ABSTRACT

Cothron, Caleb Phillip. Distribution and Diversity of Oomycete root pathogens affecting Fraser fir Christmas tree production in the Southern Appalachian Mountains and Evaluation of elite poplar genotypes performance against *Phytophthora* inoculation (Under the direction of Dr. Justin Whitehill).

Fraser fir (*Abies fraseri*) is the most economically significant Christmas tree species grown in the Southern Appalachian Mountains, particularly in North Carolina, where it accounts for most of the production. However, its cultivation is increasingly threatened by Phytophthora root rot (PRR), caused by multiple species of *Phytophthora*, which results in severe economic losses for growers. This study aimed to assess the distribution and diversity of *Phytophthora* species affecting Fraser fir, as well as evaluate potential alternative crops for cultivation on PRR-contaminated land. Symptomatic trees were sampled across the region, yielding 151 *Phytophthora* isolates. Six different species were identified including: *P. cactorum, P. cinnamomi, P. citricola, P. cryptogea, P. europaea,* and *P. plurivora* with *P. cinnamomi* being most prevalent. Phylogenetic analysis of *P. cinnamomi* isolates revealed low variation which may reflect the low diversity of *P. cinnamomi* lineages in North America, low variation among the genes chosen for sequencing, or both. In a greenhouse experiment, Fraser fir seedlings were inoculated with different *Phytophthora* isolates to compare the aggressiveness of *Phytophthora* species. Seedlings inoculated *P. cinnamomi* exhibited the most disease symptoms and mortality while seedlings inoculated with other *Phytophthora* species exhibited varying lesser symptoms and mortality. Mock-inoculated seedlings, and seedlings inoculated with non-*Phytophthora* Oomycete isolates did not exhibit any disease symptoms or mortality. A greenhouse inoculation experiment was also conducted to evaluate various elite clonal and hybrid cross poplar (*Populus*) genotypes as a potential alternative short rotation woody crop to be deployed on PRR contaminated land. Eight different clonal genotypes were inoculated with five different

Phytophthora species and height growth was measured over a 16-week period. No statistically significant differences in growth rate were detected between treatments, regardless of clone or cross. These findings contribute to our understanding of what pathogens are limiting the production of Fraser fir, how the diversity of those pathogens is changing, as well as what options growers have that can address those limitations.

Distribution and Diversity of Oomycete root pathogens affecting Fraser fir Christmas tree production in the Southern Appalachian Mountains and Evaluation of elite poplar genotypes performance against *Phytophthora* inoculation

by
Caleb Phillip Cothron

A Thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Forestry

Raleigh, North Carolina
2025

APPROVED BY:

_____	_____
Dr. Justin Whitehill Committee Chair	Dr. Elizabeth Nichols
_____	
Dr. Jean Ristaino	

DEDICATION

To my loving and ever charitable wife Cameron, who faithfully and graciously stood by my side both in times of joy and despair. Your love is a treasure to me. Also, to our son, my heart is bursting at the thought of you and you're not even here yet. I can't wait to meet you. Finally, to my friends from another time: Boethius, Augustine and Athanasius, in whose writings I found a quiet companionship, challenging reflections, affirmation of the world's beauty, and a comforting encouragement to carry on.

BIOGRAPHY

Caleb Cothron was born in Charlotte, North Carolina on March 30th, 1996, to Derly and Priscila Cothron. His interests in the natural world and particularly forests grew from his time hiking and camping in the mountains of western North Carolina. He received a Bachelor of Science in Forest Management from North Carolina State University in December 2019. Caleb worked professionally as a forester first in private industry then for the North Carolina Forest Service before joining the Christmas Tree Genetics program at North Carolina State University in August 2022 where he is pursuing a Master of Science in Forestry and minoring in Plant Pathology.

ACKNOWLEDGMENTS

Special thanks to Dr. Justin Whitehill for his advising and providing this opportunity and work and study in the Christmas Tree Genetics lab. Many thanks to my committee members for their consistent patience, wisdom, and assistance. Additionally, I would like to thank those members of NC Cooperative Extension, without whose help this project would not have been possible, especially Jeffrey Owen, Jerry Moody, Paige Pattersson, Brad Edwards, Blake Williams, Minda Daughtry, Shane Biddix, Bart Renner, and Christy Bredenkamp. Special thanks to the many growers who graciously accommodated, supported, and allowed me to collect samples from their fields, especially Rusty Barr and Joe Freeman. Thank you to Solomon Ghezehei Ph.D. for his extensive knowledge of poplar, help collecting cuttings and materials, and constant encouragement. Thank you also to Amanda Saville and Amanda Mainello-Land Ph.D. for patiently answering all my emails and meeting with me to discuss Phylogenetics. Finally, thank you to the members of the Christmas Tree Genetics program for their help, guidance, and friendship. Especially Will Kohlway Ph.D., Will Baldwin, Adarsha Devihalli, Sai Karthik Gade, Priya Rajarapu Ph.D., John Bagwell Ph.D., Jesse Hickman, Kaley Patton, Estefania Elorriaga Ph.D. and Angela Chiang.

TABLE OF CONTENTS

LIST OF TABLES vi
LIST OF FIGURES viii

Chapter 1: Background and Literature Review 1
1.1 Christmas trees – A Brief History 1
1.2 Christmas Tree Production 2
1.3 Challenges to Christmas Tree Production 4
1.4 Need for Further Research 11
Chapter 2: Distribution and Diversity of Oomycete Root Pathogens Affecting Fraser Fir (*Abies fraseri*) Christmas Tree Production in the Southern Appalachian Mountains 13
2.1 Introduction 13
2.2 Materials and Methods 16
2.3 Results 24
2.4 Discussion 28
Chapter 3: Inoculation of Poplar (Populus) Clones with Phytophthora Isolates for Evaluation of Deployment on Phytophthora Root Rot Contaminated Land 51
3.1 Introduction 51
3.2 Materials and Methods 53
3.3 Results 57
3.4 Discussion 58
Chapter 4: Conclusions and Future Opportunities 76
4.1 Summary and Future Goals 76
References 79
Appendices 87
Appendix A. Additional Material for Chapter 2 87
Appendix B. Additional Material for Chapter 2 98

LIST OF TABLES

Chapter 2

Table 2.1 Primer sequences used for amplification of target genes including the annealing temperature of the primers, the expected amplified fragment length, and the published source for the original sequence..... 33

Table 2.2 Symptom ratings of sampled symptomatic Fraser fir trees. 34

Table 2.3 Percent of tree samples by county in each symptom severity rating for each symptom category. Foliage chlorosis ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% foliage chlororsis. Stem canker ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% canker width relative to stem circumference. Root rot rating is on a scale of 0-3 where 0=0%, 1=1-33%, 2=34-66%, and 3=67-100% of root necrosis. 35

Table 2.4 Percent of tree samples by recovered *Phytophthora* spp. in each symptom severity rating for each symptom category. Foliage chlorosis ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% foliage chlororsis. Stem canker ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% canker width relative to stem circumference. Root rot rating is on a scale of 0-3 where 0=0%, 1=1-33%, 2=34-66%, and 3=67-100% of root necrosis..... 36

Table 2.5 *Phytophthora* isolates recovered from symptomatic Fraser fir roots from Christmas tree plantation sites in the Southern Appalachian mountains and identified in the initial PCR screening. Isolates are identified by their clade (1a, 2c, 7a,7c, and 8a) (Yang, Tyler, & Hong, 2017). Other non-*Phytophthora* Oomycete species of interest are included at “Other Oomycetes”.. 37

Table 2.6 *Phytophthora* isolates by species and county. Isolates were identified using sequences from 5 DNA gene sequences (β-tub, Ef-1α, ITS, Ypt1, and Cox1).. 38

Table 2.7 Population statistics calculated for *Phytophthora cinnamomi* isolate sequence alignments of target loci..... 39

Chapter 3

Table 3.1 Shapiro-Wilk test of normality for RGR by treatment and clone at each timepoint. All timepoints had significant differences between indicating that the data is not normally distributed. Significance was $\alpha < 0.05$.. 60

Table 3.2 Levene’s test for homogeneity of variance of RGR by treatment and clone at each timepoint. All but one timepoints had significant differences indicating that the variances between the groups were not equal. Significance was $\alpha < 0.05$. 61

Table 3.3 Summary Statistics for each variable (clone, cross, treatment, and timepoint) 62

Table 3.4 Kruskal-Wallis test of RGR by clone. Clones were significantly different at all timepoints and across all timepoints. Significance was $\alpha < 0.05$. 63

Table 3.5 Kruskal-wallis test of RGR by treatment and clone. There were no significant differences between groups at any timepoint and across all timepoints. Significance was $\alpha < 0.05$ 64

Appendices

Table A.1 Population statistics calculated for *Phytophthora cinnamomi* isolate sequence alignments of target loci..... 87

