SMALL STEM ASSAY FOR CHESTNUT BLIGHT RESISTANCE IN SEGREGATING HALF-SIB AND FULL-SIB FAMILIES OF F2 AND BACKCROSS HYBRID CHESTNUT TREES

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ABSTRACT

Chestnut breeders routinely screen hybrid chestnut seedling progeny for resistance to chestnut blight in an orchard setting – a process that takes five to seven years. A novel technique that allows for screening in the first and second growing season is known as a small stem assay. We screened 110 first-backcross seedlings and 98 third-backcross seedlings in a completely randomized design in 2017 (Gentner, 2018). In 2018, an additional 391 F2 hybrid chestnut seedlings were screened in a randomized complete block design. All seedlings were inoculated with the chestnut blight causal organism *Cryphonectria parasitica* when stem diameters were greater than 4mm. The 2017 trial recorded the day on which the plant wilted, during both the 2017 and 2018 trial, canker length was measured and recorded. The 2017 trial gave insight to the rate at which different seed types wilted and the 2018 trial selected the very best of the F2s.

DEDICATION

This thesis is dedicated to the devoted scientists, volunteers, and enthusiasts of The American Chestnut Foundation.

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I have many to thank for their help with this work. I would like to thank the many volunteers of The American Chestnut Foundation who helped with inoculation and screening of the chestnut trees, without them this study would have posed to be much more difficult. To the undergraduate students who showed up to help with planting, inoculation, and screening I appreciate your participation in those tedious time-consuming tasks. Trent Deason, Scotty Smith, and Paola Zannini were an essential part to the success of this project, they went above and beyond in helping with sterilizing pots, planting seeds, watering plants, moving plants (hundreds of plants, more than once), inoculating and screening. Without them, I can say with confidence I would have been lost. Thank you to Paul Sisco for donating seeds to the study. Caleb Powell volunteered some of his time to teaching me how to use RStudio and without that assistance the statistical analysis would have taken even longer (if that is possible). Jared Westbrook and Dr. Mark Schorr helped me with understanding the statistics. Thank you to Dr. Joey Shaw and Dr. Jose Barbosa for agreeing to be on my committee and making suggestions to the manuscript.

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LIST OF ABBREVIATIONS

TACF, The American Chestnut Foundation

UTC, The University of Tennessee at Chattanooga

C. parasitica, *Cryphonectria parasitica*

C. mollissima, *Castanea mollissima*

C. dentata, *Castanea dentata*

C. pumila, *Castanea pumila*

CH, Chinese chestnut

AM, American chestnut

F1, first filial generation

F2, second filial generation

B1, first-backcross generation

BB1, better backcrosses

B2, second-backcross

B3, third-backcross

B3F2, third-backcrosses intercrossed

B3F3, third filial generation of third-backcrosses intercrossed

SSA, Small stem assay

NaClO, Sodium hypochlorite

OP, open pollinated

ml, milliliter

mm, millimeter

ANOVA, analysis of variance

F, test statistic for ANOVA

t, test statistic for Welch's two samples *t*-test

HSD, honestly significant difference

p, probability value

SS, sum of squares

df, degrees of freedom

MS, mean squares

CI, confidence interval

LIST OF SYMBOLS

μ_1, population mean of population one

μ_2, population mean of population two

μ_k, population mean of the number of different groups

μ_i, population mean of hypothetical group

$\neq$, does not equal

=, equals

<, less than

$\leq$, less than or equals to

CHAPTER I

INTRODUCTION

Efforts to restore the American chestnut began in the 1920s with attempts to introduce blight resistance into the American chestnut species by hybridizing it with the Asian species. In 1981 Charles Burnham hypothesized that three generations of backcrossing and selection for blight resistance would be enough to recover trees with American chestnut morphology and Asian chestnut levels of disease resistance. The American Chestnut Foundation (TACF) was founded in 1983 to test Burnham's proposal. TACF routinely screens hybrid trees for resistance to chestnut blight in an orchard setting – a process that takes five to seven years to grow the trees to an adequate size for inoculation, requires large commitments of land and other resources, and requires that the inoculated stem be at least eight to ten cm in diameter to support development of the blight cankers for the duration of the observation period that could be up to another one or two years. In recent years TACF has been experimenting with a novel technique for screening hybrid seedling progeny during their first and second growing season, known as a small stem assay. The present study focuses on the result of two years of small stem assay trials. In 2017 UTC undergraduate student Kevin Gentner and volunteers screened 110 first back-cross seedlings and 98 third-backcross seedlings in a completely randomized design (Gentner, 2018). In 2018, myself and volunteers screened an additional 391 F2 hybrid seedlings derived from the 'Nanking' Chinese chestnut source of resistance, in a randomized complete block design. All seedlings were inoculated with the chestnut blight causal organism *Cryphonectria parasitica* in

early summer when stem diameters were greater than 4mm. During the 2017 trial seedlings were observed 3-4 days every week for 15 weeks, canker lengths were measured and the day on which seedlings wilted was recorded. Gentner (2018) analyzed canker length measurements by seed type and therefore this study only analyzes the days to wilt data and canker lengths by family that Gentner recorded.

The days to wilt data from 2017 needed to be cleaned as there were several blanks in the data set. Therefore, this study only analyzes plants that were given a specific day to wilt. After the data were cleaned this resulted in very small samples sizes therefore no statistical differences were found between seed types and days to wilt. However, a box and whisker plot gave insight as to the rate at which different seed types wilted.

Seedling survival and canker lengths were measured at eight weeks and again at sixteen weeks post inoculation during the 2018 trial. The results of the 2018 trial suggest that canker length can be a misleading measure of resistance in a small stem assay. However, survivorship of seedlings was as expected, and we were able to retrieve the very best of the sample F2 population using a small-stem assay on container-grown seedlings in the nursery.

CHAPTER II

LITERATURE REVIEW

Castanea dentata: The American Chestnut

The American chestnut tree, *Castanea dentata* (Marsh.) Bork., belongs to the family Fagaceae. The genus *Castanea* represents 7-13 species (Nixon, 1997; Mellano et al., 2012). Species within the Fagaceae dominate temperate forests of the northern hemisphere (Manos et al., 2001). Two species in the genus *Castanea* are found in North America (*C. dentata* (Marsh.) Bork. and *C. pumila* Mill.) (Johnson, 1988). *Castanea dentata* is a monoecious deciduous tree that was once a widespread species in the eastern deciduous forests of North America. In the early twentieth century *C. dentata* had a native range about 309,000 square miles, about one-third of eastern North American forests (Faison & Foster, 2014). Before the demise of the *C. dentata* by chestnut blight some forests in the eastern United States contained 25 to 50% chestnut timber (Russell, 1987).

Decline of *Castanea dentata*

Chestnut blight, triggered by *Cryphonectria parasitica* (Murrill) Barr, was introduced to *Castanea dentata* before 1904 and decimated roughly four billion trees (Roan et al., 1986). *Cryphonectria parasitica* is an ascomycete fungus that causes a canker consisting of lesions and swelling of the stem (Anagnostakis, 1987). This canker then girdles the stem killing all vegetation distal to the canker (Anderson, 1914). By the 1950s this pathogen killed

approximately four billion *C. dentata* trees across the eastern United States (Newhouse, 1990). This species was considered a foundational species in its ecosystem and was a beneficial food source for other animals in the region due to its high mast production (Anagnostakis 1987; Ellison et al., 2005). The tragic loss of the American chestnut has been thoroughly reviewed by Anagnostakis (1987), Ellison et al. (2005), and Griffin et al. (1983).

The Fungal Pathogen

Chestnut blight is caused by the necrotrophic pathogen *Cryphonectria parasitica*. (Murrill) Barr. *Cryphonectria parasitica* establishes itself in host tissue by entering through wounds or growth cracks in the bark (Roane et al., 1986). Both asexual and sexual spores of *C. parasitica* can germinate and cause infection (Rigling & Prospero, 2018). The pathogen infects the bark by sending fine threads of mycelia into the inner bark, destroying the vascular cambium. The destruction of the vascular cambium results in a sunken lesion. The host tree reacts by the formation of wound periderm and the lignification of cell walls. Mycelial fans can penetrate lignified cells and developing wound periderm, only fully developed wound periderm can prevent further penetration of the mycelial fans. Therefore, the rate at which the mycelial fan formation occurs appears to be essential in canker size (Ringling & Prospero, 2018). In susceptible chestnut species the formation of wound periderm is inhibited by mycelial fans via the use of toxins and cell-wall degrading enzymes (Roane et al., 1986). Oxalic acid is a toxin secreted by *Cryphonectria parasitica* that enhances cell well degradation, binds to calcium in the host tissue which leads to structural weakness (McCarroll & Thor, 1978). Havir and Anagnostaksis (1983) found that virulence of the pathogen was correlated to the amount of oxalic acid produced.

Restoration of the American Chestnut

Restoration efforts of the American chestnut began in the 1920s with attempts to introduce resistance into the species by hybridizing the American chestnut with an Asian species of the same genus, *Castanea mollissima* Blume. This Chinese species carries genes for blight resistance and these hybridizations between the species began with the efforts of two programs, one by the Brooklyn Botanic Garden and one by the United States Department of Agriculture (Burnham et al., 1986; Anagnostakis, 2012).

In 1981 Charles Burnham, a distinguished Minnesota corn geneticist, proposed to apply the same back cross breeding methodology that had been used in commercial vegetable crops to the *C. dentata*. Back cross breeding had been previously used by breeders of barley and wheat (Briggs, 1938). Burnham hypothesized that back crossing hybrids with *C. dentata* would conserve the alleles for blight resistance and introduce American chestnut tree morphology into these blight resistant hybrids. This is also known as introgression. Introgression is the movement of a gene from one species into the gene pool of another by the repeated backcrossing of an interspecific hybrid with one of its parent species. Backcrossing is the choice method used for introgression of an inherited trait into a species (Hebard, 2012). Introgression is important for conserving adaptive traits for that which we do not know how to select.

