
| Kemp Orchard Mix x opB3 | B3F2 | 10 | 'Nanking' and 'Clapper' | 2008 TNMON8 x JB271 and 2006 TNMON4 x IL332 | N/A |
| Sam's 2-J mix x opB3 | B3F2 | 11 | 'Clapper' | 2007 TNMON8 x GR210 | OP B3 |
| TN-TTU-A30 x NCDOT American | B1 | 12 | 'Gideon' | TNCLA1 x Gideon | *C. dentata* x opAm |
| TN-TTU-A30 x Talladega #2 | B1 | 13 | 'Gideon' | TNCLA1 x Gideon | *C. dentata* x opAm |
| TN-TTU-A34 x NCDOT American | B1 | 14 | 'Gideon' | TNCLA1 x Gideon | *C. dentata* x opAm |
| TN-TTU-C27 x TN-TTU-A30 | BB1 | 15 | 'Clapper' and 'Gideon' | 2004 TNSUM1 x VA89 | 2004 TNCLA1 x Gideon |
| TN-TTU-E24 x TN-TTU-A30 | BB1 | 16 | 'Clapper' and 'Gideon' | 2004 TNSUM1 x VA89 | 2004 TNCLA1 x Gideon |
| TN-TTU-K2 x TN-TTU-A30 | BB1 | 17 | 'Clapper' and 'Gideon' | TNCLA1 x GL28 | TNCLA1 x Gideon |
| TN-TTU-L13 x TN-TTU-A30 | BB1 | 18 | 'Clapper' and 'Gideon' | TNCLA1 x GL28 | TNCLA1 x Gideon |
| TN-TTU-M13 x TN-TTU-A30 | BB1 | 19 | 'Graves' and 'Gideon' | TNCLA2 x AB248 | TNCLA1 x Gideon |
| TNCOC1 x TN-TTU-A30 | B1 | 20 | 'Gideon' | *C. dentata* x opAm | 2004 TNCLA1 x Gideon |
| W7-32-147 x opB3F2 | B3F3 | 21 | 'Graves' | Meadowview B3F2 | OP B3F2 |

KEY: 'OP' = open-pollinated, 'B' = backcross, 'BB' = better backcross, 'Ch'= Chinese chestnut, 'Am'= American chestnut, *See Appendix II for information regarding the family codes used to label the trees in the greenhouse.

The seeds were stored in a refrigerator with moistened peat moss inside a perforated plastic bag for stratification. In January 2016, the germinated seeds were washed and

planted into individual D40 containers, filled with commercially available potting medium (Sun Gro Metro-mix 360) and top dressed with a teaspoon of a complete encapsulated slow release fertilizer (Osmocote). The number of seeds planted for each cross-type was dependent on the number of nuts available during harvest. To control the spread of *Phytophthora*, the seedlings were arranged in a water-containment chamber that was constructed inside the greenhouse. The seedlings were watered and monitored daily for the remainder of the study (Figure 2).

**Figure 2: An image of the chestnut families in the water-containment chamber inside the Fortwood St. greenhouse.**

## 2.3 Inoculation of the Small Containers

Isolates of *P. cinnamomi* were recovered on plates of PAR(PH) medium at 25°C, and transferred to a clarified V8 agar (cV8A) to get actively-growing colonies on the medium. The inoculum of *P. cinnamomi* was created with sterilized rice grains: 1-part 10% cV8A: 2 parts of long-grain white rice in Pyrex bottles; bottles were autoclaved for sterility. After sterilization, three 5-mm PAR(PH) agar plugs of each isolate were added

to each bottle and were incubated at 25°C, in the dark for 10-14 days. To encourage uniform colonization of the rice grains, each bottle was carefully shaken for several days after introduction. The chestnut seedlings were inoculated ~14 weeks (May 2016) after they were planted in the greenhouse. Each plant received three colonized rice grains within the perimeter of the root collar (Figure 3).

**Figure 3: An image of a chestnut seedling being inoculated with a rice grain colonized with *P. cinnamomi* within the perimeter of the root collar.**

The plants were subsequently flooded to promote disease development of the oomycete. These methods were slightly modified from the protocols developed by Jeffers et al. (2009).

## 2.4 Disease Screening for PRR in the Backcross Hybrids

In February 2017, the plants were evaluated for PRR symptoms while they were in full dormancy. Each plant was removed from their containers, one family at a time, one plant at a time, and were washed with water to remove the residual planting medium. The root

system for each plant was rated based on the levels of necrosis caused by *P. cinnamomi*. The necrotic lesions were rated using a numerical scale from 0-3, with "0" representing a plant that is completely asymptomatic, "1" representing lesions on the lateral roots only, "2" representing lesions on the lateral and on tap roots, and "3" representing plants killed by PRR (Jeffers et al., 2009). The individuals that were alive at the end of the trial were repotted and monitored for an additional year in the nursery. Survivors will be transplanted to a PRR symptomatic orchard.

## 2.5 A Brief Overview of 2017-2018 Methods

In the 2017-2018 trial, the individual containers were organized in a complete randomized block design outside of the greenhouse to ensure that results were not skewed by environmental variation. The plantlets were inoculated using a cV8A vermiculite inoculum instead of a cV8A rice grain inoculum to optimize the homogeneity of the inoculum inside the pot during watering. Finally, the plantlets were inoculated twice during the growing season, once in May 2017 and once in November 2017. The remainder of the methodology that was implemented in the 2017-2018 trial was replicated from the 2016-2017 trial.