LIST OF FIGURES

Chapter 2

Figure 2.1 Map of sites in each county where root tissue samples were collected from symptomatic Fraser fir trees. Inset table displays numbers of sites and trees sampled in each county……..... 40

Figure 2.2 Process for culture and isolation of Phytophthora from infected root tissue using PARP-v8 selective media (Jeffers & Martin, 1986). 41

Figure 2.3 Fir seedlings arranged on greenhouse benches into treatment blocks with 10 Fraser fir and 4 Momi fir seedlings per treatment block 42

Figure 2.4 Fir seedlings being inoculated with colonized rice grains. A ¼ inch thick rod is used to make 2 small holes in the potting media approximately 2.5cm from the seedling stem. Sterilized forceps are used to place 2 colonized rice grains into each hole 43

Figure 2.5 3 day-old cultures on PARP-v8 agar media inoculated with colonized rice grains used to inoculate fir seedlings. (A) non-colonized negative control, (B) 23ss04 *P. cinnamomi* positive control, (C) *P. cactorum*, (D) *P. cryptogea* isolate 1, (E) *P. cryptogea* isolate 2, (F) *P. europaea*, (G) *P. plurivora*, (H) *Elongisporangium Undulatum*, (I) *Globisporangium macrosporum* 44

Figure 2.6 Visual representation of *Phytopthora* isolate recovery by county. Pie charts represent the diversity of species isolated from symptomatic Fraser fir in each county. The size of each pie chart is scaled to the number of trees sampled in each county (ranging from 2 trees, in Cherokee and Henderson Counties, to 70 trees in Ashe County). Also included are 3 non-*Phytophthora* oomycete species of interest 45

Figure 2.7 Maximum-Likelihood tree built from aligned *Btub* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types of *P. cinnamomi* and *P. parvispora* (a closely related species) were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state 46

Figure 2.8 Maximum-Likelihood tree built from aligned *Ef1a* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types of *P. cinnamomi* and *P. parvispora* (a closely related species) were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state 47

Figure 2.9 Maximum-Likelihood tree built from aligned *Cox1* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences obtained from published mitochondrial genomes of P. cinnamomi isolates

were included in the Phylogeny (Winkworth, et al., 2022). Colored bands were added to display metadata, including county of collection and seedling source state 48

Figure 2.10 Maximum-Likelihood tree built from aligned and concatenated βtub and Ef1a sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types culture of *P. cinnamomi* were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state 49

Figure 2.11 Disease symptom progression over the 16-week experimental period for Fraser fir seedlings in each treatment group. Seedlings were observed biweekly for either disease symptoms or mortality. The proportion of seedlings exhibiting disease symptoms (such as foliage chlorosis or stem canker) or mortality was plotted at each timepoint 50

Chapter 3

Figure 3.1 Dormant cuttings collected from the Wallace, NC field trial site. 65

Figure 3.2 Cuttings were planted as deep as possible with only the top-most node/bud above the surface of the potting media. 66

Figure 3.3 Inoculation of rooted cuttings with colonized rice grains. A sterile rod is used to make two ~2cm deep holes, ~2cm from the stem of the rooted cutting, then 2 rice grains are placed into each hole. 67

Figure 3.4 (A) Arrangement of rooted cuttings in the treatment blocks. Clones and biological replicates were arranged randomly within the treatment block. (B) Rooted cuttings arranged on greenhouse benches according to experimental design. 68

Figure 3.5 There were no significant differences in starting heights between treatment blocks ($F_{5,137}$ = 0.89; P = 0.49). Significance was $\alpha < 0.05$. 69

Figure 3.6 There were no significant differences in starting heights between crosses ($F_{1,141}$ = 3.317; P = 0.0707). Significance was $\alpha < 0.05$. 70

Figure 3.7 There were significant differences in starting heights between clones ($F_{7,135}$ = 3.583; P = 0.00144). Significance was $\alpha < 0.05$. Clone 438 had the highest value at 31.77cm while clone 437 had the lowest at 16.93cm. 71

Figure 3.8 Average relative growth rate across timepoints by clone and treatment. Kruskal-Wallis test did not find significant differences between groups [$H(5)$ = 5.2752, P = 0.3832]. Significance was $\alpha < 0.05$. 72

Figure 3.9 Average relative growth rate of clones. A Kruskal-wallis test found significant differences between clones [H(7) = 22.396, P = 0.00217]. Significance was $\alpha < 0.05$. 73

Figure 3.10 Average relative growth rate across timepoints by cross. Kruskal-Wallis test did not find significant differences between groups. α = 0.05 [*H*(1) = 0.33335, *P* = 0.5637]..... 74

Figure 3.11 Average relative growth rate across timepoints by treatment. Kruskal-Wallis test did not find significant differences between groups. α = 0.05 [*H*(5) = 5.2752, *P* = 0.3832]..... 75

Appendices

Figure A.1 Maximum-Likelihood tree built from aligned *ITS* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types of *P. cinnamomi* and *P. parvispora* (a closely related species) were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state... 92

Figure A.2 Maximum-likelihood tree built from aligned Ypt1 sequences from P. cinnamomi isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019) Sequences rom an ex-type of P. cinnamomi were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state 93

Figure A.3 R script for performing chi-square test and combining duplicate experiment data for Fir inoculation.. 94

Figure A.4 Average relative growth rate across timepoints by treatment. Kruskal-Wallis test did not find significant differences between groups. α = 0.05 [*H*(5) = 5.2752, *P* = 0.3832]..... 96

Figure B.1 R script for ANOVA of start heights of treatment groups, clones, and crosses..... 98

Figure B.2 R script for testing normality of RGR data.. 102

Figure B.3 R script for kruska-wallis test of RGR by treatment, clone, and cross.. 103

CHAPTER 1

Background and Literature Review

1.1 Christmas Trees – A Brief History

The use of evergreen decorations has existed since ancient times. Both Christian and pagan traditions used evergreens as a symbol of hope in waiting for a coming spring or a representation of everlasting life during a cold and dark winter. Unfortunately, not much is recorded that links the use of evergreen trees to specific ancient practices other than vague references to their use. For example, the 2nd century Carthaginian Christian theologian, Tertullian, urged Christians to not engage in pagan practices which included, among other things, adorning houses with branches of evergreen trees (Churco, 1938). The first use of Christmas trees as we know them today, however, comes much later in the 16th century among German Lutherans (Perry, 2010).

Regardless of the origins, setting up a tree for the Christmas holiday has become a tradition enjoyed by people of many beliefs around the world. Specifically in North America, the practice of setting up a Christmas tree was thought to have been introduced as early as the 1780's by Hessian Mercenaries during the American Revolutionary War (Werner, 2006). Christmas trees became common in the US by the middle of the 19th century. Initially, trees were harvested from local forests. However, the commercial cultivation of Christmas trees has become a major industry throughout the country, especially in certain states like Oregon and North Carolina.

1.2 Christmas Tree Production

Cultivated Christmas Tree Species

Real Christmas trees are used by consumers around the world, as a result, a wide variety of conifer species are cultivated for use as Christmas trees and in the production of greenery. The most popular species include members from the Pinaceae such as spruces (*Picea* spp.), pines (*Pinus* spp.), true firs (*Abies* spp.), and Douglas fir (*Pseudotsuga menziesii*). Members of the Cupressaceae including cypresses (*Cupressus*) and junipers/redcedars (*Juniperis*) are also popular in certain regions (McKinley, Hazel, & Kohlway, Christmas Tree Species, 2019).

Historically, Norway spruce (*Picea abies*) has been heavily cultivated in Europe and the US as a Christmas tree species although its popularity continues decline. However, true fir (*Abies* spp.) species are the most popular group of real Christmas trees produced around the world. True firs are considered to possess many desirable qualities as a Christmas tree including elite post-harvest needle retention, strong festive aromas, and soft needles. In Europe, Nordmann Fir (*A. nordmanniana*) is the most popular commercial species. Conversely, Fraser fir (*A. fraseri*) is the most popular Christmas tree species in the US where it dominates on the east coast, while noble fir (*A. procera*) and Douglas fir are the most popular species on the west coast (USDA National Agricultural Stattistics Service, *2022 Census of Agriculture*. Complete data available at http://www.nass.usda.gov/AgCensus). Balsam fir (*A. balsamea*) is also a popular species although its cultivation is limited to the New England area of the US and eastern Canada (Chastagner & Benson, 2000). Other conifer species commonly grown as Christmas trees in the US include concolor fir (*A. concolor*), Colorado blue spruce (*P. pungens*), white spruce (*P. glauca*), Eastern white pine (*P. strobus*), scots pine (*P. sylvestris*), Virginia pine (*P. virginiana*), Leyland cypress (x *Cupressocyparis leylandii*), Arizona cypress (*Cupressus arizonica*), eastern

redcedar (*Juniperis virginiana*), and many others (McKinley, Hazel, & Kohlway, Christmas Tree Species, 2018).