The American Chestnut Foundation was founded in 1983 to help with Burnham's proposal (Burnham et al., 1986). The foundation was founded by Nobel Prize-winning plant breeder Dr. Norman Borlaug, director of the Missouri Botanical Garden, Dr. Peter Raven and independent chestnut researcher Philip Rutter, Dr. Charles Burnham and his former student Dr. Larry Inman. The breeding methodology was described by Burnham (1988) and Hebard (2006). The American Chestnut Foundation is composed of 16 chapters located throughout the native

range of the American chestnut. Volunteers in each chapter work to support regional breeding programs, independent research and educational outreach.

Breeding for Chestnut Blight Resistance

The backcross breeding process begins by crossing *C. dentata* with *C. mollissima*. The progeny of this cross, the F1 generation, is one-half *C. dentata* and one-half *C. mollissima*. Burnham et al. (1986) presumed that inheritance of chestnut blight resistance is incompletely dominant, based on observations that the heterozygous F1s have a canker length that is about the average length between their *C. dentata* and *C. mollissima* parents' canker lengths (Burnham, 1988; Steiner et al., 2016). Early evidence indicated that blight resistance in *C. mollissima* was controlled by alleles at a minimum three loci (Kubisiak et al., 1997; Kubisiak et al., 2013; Hebard, 2006). The F1 generation is crossed with *C. dentata* chestnut to create the first backcross (B1) generation. B1s are screened for blight resistance and the most resistant individuals are advanced to the second backcross generation. Each subsequent backcross dilutes the *C. mollissima* genes by a factor of one-half. Trees in the third backcross generation are, on average, one-sixteenth *C. mollissima* and fifteenth-sixteenths *C. dentata*. Each generation of backcrosses is screened for blight resistance by evaluating canker symptoms after an inoculation with *Cryphonectria parasitica*. The very best backcross trees, in any generation, are only expected to be of intermediate blight resistance (because the recurrent backcross parent is the susceptible *C. dentata*). To recover full resistance (equivalent to *C. mollissima* levels) Burnham et al. (1986) proposed intercrossing selected B3s to generate a segregating population of B3F2s. Very few of the B3F2s (1/64 in the 3-locus model) are expected to be fixed for blight resistance (homozygous resistant at all loci). After screening (inoculation and selection) of the B3F2s, a second intercross

generation (B3F3) should be true breeding, in that all future progeny of B3F3 crosses would express the resistant phenotype. The B3-F3s are the trees that will be used for reintroduction experiments of *C. dentata* into the eastern deciduous forests. This process is outlined in Figure 1.

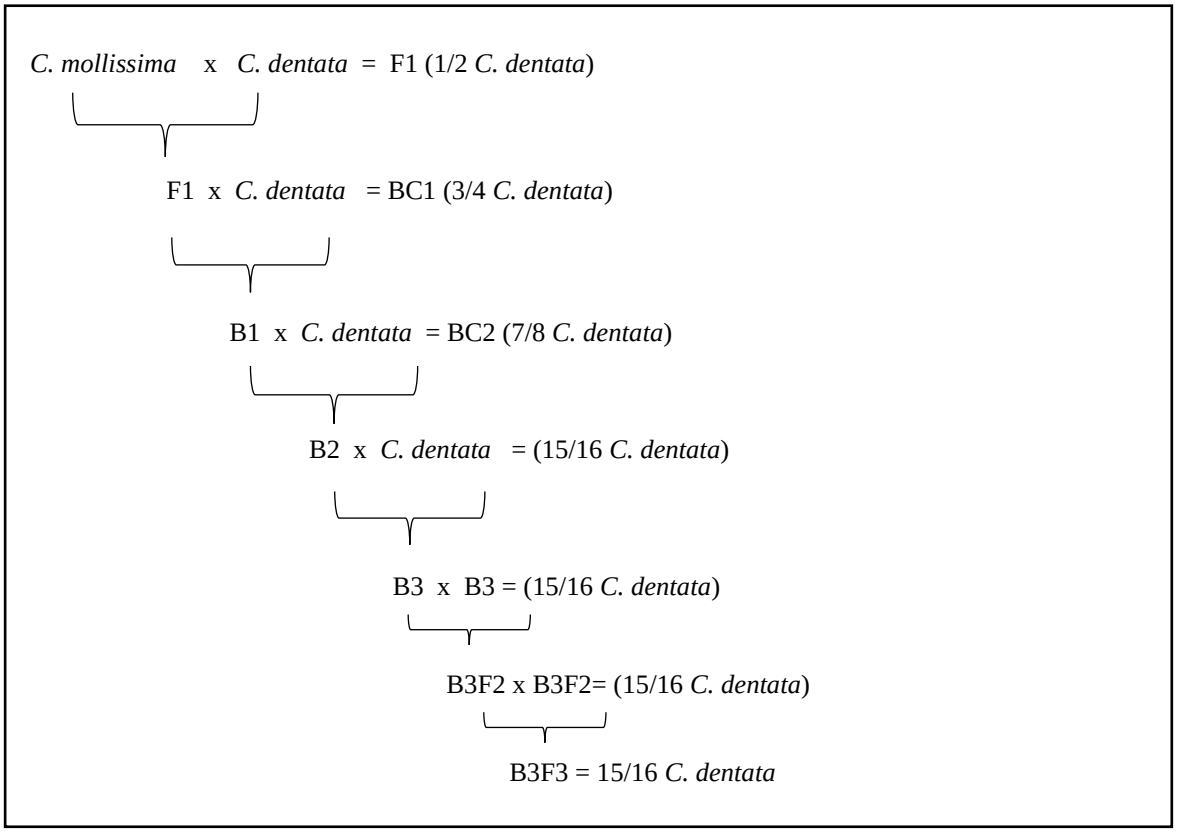

Figure 1 Backcross breeding schematic for one hybrid line
Charles Burnham proposed through backcrossing hybrids of *C. dentata* and *C. mollissima* to *C. dentata* and selection of blight resistance of these backcrosses that trees at the BC3F3 level would be true breeding for blight resistance.

Mendelian Genetics Applied to Inheritance of Chestnut Blight Resistance

The Burnham hypothesis predicts chestnut blight resistance is partially dominant (Hebard, 2006), therefore the F1 population will have an intermediate blight resistance between their parent species. Early research showed evidence of at least three genes influencing blight resistance (Kubisiak et al., 1997). Mendelian genetics has shown that crossing two heterozygotes for one trait, a monohybrid cross, will have a genotypic ratio of 1:2:1. When crossing heterozygotes for three traits, a trihybrid cross, the chances of obtaining an individual which is homozygous at each locus for blight resistance is 1 per 64 individuals. Although TACF has pursued a backcross strategy based on a three-locus model, recent genomic analyses of resistance at the B3F3 population level (progeny of selected B3F2s) that were expected to be highly resistant show evidence of polygenic inheritance (Craddock & Perkins, 2020; Westbrook et al., 2020). Therefore, the F2s should display a full range of resistance, from high to low resistance to the effects of chestnut blight with most individuals having an intermediate resistance.

TACF's chief scientist, Fred Hebard, started to create hybrid lines in 1989. Hebard decided that a minimum of twenty lines were needed per source of resistance to capture a reasonable amount of genetic diversity for a geographic region. Two original sources of resistance, 'Clapper' and 'Graves' are BC1 trees from early breeding programs and were bred as distinct sources of resistance. Another source of resistance is known as 'Nanking', which is a highly resistant graft-propagated *C. mollissima* cultivar. 'Clapper' and 'Graves' BC1 trees were crossed with an American chestnut tree. In 1989 he began crossing Clapper trees with *C. dentata* found at the Connecticut Agriculture Experiment Station. He then began using *C. dentata* found in old clear cuts in the Jefferson National Forest from various elevations to begin other hybrid lines. Each one of these *C. dentata* trees crossed to the original sources of resistance was defined

as the ancestor of a line. Each state chapter of TACF has at least two lines, using up to twenty different local *C. dentata* per source of resistance, to ensure that trees coming from their state's breeding program will be most ideally adapted to their region. These chapter lines are being selected and backcrossed in backcross orchards. After third-backcross trees are screened they are intercrossed with each other in the backcross orchard. The seeds from these intercrosses (B3F2s) are then planted in a high-density seed orchard design.

Many chapters are now planting seed orchards. Seed orchards can serve many purposes, including progeny testing and seed production. After screening of the B3F2s they interbreed with one another to create the B3F3 generation. For progeny testing, different families of B3F3s are screened to determine which B3F2 tree has the best offspring. A family comprises all the seeds from a specific mother tree. The designation of families helps chestnut researchers determine which mother has the best chance of transmitting blight resistance. The trees of a specific family are either half siblings or full siblings. Half sibling families are trees that have been open pollinated, where the pollen parent is a mixture of all other breeding trees in the orchard. Full sibling families have been produced through a controlled hand pollination, or are seeds collected from two isolated, adjacent trees, close enough together to cross pollinate but far enough away from other chestnut trees to prevent pollen contamination.

Screening for Blight Resistance

To screen B3F2 and B3F3 hybrids for blight resistance the trees must first be inoculated with *Cryphonectria parasitica*. The standard method for inoculation is the cork borer, agar-disk method used to inoculate chestnut trees with *C. parasitica* (Griffin et al., 1983). Trees are screened for blight resistance at three to five years old because that allows for a high resolution;

the ability to separate intermediate levels of resistance. The inoculation with chestnut blight produces a canker that is then measured. This method requires that trees be at least 4 cm in diameter (Powell et al., 2007). Reaching this diameter typically takes three or four years. Recently many chapters have participated in a small stem assay. This is the inoculation of chestnut seedlings during their first growing season. The small stem assay can allow for early progeny testing of hybrids. Progeny testing has been used by TACF to assess a hybrid's resistance to blight. In progeny testing at the F2 and F3 level, we may not need high resolution because we are only interested in progeny with high levels of resistance. Large amounts of seed progeny of trees of interest are planted and inoculated. The family whose progeny have the smallest average canker severity are selected. The small stem assay has allowed for more hybrids to be screened each year shortening the amount of time to determine the resistance of a family. Seedlings have been inoculated on stems as small as 3 mm, to reach this diameter typically only requires 12 weeks to reach this diameter, hence saving time for the efforts of chestnut researchers. This technique can greatly reduce the amount of B3F2s planted in seed orchards. The small stem assay technique is described by Powell et al. (2007).