## 2.6 Statistical Analyses

*Survival Quotient and Seedling Mortality by Family:*

As a preliminary analysis, the strength of resistance in each hybrid chestnut family was determined by calculating the Survival Quotient (SQ), which is expressed as a percentage (Jeffers et al., 2009). Because PRR resistance in chestnut is thought to be under the

control of multiple genes (Santos et al., 2017), Asian-American BC1 hybrids are expected to retain more alleles for resistance than later generation backcrosses to *C. dentata* if parent trees were not selected for PRR resistance at every generation, as was the case with the BC2, BC3, and B3F3 progeny screened by Jeffers et al. (2009). Families with SQ values that are closer in proximity to the SQ value of *C. mollissima* will be interpreted as highly disease resistant families. The SQ was calculated using the following equation:

SQ = ([(1x n0) + (0.5x n1) + (0.25 x n2)]/ (total # of seedlings)) x 100

where n0, n1, and n2= # of seedlings rated 0, 1, and 2, respectively.

In addition, a record of the greenhouse seedling inventory was maintained through the years, 2016 to 2018. The number of chestnut seedlings that germinated and survived the first-year post-inoculation was recorded in February 2017. Likewise, the number of chestnut seedlings that survived a second year in the greenhouse post-inoculation was recorded in February 2018.

*Fisher's F-Test and Independent Two-Sample T-Test:*

Differences in the average root rating between the American and Chinese chestnut control groups was evaluated for significance using a two-sample t-test. Before proceeding with the t-test, the sample variances of the two groups was evaluated using a Fisher's F-test to verify the homogeneity of the population variances, where $H_0$: $\sigma^2 1 = \sigma^2 2$. The two-sample t-test will be employed to validate that the fully resistant Chinese chestnut seedlings and the fully susceptible American chestnut seedlings are significantly

different from each other, with respect to average root rating. The null hypothesis will state that differences in the average root rating between the two-control groups (*C. dentata* and *C. mollissima*) will not be statistically significant, at a significance level of 0.05. All results will be generated through the statistical software: RStudio Version 1.1.419. (RStudio, 2018)

*Tukey's Multiple Comparison Analysis in ANOVA:*

Tukey's multiple comparison analysis method tests for pairwise differences in means between experimental groups and control groups. The Tukey method is ideal for testing for differences in groups of unequal sizes and for minimizing the probability of making a false claim of significance (Type I error). By utilizing a conservative estimate of alpha, Tukey tests all the contrasts as a family (familywise error rate) to warrant the unlikelihood of making a Type I error. In this experiment, Tukey's Multiple Comparison Analysis was used to test for significant differences between the average root ratings among families and generations. Chestnut families with average root ratings that were significantly different from the American chestnut and were statistically insignificant to the Chinese chestnut controls were interpreted as highly disease resistant families. Finally, it is hypothesized that there will be no significant differences in average root ratings between generations of chestnut backcross hybrids and *C. dentata*. All results were analyzed through the statistical software: RStudio Version 1.1.419. (RStudio, 2018)

# Chapter 3

# Results

*Purpose:*

The research objectives for the study were to identify PRR resistant hybrid crosses and to diversify sources of resistance by early phenotypic evaluation for future breeding efforts. This study tested the efficacy of implementing early and reliable screening methods for identifying PRR-resistant hybrid chestnuts in a greenhouse setting. Although experiments were conducted during two consecutive growing seasons, results of only the 2016 trial are presented here due to problems with the 2017 inoculations and exceptional freeze damage to the container-grown seedlings during the 2017-2018 winter that confounded evaluations of PRR symptoms.

*Strength of PRR-Resistance in Backcross Hybrid Families:*

The strength of resistance in each hybrid chestnut family was determined by calculating the Survival Quotient. In Table 2, the survival quotients of the American-Chinese backcross families ranged from 8.33% to 39.39%, while the survival quotients of the American and Chinese controls were 20% and 58.33%, respectively.

**Table 2: Strength of resistance in 21 hybrid chestnut families screened in 2017.** A survival quotient was computed for each family based on the number of seedlings in each of three symptom severity rating (0, 1, and 2).

| Family Code | Generation | Source of Resistance | SQ (%) |
|---|---|---|---|
| **11** | B3F2 | "Clapper" | 8.33 |
| **12** | B1 | "Gideon" | 10.77 |
| **20** | B1 | "Gideon" | 14.58 |
| **21** | B3F3 | "Graves" | 15.63 |
| **16** | BB1 | "Clapper" and "Gideon" | 18.15 |
| **1** | Am | None | 20.00 |
| **17** | BB1 | "Clapper" and "Gideon" | 21.51 |
| **6** | B1 | *C. mollissima* x opCh | 23.91 |
| **19** | BB1 | "Graves" and "Gideon" | 24.11 |
| **9** | B1 | "Lindstrom-67" | 24.32 |
| **14** | B1 | "Gideon" | 24.61 |
| **8** | B1 | "Gideon" | 26.25 |
| **18** | BB1 | "Clapper" and "Gideon" | 27.50 |
| **13** | B1 | "Gideon" | 27.72 |
| **15** | BB1 | "Clapper" and "Gideon" | 28.33 |
| **5** | B1 | "Amy" | 28.81 |
| **7** | B1 | *C. mollissima* x opCh | 29.72 |
| **10** | B3F2 | "Nanking" and "Clapper" | 30.56 |
| **3** | B1 | "Gideon" | 37.04 |
| **4** | B1 | "Lindstrom-99" | 39.39 |
| **2** | Ch | *C. mollissima* x opCh | 58.33 |

Over a two-year period (2016-2018), of the 749 seedlings planted in the greenhouse: 703 seedlings (94%) were inoculated, 550 seedlings (78%) survived the inoculum in 2017 and 350 seedlings (64%) of the 2017 survivors persisted to 2018 (Table 3). The surviving individuals (with symptom severity ratings ranging from 0-2) will be replanted in the field for further evaluation.