Fraser fir and Christmas Tree Production in North Carolina

Fraser fir makes up 96% of all Christmas trees grown in North Carolina (Owen, North Carolina Christmas Trees by the Numbers, 2016). However, in recent years, North Carolina Christmas tree growers have expanded the species they cultivate as Christmas trees and includes hybrid and exotic firs like canaan (*A. balsamea var. phanerolepis*), Korean (*A. koreana*), Nordmann (*A. nordmanniana*), Trojan (*A. nordmanniana subsp. equi-trojani*), Turkish (*A. nordmanniana subsp. bornmuelleriana*), and momi fir (*A. firma*) as well as others. This expansion of species is primarily due to an increase in pressure of pests and pathogens in a changing climate. Additionally, growers in lower elevation regions in North Carolina cannot cultivate firs and therefore grow more heat tolerant species like eastern white pine, Virginia pine, Leyland cypress, eastern red cedar, and Arizona cypress.

Fraser fir is the only fir species native to North Carolina and is also one of the most commercially important Christmas tree species in the country (Owen, North Carolina Christmas Trees by the Numbers, 2016). The tree is a unique relic of the last period of glaciation when the climate was much colder. Around 8000 years ago, as the climate slowly warmed and glaciers retreated northwards, cold adapted plant species (like firs) migrated northwards. Some of these firs retreated to the high peaks of the southern Appalachian Mountains where they were trapped in island-like mountain top communities, unable to migrate further north. Over thousands of years these firs diverged into their own species unique from the more northern balsam fir species (Delcourt & Delcourt, 1984).

In addition to its unique ecology, Fraser fir is prized as a Christmas tree species. Fraser fir has a naturally conical form that requires minimal shearing and grows soft, deep-green, thick, and full foliage as well as sturdy yet pliable branches which are perfect for decorating. It also has excellent post-harvest needle and moisture retention so it does not drop its needles as quickly as other species and stays green long after it has been cut. These unique traits allow Fraser fir to be harvested earlier than other species of conifers and can be stored and displayed longer. Fraser fir has a strong, pleasant aroma that many consumers consider as one of the most desirable characteristics of the tree. All of these attributes combine to make Fraser fir a premium Christmas tree species preferred by consumers all over the country (Owen, 2011).

According to the 2022 USDA census of agriculture, North Carolina is ranked second in the country for number of trees harvested. In fact, North Carolina produces 22% of all real Christmas trees grown in the United States. Whole Christmas tree sales in North Carolina (not including other greenery products like wreaths, roping, and other decorative products) totaled $144 million in 2022 alone, with over 3.2 million trees harvested that year (USDA National Agricultural Statistics Service, 2022). The Fraser fir has made western North Carolina a central hub for Christmas tree production in the US and is admired around the world.

1.3 Challenges to Christmas Tree Production

Pests and Diseases Limiting Christmas Tree Production in North Carolina

There are nearly 1000 Christmas tree growers in the state growing 50 million+ trees on over 33,000 acres of land (USDA National Agricultural Statistics Service, 2022). Christmas trees are a very important bioeconomy in North Carolina and specifically to rural areas where there are few other agricultural opportunities primarily due to climate and topography. Production of

Fraser fir Christmas trees in many of these areas is increasingly limited by numerous pests and diseases. Historically, Fraser fir has been heavily affected by the invasive balsam woolly adelgid (*Adelges piceae*), even affecting natural populations of Fraser fir. In recent years, other insect and mite pests have created challenges for production, distribution, and sale of Fraser fir Christmas trees such as elongate hemlock scale (*Fiorinia externa*), cryptomeria scale (*Aspidiotus cryptomeriae*), spruce spider mite (*Oligonychus ununguis*), and balsam twig aphid (*Mindarus abietinus*) (Sidebottom & Bookewalter, 2023). However, Phytophthora root rot disease is the biggest challenge to Fraser fir production caused by the fungal-like soil borne pathogen in the genus *Phytophthora*.

The *Phytophthora* Life Cycle

The name *Phytophthora* was first given to the genus in 1876 by German mycologist Anton de Bary while investigating the causal agent of late blight of potato (*Phytophthora infestans*). Late blight of potato was responsible for the Great Irish Famine of the mid-19th century and one of the most devastating plant pathogens to humanity. The word '*Phytophthora'* derives from the Greek words for 'plant' (*phytón*) and 'destruction' (*phthora*). The genus is one of the most studied and important groups of plant pathogens as many of its members cause serious damage to both crops and natural ecosystems (Abad & Abad, 2007).

Members of the genus *Phytophthora* are fungal-like organisms but are not *fungi*. They share many structural and reproductive characteristics with *fungi* including hyphal growth and spores but differ in significant ways. The genus *Phytophthora* belongs to the phylum *Oomycota* within the kingdom *Chromista*. This kingdom also includes several groups of algae and diatoms. Despite similarities in structure, *Phytophthora* spp. differ from fungi by having cellulose-based

cell walls, diploid cells, and hyphae lacking septa. *Fungi* by contrast have chitin-based cell walls, are either haploid or dikaryotic, and may or may not have septate hyphae (Ho, 2018).

Phytophthora species can be soilborne, airborne, or both but often move through water such as streams, runoff, and groundwater. When conditions are favorable (i.e. when it is warm and wet) *Phytophthora* will form structures called *sporangia* which release motile zoospores. Zoospores are biflagellate with one flagellum serving as a sensing organ to detect exudates from actively growing plant roots in the soil, while the other flagellum is used to propel the zoospore short distances through water in the soil towards a susceptible host root. Once the zoospore reaches a host root, it attaches and encysts on the root surface where it can begin growing within the root cortex and acquiring nutrients from host's cells. (Hardham, 2017). For sexual reproduction to occur *Phytophthora* forms oogonia which hybridizes with antheridium either from the opposite mating type of the same species (for heterothallic species such as *P. cinnamomi*) or, if it is a homothallic species, it can hybridize with itself (Turkensteen, Flier, Wanningen, & Mulder, 2008). During asexual reproduction, *Phythopthora* can form a specialized thick-walled survival structure called a chlamydospore when conditions become too cold or dry (i.e. unfavorable). These are not true spores but chlamydospores will remain dormant in the soil for months or even years. When conditions become favorable for growth, chlamydospores can become active again, growing and producing more zoospores that can then infect susceptible plant roots (Jung, Colquhoun, & Hardy, 2013). This makes *Phytophthora* nearly impossible to completely remove from a site and very difficult to manage once an infection has occurred.

Anthropogenic and Climatic Factors Influencing the Spread of *Phytophthora*

Like many other plant pathogens, *Phytophthora* has been shown to move via contaminated plant materials and through soil as part of the global nursery industry. The movement of infested materials by humans is generally considered the main driver of spread for these plant pathogens (Brasier, 2008). As of 2023, there are 223 species described in the genus *Phytophthora*, most of which are plant pathogens (Abad G. Z., et al., 2023). Notably, *Phytophthora* spp. have caused significant damage to forest trees and woody perennial crops around the world. Some of the most devastating forest tree diseases caused by these pathogens include littleleaf disease of pine (*Pinus*) in the southern US caused by *P. cinnamomi* (Mistretta, 1984); *Phytophthora* root rot of avocado (*Persea americana*) in Florida caused by *P. cinnamomi* (Eskalen, Bender, Faber, & Rios, 2017); sudden oak death in California caused by *P. ramorum* (Rizzo & Garbelotto, 2003); and ink disease of *Castenea* species in Europe and the US caused by *P. cinnamomi* and *P. cambivora* (Fonseca, Abreu, & Parresol, 2004).

Global climate change is causing increased temperatures and changes in precipitation patterns. These climatic changes as well as warmer and wetter winters create opportunities for *Phytophthora* species to become established and persist in areas where they previously could not. Generally, *Phytophthora* species are limited by environmental conditions. Many *Phytophthora* species require warm and wet conditions and may be excluded from some areas based on their inability to adapt to the environment. More frequent extreme weather events such as flooding and hurricanes pose a potential risk of short and medium distance spread of *Phytophthoras* through movement of water (Contreras-Cornejo, Larsen, Fernandez-Pavia, & Oyama, 2023). These challenges contribute to the difficulties in managing *Phytophthora* as once it is introduced to a site it is very difficult, if not impossible, to remove.

Phytophthora Root Rot of Fraser fir Christmas Trees in North Carolina

Phytophthora root rot (PRR) is the most important disease impacting the cultivation of Fraser fir Christmas trees in North Carolina. Fraser fir is extremely susceptible to PRR and lacks any natural resistnace. *P. cinnamomi* was first reported infecting Fraser fir in North Carolina in 1963 in Transylvania County (Kuhlman & Hendrix, 1963). *P. cinnamomi* has spread throughout the region as growers have planted more and more locally produced seedlings over the decades. By 1998, *P. cinnamomi* was detected at an incidence rate of approximately 9% in at least 5 counties in Western North Carolina (Benson & Grand, 2000).