Small Stem Assay at TACF

The small stem assay can vastly increase the number of hybrid seedlings screened each year to accelerate the process of progeny testing.

In 2017, TACF partnered with the U.S. Forest Service Resistance Screening Center in Asheville, NC to conduct a small stem assay on 68 B3F3 families, as progeny testing, along with *C. dentata* and *C. mollissima* controls. Thirteen of the 68 B3F3 screened had previously been screened in orchards, this replication was done to determine if the family averages for canker

severity would be correlated in small stem assays and in orchard tests. Half of the seedlings were inoculated with a highly pathogenic strain (Ep155) and the other half were inoculated with a weakly pathogenic strain (SG2-3). Canker lengths in the small stem assay were strongly correlated with canker lengths in the orchards and confirmed that genetic differences in blight resistance were detectable at the seedling stage. Canker lengths on *C. mollissima* seedlings were significantly shorter than canker lengths on *C. dentata* 15 weeks after inoculation with Ep155 and 24 weeks after inoculation with SG2-3. This was also the case with the variation in canker lengths on B3F3 seedlings. TACF decided to inoculate all trees with the highly pathogenic strain Ep155 in future small stem assays based on the results of this 2017 trial. The results from this experiment provided proof-of-concept for state chapters to use small stem assays to screen progeny from seed orchards (Westbrook & Jarrett, 2018).

In 2018 TACF, Penn State University and cooperators conducted small stem assay progeny tests of Meadowview seed orchard B3F3s, F1 hybrids, *C. dentata* and *C. mollissima* controls (Saielli & Levine, 2019). A highly virulent (Ep155) strain of *Cryphonectria parasitica* was used and resistance was measured by monitoring "time to wilt" (this measures the number of days before seedlings wilt/die due to susceptibility of chestnut blight). The B3F3 hybrid families and controls segregated as expected, Chinese chestnuts were the most resistant and the American chestnuts were most susceptible, hybrid chestnuts and F1's ranged from susceptible to intermediately-resistant. Ten out of the 107 B3F3 families tested were more resistant than F1 hybrids. The results of this small stem assay helped to determine which B3F2 parents are adequately blight resistant and which families need to be culled from the program (Saielli & Levine, 2019).

Other Strategies for Restoration

TACF is currently applying a combination of scientific restoration strategies to help restore the species. Along with breeding for resistance, the fields of biocontrol and genomics are also helping with the mission of TACF.

Biocontrol

The biocontrol method being used by scientists of TACF is hypovirulence. Hypovirulence is the use of one pathogen to control another. Mycoviruses are common throughout taxonomic groups of fungi typically infecting their host without any phenotypic changes, therefore mycoviruses that change the virulence of their host are exceptions (Buck, 2018). Some plant pathogenic fungi that are mediated by mycoviruses include chestnut blight, white root rot, dutch elm disease and victoria blight of oats (Nuss, 2005). Infection of *Cryphonectria parasitica* by RNA hypoviruses reduces the virulence of *Cryphonectria parasitica* (Anagnostakis, 1982). Attenuation of virulence is achieved by phenotypic changes such as stopping the production of oxalic acid (Havir & Anagnostakis, 1983). As previously stated, oxalic acid is a toxin utilized by *Cryphonectria parasitica* that attributes to the virulence of the pathogen by enhancing cell wall degradation. Therefore, reducing the production of this toxin is a great defense against the pathogen and a great resource for TACF.

Biotechnology

Since 1990 the State University of New York's College of Environmental Science & Forestry (SUNY-ESF) began working to add new genes transgenically to the American chestnut tree to give the species tolerance to chestnut blight. Oxalic acid is one the of strongest organic

acids and can decrease the pH of tissues (McCarroll & Thor, 1978). Fungal enzymes, such as oxalate oxidase, work best at lower pH values (Dutton & Evans, 1996). Oxalate oxidase (OxO) is an enzyme that breaks down oxalic acid by oxidizing oxalate into hydrogen peroxide and carbon dioxide (Bolwell & Wojtszek, 1997). The OxO gene is non-allergenic and is naturally occurring in many food crops such as wheat and barley (Zhang et al., 1995, Hurkman & Tanaka 1996). Along with breaking down oxalic acid Welch et al. 2007 found that the oxalate oxidase may also enhance lignin formation, making it an excellent option for genetic transformation of the American chestnut tree. In 2006, the first transgenic American chestnut trees were planted outside, these earlier lines demonstrated enhanced blight resistance to levels of intermediate between susceptible American chestnut and resistant Chinese chestnut according to leaf assays and stem assays of older trees in the field (Newhouse et al., 2014). Newer lines of transgenic trees have shown higher levels of blight resistance resembling that of the Chinese chestnut according to leaf assays and small stem assays (Zhang et al., 2013).

Problem Statement

TACF state chapters that are participating in the breeding program have been challenged by the logistical difficulty, costs, and long time periods required to screen large families of hybrid seedling progeny for blight resistance. Screening and selection processes can require up to five or more years, extensive plots of land, labor, and other resources. The small stem assay can reduce the amount of time and labor needed for screening, land needed for plantings, improve the quality of trees planted in the seed orchard, and help with the screening of the promising transgenic trees. Although the small stem assay is an attractive alternative to traditional screening practices it is a novel technique in which the methodology is continuously evolving. TACF has

experimented with using both canker length and days to wilt to determine if significant differences can be observed between known resistant and susceptible chestnut species (Westbrook & Jarrett, 2018, Saielli & Levine, 2019). More research concerning methodology of the small stem assay needs to be done to determine the best practices for screening blight resistance in a small stem assay.

Objectives

The objective of this study was to focus on the results of two years of trials that included the small-stem inoculation of 391 F2 seedlings during their first and second growing season. In 2017 Kevin Gentner and volunteers screened 258 first-backcross seedlings and 261 third-backcross seedlings in a completely randomized design (Gentner, 2018). In 2018, myself and volunteers screened 391 F2 hybrid seedlings derived from 'Nanking' Chinese, in a randomized complete block design. During this study I:

(1) Analyzed whether the F2 population in the 2018 trial segregates as we expect with most trees having intermediate resistance to that of the F1s and very few trees having similar resistance to that of *C. mollissima* and *C. dentata*. Specifically, I analyzed if this segregation can be observed in canker length, such that more susceptible trees have larger cankers and more resistant trees have smaller cankers.

(2) Compared average canker lengths by family to determine if there was significant variation between family types of the same generation of the 2017 trial.

(3) Analyzed the days to wilt data from the 2017 trial to determine the accuracy of days to wilt compared to canker length in determining resistance to chestnut blight.

CHAPTER III

RESEARCH METHODS

Study Site and Experimental Design

The location of the 2018 and 2017 studies was the chestnut research greenhouse and nursery at the University of Tennessee at Chattanooga (UTC). The facility consists of a heated greenhouse and a space outside for container-grown plants on a drip irrigation system. The greenhouse and the drip irrigation were both used in these studies. The 2017 trial was organized into a completely randomized design consisting of 38 families from seven cross types including: AM, CH, B1, BB1, F1, F2, B3F2 (Figure 1 and Table 1). BB1s "better backcrosses" a term coined by Paul Sisco are the progeny of a straight F1 crossed with a selected B3 hybrid (instead of *C. dentata*). This is advantageous because the selected B3 tree carries at least some of the resistance alleles inherited from its *C. mollissima* ancestor, which increases the average resistance of the progeny when compared to a normal B1 cross (Hebard, 2006). In 2018 a randomized complete block design was used. Plants were arranged into three non-adjacent blocks consisting of roughly 200 plants per block. Eight families were planted from four cross types: F2, F1, *C. dentata* and *C. mollissima* (Table 2). Each block of 200 was organized on one row of drip irrigation and in between each block there was a guard row of 200 other plants on a row of drip irrigation to take precaution against a pseudo replication.

Planting

2017

Seeds obtained from contributing scientists of TACF were planted from 38 different families (Table 1) in January and February 2017. A potting medium consisting of 50 – 60% composted pine bark, Canadian sphagnum peat moss, perlite, vermiculite, and dolomitic lime was used for planting (Sungrow Horticulture). A complete slow release fertilizer with micronutrients was applied as a top-dressing at planting (Osomocote Plus 15-9-12, 8-9 months). Seeds were planted in two types of containers, Stuewe & Sons 656ml and 7.65 liters. Seedlings remained inside the greenhouse until the danger of frost had passed and weather outside was warm enough to support healthy growth in mid-April.

2018

Before seedlings were planted, all 7.65-liter containers were sterilized in a solution of Sodium hypochlorite (NaClO) and water using the recommended ratio of 177ml NaClO to 3.8 liters water. In early February to early March seeds from 14 different families (Table 2) were planted. These seeds are from two cross types (F2 and F1s), and American chestnut and Chinese chestnut controls. They were donated to this study by contributing scientists Paul Sisco and Hill Craddock, of The American Chestnut Foundation. The seeds were planted in February and March 2018 in the UTC Fortwood Greenhouse directly into the Stuewe & Sons 7.65-liter pots. The potting medium consisted of 50-60% composted pine bark, Canadian Sphagnum peat moss, perlite, vermiculite, and dolomitic lime (Sungrow Horticulture). A complete slow release fertilizer with micronutrients was applied as a top-dressing at planting (Osomocote Plus 15-9-2, 8-9 months). Seedlings remained inside the greenhouse until early June.

The two families of F2 seedlings used in the trial were the progeny of two full sibling 'Nanking' F1s from an isolation plot at Warren Wilson College. Both F1 trees were from a single seed lot made at Meadowview Farms (GR119 x KH2UU). GR119 is the standard 'Nanking' ramet used at Meadowview. KH2UU was an American chestnut tree in the Mount Rogers National Recreation Area. Both F1s had Chinese cytoplasm and are male fertile. 'Nanking' 5 was the open pollinated progeny of WWC67 (one of the F1s) and 'Nanking' 6 was the open pollinated progeny of WWC70 (the other F1). The two F1s were 7 feet apart with over lapping branches (Paul H Sisco, personal communication).