**Table 3: Chestnut seedling mortality by family in the greenhouse setting (2016-2018), including: the number of seeds planted, the number of plants inoculated (and percentages), and the number of survivors at one and two years post-planting (and percentages).**

| Family Code | No. Seeds Planted (Feb.2016) | No. Plants Inoculated (May 2016) | No. Individuals Alive (Feb. 2017) | No. Individuals Alive (Feb. 2018) |
|---|---|---|---|---|
| **1** | 16 | 15 (94%) | 11 (69%) | 6 (38%) |
| **2** | 12 | 12 (100%) | 12 (100%) | 10 (83%) |
| **3** | 27 | 27 (100%) | 18 (67%) | 16 (59%) |
| **4** | 66 | 63 (95%) | 50 (76%) | 28 (42%) |
| **5** | 59 | 54 (92%) | 48 (81%) | 33 (56%) |
| **6** | 23 | 23 (100%) | 20 (87%) | 14 (61%) |
| **7** | 53 | 48 (91%) | 40 (75%) | 19 (36%) |
| **8** | 20 | 17 (85%) | 14 (70%) | 7 (35%) |
| **9** | 74 | 72 (97%) | 56 (76%) | 27 (36%) |
| **10** | 9 | 9 (100%) | 9 (100%) | 8 (89%) |
| **11** | 12 | 9 (75%) | 4 (33%) | 0 (0%) |
| **12** | 65 | 54 83%) | 25 (38%) | 15 (23%) |
| **13** | 46 | 46 (100%) | 45 (98%) | 24 (52%) |
| **14** | 64 | 61 95%) | 55 (86%) | 48 (75%) |
| **15** | 30 | 27 (90%) | 24 (80%) | 14 (47%) |
| **16** | 73 | 68 (93%) | 41 (56%) | 35 (45%) |
| **17** | 43 | 42 (98%) | 34 (79%) | 20 (47%) |
| **18** | 10 | 9 (90%) | 8 (80%) | 1 (10%) |
| **19** | 27 | 27 (100%%) | 24 (89%) | 18 (67%) |
| **20** | 12 | 12 (100%) | 7 (58%) | 4 (33%) |
| **21** | 8 | 8 (100%) | 5 (63%) | 3 (38%) |

*Fisher's F-Test for the Equivalence in Variances:*

The results of the Fisher's F-test suggest that there is enough evidence to support the null hypothesis that the two-control group (*C. dentata* and *C. mollissima*) population variances are equal. The differences in sample variances of the two-control groups are statistically insignificant, $F(11, 15) = 0.46$, $p = 0.16$, $n < 30$, at the 0.05 significance level. The results of the Fisher's F-test are listed in Table 4.

**Table 4: F-values and P-values obtained using a two-tailed Fisher's F-Test for the equivalence in variances between *C. dentata* and *C. mollissima*.**

| | M | n | df | $\sigma^2$ | F | F-crit | α | p |
|---|---|---|---|---|---|---|---|---|
| ***C. dentata*** | 2.25 | 16 | 15 | 0.33 | 0.46 | 0.14 | 0.05 | 0.16 |
| ***C. mollissima*** | 1 | 12 | 11 | 0.73 | 0.46 | 1.38 | 0.05 | 0.16 |

$H_0$: $\sigma^2 1 = \sigma^2 2$
$H_a$: $\sigma^2 1 \neq \sigma^2 2$
Reject $H_0$ if: $F < 0.14$ or $F > 1.38$; $p < 0.05$

*Independent Two-Sample T-Test:*

An independent-samples t-test was conducted to compare the average PRR root rating in *C. dentata* and *C. mollissima*. There was a significant difference in the average PRR root rating for the *C. dentata* (M=2.25, SD= 0.58) and *C. mollissima* (M=1, SD=0.85) control groups; t (26) =4.63, p <<0.05. The results of the independent-samples t-test are listed in Table 5.

**Table 5: Results of T-Test and Descriptive Statistics for PRR Root Rating by Control Group**

| | Control Group | | | | | | 95% CI for Mean Difference | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***C. dentata*** | | | ***C. mollissima*** | | | | | |
| | M | SD | n | M | SD | n | | t | df |
| PRR Root Rating | 2.25 | ±0.58 | 16 | 1 | ±0.85 | 12 | 0.69, 1.81 | 4.63* | 26 |

*p<<0.05

*Tukey's Multiple Comparisons of Means in ANOVA:*

The Tukey HSD post hoc analysis was conducted on all possible pairwise contrasts. The following pairs of generations were found to be significantly different ($p < 0.05$): Chinese chestnut (M= 1, SD= 0.85) and BB1 generation (M= 2.08, SD= 0.67), Chinese chestnut (M= 1, SD= 0.85) and American chestnut (M= 2.25, SD= 0.58), Chinese chestnut (M= 1,

SD= 0.85) and B3F3 generation (M= 2.31, SD= 0.48), Chinese chestnut (M= 1, SD= 0.85) and B1 generation (M= 1.95, SD= 0.77), and Chinese chestnut (M= 1, SD= 0.85) and B2F2 generation (M= 2.17, SD= 0.62). There were no significant differences in average PRR root rating between American chestnut and the backcross hybrids. Table 6 lists the pairwise comparison results of the Tukey HSD post hoc analysis by generation of chestnut.