As PRR spread throughout Fraser fir plantations, many growers started to import seedlings from out-of-state providers. However, these other regions producing seedlings also have well-documented infections with multiple *Phytophthora* species that all negatively affect Christmas tree production (McKeever & Chastagner, 2016). Since 1998, it is likely that the practice of importing seedlings from out of state has resulted in the unintentional spread of other *Phytophthora* species on these imported seedlings. For example, a field survey conducted in 2014 revealed six new *Phytophthora* species isolated from symptomatic Fraser fir Christmas tree plantations in the Southern Appalachian Mountain region (Pettersson, et al., 2017). Unfortunately, the practice of importing planting materials from out-of-state has not decreased. In fact, the growing demand for North Carolina Fraser fir Christmas trees has likely increased the importation of out of state seedlings.

Management of Phytophthora Root Rot

The most important management action that can be taken is to prevent the introduction and spread of *Phytophthora* into new locations. *Phytophthora* is impossible to remove once introduced into a Christmas tree plantation. No treatments are available to 'cure' a Fraser fir once it has become infected with *Phytophthora*. However, two measures can be taken to prevent infection and include: 1) proper site selection; and 2) phytosanitary practices that prevent the introduction of potential soil pathogens.

Sites that are well-drained and not likely to flood from nearby streams or rivers are ideal when establishing a Fraser fir plantation. *Phytophthora* spp. thrive in saturated soil conditions so low laying and poorly drained sites should be avoided (Chastagner & Benson, 2000). Soils that are high in clay can decrease the flow of water and hold onto moisture longer due to clay particles' high cationic exchange capacity (Sanchez-Cuesta, Gonzalez-Moreno, Cortez-Marquez, Navarro-Cerrillo, & Ruiz-Gomez, 2022). Shallow soils or soils with compacted layers (i.e. plowpans/hardpans) can increase the risk of PRR. These soils do not allow ground water to drain deeper into the soil and tree roots remain saturated for longer periods of time (Rhoades, Brosi, Dattilo, & Vincelli, 2003). Clay content and depth of soil were identified previously as the two most important factors contributing to *Phytophthora* incidence (Kohlway, Cothron, & Whitehill, 2019).

Healthy seedlings free of *Phytophthora* should be the most important consideration when establishing a new site. During seedling production, *Phytophthora* can spread very quickly in seed beds and transplant beds. Seedling production should be kept to well drained areas that won't flood although ideally raised beds should be used. Seed/transplant beds should also use sterile soil media with low clay content. Irrigation should not rely on natural bodies of water like

streams and ponds. Instead, irrigation should only be done using treated or well water. Soil fumigants can be used in seedling/transplant beds prior to sowing or planting to reduce risk of PRR. Fungicides such as Subdue MAXX or Aliette can be applied to provide temporary protection. However, these fungicides cannot protect trees that have already begun dying from PRR (Kohlway, Cothron, & Whitehill, 2019). Ultimately, unhealthy seedlings should not be used, this represents the best method to control PRR spread.

Best planting practices that avoid stressing the tree root system are important to ensure seedlings are healthy. If PRR does develop in a field, it is important to quarantine the area to avoid the spread of disease to other areas of the field or farm. Great care should be taken to not disturb the soil in these contaminated spots. Infected root systems should not be dug up and walking or operating equipment in the contaminated area should be avoided, especially when the soil is saturated. Fraser fir or other highly susceptible species should not be planted in contaminated areas. Instead, tolerant and resistant species should be selected. Fraser fir grafted onto resistant root stock (such as momi or Turkish fir) has been effective on sites infected with *Phytophthora* (Kohlway, Cothron, & Whitehill, 2019).

Alternative Species and Grafting

The most susceptible species to PRR include Fraser, Balsam, noble, grand (*A. grandis*), red (*A. magnifica*), and Shasta fir (*A. magnifica var. shastensis*). Tree species grown as Christmas trees with some resistance to PRR include Eastern white pine, Nordmann fir, Turkish fir, Trojan fir, and momi fir (Frampton & Benson, 2012; Chastagner & Benson, 2000) . Many of these species are grown for greenery and as Christmas trees in North Carolina. However, consumers and growers still prefer the elite characteristics of Fraser fir. To continue to grow Fraser fir on infected sites, growers often graft susceptible fir species onto PRR resistant

rootstock. Grafting Fraser fir onto PRR resistant momi fir rootstock is becoming a more common practice in North Carolina. However, grafting is labor intensive, requires skill, and increases costs significantly. Additionally, the supply of momi fir rootstock is limited in the US. Still many growers in North Carolina use grafting in areas where PRR has prevented Christmas tree cultivation (Hibbert-Frey, Frampton, Blazich, & Hinesley, 2010).

Unfortunately, in some cases growers may have large farms acreages so heavily affected by PRR that Christmas tree production is no longer possible. This presents a major challenge as there are few if any short rotation woody crops as profitable as Fraser fir Christmas trees. However, growing *Populus* species as a high-value and flexible fiber crop on PRR infected sites has potential as an alternative to Fraser fir. *Populus* can be cultivated profitably on rotations as short as 12 years even on marginal lands (Ghezehei, Ewald, Hazel, Zalesny, & Nichols, 2021). There is not much information on the impact of *Phytopthora* species on *Populus* growth. In Europe, there is evidence that some hybrid *Populus* clones such as I-214 and Pánnonia (hybrids between *P. nigra* and *P. deltoides*) experienced decline in growth and even mortality when exposed to different *Phytophthora* species which included *Phytophthora plurivora, P. pini, P. polonica, P. lacustris, P. cactorum*, and *P. gonypodyides* (Milenković, et al., 2018). However, other studies have found *Populous* species have tolerance and potentially resistance to *Phytophthora* species (Cerny, et al., 2022).

1.4 Need for Further Research

To develop better strategies to manage the spread of Phytophthora in North Carolina it is important to understand the diversity and distribution of species present on Fraser fir Christmas tree farms. Also, how these species contribute to the mortality of Fraser fir and other alternative

species and crops is crucial if we are to continue developing strategies to improve and preserve the important bioeconomies of western North Carolina. Therefore, the objectives of this thesis project are to: 1) determine the diversity of *Phytophthora* species present on Christmas tree farms in western North Carolina; 2) determine if the species present on Christmas tree farms can cause disease on Fraser fir; 3) explore the use of alternative species such as high-value veneer *Populus* hybrid clones on PRR infected Christmas tree farms.

Chapter 2

Distribution and Diversity of Oomycete Root Pathogens Affecting Fraser fir (*Abies fraseri*) Christmas Tree Production in the Southern Appalachian Mountains

2.1 Introduction

Fraser fir (*Abies fraseri*) is the most economically important species grown for Christmas tree production in the southern Appalachian Mountains. Fraser fir is the only true fir (*Abies* spp.) species native to the high-elevation spruce-fir forest ecosystems (Busing, White, & MacKenzie, 1993). Fraser fir is especially important to the North Carolina Christmas tree industry where it makes up nearly 96% of all commercially grown Christmas trees (Owen, 2016). In 2022 alone, over 3.2 million whole trees were harvested in the state with a market value of $144 million (USDA National Agricultural Statistics Service, 2022).

Fraser fir Christmas tree production is limited to high-elevation land which is limited. This land is also not very productive due to thin soils and cool conditions which limits the agronomic potential for these communities. Fraser fir is often grown commercially at lower elevations (>3000 ft and above) outside of its natural range (>5000 ft). These lower elevation soils are warmer and more productive. However, these soils often have poor drainage and are more favorable for pests and diseases which can limit Christmas tree production. The most devastating disease impacting Fraser fir production and thrives in poorly drained soils is Phytophthora root rot (PRR). PRR was first reported affecting Fraser fir in North Carolina in 1963. The causal agent was identified as *Phytophthora cinnamomi* (Kuhlman & Hendrix, 1963). Since that time PRR has spread rapidly and become the most significant threat to Christmas tree growers in the region. Fraser fir infected with PRR can exhibit a range of symptomology

including: branch dieback especially of the lower branches (i.e. flagging), stem cankers typically found near the root collar, stunted growth, chlorotic foliage, wilting of current season growth, and root necrosis (i.e. root rot) (Chastagner & Benson, 2000). PRR induced tree mortality results from a combination of loss of fine root mass and stem cankers that girdle the tree. Girdling cankers restrict the flow of water and nutrients between the roots and foliage which ultimately causes tree death.