Table 1 Thirty-eight families that entered the 2017 trial, including cross type, source of resistance, pedigree of mother, and pedigree of father; Trees that are open pollinated are designated (OP) (Gentner 2018)

Family	Cross type	Source of Resistance	Pedigree of Mother	Pedigree of Father
Haun (AM)	AM	ø	*C. dentata*	*C. dentata*
CAT 33 x Pryor 180 (AM)	AM	ø	*C. dentata*	*C. dentata*
CAT-275 x Neel 4-195 (B1)	B1	Amy	*C. dentata*	2004 TN-BF1-E10 x Amy
CAT-273 x TTU A29 (B1)	B1	Gideon	*C. dentata*	2004 TNCLA1 x Gideon
TN-TTU-A34 x NCDOT (B1)	B1	Gideon	2004 TNCLA1 x Gideon	*C. dentata*
CG61 x Pryor 180 (B1 NK2)	B1	Nanking	Ted Farmer B x GR 199 'Nanking'	*C. dentata*
CG61 x NCDOT (B1 NK4)	B1	Nanking	Ted Farmer B x GR 199 'Nanking'	*C. dentata*
CAT-273 x TN-CN 9-153 (B1)	B1	Chinese	*C. dentata*	Whiteside x opCh
TN-SM1-Q/S58 x OP (B3F2)	B3F2	Clapper	2002 TNBLO1 x GL103	OP
TN-SM2-C37 x OP (B3F2)	B3F2	Clapper	2007 AG387 x TNMAC2	OP
TN-SM2-E29 x OP (B3F2)	B3F2	Clapper	2006TNMON5 x HE416	OP
TN-SM2-G27 x OP (B3F2)	B3F2	Clapper	2006 TNMON4 x IL332	OP
TN-SM2-G44 x OP (B3F2)	B3F2	Clapper	2007 VA89 x TNJAC5	OP
TN-SM2-G-56 x OP	B3F2	Clapper	2007 VA89 x TNJAC5	OP
TN-SM2-H37 x OP	B3F2	Clapper	2007 GL367 x TNGSMNP1	OP
TN-SM2-H56 x OP	B3F2	Clapper	2007 VA89 x TNJAC5	OP

Family	Cross type	Source of Resistance	Pedigree of Mother	Pedigree of Father
TN-SM2-I28 x OP	B3F2	Clapper	2007 NCGRA1 x GL96	OP
TN-SM2-I31 x OP	B3F2	Clapper	2007 NCGRA1 x GL96	OP
TN-SM2-I33 x OP	B3F2	Clapper	2007 NCGRA1 x GL96	OP
TN-SM2-J28 x OP	B3F2	Clapper	2007 TNMON8 x GR210	OP
TN-SM2-J39 x OP	B3F2	Clapper	2007 TNMON8 x GR210	OP
TN-TTU-M13 x OP	B3F2	Graves	2004 TNCLA2 x AB248	OP
TN-TTU-C27 x TN-TTU-A30	B3F2	Clapper and Gideon	2004 TNSUM1 x VA89	2004 TNCLA1 x Gideon
TN-TTU-E6 x Neel 5-275	BB1	Clapper, Meiling and Lindstrom 67	2004 TNSUM1 x VA89	2004 TN-BF3-L10 [1996 TN-BF1-D5 (American) x AP1-1 (Meiling x American)] x Lindstrom 67
TN-TTU-E6 x TN-TTU-A30	BB1	Clapper and Gideon	2004 TNSUM1 x VA89	2004 TNCLA1 x Gideon
TN-TTU-M10 x A30	BB1	Graves and Gideon	2004 TNCLA2 x AB248	2004 TNCLA1 x Gideon
TN-TTU-M13 x TN-TTU-A30	BB1	Graves and Gideon	2004 TNCLA2 x AB248	2004 TNCLA1 x Gideon
TN-SM2-I28 x OP	B3F2	Clapper 2007	NCGRA1 x GL96	OP
Smith Farm Chinese	CH	*C. mollissima*	*C. mollissima*	*C. mollissima*
Princeton MA Chinese	CH	*C. mollissima*	*C. mollissima*	*C. mollissima*
NCBUN10 x CC-PR05-4-42	F1	*C. mollissima*	*C. mollissima*	*C. mollissima*
TNCOC1 x Nanking	F1	*C. mollissima*	*C. dentata*	*C. mollissima*
WWC67 x OP (NK5)	F2	Nanking	GR119 'Nanking' x KH2UU	GR119 'Nanking' x KH2UU
WWC70 x OP (NK6)	F2	Nanking	GR119 'Nanking' x KH2UU	GR119 'Nanking' x KH2UU
TN-SM1-C59 x OP	F2	Ginyose	2008 TNMON7 x Ginyose	OP
TN-SM1-D41 x OP	F2	Sleeping Giant	2005 KYADA1 x Sleeping Giant	OP
TN-TTU-A10 x OP	F2	Gideon	2004 TNCLA1 x Gideon	OP
NJ Paris F1	F1	Paris	Paris AM	Paris opCH
Greg Miller Chinese	CH	*C. mollissima*	Greg Miller Chinese Mix	opCH

Family	Generation	Source of Resistance	Pedigree of Mother	Pedigree of Father
Nanking 5	F2	Nanking	WWC67	OP
Nanking 6	F2	Nanking	WWC70	OP
Pryor 180 x GR119	F1	Nanking	Pryor 180	GR 119
Pryor 90 x OP	AM	*C. dentata*	Pryor 90	OP
YGF x OP	CH	*C. mollissima*	CH YGF	OP
TN-SF H38	CH	*C. mollissima*	TN-SF-H38	
C. mollissima Princeton MA	CH	*C. mollissima*		
CH Qing x OP	CH	*C. mollissima*	Qing Seedling x OP	OP

Table 2 Families planted for the 2018 small stem assay. "OP" represents trees that are open pollinated

Watering

Plants from each year were required to remain inside the greenhouse until the weather was warm enough for them to be moved outside. Inside the greenhouse the same watering method was used for both the 2017 and 2018 trials. Watering in the greenhouse was done by hand; each plant was watered until saturation, by the greenhouse staff (myself, Trent Deason, Paola Zannini, Scotty Smith and Hill Craddock) as needed, according to nursery practice. During the 2017 trial the seedlings planted in 656 ml containers were watered by hand once placed outside the greenhouse by Hill Craddock, me, Kevin Gentner, and Paola Zannini. When the plants were moved outside the 7.65-liter containers were assembled on a drip irrigation system. The drip irrigation system (Netafirm non-pressure compensating spray stakes (black 7.0 GPH)) is designed so that each plant receives as close to the same amount as possible. The 7.65-liter containers were watered as needed by the nursery staff. The container-grown plants needed every

day watering during the hottest months (June, July, and August). Once the weather cooled down the plants were watered two or three times a week.

Inoculation

2017

Seedlings were inoculated during the first week of July by Kevin Gentner with the help of the Fortwood Greenhouse Crew and several volunteers. Of the 1,299 seeds that were planted 1,132 seedlings were inoculated. Minimum growth requirements for inoculation required each seedling to be at least 25cm tall, and greater than 3mm in diameter at 10cm above the root collar. Seedlings that did not meet the growth requirements or were infected by other plant diseases were removed from the trial. *Cryphonectria parasitica* grown on a potato dextrose agar was used for the inoculation. The immature bark of each seedling was sliced open roughly 10 cm above the root collar using a nitpicker (this distance was adjusted as needed so the inoculation point was away from axillary branches). Seven-day-old plates of *Cryphonectria parasitica* were used for inoculation. A cork borer method was used to apply a 4mm plug of the fungal mycelium to the open wound with ethanol sterilized tools and secured with a piece of parafilm.

2018

Inoculation was done by me, Hill Craddock, Trent Deason, Scotty Smith, Paola Zannini, students of UTC and several TACF volunteers, at the end of July approximately four months after planting. An isolate of EP-155 was used for the inoculation. This is a highly virulent strain of *Cryphonectria parasitica* obtained from the TACF lab in Meadowview, Virginia. Each seedling had to have a stem diameter of 4 mm to be inoculated. Seedlings that did not meet the

stem diameter requirements or that were dead before the inoculation were removed from the trial. Of the 666 seeds planted, 537 seedlings were inoculated. *Cryphonectria parasitica* was grown on a potato dextrose agar. The bark of the seedlings was cut with an ethanol sterilized knife. Dr. Craddock cut a rectangular incision that was exactly 10 mm long and 1-2 mm wide on each seedling (Figure 2b). Two inoculations were made per seedling to obtain a higher resolution. Incisions were cut through the phloem to allow the pathogen access to the xylem. The fungus was then applied to the incision with ethanol sterilized tools. The outer edge of the mycelium of the inoculum was cut into fragments roughly the size of the incision (Figure 2a). The outer edge of the mycelium was used to obtain fast-growing hyphal tip that will continue to grow in the xylem of each seedling. After the inoculum was applied to the incision a piece of parafilm was wrapped around the stem at the point of inoculation (Figure 2c).