**Table 6: Tukey HSD pairwise comparison for PRR resistance in generation of chestnut.**

| Pair | Contrast | Lower bound | Upper bound | p(tukey) |
|---|---|---|---|---|
| **Ch-BB1** | -1.086 | -1.715 | -0.458 | 0.000* |
| **Ch-Am** | -1.250 | -2.056 | -0.444 | 0.000* |
| **Ch-B3F3** | -1.308 | -2.153 | -0.462 | 0.000* |
| **Ch-B1** | -0.947 | -1.564 | -0.330 | 0.000* |
| **Ch-B3F2** | -1.167 | -1.954 | -0.380 | 0.000* |
| **BB1-B1** | 0.140 | -0.043 | 0.322 | 0.244 |
| **B3F3-B1** | 0.361 | -0.233 | 0.954 | 0.507 |
| **B1-Am** | -0.303 | -0.839 | 0.233 | 0.589 |
| **B3F2-B1** | 0.220 | -0.287 | 0.726 | 0.818 |
| **BB1-B3F3** | -0.221 | -0.827 | 0.385 | 0.903 |
| **BB1-Am** | -0.164 | -0.714 | 0.387 | 0.958 |
| **B3F3-B3F2** | 0.141 | -0.627 | 0.910 | 0.995 |
| **BB1-B3F2** | -0.080 | -0.601 | 0.441 | 0.998 |
| **B3F2-Am** | -0.083 | -0.809 | 0.642 | 0.999 |
| **B3F3-Am** | 0.058 | -0.731 | 0.846 | 1.000 |

*$p<0.05$

Additionally, the Tukey HSD post hoc analysis was conducted to denote significant differences in average root rating between families. Using the compact letter display approach, families with the same letter are not significantly different at 0.05 significance level. The results are represented by Table 7 and Figure 4.

**Table 7: Tukey HSD comparison for PRR resistance in hybrid families of chestnut**

| Pedigree | Generation | n | M | SE | tukey |
|---|---|---|---|---|---|
| *C. mollissima* x opCh | Ch | 12 | 1 | 0.20 | ***d*** |
| Cataloochee 2007 Tree 33 x Neel 2-127 | B1 | 65 | 1.58 | 0.09 | ***cd*** |
| Cataloochee 2007 Tree 273 x TN-TTU-A29 | B1 | 27 | 1.70 | 0.14 | ***bd*** |
| TN-TTU-C27 x TN-TTU-A30 | BB1 | 26 | 1.73 | 0.14 | ***bd*** |
| Kemp Orchard Mix x opB3 | B3F2 | 9 | 1.78 | 0.24 | ***abd*** |
| Cataloochee 2007 Tree 80 x Pryor Seed Orchard Tree 3-50 | B1 | 51 | 1.82 | 0.10 | ***bc*** |
| Cataloochee 2007 Tree 80 x Neel 4-195 | B1 | 58 | 1.86 | 0.09 | ***bc*** |
| TN-TTU-L13 x TN-TTU-A30 | BB1 | 9 | 1.89 | 0.24 | ***abd*** |
| TN-TTU-A30 x Talladega #2 | B1 | 47 | 1.89 | 0.10 | ***bc*** |
| TN-TTU-A34 x NCDOT American | B1 | 62 | 1.98 | 0.09 | ***bc*** |
| Cataloochee 2007 Tree 80 x TN-TTU-A30 | B1 | 20 | 2 | 0.16 | ***abc*** |
| TN-TTU-M13 x TN-TTU-A30 | BB1 | 26 | 2 | 0.14 | ***abc*** |
| GABE001-165 x GAHA14 | B1 | 73 | 2.03 | 0.08 | ***b*** |
| Cataloochee 2007 Tree 80 x Neel 8-192 | B1 | 23 | 2.04 | 0.15 | ***abc*** |
| TNCOC1 x TN-TTU-A30 | B1 | 8 | 2.12 | 0.25 | ***abd*** |
| TN-TTU-K2 x TN-TTU-A30 | BB1 | 42 | 2.14 | 0.11 | ***ab*** |
| TN-TTU-E24 x TN-TTU-A30 | BB1 | 82 | 2.22 | 0.08 | ***ab*** |
| *C. dentata* x opAm | Am | 16 | 2.25 | 0.18 | ***abc*** |
| W7-32-147 x opB3F2 | B3F3 | 13 | 2.31 | 0.20 | ***abc*** |
| TN-TTU-A30 x NCDOT American | B1 | 56 | 2.50 | 0.09 | ***a*** |
| Sam's 2-J mix x opB3 | B3F2 | 9 | 2.56 | 0.24 | ***ab*** |

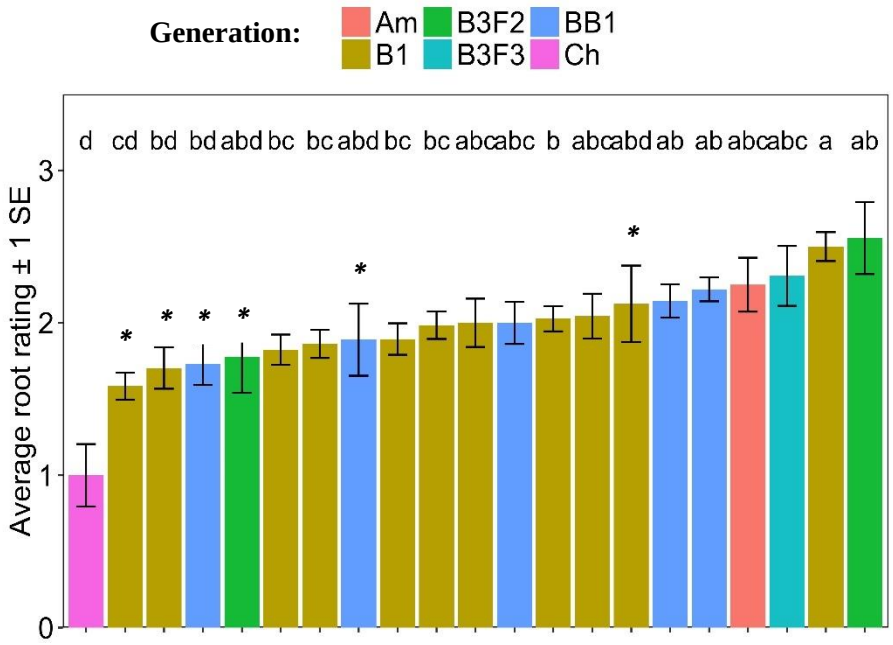


**Figure 4: Tukey HSD comparison for PRR resistance in hybrid families of chestnut.** Families are displayed in ascending order (lowest average symptom severity score ± 1 SE – highest average symptom severity score ± 1 SE) to visualize significant differences between families and generations for PRR resistance.