The incidence of PRR in the southern Appalachian region has been investigated through several surveys since the 1990's. A survey conducted from 1997 to 1998 found an average PRR incidence of 9% across 58 Fraser fir plantations in five North Carolina counties (Benson & Grand, 2000). This initial survey found very little species diversity and nearly all isolates were identified as P. cinnamomi. Only one isolate was left as an unidentified Phytophthora species. The authors of this study also sampled from nursery transplant beds, finding a mean PRR incidence of 2%, and identified 3 Phytophthora species: *P. cinnamomi, P. cactorum,* and *P. dreschleri* as well as one unidentified *Phytophthora* species. Growers began to import seedlings produced out of state to reduce the spread of PRR within western North Carolina Fraser fir Christmas tree farms following the results of the 1997 and 1998 survey. A subsequent survey conducted in 2015 of 89 growers from North Carolina, Tennessee, and Virginia found that 64% of respondents were planting seedlings produced and imported from out-of-state. Of the respondents, 83% of growers with more than 50 acres of Christmas tree production relied on out-of-state Fraser fir planting stock.

The spread of PRR continued to accelerate throughout western NC in subsequent years despite an increase in the number of seedlings produced out of state. A study was conducted in 2014 to evaluate the diversity of Phytophthora species infecting Fraser fir across 14 counties in

North Carolina, Tennesee, and Virginia to assess the severity of the expanding PRR infections (Pettersson, et al., 2017). In total, 103 sites were sampled of which 80% were positive for *Phytophthora* spp. A total of six *Phytophthora* spp. were isolated from Fraser fir exhibiting symptomology consistent with PRR infection including *P. cinnamomi*, *P. cryptogea*, *P. europaea*, *P. citrophthora*, *P. pini*, and *P. sansomeana*. While 70.3% of the recovered isolates were identified as *P. cinnamomi*, another 23.1% were identified as *P. cryptogea* representing a major change in the diversity of oomycete pathogens affecting Fraser fir production in the region. The shift away from seedlings produced within NC to those grown out of state likely provided an opportunity for the introduction of new pathogen species not previously found in the region. For instance, most of the imported materials were produced within regions known to have an abundant diversity of *Phytophthora* spp. with the potential to cause significant disease on fir species (McKeever & Chastagner, 2016)

Unfortunately, since the 2014 survey there have been no indications that the importation of out-of-state planting materials has decreased. Additionally, the impacts of accelerated climate change have continued to exacerbate conditions that favor the spread of *Phytophthora* spp. Conditions that favor *Phytophthora* includes milder winters, changes in precipitation patterns, and more frequent extreme flooding events. Therefore, we hypothesize that the diversity and distribution of *Phytophthora* species affecting Fraser fir in the Southern Appalachian region has continued to increase. The objectives of this study were to determine what *Phytophthora* spp. are present on Christmas Tree farms in western North Carolina. Here we assess whether the number of species is increasing or becoming more widespread, and if all species recovered can cause disease on Fraser fir.

2.2 MATERIALS AND METHODS

Field site selection

Previous surveys used a weighted method based on the acreage of Christmas trees in each county to determine the number of sample sites in each county (Pettersson, et al., 2017) and the same method was employed here. Sample sites were chosen based on the presence of multiple symptomatic trees within the same vicinity (100 feet or less from one another). While an attempt was made to revisit sites from the 2014 survey, only nine of the 89 sites sampled were consistent with that study due to many of the previous sites no longer being used for the cultivation of Fraser fir Christmas trees. Often this meant either, growers switched to a different species for that site such as eastern white pine or abandoned the site altogether.

Root sample collection from Fraser fir plantation trees

Putatively PRR infected Fraser fir root tissues were sampled between April and September 2023. A total of 332 Fraser fir trees exhibiting symptoms consistent with active PRR infections were collected from 89 sample sites and 16 counties in North Carolina, eastern Tennessee, and southwestern Virginia (**Figure 2.1**). Metadata for each tree sampled was also collected and includes the origin from which each seedling was received and the year each tree was planted in the field. However, this information was not known for most sample sites as these trees were evaluated on active Christmas tree farms and not all information was shared or recorded by farm personnel. PRR disease symptoms for each tree were rated based on the presence of foliar chlorosis, stem cankers, and/or root necrosis according to the protocol described in Pettersson, et al., 2017. These ratings were on a scale of 1-4 for foliage chlorosis severity and stem canker width, and a scale of 1-3 for root necrosis severity. For sample collection, root tissues were collected from three to five symptomatic trees at each site.

Additionally, root tissues were collected from one to three asymptomatic trees per site. GPS coordinates were recorded for each tree sampled using a Juniper Systems Archer 2 handheld data collector. Root tissue for each sample was placed in a labeled resealable plastic bag. Each sample bag was stored in a cooler filled with ice and transported back to the laboratory at North Carolina State University for sample processing. All root samples were stored at 4°C and were processed with four weeks of collection.

Isolation of *Phytophthora* spp. from Fraser fir roots

Root tissues were gently rinsed with sterile water to remove excess soil. Root tissues were finely chopped into small pieces using a clean razor blade. Chopped root tissues were then placed on to selective PARP-cV8 media (**Figure 2.2**) (Jeffers & Martin, 1986). Cultures were incubated in the dark at 25ºC for 3-5 days. Colonies that formed were observed for oomycete characteristics such as coenocytic hyphae (Fry & Grünwald, 2010). Next, a small block of agar from the leading edge of active mycelial growth containing hyphae was removed using a sterilized scalpel and placed onto a fresh petri dish containing PARP-cV8 media. After three to five days of incubation in the dark at 25 ºC a block of media containing hyphae was excised from the leading edge of mycelial growth and transferred to a petri dish containing low nutrient media (water agar). Water agar cultures were incubated for an additional three to five days at room temperature. Once individual hyphal strands could be observed with an inverted light microscope, a block of agar containing a single hyphal tip was carefully removed using a scalpel and placed onto a new petri dish containing clarified V8 agar media to produce a pure hyphal tip culture of each isolate.

Molecular confirmation of *Phytophthora* spp. isolates

Initial screening and identification of potential oomycete cultures were based on visual confirmation of morphological characteristics observed using an inverted light microscope. Next, PCR was performed to determine the *Phytopthora* spp. isolated. The internal transcribed spacer region of the ribosomal DNA (rDNA) was amplified from each isolate using the universal primers ITS1 (5' - TCCGTAGGTGAACCTGCGG - 3') and ITS4 (5' - TCCTCCGCTTATTGATATGC - 3') (White, Bruns, Lee, & Taylor, 1990). PCR amplification of the ITS region was performed directly using mycelial tissues from pure culture isolates with the Thermo Scientific™ Phire Plant Direct PCR kit (Thermo Fisher Scientific, 2024). Reagents and PCR conditions followed the manufacturer's specifications for a 20 µL. PCR conditions included initial denaturation at 98ºC for 5 minutes followed by 24 cycles of 5 seconds at 98ºC, 5 seconds at 56ºC, and 20 seconds at 72ºC, with a 1 minute extension time at 72ºC. The amplified products were run on 1% agarose gel to ensure sufficient DNA amplification and assess contamination. PCR reactions were performed on a Biorad C1000 Touch Thermal Cycler. PCR products were purified using the New England Biolabs Monarch® Spin PCR & DNA Cleanup Kit. Purified PCR products were submitted for sequencing using the Azenta Life Sciences Genewiz® Sanger sequencing service (Azenta Life Sciences, 2024) with only the forward primer (ITS1) for initial screening. Sequencing results were trimmed using Qiagen CLC Main Workbench software (https://digitalinsights.qiagen.com). Trimmed sequences were analyzed using nucleotide BLAST (Basic Local Alignment Search Tool) to identify homologous sequences in the NCBI database (Altschul, Gish, Miller, Myers, & Lipman, 1990; Camacho, et al., 2009). Using this approach, isolates were not identified to the species level but instead identified to subclade as described in (Yang, Tyler, & Hong, 2017). Species ID were verified by

querying the TBAS *Phytophthora* tool (Coomber et al, 2023) and IDPhy (Abad G. Z., et al., 2023)

Molecular identification of *Phytophthora* spp.

151 isolates were identified as *Phytophthora* spp. in the initial screening. From these isolates, 69 were selected for further molecular analysis. For each isolate, 100mg of mycelia was flash frozen using liquid nitrogen and ground to a fine powder with a mortar and pestle. DNA was extracted from ground mycelia with the QIAGEN DNeasy plant pro DNA extraction kit following the manufacturer's instructions (QIAGEN, 2024). Next, PCR was used to amplify five target loci from each putative *Phytophthora* spp. isolate using the cleaned DNA extracts. Target loci were chosen based on their common use for identification of *Phytophthora* spp. (Abad G. Z., et al., 2023). Target loci targeted in this study include β-tubulin (βtub), elongation factor 1-alpha (EF1a), internal transcribed spacer region (ITS), ras-like GTP-binding protein Ypt1 (Ypt1), and cytochrome x oxidase subunit 1 (Cox1). A total volume of 20 µL was used in each reaction including: 10 µL of Thermo ScientificTM Phire plant direct PCR master mix (Thermo Fisher Scientific, 2024), 1 µL of dimethyl sulfoxide, 1 µL of 10 µM forward Primer, 1 µL of 10 µM reverse primer, 6 µL of sterile deionized H2O, and 1 µL of extracted DNA solution were used in each reaction. PCR products were obtained for both the forward and reverse primers for each of the five target loci. **Table 2.1**. Amplified PCR products were submitted in 96-well plates to the NC State University Genomic Science Laboratory for cleanup and Sanger sequencing. Forward and reverse Sanger sequencing reads were cleaned assembled using CLC Main Workbench version 23.0.4 (Qiagen Digital Insights, 2023) and aligned to reference sequences identified through a nucleotide BLAST search (Altschul, Gish, Miller, Myers, & Lipman, 1990; Camacho,

et al., 2009). A species identity was assigned to each isolate based on consensus of BLAST identities for each sequence with a minimum nucleotide match ≥99% and an E-value ≤1e-5 (Choudhuri, 2014).