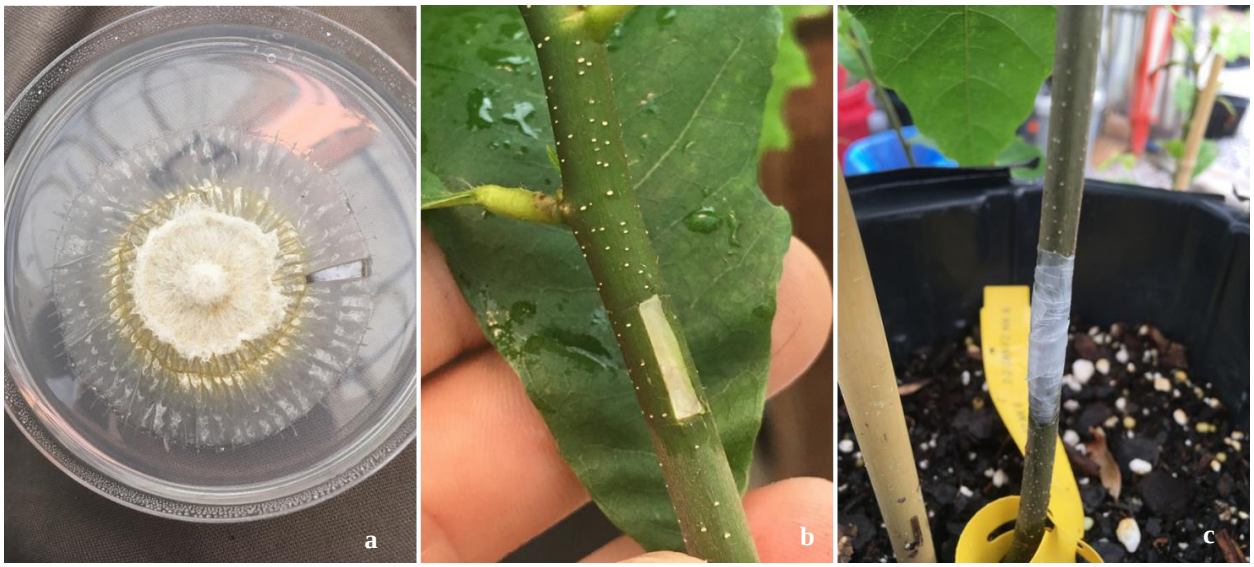

Figure 2 Process of Inoculation
The outer edge of the mycelium of the inoculum was cut into fragments roughly the size of the incision (a) J. Hill Craddock cut a rectangular incision that was exactly 10 mm long and 1-2 mm wide on each seedling (b) Parafilm was wrapped around the stem at the point of inoculation (c)

Screening

2017

Seedlings were observed 3-4 days a week for 15 weeks. Inoculated plants were examined for wilting, discoloration of the leaves and death of distal vegetative growth caused by the lesion. The day on which the seedlings wilted was recorded and used to calculate days to wilt. Canker length was also measured on the day the plant wilted. Fifteen weeks after inoculation all canker lengths were recorded.

When screening in 2017 there were an unusual amount of no takes, trees were inoculated however there was no sign of infection or any fungal presence. These no takes greatly reduced the sample sizes.

2018

Cankers were measured at eight weeks and sixteen weeks after inoculation. Several volunteers gathered on both screening days to measure cankers. The infected tissue of a canker appears orange in color and there is a distinction between the canker and the healthy bark (Figure 3b). Sometimes the distinction between canker and healthy bark can be blurred. This could cause some variation in canker measurements dependent upon the person measuring the canker. Each seedling had two inoculations therefore a measurement was taken for each inoculation. Due to there being two inoculations (some points of inoculation were close together) the cankers had sometimes fused to become one large canker. When this was the case the entire canker was measured, and this measurement was divided by two (the number of inoculations) giving an estimate of the size of each canker. These two measurements were then recorded. Before analyzing statistics, the average canker length for each seedling was calculated, this average represented the canker length for each plant.

Figure 3 Developing Cankers
Within a few days the orange fungus can be seen spreading from the wound site (a)
Seedlings with little to no resistance develop a canker at the point of inoculation (b)

Analysis

The software package RStudio was utilized for the statistical analysis of both trials (recommended by Jared Westbrook, Director of Science at TACF). All significance decisions made during analyses and conclusions used an alpha level of 0.05. Data were analyzed utilizing a Welch's two samples *t*-test, ANOVA, and Tukey's Honestly Significantly Difference tests (Elliott et al., 2007, Zar 1984). Assumptions of normal distribution were checked using boxplots and histograms for appropriate data sets.

Welch's Two Sample *t*-test

Welch's two sample *t*-test was used to determine whether two means from two different populations or sample sizes are different from each other. This test can be used when populations

or sample sizes have unequal variances or sample sizes (Ruxton, 2006). Welch's two sample test was used to heck for significant differences between the two different F2 families (2018 trial). The null hypothesis in this experiment is that the sample mean group one is not significantly different from group two. The alternative hypothesis is that the sample mean of group one is not the same as group two.

Analysis of Variance

Analysis of Variance (ANOVA) is an extension of the independent samples *t*-test used to determine whether there are differences among more than two group means (Elliott et al., 2007). ANOVA is a compilation of statistical models that can be applied when determining difference between group means and their variation among and between groups. This test was used for testing significance in both the 2017 and 2018 trial. The one-way ANOVA was used to test for significance in different comparisons:

(1) Determine if there was significant difference between average days to wilt and cross type (2017).
(2) Determine if there was significant difference between average canker length and families of the same generation (2017).
(3) Determine if there was significant difference between average canker length and the different Chinese families (2018).
(4) Determine if there was significant difference between average canker length and the different generations of seedlings (2018).

The null hypothesis of the ANOVA is: $\mu_1 = \mu_2 = \ldots \mu_k$. The alternative hypothesis is at least one μ_i is different from at least one other μ_i. A randomized complete block design was used in the

2018 trial therefore a two-way ANOVA was utilized to determine if there was a significant effect of block on canker length and if the interaction of cross type and block had a significant effect on canker length.

Data Cleaning

The days to wilt data from the 2017 trial had to be cleaned before statistical analysis. Several trees were not given a day to wilt therefore only trees that were given a day to wilt were used in the analysis.

Tukey's Honestly Significant Difference Test

Tukey's Honestly Significant Difference Test (Tukey's HSD) is a multiple-comparisons test (Zar, 1984). This test is used if a significant difference was determined after an ANOVA. Tukey's HSD compares each possible combinations of groups' (cross types or generations) means with each other to find where the significance found during the ANOVA was stemming from.

CHAPTER IV

RESULTS

2018

Before statistical analysis, I compared the differences between the mean canker lengths of the Chinese families with an ANOVA. No significant difference was found at the eighth week measurement between the four Chinese families (F=1.036, $p > 0.05$) therefore Chinese families were grouped for all analyses (Table 3). No significant difference was found at the sixteen-week measurement between the four Chinese families (F=0.693, $p > 0.05$) therefore Chinese families were grouped for all analyses (Table 4). I also compared the differences between the mean canker lengths of the two F2 families with a *t*-test. No significant difference was found at the eight-week measurement ($p > 0.05$) and the two F2 families were grouped together in further analyses (Table 5). No significant difference was found at the sixteen-week measurement ($p > 0.05$) and the two F2 families were grouped together in further analyses (Table 6).

Table 3 Results of one-way ANOVA of mean canker length between different families of *C. mollissima* at eight weeks

Source of Variation	**SS**	**df**	**MS**	***F***	***p*-value**
Between Groups	1,665	3.000	555.0	1.036	0.393
Within Groups	13,930	26.00	535.8		
Total	15,590	29.00			

Table 4 Results of one-way ANOVA of mean canker length between families of *C. mollissima* at sixteen weeks

Source of Variation	**SS**	**df**	**MS**	***F***	***p*-value**
Between Groups	1,139	3.000	379.7	0.693	0.565
Within Groups	15,380	26.00	547.9		
Total	16,520	29.00			

Table 5 Results of *t*-test and descriptive statistics for eight week canker length between Nanking 5 and Nanking 6 (F2s)

	Families							
	Nanking 5		Nanking 6		95% CI for the Mean Difference			
	M	n	M	n		*t*	df	*p*-value
Canker Length	102.30	67	97.51	324	-2.82, 12.40	1.25	88.40	0.214

Table 6 Results of *t*-test and descriptive statistics for sixteen week canker length between Nanking 5 and Nanking 6 (F2s)

	Families							
	Nanking 5		Nanking 6		95% CI for the Mean Difference			
	M	n	M	n		*t*	df	*p*-value
Canker Length	127.30	10	107.40	91	-5.22, 45.00	1.75	10.50	0.108

One-Way Analysis of Variance

A one-way ANOVA was conducted to determine if there was significant difference in mean canker length between all groups *C. dentata*, *C. mollissima,* F1 and F2 seedlings. There was a significant difference found at eight weeks and sixteen weeks between cross type and canker length shown in Table 7 and Table 8.

Table 7 Results of one-way ANOVA of eight week mean canker length between all families 2018

Source of Variation	**SS**	**df**	**MS**	***F***	***p*-value**
Between Groups	16,640	3.000	5,487	8.041	0.000*
Within Groups	340,500	499.0	682.0		
Total	357,100	502.0			

*$p < 0.05$

Table 8 Results of one-way ANOVA of sixteen week mean canker length between all families 2018

Source of Variation	**SS**	**df**	**MS**	***F***	***p*-value**
Between Groups	32,690	3.000	1.090e04	12.32	0.000*
Within Groups	121,200	137.0	884.0		
Total	153,900	140.0			

*$p < 0.05$

Tukey's Multiple Comparisons of Means

The Tukey HSD analysis was conducted on all possible pairwise comparisons. Table 9 and Table 10 lists all the pair wise comparison results of the Tukey HSD analysis by groups of chestnut. The only pair that was found to be significantly different at the eight-week measurement (Table 9, $p < 0.05$) was the comparison between the Chinese and F2 generations. Significant differences were between AM and CH and between CH and F2 at the sixteen-week measurement (Table 10, $p < 0.05$)

Table 9 Tukey HSD pairwise comparison for eight week canker length between groups of chestnut seedlings

Pair	**Contrast**	**Lower bound**	**Upper bound**	***p*-value**
AM-CH	13.28	-1.535	28.05	0.097
F2-CH	21.86	9.105	34.62	0.000*
F1-CH	15.00	-6.293	36.29	0.267
F2-AM	8.605	-0.298	17.51	0.063
F1-AM	1.743	-17.491	20.98	0.996
F1-F2	-6.862	-24.58	10.85	0.750

*$p < 0.05$

Table 10 Tukey HSD pairwise comparison for sixteen week canker length between groups of chestnut seedlings

Pair	Contrast	Lower bound	Upper bound	*p*-value
AM-CH	-55.12	-102.0	-8.280	0.013*
F2-CH	36.16	20.08	52.24	0.000*
F1-CH	22.14	-10.33	54.61	0.290
F2-AM	-18.96	-64.27	26.36	0.697
F1-AM	-32.98	-86.35	20.30	0.378
F1-F2	-14.02	-16.21	44.25	0.624

*$p < 0.05$

Two-Way ANOVA

A two-way ANOVA was conducted to determine if there was a significant effect of block on canker length or between the interaction of block and seed type on canker length. Canker length was not affected by block and there was no significant effect found between the interaction of cross type and block on canker length at the eight-week measurement (Table 11). Canker length was not affected by block and there was no significant effect found between the interaction of cross type and block on canker length at the sixteen-week measurement (Table 12).