*****Families statistically similar to *C. mollissima*, with respect to average root rating.

# Chapter 4

# Discussion and Future Directions

## 4.1 Overview

The research objectives for the study were to identify PRR resistant hybrid crosses and to diversify sources of resistance by early phenotypic evaluation for future breeding efforts. This study tested the efficacy of implementing early and reliable screening methods for identifying PRR-resistant hybrid chestnuts in a greenhouse setting. The results obtained from the tests conducted in 2016-2017 trial, at a 0.05 significance level, stating that: there were significant differences in the average root rating between *C. mollissima* and *C. dentata*; screening by generation did not provide statistically significant data pertaining to PRR resistance by the symptom severity scale; significant differences in the average root rating between families, intragenerational and intergenerational, were observed in the backcross hybrid families; and insignificant differences between the average root rating in Chinese chestnut and backcross hybrid families: 3, 4, 10, 20, 15, and 18. These families were deemed the most resistant by the Tukey HSD post hoc analysis. Families that were interpreted to have any degree of PRR resistance were under the assumption that a PRR resistance allele inherited from *C. mollissima*. Additionally, the results may have been skewed due to external influences such as: freeze damage, inoculum strength, and other environmental variations. In the future, seedlings grown for disease screening studies should be maintained in a uniform environment to control for temperature variations, homogeneity in direct light exposure, levels of saturation, and pathogenic spread.

### 4.1.1 Strength of PRR-Resistance in Backcross Hybrids:

The strength of resistance in each hybrid chestnut family was determined by calculating the Survival Quotient. Of the 21 families evaluated (Table 2), three families (14 percent) had a SQ between 0.1 and 15.0 percent; 14 families (67 percent) had a SQ between 15.1 and 30.0 percent; three families (14 percent) had a SQ between 30.1 and 40.0 percent; and one family had a SQ over 50 percent (*C. mollissima* x opCh, SQ = 58.33 percent). The higher SQs of several backcross families relative to the *C. dentata* SQ suggests that PRR resistance was inherited by a large portion of backcross progeny in these families. The 58.33% SQ observed in the *C. mollissima* implies that high disease pressure existed in the greenhouse screening conditions. Over a two-year period (2016-2018), of the 749 seedlings planted in the greenhouse: 703 seedlings (94%) were inoculated, 550 seedlings (78%) survived the inoculum in 2017 and 350 seedlings (64%) of the 2017 survivors persisted to 2018 (Table 3). The surviving individuals will be replanted in the field for further evaluation. On average, the individuals with a PRR root rating of 0 or 1 have survived better than those with a rating of 2. The phenotypic data from this study currently supports the genotypic data being collected at Clemson University. However, of the three families selected for their resistance to *C. parasitica* (generation: B3F2 and B3F3; Source of Resistance: ‘Clapper’ and ‘Graves’) exhibited very low levels of PRR resistance (SQ= 8.33% and 15.36%, respectively. Preliminary results based on seedling mortality and SQ, through early disease screening, suggests that identical crosses of the families deemed to have PRR resistance traits will need to be performed, phenotyped and genotyped for resistance to control for environmental variation.

### 4.1.2 Fisher's F-Test for the Equivalence in Variances:

The results of the Fisher's F-test suggest that there is enough evidence to support the null hypothesis stating that the two control group population variances are equal. The differences in the sample variances of the two control groups are statistically insignificant, F (11, 15) = 0.46, p =0.16, at the 0.05 significance level (Table 4). With a p-value greater than 0.05 and an F-value within the range of the critical values for the 0.05 level of significance, in a two-tailed F distribution, it can be assumed that the two population variances are homogeneous. Thus, verifying that the t-test will operate under the assumption of equal population variances.

### 4.1.3 Independent Two-Sample T-Test:

An independent-samples t-test was conducted to compare the average PRR root rating in *C. dentata* and *C. mollissima*. There was a significant difference in the average PRR root rating for the *C. dentata* (M=2.25, SD= 0.58) and *C. mollissima* (M=1, SD=0.85) control groups; t (26) =4.63, p <<0.05 (Table 5). These results suggest that the fully resistant Chinese chestnut seedlings and the fully susceptible American chestnut seedlings are significantly different from each other, with respect to average root rating. Specifically, these results suggest that there is enough evidence to reject the null hypothesis stating that the differences in the average root rating between the two-control groups (*C. dentata* and *C. mollissima*) are not statistically significant, at a significance level of 0.05.

### 4.1.4 Differences in Average Root Rating by Generation:

The Tukey HSD post hoc analysis was conducted on all possible pairwise contrasts. The following pairs of generations were found to be significantly different ($p < 0.05$): Chinese chestnut (M= 1, SD= 0.85) and BB1generation (M= 2.08, SD= 0.67), Chinese chestnut

(M= 1, SD= 0.85) and American chestnut (M= 2.25, SD= 0.58), Chinese chestnut (M= 1, SD= 0.85) and B3F3 generation (M= 2.31, SD= 0.48), Chinese chestnut (M= 1, SD= 0.85) and B1 generation (M= 1.95, SD= 0.77), and Chinese chestnut (M= 1, SD= 0.85) and B2F2 generation (M= 2.17, SD= 0.62) (Table 6). In other words, each generation of chestnut (Am, B1, BB1, B3F3, and B3F2) was denoted to be significantly different by average root rating than the Chinese chestnut, while there were no significant differences in average PRR root rating between American chestnut and the backcross hybrids, there is enough evidence to reject the null hypothesis stating that there would be no significant differences in average root rating between the backcross hybrids and *C. dentata*. In this study, screening by generation did not provide statistically significant data pertaining to PRR resistance by the symptom severity scale, implying that generation may have no effect on PRR resistance in American chestnut.