Phylogenetic analysis of *Phytophthora cinnamomi* isolates

A phylogenetic analysis was performed on isolates identified *as Phytophthora cinnamomi*. An additional isolate of *P. cinnamomi* (23ss04) routinely used in the Whitehill lab for resistance screening studies and known to be highly virulent on Fraser fir was included in the phylogenetic analysis (Kohlway, Whetten, Benson, & Frampton, 2017). Alignments of the isolate sequences were made for each loci using MEGA (Molecular Evolutionary Genetics Analysis Software) version 11.0.13 (Tamura, Stecher, & Kumar, 2021). The MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm was then used to build alignments (Edgar, 2004). Sequences from ex-type specimens of clade 7c species curated by the USDA-APHIS-PPQ Plant Pathogen Confirmatory Diagnostics Laboratory were also included with the alignments when available (Abad G. Z., et al., 2023). Sequences from an isolate of *P. cactorum* (cl3a) were also included to serve as an outgroup for rooting in later steps. Basic population statistics including haplotype diverisity, nucleotide diversity and Tajima's D test statistic were generated for each loci alignment using the R packages "ape", "pegas", and "seqinr" (Paradis & Schliep, 2019; Paradis, 2010; Charif & Lobry, 2007). In addition to the single loci alignments, a concatenated alignment of nuclear genes β-tub and Ef1a was made using MEGA version 11.0.13 software after these loci were determined to have higher variation. Maximum-likelihood trees were constructed for each alignment using RAxML (Stamatakis, 2006). The trees were

visualized with associated metadata using the “upload tree” feature in T-BAS version 2.3 (Carbone, et al., 2019).

Experimental Design for evaluating Koch’s postulates

A 16-week inoculation experiment was performed in the summer of 2024 to evaluate the pathogenicity of isolates collected in summer 2023 on Fraser fir. Eight isolates representing the diversity of *Phytophthora* spp. and other oomycetes were selected to be used in the experiment. The experiment was conducted at the NC State Horticultural field lab starting on 19 June and ending 9 October 2024. Seven treatment groups and two control groups were evaluated. One control group served as a negative control while the second as a positive control group. Each group consisted of ten Fraser fir seedlings and four resistant momi fir (*Abies firma*) seedlings (**Figure 2.3**). *Phytophthora* resistant momi fir were included to serve as a positive control to ensure that the inoculation treatments and greenhouse conditions were optimal, and trees were not stressed during the experiment. All Fraser fir seedlings used in the experiment were progeny of Family 112 and provided by North Carolina Premium Fraser Fir Seed Coop. The negative control group received a mock-inoculation treatment. The mock-inoculated group served to ensure no contamination occurred during the experimental timeframe. The positive control group was inoculated with a known aggressive strain of *P. cinnamomi* (23ss04) and served as a comparison against the unknown isolates. Each of the remaining seven treatment groups were inoculated with the oomycete species isolates collected from symptomatic Fraser fir during the 2023 survey. The species identities of the isolates used in the experiment include *P. cactorum, P. europaea, P. citricola, P. cryptogea* isolate 1, and *P. cryptogea* isolate 2. Two non-*Phytophthora* oomycete species isolated from symptomatic Fraser fir during the 2023 survey were also

evaluated and include the species *Elongisporangium undulatum* and *Globisporangium macrosporum*.

Preparation of *Phytophthora* spp. inocula for evaluating Koch's postulates

Seedlings were inoculated using colonized rice grains according to the protocol described in Frampton & Benson, 2012. Inocula was prepared two weeks prior to the application of treatments. Agar plugs from actively growing colonies of the eight oomycete species being evaluated were used to produce rice grain inocula following standard protocols (Holmes & Benson, 1994). Briefly, three agar plugs containing mycelia from each isolate were transferred to a flask of twice-autoclaved rice grains. Flasks containing inoculated rice-grains were incubated at 30ºC for two weeks. Flasks were shaken daily to ensure that the *Phytophthora* inoculum was evenly distributed amongst the rice grains. The mock-inoculum (negative control) flask containing twice-autoclaved rice grains were treated in the same fashion using sterile PARP-V8 agar plugs.

Inoculation of Fraser fir with *Phytophthora* spp. isolated from symptomatic field trees

Seedlings were inoculated following established protocols developed by the NC State Christmas Tree Genetics program (Frampton & Benson, 2012; Holmes & Benson, 1994; Kohlway, Whetten, Benson, & Frampton, 2017). Briefly, each biological replicate within each treatment group received three grains of either (1) mock-inoculated rice (i.e. negative control group) or (2) *Phytophthora*-infected rice (**Figure 2.4**). Rice grains were buried in ~2.5 cm deep divots located ~1-2 cm away from base of the stem. Seedlings were then heavily watered so that their containers were flooded. Seedlings were watered in this manner three times per week for

the remainder of the experiment. Three rice grains per treatment were placed on PARP-v8 media and cultured for three to five days (**Figure 2.5**). A PCR assay (following the protocol described in the initial screening of isolates section of the materials and methods) was used to confirm that the rice grains were colonized.

Assessment of Phytophthora inoculated Fraser and momi fir

Seedlings were observed for disease symptoms and mortality every two weeks. Each seedling was assigned a rating of "A" (healthy), "D" (diseased), or "M" (dead) based on a visual assessment of the individual seedling at each timepoint. The experiment was conducted in duplicate to ensure consistency in results. A chi-square test was conducted to ensure that the two replicated experiments were not significantly different, and the data was subsequently merged.

Re-isolation and identification of *Phytophthora* from treated fir

Root samples were collected randomly from one inoculated Fraser fir seedling and one inoculated momi fir seedling from each treatment group. Roots from each sampled tree were rinsed with sterile water to remove excess soil then finely chopped with a sterile razor blade and plated onto *Phytophthora* selective PARP-V8 media in three small piles (Jeffers & Martin, 1986). The plates were then incubated at 25ºC and were observed daily for a maximum of five days for signs of hyphal growth. Hyphae characteristics of new growth was compared to a lab culture of each isolate and standard *Phytophthora spp*. references on IDphy (Abad G. Z., et al., 2023) to morphologically identify sample.

The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was PCR amplified from each culture with the Phire™ Plant Direct PCR Kit (Thermo Fisher Scientific,

2024). A sterile pipet tip was used to collect a tuft of mycelia (~3mm in diameter) into a 200 µl microcentrifuge tube with 20 µl of dilution buffer and incubated at 98ºC for 10 minutes. 1.5 µl of the incubated sample was then used as template for PCR with the ITS1 and ITS4 primers following the manufacture's recommendations (White, Bruns, Lee, & Taylor, 1990). PCR conditions included initial denaturation at 98°C for 5 min followed by 24 cycles of 1 min at 98°C, 1 min at 56°C and 1 min at 72°C, with a final 10 min extension cycle at 72°C. The PCR products were purified with the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA). Purified PCR products were then submitted for Sanger sequencing at Genewiz (Azenta Life Sciences, 2024). DNA sequences were trimmed and assembled with CLC Main Workbench 23.0.4 (Qiagen Digital Insights, 2023). Sequence similarity searches with the assembled ITS sequences were performed with the NCBI BLAST tool (Altschul, Gish, Miller, Myers, & Lipman, 1990; Camacho, et al., 2009) to determine the sample's species.

2.3 RESULTS

PRR disease symptom rating of Fraser fir survey trees

All 332 Fraser fir trees exhibiting symptoms of PRR infection were rated based on the presence of foliar chlorosis, stem cankers, and/or root necrosis. For foliar chlorosis, 0% of trees sampled exhibited foliar chlorosis; 43% of the sampled trees exhibited 1-25% foliar chlorosis; 32% exhibited 24-50%; 10% exhibited 51- 75%; and 15% exhibited 76-100%. For stem cankers, 15% had no stem canker present; 14% had stem canker widths that were 1-25% of the stem circumference; 21% had stem cankers that were 26-50% of the stem circumference; 18% had stem cankers that were 51-75% of the stem circumference; and 31% had stem cankers that were

76-100% of the stem circumference. For root necrosis, 29% had root necrosis affecting 1-33% of the root system; 43% had root necrosis affecting 34-66% of the root system; and 28% had root necrosis affecting 67-100% of the root system (**Table 2.2**). Disease symptom ratings by county are also recorded in **table 2.3** and symptom ratings by Phytophthora spp. are recorded in **table 2.4**.