Table 11 Results of two-way ANOVA of the interaction of block and seed type on canker length at the eight-week canker length 2018

Source of Variation	**SS**	**df**	**MS**	***F***	***p*-value**
Block	2,247	2.000	1,123	1.600	0.193
Seed Type	16,410	3.000	10,760	8.038	0.000
Interaction	4,090.0	6.000	682.0	1.001	0.424

*$p < 0.05$

Table 12 Results of two-way ANOVA of the interaction of block and seed type on canker length at the sixteen-week canker length 2018

Source of Variation	**SS**	**df**	**MS**	***F***	***p*-value**
Block	2,247	2.000	1,123	1.651	0.190
Seed Type	16,410	3.000	5,471	8.041	0.000
Interaction	4,091	6.000	682	1.000	0.424

*$p < 0.05$

2017

One-Way ANOVA Between Days to Wilt

A one-way ANOVA was conducted to determine if there was significant difference in days to wilt between all generations of the 2017 trial: *C. dentata, C. mollissima*, F1, F2, B1, BB1, and B3F2 seedlings. No significant difference was found ($p = 0.442$) between seed type and days to wilt shown in Table 13.

Table 13 Results of one-way ANOVA between days to wilt and all groups

Source of Variation	**SS**	**df**	**MS**	***F***	***p*-value**
Between Groups	2,382	5.000	476.4	0.966	0.442
Within Groups	53,250	108.0	493.0		
Total	55,630	636.0			

*$p < 0.05$

One-Way ANOVA Between Families

One-way ANOVA was conducted on all families of the same generation and canker length at the eight-week measurement. There was no significant difference found between families of the same generation and canker length (table 15).

Table 14 ANOVA *p*-values for mean canker length comparisons to families of the same generation

Families	***p*-values**
B1	0.080
B3F2	0.103
BB1	0.389
F2	0.975

CHAPTER V

DISCUSSION AND CONCLUSIONS

Objectives of Study

The research objectives for the study were to focus on the results of two years of trials that included the inoculation of 391 F2 hybrid seedlings (2018) and the inoculation of 258 first back-cross and 261 third-backcross seedlings (2017) during their first and second growing seasons. This study:

(1) Analyzed whether the F2 population in the 2018 trial segregates as we expect with most trees having intermediate resistance to that of the F1s and very few trees having similar resistance to that of *C. mollissima* and *C. dentata*. Specifically, I analyzed if this segregation can be observed in canker length, such that more susceptible trees have larger cankers and more resistant trees have smaller cankers.

(2) Compared average canker lengths by family to determine if there was significant variation between family types of the same generation of the 2017 trial.

(3) Analyzed the days to wilt data from the 2017 trial to determine the accuracy of days to wilt compared to canker length in determining resistance to chestnut blight.

A Welch's two sample *t*-test and a one-way ANOVA, at a 0.05 significance level, detected that there were no significant differences between the two F2 families (Nanking 5 and Nanking 6) and no significant differences between the four Chinese families at the eight and sixteen week canker lengths. Thus, the Chinese families and F2 families were grouped in all

statistical analyses. The results obtained from the tests conducted in the 2018 trial, at a 0.05 significance level, stated that: there were significant differences found between *C. dentata* and *C. mollissima* controls at the end of the sixteen weeks (no significance was detected at eight weeks between *C. dentata* and *C. mollissima* controls) and between cross type and canker length at both sixteen weeks and eight weeks. The significant difference found between cross type and canker length was between the *C. mollissima* and F2 seedlings at both sixteen weeks and eight weeks. A two-way analysis of variance showed no significant difference between the interaction of cross type and block on canker length at either the eighth or sixteenth week.

The results obtained from the tests conducted in the 2017 trial, at a 0.05 significance level, showed that: there was no significant difference found between families of the same generation and length and there was no significant difference between cross type and days to wilt.

Segregation of F2 Population 2018

Blight resistance in *C. mollissima* was thought to be primarily controlled by alleles at a minimum of three loci (Kubisiak et al., 1997; Kubisiak et al., 2013; Hebard, 2006,). Burnham et al. (1986) presumed, based on early observations of the F1s having a canker length intermediate between their *C. dentata* and *C. mollissima* parents, that the inheritance of chestnut blight was incompletely dominant. Due to the observed genetic inheritance of chestnut blight resistance, after the breeding process eventually created a B3F2 hybrid, he predicted only 1/64 B3F2 hybrids to be homozygous at all three loci for chestnut blight resistance. Therefore, most of the F2 hybrids (straight F2s or backcrossed F2s) should display a full range of resistance, from highly resistant to low resistance to the effects of chestnut blight with most individuals having an

intermediate resistance. In a bar graph this distribution would resemble a bell curve. More recent research (Westbrook et al., 2020) shows evidence that inheritance of blight-tolerance is polygenic (more than three loci involved), the distribution of the F2s would still resemble a bell curve. The F2 bell curve observed in canker length at the end of the sixteen weeks (Figure 4) is misleading. If we are assuming that more resistant trees have smaller cankers the distribution of F2 canker lengths is telling a different story, as most of the surviving F2s had large cankers (Figure 4). However, very few of the F2 seedlings survived (Figure 5) as expected and supports polygenic inheritance of blight resistance.

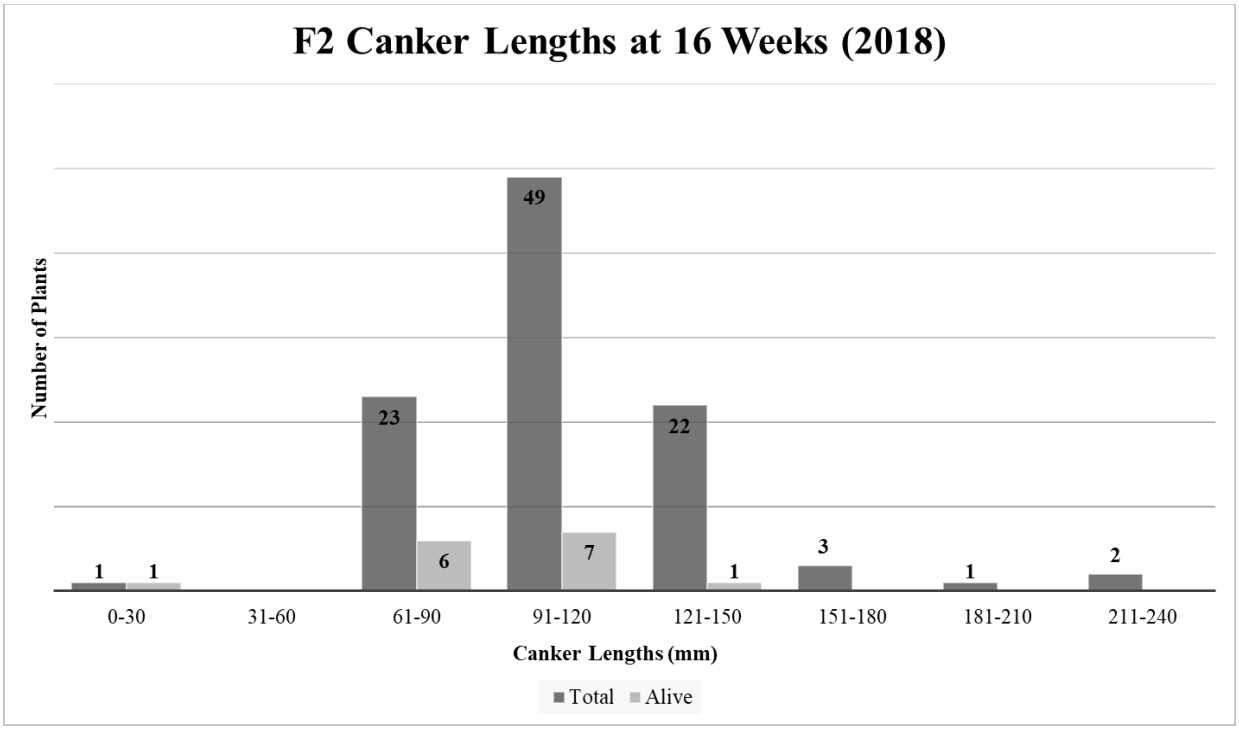

Figure 4 Frequency of the different canker lengths of dead and alive F2 seedlings at sixteen weeks
The canker length range of all seedlings (dead and alive) with the highest frequency is 91 to 120mm.
The canker length range of alive seedlings with the highest frequency is 91 to 120 mm.