**4.1.5 Differences in Average Root Rating by Family:**

The Tukey HSD post hoc analysis was conducted to denote significant differences in average root rating between chestnut families Using the compact letter display approach, families with the same letter are not significantly different at 0.05 significance level. Chestnut families with average root ratings that are significantly different to the American chestnut and are statistically insignificant to the Chinese chestnut were interpreted as highly disease resistant families. The results of the test yielded insignificant differences between the average root rating in Chinese chestnut and backcross hybrid families: 3, 4, 10, 20, 15, and 18 (Table 7 and Figure 4) with the only family significantly different from *C. dentata* being *C. mollissima*. However, significant differences in the average root rating between families, intragenerational and intergenerational, were

observed in the backcross hybrids. Families with smaller sample sizes, relative to the families with larger sample sizes, tended to demonstrate larger values of standard error. Outside of environmental discrepancies, the variant levels of PRR resistance that was expressed between the hybrid families were likely due to the *C. mollissima* cultivars where their genes that influence resistance were inherited from. Of the hybrid families that were interpreted as highly disease resistant at a 0.05 significance level, four of the six families inherited PRR resistance from 'Gideon' and one of the six families inherited PRR resistance from 'Lindstrom-99', TN cultivars not previously utilized by TACF to date. These results suggest that future crosses with Chinese cultivars "Gideon" and 'Lindstrom-99' will need to be performed to identify whether they are a consistently reliable source of PRR resistance traits for the American chestnut backcross hybrids.

## 4.2 Critique of Methods

### 4.2.1 Experimental Design

In this study, two separate trials were performed in the years 2016 and 2017. The conditions of the 2016 trial are thoroughly explained in the Materials and Methods section of this paper. In the 2017 trial, the individual containers were organized in a complete randomized block design outside of the greenhouse to ensure that results were not skewed by environmental variation. Consequently, the results of the ANOVA and independent t-tests exhibited no significant differences between the blocks, the hybrid chestnut families, the generations, and the control groups. It is hypothesized that these results were caused by an inoculum failure (See section 4.2.2) and freeze damage. Due to sustained cold temperatures during the winter of 2017-2018, most of the *C. mollissima*

seedlings sustained freeze damage of the roots even though the nursery was previously winterized. In addition, *Phytophthora* was spread throughout the nursery by not utilizing the water-containment chamber (Figure 2). In both trials, limitations were present due to the variance in sample sizes across families. The number of individuals present in the study was directly dependent on the number of nuts available during harvest and the proportion of those nuts that successfully germinated after planting. Finally, a proportion of the individuals within certain families were simultaneously infected with *C. parasitica* and *P. cinnamomi*. This poses the question on whether the PRR resistance strength of the seedlings, that succumbed to both disease pressures, was compromised by their levels of susceptibility to *C. parasitica*. Overall, seedlings grown for disease screening studies should be maintained in a uniform environment to control for temperature variations, homogeneity in direct light exposure, levels of saturation, and pathogenic spread.

### 4.2.2 Inoculum Failure and Fungal Contamination

In the 2017 screening trial, seedling dieback in C. dentata was not occurring at a rate consistent with previous studies post-inoculation. A second batch of inoculum was prepped in September 2017 to ensure infection of PRR across families. The inoculum was tested for Koch's postulates to verify the presence of *Phytophthora* and to confirm the absence of other microorganisms. Koch's postulates revealed that there was in fact other microbe species present in the inoculum; therefore, a third batch of inoculum had to prepped. After inoculum confirmation, the seedlings were subjected to a second inoculation in early November 2017. Because PRR screening began in February 2018, it was not likely that the second inoculum would yield reliable results, as the seedlings were preparing for dormancy. In future studies, individuals will benefit from additional

inoculations, after inspecting the inoculum for purity, to increase the mortality rates of susceptible individuals.

## 4.3 Future Directions

### 4.3.1 Orchard Studies on Pre-Screened Resistant Hybrids

Individuals identified as highly PRR resistant through early screening will be replanted in symptomatic orchard settings for further evaluation of disease response over time. The PRR screening efforts will continue at the Bent Creek Experiment Station (USDA Forest Service) greenhouse, "Rust Screening Center", in Asheville, NC. One of the future directions for TACF PRR screening programs includes the continuation of early screening in individual containers to explore untested novel sources of resistance and to repeat identical crosses that displayed low average root ratings on the symptom severity scale. These disease screening methods can facilitate the efficient introgression of PRR resistance from Chinese chestnut into potentially blight resistant advanced hybrid populations (B3F3 and B3F2) and by diversifying sources of resistance in the chestnut breeding program by phenotypic evaluation.

### 4.3.2 Quantitative Trait Locus Mapping

The most resistant backcross progeny, identified through early screening in TACF, will be genotyped for the presence of the molecular marker on linkage group E, previously identified and evaluated by Zhebentyayeva et al. (2014). Linkage group E is believed to be one of the chromosomes on which reside loci that encodes for PRR resistance in the chestnut genome. Quantitative trait locus (QTL) mapping can be used to determine whether the same loci confer PRR resistance in different *C. mollissima* cultivars, and

whether the *C. dentata* parent in each cross contributes a genetic influence on PRR resistance strength in the backcross progeny. For example, performing separate crosses with the same Chinese chestnut parent and different American parents to identify the effects the American cultivar has on PRR resistance. Additional loci are being evaluated for their influence on PRR resistance in pre-screened highly resistant chestnut hybrid populations.

## References:


Acf.org. (2018). *Saving the American Chestnut Tree | The American Chestnut Foundation*. [online] Available at: https://www.acf.org/ [Accessed 16 Apr. 2018].

Anagnostakis S.L. 1987. Chestnut blight: the classical problem of an introduced pathogen. *Mycologia* 79: 23–37.

Australian Government. 2014. *Threat Abatement Plan for Disease in Natural Ecosystems Caused by Phytophthora cinnamomi*. Canberra: Commonwealth of Australia.