Phytophthora recovery and identification

Following the initial screen we identified 151 isolates as *Phytophthora* (**Table 2.4**). Of those, 134 (88.7%) were placed in clade 7c; 9 (6%) in clade 8a; 3 (2%) in clade 2c; 3 (2%) in clade 7a; and 2 (1.3%) in clade 1a. The highest diversity of clades was identified from Ashe County, NC and represented clades 1a, 2c, 7a, 7c, and 8a. The most *Phytophthora* isolates were identified from Avery County, NC with 33 present and all belonging to clade 7c. Carter County, TN was the only county where no *Phytophthora* isolates were identified. From the symptomatic trees samples, we isolated three non-*Phytophthora* oomycete species. These species were identified as *Elongisporangium undulatum*, *Globisporangium irregulare*, and *Globisporangium macrosporum*. *Elongisporangium undulatum* is the first reported detection in Fraser fir in Western North Carolina based on published literature. A map detailing the location from where isolates were recovered following the initial screening assay are outlined in **Figure 2.6**. We selected a subset of 69 isolates representative of the sites and locations sampled for further detailed molecular evaluation. Amplification of five target loci led to the identification of six species of *Phytophthora* (**Table 2.5**). Of the 69 evaluated, 53 isolates were identified as *P. cinnamomi*; eight isolates were identified as closely related species in subclade 8a (*P. cryptogea, P. pseudocryptogea*, and *P. kelmanii*) which were considered "*P. cryptogea*-like" species for the

purposes of this study; two isolates were identified as *P. europaea*; two isolates were identified as *P. cactorum*; two isolates were identified as *P. citricola*; and one isolate was identified as *P. plurivora*. We did not recover *Phytophthora* or any other oomycete species from the root tissues collected from the one to three asymptomatic trees sampled from each field site.

Phylogenetic Analysis of *Phytophthora cinnamomi* Isolates

Of the five target loci, both the haplotype and nucleotide diversity tend to be moderate and high. Cox has the highest haplotype diversity (0.99) while ITS and Ypt have the lowest (0.63). Nucleotide diversity also tended to be moderate to high for all the loci with Cox having the highest (0.019) and Ef1a having the lowest (0.0019). The Tajima's D neutrality statistics for each loci mostly tended to be negative. βtub, Ef1a, ITS, and Cox all had Tajima's D statistics below -2 indicating either a purifying selective pressure or expansion after a bottleneck event. Ypt was more neutral (-1.11) indicating more balanced evolution (**Table 2.7**).

ITS and Ypt phylogenies had very low bootstrapping support while the βtub, Ef1a, and Cox phylogenies had sufficient bootstrapping support separating the outgroups from the *P. cinnamomi* sequences (**Figures 2.7-2.9)**. The βtub and Ef1a phylogenies also had sufficient bootstrap support for a small clade, however, when alignments for these two loci were concatenated together and used to construct a tree there was less consistency and low bootstrap support, even for the outgroup (**Figure 2.10**).

Pathogenicity of Isolates

No disease symptoms or mortality were observed in any of the momi fir seedlings in any treatment group in either of the two experimental replicates. Therefore, only observations of

disease symptom development and mortality in Fraser fir seedlings are reported here. Proportions of healthy, diseased, and dead Fraser fir seedlings in each treatment group and at each timepoint for the merged data of both experiment replicates are displayed in **Figure 2.13**.

The mock-inoculated negative control group, the *Elongisporangium undulatum* treatment, and the *Globisporangium macrosporum* treatment exhibited no disease symptoms or mortality for the duration of the 16-week observation period.

The positive control group treated with the highly pathogenic *P. cinnamomi* strain 23ss04 began exhibiting disease symptoms at 4 weeks post inoculation (wpi), and mortality at 8 wpi. At 16 wpi, 15 of the 18 seedlings were dead, 2 exhibited disease symptoms, and 1 was apparently healthy.

In the *P. cactorum* treatment group, disease symptoms were only observed only in 1 seedling and only at 16wpi. No mortality was observed. All other seedlings were apparently healthy.

In the *P. cryptogea*-1 treatment group, disease symptoms were first observed at 6 wpi, with mortality recorded at 8 wpi. By 16 wpi, 4 of the 20 seedlings had died, 2 exhibited disease symptoms, and 14 were apparently healthy.

In the *P. cryptogea*-2 treatment group, disease symptoms were first observed at 6 wpi, with mortality recorded at 10 wpi. By 16 wpi, 4 of the 20 seedlings had died, 1 exhibited disease symptoms, and 15 were apparently healthy.

In the *P. europaea* treatment group, disease symptoms were first observed at 6 wpi, with mortality recorded at 10 wpi. By 16 wpi, 1 of the 20 seedlings had died and all other seedlings were apparently healthy.

In the *P. plurivora* treatment group, disease symptoms were first observed at 6 wpi, with mortality recorded at 8 wpi. By 16 wpi, 1 of the 20 seedlings had died, 4 exhibited disease symptoms, and 15 were apparently healthy.

2.4 Discussion

Disease Symptoms of Samples and Recovery from Samples

All 332 sample trees exhibited symptoms of PRR but the number of *Phytophthora* isolates identified in the initial screening was only 151. Additionally, isolates recovered and used in the final identification and phylogenetic analysis were isolated from only 66 (20%) of the 332 trees sampled. The detection methods used likely led to an underrepresentation of the *Phytophthora* present at the survey sites since detection relied on isolation of *Phytophthora*s from root tissue and sequencing of culture DNA. Initial screening of samples with a rapid detection tool such as Agdia, Inc. Immunostrip® for *Phytophthora* (Agdia, Inc., 2024) might have provided a better representation of which sites had *Phytophthora* present before moving on to identification of *Phytophthora* spp.

Many of the species recovered are consistent with species previously identified as infecting Fraser fir in the region. It is not surprising that most isolates are *P. cinnamomi* considering it has been present on North Carolina Fraser fir Christmas tree farms since 1963 (Kuhlman & Hendrix, 1963). It has historically been the most prevalent species (Benson & Grand, 2000; Pettersson, et al., 2017), and has been found to be the most aggressive species against Fraser fir (Kohlway, Whetten, Benson, & Frampton, 2017). The identification of *P. cryptogea* as the second most common species affecting Fraser fir is consistent with previous survey work. *P. europaea, P. cactorum,* and *P. citricola* have also been identified in previous

literature (Pettersson, et al., 2017; Benson & Grand, 2000; Shew & Benson, 1981). The only species identified in this study which has not been previously identified infecting Fraser fir Christmas trees in North Carolina is *P. plurivora*. This species is closely related to *P. citricola* and was described as a new species in 2009 (Jung & Burgess, 2009). It is likely that this "new" detection was only previously identified as *P. citricola* although identification of isolates in this study supports the presence of both species in North Carolina Christmas tree production fields.

Phylogeny of *Phytophthora cinnamomi* Isolates

The haplotype and nucleotide diversity of the isolates tended to be on the moderate to higher side which is not typically associated with purely clonal populations. These diversity values could be inflated due to sequencing errors or small sample sizes. However, the presence of multiple clonal lineages introduced over time or geographically structured populations can also increase diversity. The Tajima's D results seem to also indicate structured populations, more negative results indicate an expansion after a bottleneck event which is consistent with the hypothesis that these pathogens were introduced and expanded over several decades.

βtub and Ef1a had moderate variation and good bootstrap and, when phylogenies were constructed for them, exhibited consistent clades which provide insights into what genes might be useful targets for intra-species phylogenetic analysis. However, when a phylogeny is constructed from a concatenated alignment of these two genes the bootstrapping support is actually lower and the branching is less consistent compared to the individual gene trees. Phylogenies of other loci were less useful with low bootstrapping support and varying diversity. This provides insights into regions that may or may not be useful for evaluating intraspecific variation and which regions are useful for species identification. These results also may reflect

the low diversity of *P. cinnamomi* in North America. Recent phylogeographic analysis of the species globally revealed only two clonal lineages present in North America (Shakya, et al., 2021). Future work evaluating relationships among *P. cinnamomi* isolates infecting Fraser fir may be more successful through other methods such as evaluating microsatellite data (Engelbrecht, Duong, & Berg, 2017).

Unfortunately, too little data regarding seedling source or planting year of trees sampled was available to make many phylogenetic inferences. Of the Isolates which clustered in the previously mentioned clade, four were collected in Avery County, and three of those were collected from trees that originated from one seedling producer in Pennsylvania. However, isolates collected from trees in Avery County originating from that same seedling producer were also present outside the clade.