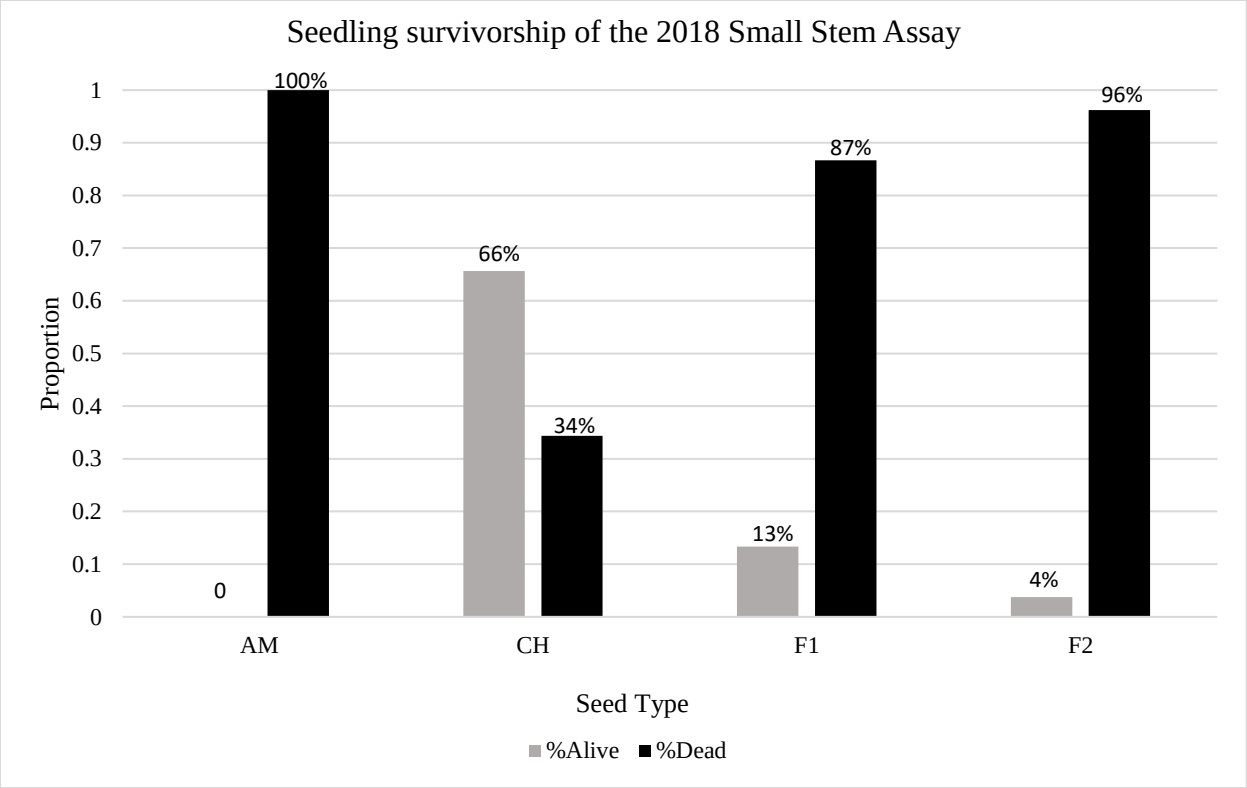

Figure 5 2018 Number of inoculated and surviving chestnut hybrids, F1s and F2s, and control groups, *C. dentata* and *C. mollissima* seedlings at the end of the sixteen weeks

Due to the polygenic inheritance of blight resistance Westbrook (2018) suggests that blight resistance may be improved with additional generations of intercrossing and recurrent selection. However, homozygosity at all loci involved is highly improbable. Selection of backcross trees will still take place, and once resistance levels of backcross allows trees to compete in forests, natural selection may continue to improve resistance (Westbrook et al., 2020)

Inoculum of the 2017 Trial

During the 2017 trial there was an issue with the inoculum that led to a high rate of inoculation failure, recorded as “no takes” by Gentner (2018). After further investigation it was

discovered that inoculum received from the research lab at Meadowview, VA was possibly contaminated with *Trametes versicolor*. J. Hill Craddock noticed that the fungus was not orange and decided to use a wild-type strain he collected and grew from Lula Lake.

Canker Length by Families of the Same Generation 2017

Table 1 shows the results of the one-way ANOVA showing the effects of families of the same generation on canker length. No significant difference was found between families of the same generation and canker length during the eighth week. Families were not different from one another regarding canker length, meaning we were not able to differentiate between families during the eighth week. Therefore, we were unable to determine the differences in canker length during the eighth week from different families of B1, BB1, B3F2, and F2s.

Days to Wilt 2017

A one-way ANOVA found no significant difference ($p = 0.442$) on the effect of generation on days to wilt. The very small sample sizes did not allow for us to see more significant results from the 2017 trial. However, Figure 6 shows the different rates that different seed types wilted. As expected, *C. dentata* on average died faster than all other seed types. The B3F2 and the B1 seedlings both wilted on average around eighty days. The B1s (first backcrosses) can only be expected at best to have intermediate blight resistance similar to that of the F1s. Due to the polygenic inheritance of blight resistance most of the F2s (straight F2s or B3F2s) are expected to have intermediate blight resistance also similar to that of F1s. It is promising that we were able to see this similarity in the box and whisker plot.

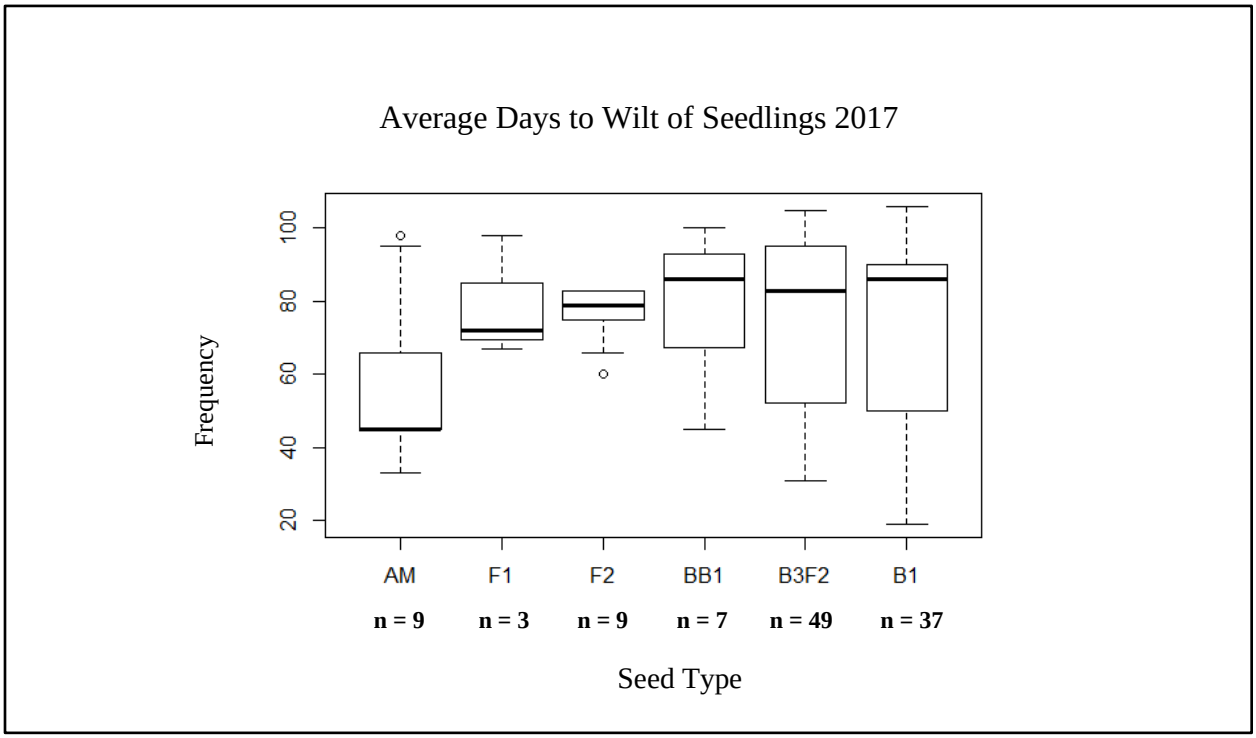

Figure 6 Distribution of days to wilt between the different groups of seedlings 2017 Chinese seedlings are not present as none of the Chinese wilted.

Days to Wilt as Determining Resistance

During the 2018 trial Chinese seedlings although having resistance to the effects of chestnut blight have very large cankers, the average canker length of surviving Chinese seedlings at the end of the 16 weeks was 73.22 mm with highest frequency of canker lengths lying within the range of 30 to 90 mm with some seedlings living with cankers ranging from 91 to 120 mm (Figure 7). The average length of *C. dentata* seedling cankers at eight weeks was 89.72 mm which lies within the range of the highest frequency of canker lengths in the Chinese during the eighth week (Figure 9). Furthermore, the F2s that were alive at the end of the trial also had very large cankers (Figure 5). This was unexpected as the very best of the F2s were expected to have smaller cankers. Vice versa there were F2s that did not survive and had very large cankers. These

observations have led to a discussion as to whether canker length is the most effective way to determine blight resistance given that the Chinese can live with chestnut blight therefore the fungus can continue to grow on a living tree producing a larger canker. More *C. dentata* die than *C. mollissima* (Figure 4) and therefore can have small cankers (Figure 8). This can become problematic if we are only using canker length to select the best families of F2s, as both living and dead trees have similar canker sizes.

Another metric being used by chapters of TACF is days to wilt. This is a logical way to determine resistance since *Cryphonectria parasitica* infects the bark by sending fine threads of mycelia into the inner bark and destroying the vascular cambium. The degradation of the vascular cambium via oxalic acid causes structural weakness and prohibits the flow of nutrients to parts distal of the lesion (McCarroll & Thor, 1978) which would cause a plant to wilt. This technique has shown success in other trials. Saielli & Levine (2018) were able to differentiate between the *C. dentata* and *C. mollissima* and were able to detect the ten best families of B3F3s out of the 107 families tested. The results from this trial helped in determining which B3F2 parents need to be culled from the program. Trials at Pennsylvania State University have also had more success using a days to wilt metric and have found canker lengths in a small stem assay to be problematic (Sara Fitzsimmons, personal communication).