Baxter, B.N. 2009. An Oral History of the American Chestnut in the Southern Appalachia. Master's thesis. University of Tennessee at Chattanooga, Department of Biological and Environmental Sciences. Chattanooga, TN.

Branzanti, M.B. and Rocca, E., Pisi, A. 1999. Effect of ectomycorrhizal fungi on chestnut root rot." *Mycorrhiza* 9: 103–109.

Brown, M., and Davis, D. 1992. Trail history notebook. Great Smoky Mountains Natural History Assoc., Gatlinburg, Tennessee. 182 p.

Burnham, C.R. 1988. The restoration of the American chestnut. *American Scientist* 76: 478-487.

Cahill, D.M., Rookes, J.E., Wilson, B.A., Gibson, L. and McDougall, K.L. 2008. *Phytophthora cinnamom*i and Australia's biodiversity: impacts, predictions and progress towards control. *Aust. J. Bot.* 56, 279–310.

Condon, T. 1994. Chestnut top trail. P. 166-168 in Hiking Trails of the Smokies, DeFoe, D., et al. (eds.). Great Smoky Mountains Natural History Assoc., Gatlinburg, Tennessee.

Cook, R. and Forest, H.S. 1979. The American chestnut II: chestnuts in the Genessee Valley region, 1978. The Rochester Committee for Scientific In Formation Bull. 226:1-9.

Davis, D.E. 2006. Historical significance of American chestnut to Appalachian culture and ecology. *Restoration of American chestnut to forest lands* (ed. By K.C. Steiner and J.E. Carlson) 53-60. Natural Resources Report NPS/NCR/CUE/NRR-2006/001, National Park Service, Washington, DC.

Faison, E.K., and Foster, D.R. 2014. Did American Chestnut Really Dominate the Eastern Forest? *Arnoldia* 72 (2): 18-32.

Fei, S., Liang, L., Paillet, F. L., Steiner, K. C., Fang, J., Shen, Z., . . . Hebard, F.V. 2012. Modelling chestnut biogeography for American chestnut restoration. *Diversity and Distributions, 18*(8), 754-768.

Forest, H.S. 1978. The American chestnut in New York, p. 25-26. In The Conservationist, New York Dept. Env. Conservation, July-Aug.

Giddens, N.J. 1912. Untitled report on chestnut blight. P. 173-174 *in* Proc. of conf. on Chestnut blight. Harrisburg, Pennsylvania, February 20-21.

Gravatt, G.F., and Crandall, B.S. 1945. The Phytophthora root disease of chestnut and chinkapin. Ann. *Rep Northern Nut Grower's Association* 35: 83-87.

Hardham, A.R., and Blackman, L.M. 2017. Phytophthora cinnamomi. *Molecular Plant Pathology, 19*(2), 260-285.

Hebard F.V. 2006. The backcross breeding program of the American Chestnut Foundation. *Journal of The American Chestnut Foundation* 19: 55–77.'

Hill, J.M. 1993. Wildlife value of *Castanea dentata* past and present, the historical decline of the chestnut, and its future use in restoration of natural areas. Randolph Macon College, Lynchburg, Virginia.

Jeffers S.N., James F. B., and Sisco P. H. 2008. Screening for resistance to *Phytophthora cinnamomi* in hybrid seedlings of American chestnut. *Clemson University, Chestnut Return, The American Chestnut Foundation*: 1-23.

Jeffers, S.N., James, J.B., and Sisco, P.H. 2009. Screening for resistance to Phytophthora cinnamomi in hybrid seedlings of American chestnut. Proc. Fourth Meeting of the International Union of Forest Research Organization (IUFRO) Working Party S07.02.09: Phytophthoras in Forests and Natural Ecosystems. E.M. Goheen and S.J. Frankel (tech. coords.), Gen. Tech. Rep. PSW-GTR-221. USDA, Forest Service, Pacific Southwest Research Station. Albany, CA. pp. 188-194.

Johnson, G.P. 1988 Revision of *Castanea* sect. Balanocastanon (Fagaceae). *Journal of the Arnold Arboretum (USA)*, 69, 25-49.

Jung, T., Colquhoun, I.J. and Hardy, G.E.S.J. 2013. New insights into the survival strategy of the invasive soilborne pathogen *Phytophthora cinnamom*i in different natural ecosystems in Western Australia. *Forest Pathol.* 43, 266–288.

Kuhlman, E.G. 1978. The devastation of American chestnut by blight. P. 1-3 *in* Proc. of the American chestnut symposium, MacDonald, W.L., et al. (eds). West Virginia University Press, Morgantown, WV.

Lutts, R.H. 2004. Like manna from god: the American chestnut trade in southwestern Virginia. *Environmental History*, 9, 497-525.

MacDonald, W.L. 1978. Ed. Proceedings of the American chestnut symposium. West Virginia University Press, Morgantown, WV.

McCarthy, M.A., and Meredith, F.I. 1988. Nutrient data on chestnuts consumed in the United States. *Economic Botany* 42(1): 29-36.

Ousmane, S., Bosland, P.W., and Steiner, R. 2005. Inheritance of Phytophthora Stem Blight Resistance as Compared to Phytophthora Root Rot and Phytophthora Foliar Blight Resistance in *Capsicum annuum American Society of Horticultural Science*, 130(1):75-78.

Perkins, M.T. 2012. The effect of phosphite on mycorrhiza formation in American chestnut (*Castanea dentata*). Honors thesis. University of Tennessee at Chattanooga, Department of Biological and Environmental Sciences. Chattanooga, TN.

Perkins, M.T. 2016. Chloroplast DNA phylogenetics of the North American chestnuts and chinquapins (Castanea Mill., Fagaceae). *Masters Theses and Doctoral Dissertations.*

Perkins, M.T., Robinson A.C., and Craddock J.H. 2017. Identifying novel sources of resistance to Phytophthora root rot in backcross American-Chinese hybrid chestnuts, in submission.

Peterson, A.T. 2011. Ecological niche conservatism: a time structure review of evidence. *Journal of Biogeography*, 38, 817-82.

RStudio.2018. RStudio: Integrated development environment for R (Version 1.1.419) [Computer software]. Boston, MA.

Russell, E. W. 1987. Pre-Blight Distribution of Castanea dentata (Marsh.) Borkh. *Bulletin of the Torrey Botanical Club, 114* (2), 183.

Russell, G. E., 1978. Plant breeding for pest and disease resistance. Butterworths, London, UK: 1-485.

Santos, C., Machado, H., Correia, I., Gomes, F., Gomes-Laranjo, H., and Costa, R.L. 2015 Phenotyping *Castanea* hybrids for *Phytophthora cinnamomi* resistance. *Plant Pathology*. 64(4): 901-910.

Santos, C., Nelson, C.D., Zhebentyayeva, T., Machado, H., Gomes-Laranjo, J., and Costa R.L. 2017. First interspecific genetic linkage map for *Castanea sativa* × *Castanea crenata* revealed QTLs for resistance to *Phytophthora cinnamomi*. PLOS ONE 12(9): e0184381.

Sidebottom, J.R., Jones, R., Benson, M., and Ivors, K. 2004. Management of *Phytophthora* root rot in fraser fir Christmas trees. *North Carolina Cooperative Extension*. Online.

Smith, A.S. 2012. Breeding for resistance: TACF and the Burnham hypothesis. *Journal of the American Chestnut Foundation* 26 (2): 11-15.

Sharpe, S. R. 2017. *Phytophthora* Species Associated with American, Chinese, and Backcross Hybrid Chestnut Seedlings in Field Sites in the Southeastern United States. Master's thesis. Clemson University, Department of Plant and Environmental Sciences. Clemson, South Carolina.

Zentmeyer, George A. 1980. *Phytophthora cinnamomi* and the diseases it causes. *American Phytopathological Society* 10: 1-96.

Zhang, B., Oakes, A., Newhouse, A., Baier, K., Maynard, C. and Powell, W. 2013. A threshold level of oxalate oxidase transgene expression reduces Cryphonectria parasitica-induced necrosis in a transgenic American chestnut (Castanea dentata) leaf bioassay. *Transgenic Research*, 22(5), pp.973-982.

Zhebentyayeva, T., Chandra, A., Abbott, A.G., Olukolu, B.A., Jeffers, S.N., James, J.B., Station, M.E., Hebard, F.V., Georgi, I., Sisco, P.H., and Nelson, C.D. 2014. Genetic and genomic

resources for mapping resistance to *Phytophthora cinnamomi* in chestnut. *Proceedings 5ht International Chestnut Symposium*: 263-270.

# Appendix I

## V-8 Vermiculite Inoculum

Developed by (Jeffers, 2009)

**Objective** To prepare inocula of *Phytophthora* spp. on vermiculite moistened with V8 broth (V8B) for experiments on *Phytophthora* root & crown rot or Phytophthora foliage/aerial blight

### Inoculum Preparation

1. Recover isolates of *Phytophthora* sp. on plates of PAR(PH) medium at 25°C; 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 + 1.0 g CaCO3 + 900 ml distilled water; do not sterilize before use
4. Use fine-textured, horticultural-grade vermiculite; store in a dry place
5. Pyrex bottles were used to store the inoculum
6. Place lids loosely on bottles to allow for ventilation during autoclaving
7. Autoclave bottles for 30-45 min; remove bottles from autoclave soon after autoclaving to prevent evaporation of V8 broth and subsequent desiccation of vermiculite; allow bottles to cool overnight
8. The following day, cover tops and necks of bottles with aluminum foil, place bottles in a brown paper bag, fold the top of the bag and staple it closed, and then autoclave the bag(s) with bottles again for 30-45 min
9. When bottles have completely cooled—aseptically, seed each bottle with (1) 5-mm plug of each isolate per bottle 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, CMA, PDA, etc.)
10. Incubate cultures in bottles at 25°C (dark) for 10-14 days

11. After several days, 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; repeat this process several times during the incubation process
12. Before using inoculum, check each bottle for purity—this is essential!! • autoclave spatulas needed to sample the V8-vermiculite
    i. aseptically in a laminar-flow hood—remove foil and lid from each bottle—one at a time
    ii. with a sterile spatula, remove a small amount of vermiculite and sprinkle it on a plate of nonamended CMA or cV8A—without antibiotics
    iii. place these plates at 25°C for 24-72 hr—check for growth of *Phytophthora* sp. from each and every piece of vermiculite and for any evidence of contamination—fungi or bacteria
    iv. if contamination is present—modify sterilization procedure or aseptic technique accordingly
    v. do not use inoculum from any bottle that is heavily contaminated

# Appendix II

## Family Codes

Codes were assigned to each cross in order to differentiate between individuals in the greenhouse with respect to their genetic family. However, the codes that were written on the tags of each family in the greenhouse differ from the 'Family Codes' denoted in this paper. The codes were modified to adjust for the chestnut families in the greenhouse that were not utilized in the study and to organize the families sequentially to avoid confusion regarding the missing family codes.

| Family Code No. | Greenhouse Family Tag No. |
|---|---|
| 1 | 99 |
| 2 | 100 |
| 3 | 4 |
| 4 | 5 |
| 5 | 3 |
| 6 | 20 |
| 7 | 1 |
| 8 | 2 |
| 9 | 8 and 11 |
| 10 | 24 |
| 11 | 10 |
| 12 | 6 |
| 13 | 13 |
| 14 | 17 |
| 15 | 18 |
| 16 | 23 |
| 17 | 14 |
| 18 | 7 |
| 19 | 22 |
| 20 | 12 |
| 21 | 15 |