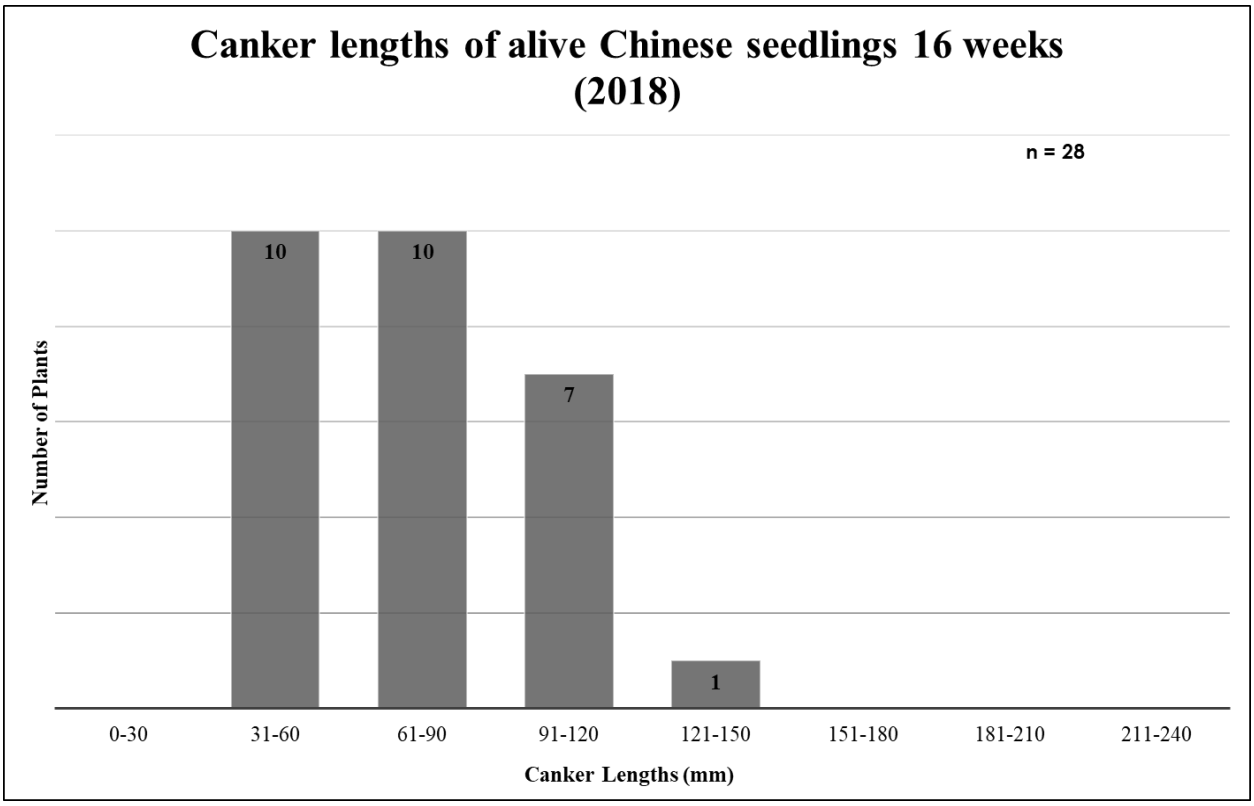

Figure 7 Frequency of the different canker lengths of alive *C. mollissima* seedlings at sixteen weeks
The canker length ranges with the highest frequency is 31 to 61mm and 61 to 90 mm.

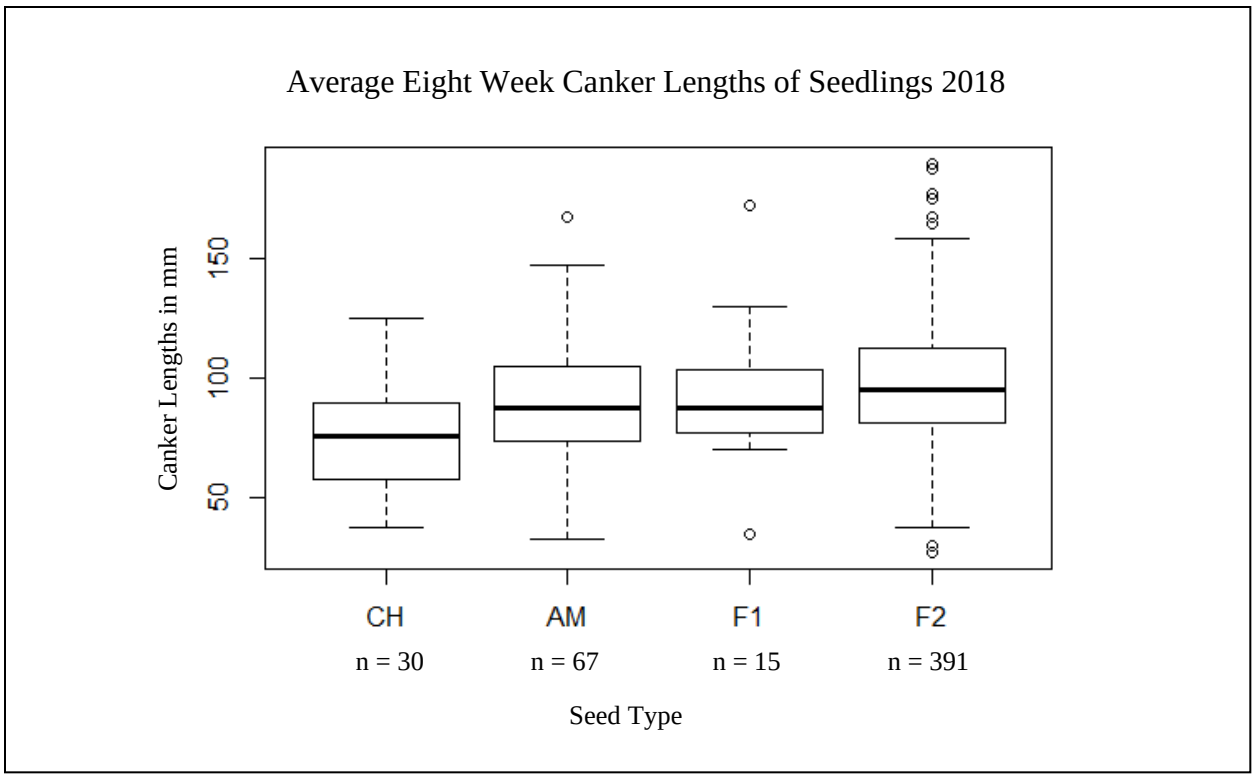

Figure 8 2018 Distribution of eight-week canker lengths between the different groups of seedlings at eight weeks

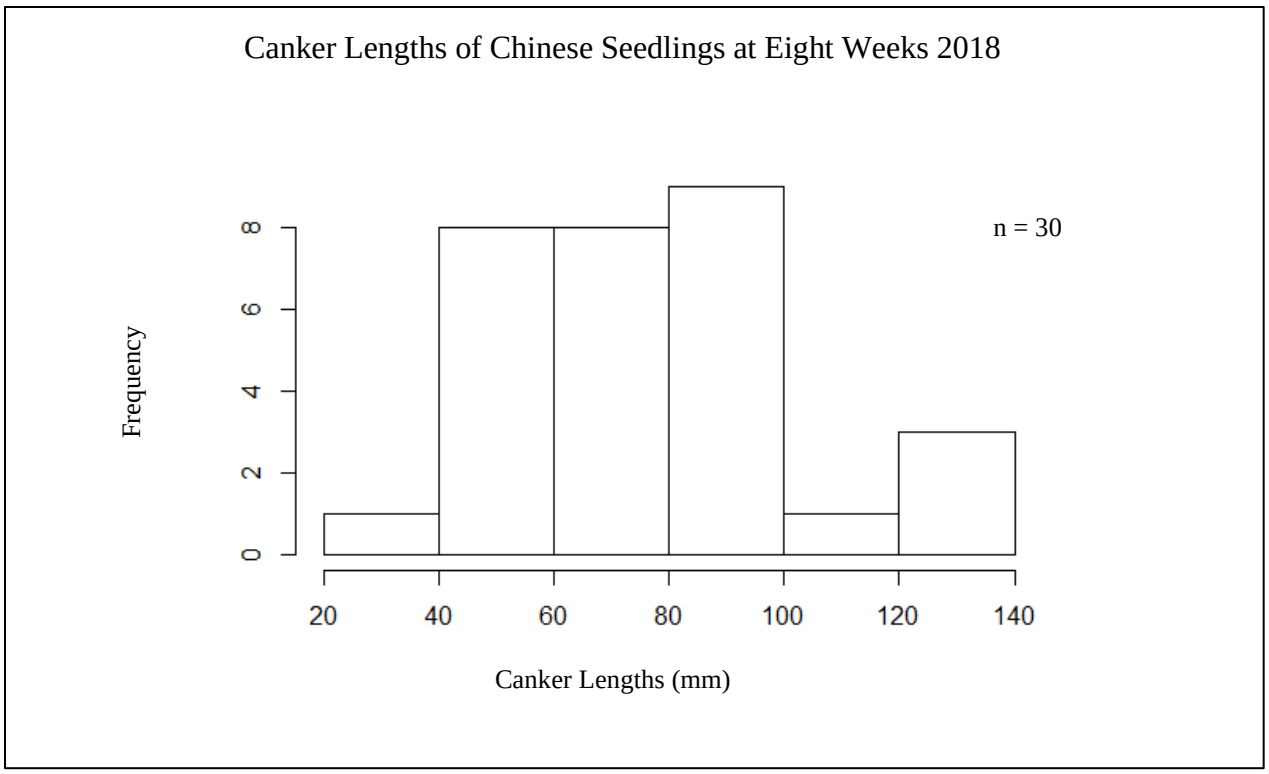

Figure 9 Frequency of the different canker lengths of *C. mollissima* seedlings at eight weeks The canker length range with the highest frequency is 80 to 100mm.

Conclusion

The small stem assay is very harsh and produces very large cankers. The ability to differentiate between intermediate levels of resistance proves to be difficult with this type of a trial. However, the small stem assay does look to be promising for progeny testing and to select the very best of the B3F2s to plant into the seed orchard.

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VITA

Margaret Miller was born 8 July 1991 in Tupelo, Mississippi. Early during childhood, she moved to Jackson, Tennessee where she attended high school. After graduating high school, she began college at The University of Tennessee at Chattanooga and graduated in 2014 with a bachelor's degree in Spanish. A few years after graduation she decided that she needed to follow her true passion of working with and studying plants. She didn't know the best way to pursue this passion, but she knew had to return to school to enhance her scientific background. While attending UTC as a post-baccalaureate student she started working with Dr. Hill Craddock in nursery on campus and really enjoyed working with him. Dr. Craddock one day asked her if she would like to continue working for him doing chestnut research while receiving her master's degree in Environmental Science. She excitedly accepted his offer and was accepted to the graduate program at UTC. This manuscript is the final requirement of her graduate work before receiving her M.S. in Environmental Science from The University of Tennessee at Chattanooga.