Pathogenicity of Isolates

While pathogenicity against Fraser fir has been confirmed for most of these species, there have been few studies which evaluate the comparative aggressiveness of the different species (Kohlway, Whetten, Benson, & Frampton, 2017). Results from the positive and negative control groups as well as the lack of symptoms present on momi fir seedlings followed our expectations. Additionally, a slower disease progression and lower mortality among seedlings inoculated with other *Phytophthora* species other than *P. cinnamomi* is consistent with previous studies.

The lack of disease symptoms among the *Elongisporangium undulatum* [previously *Pythium undulatum* (Uzuhasi, Tojo, & Kakishima, 2010)] inoculated seedlings is surprising. Previous literature indicates that this species has a potential to be highly aggressive, in some cases more aggressive than *P. cinnamomi*, when infecting conifer species including *Abies* species

in European Christmas trees (Weber, Sulzer, & Haarhaus, 2004; Shafizadeh & Kavanagh, 2005). This might indicate that the inoculation method is not suitable for *E. undulatum*, or that Fraser fir is unaffected or only opportunistically affected by *E. undulatum*.

Though the chi-squared test determined the differences between the two replicates of the experiment were not statistically different, there were still minor differences. Disease progress was slower, and mortality was lower in the second replicate. Additionally, some treatment groups in the first replicate exhibited or lacked disease symptoms but the same treatment group exhibited or lacked disease symptoms in the second replicate (In the case of *P. europaea* and *P. cactorum* respectively). These replicates were on separate greenhouse benches next to one another, environmental effects should be minimal to non-existent. This could be a result of inconsistency in watering, amount of inoculum on the colonized rice grains, or other unknown factors.

Conclusion

This study presents updated information on the current distribution and diversity of Phytophthora species affecting Fraser fir Christmas tree production in the Southern Appalachian mountains. It identified six different *Phytophthora* species isolated from symptomatic Fraser fir as well as one other oomycete species, *E. undulatum*, with potential to cause disease in conifer trees. A greenhouse inoculation experiment compared the relative aggressiveness of the different species against Fraser and momi fir species, notably, *E. undulatum* did not seem to cause symptoms or mortality in Fraser fir or momi fir seedlings.

An analysis of five gene targets of *P. cinnamomi* isolates suggests moderate variation and a structured population of few clonal lineages. The isolates collected in this study provide an

opportunity for future genomic and phylogenetic analysis. Future work should focus on regular monitoring and collection of more isolates and the identification of regions of high variability in the *P. cinnamomi* genome to better understand phylogenetic differences.

Chapter 2 Tables:

Table 2.1 Primer sequences used for amplification of target genes including the annealing temperature of the primers, the expected amplified fragment length, and the published source for the original sequence. Thermocycling parameters followed the manufacturer's specification and included an initial denaturation at 98ºC for 5 minutes then 24-30 cycles of 1.) denaturation for 5 seconds at 98ºC 2.) annealing for 5 seconds at the annealing temperature and 3.) extension for 20-24 seconds at 72ºC. After the 24-30 cycles a final extension step was performed for 1 minute at 72ºC.

Target Region	Primer Name	Direction	Sequence	Annealing Temperature	Amplified Fragment (bp)	Source
Internal transcribed spacer (*ITS*)	ITS1	Forward	5' - TCCGTAGGTGAACCTGCGG - 3'	56ºC	350-880	(White, Bruns, Lee, & Taylor, 1990)
	ITS4	Reverse	5' - TCCTCCGCTTATTGATATGC - 3'			
βeta-tubulin (*Btub*)	Btub_F1	Forward	5'-GCCAAGTTCTGGGAGGTCATC-3'	60ºC	~1200	(Blair, Coffey, Park, Geiser, & Kang, 2008)
	Btub_R1	Reverse	5'- CCTGGTACTGCTGGTACTCAG-3'			
Cytochrome c oxidase Subunit 1 (*Cox1*)	CoxF4N	Forward	5' - GTATTTCTTCTTTATTAGGTGC - 3'	52ºC	972	(Kroon, Bakker, Van Den Bosch, Bonants, & Flier, 2004)
	CoxR4N	Reverse	5' - CGTGAACTAATGTTACATATAC - 3'			
Ras-related GTP-binding protein Ypt1 (*Ypt1*)	Yph1F	Forward	5' - CGACCATKGGTGTGGACTTT - 3'	62.5ºC	~450	(Schena, Hughes, & Cooke, 2006)
	Yph2R	Reverse	5' - ACGTTCTCMCAGGCGTATCT - 3'			
Elongation factor 1 alpha (*Ef1a*)	Ef1a_FL	Forward	5'- GGTCACCTGATCTACAAGTGC-3'	60ºC	~1200	(Blair, Coffey, Park, Geiser, & Kang, 2008)
	Ef1a_RL	Reverse	5'- CCTTCTTGTTCACCGACTTG-3'			

Table 2.2 Symptom ratings of sampled symptomatic Fraser fir trees.

Trait	Scale	Frequency (%)
foliage chlorosis (%)	0	0
	1-25	43
	26-50	32
	51-75	10
	76-100	15
canker width to stem circumference (%)	0	16
	1-25	14
	26-50	21
	51-75	18
	76-100	31
Root Necrosis (%)	1-33	29
	34-66	43
	67-100	28

Table 2.3 Percent of tree samples by county in each symptom severity rating for each symptom category. Foliage chlorosis ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% foliage chlororsis. Stem canker ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% canker width relative to stem circumference. Root rot rating is on a scale of 0-3 where 0=0%, 1=1-33%, 2=34-66%, and 3=67-100% of root necrosis.

County	Number of Samples	Foliage Chlorosis rating					Stem Canker rating					Root Rot rating			
		0	1	2	3	4	0	1	2	3	4	0	1	2	3
Alleghany	48	0%	35%	50%	13%	2%	19%	21%	27%	13%	21%	0%	38%	31%	31%
Ashe	68	0%	46%	32%	16%	6%	13%	3%	22%	22%	40%	1%	25%	50%	24%
Avery	65	0%	49%	35%	5%	11%	6%	17%	34%	25%	18%	0%	23%	55%	22%
Buncombe	10	0%	50%	20%	0%	30%	0%	20%	50%	10%	20%	0%	30%	30%	40%
Carter	5	0%	40%	20%	40%	0%	0%	20%	0%	20%	60%	0%	20%	60%	20%
Grayson	24	0%	63%	21%	8%	8%	42%	13%	8%	8%	29%	0%	25%	54%	21%
Haywood	6	17%	17%	0%	17%	50%	17%	17%	0%	17%	50%	17%	17%	17%	50%
Henderson	2	0%	0%	0%	0%	100%	0%	50%	0%	0%	50%	0%	50%	50%	0%
Jackson	23	0%	35%	9%	4%	52%	13%	13%	9%	17%	48%	0%	30%	26%	43%
Macon	5	0%	20%	60%	0%	20%	0%	60%	0%	40%	0%	0%	60%	40%	0%
Madison	7	0%	14%	14%	0%	71%	14%	0%	0%	0%	86%	0%	29%	29%	43%
Mitchell	15	0%	47%	27%	7%	20%	20%	20%	13%	20%	27%	0%	33%	40%	27%
Transylvania	5	0%	0%	20%	40%	40%	0%	0%	20%	40%	40%	0%	0%	40%	60%
Watauga	27	0%	52%	37%	7%	4%	30%	11%	11%	15%	33%	0%	30%	37%	33%

Table 2.4 Percent of tree samples by recovered *Phytophthora* spp. in each symptom severity rating for each symptom category. Foliage chlorosis ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% foliage chlororsis. Stem canker ratings are on a scale of 0-4 where 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, and 4=76-100% canker width relative to stem circumference. Root rot rating is on a scale of 0-3 where 0=0%, 1=1-33%, 2=34-66%, and 3=67-100% of root necrosis.

***Phytophthora* Species**	**Number of Samples**	**Foliage Chlorosis rating**				**Stem canker Rating**				**Root Rot Rating**		
		1	2	3	4	1	2	3	4	1	2	3
P. cactorum	2	0%	50%	50%	0%	0%	0%	100%	0%	0%	50%	50%
P. cinnamomi	53	36%	34%	13%	17%	6%	9%	19%	25%	28%	40%	32%
P. citricola	2	0%	100%	0%	0%	0%	0%	0%	0%	0%	50%	50%
P. cryptogea	8	25%	38%	25%	13%	13%	0%	0%	25%	38%	38%	25%
P. europaea	3	33%	33%	33%	0%	0%	0%	33%	0%	33%	33%	33%
P. plurivora	1	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	100%

Table 2.5 *Phytophthora* isolates recovered from symptomatic Fraser fir roots from Christmas tree plantation sites in the Southern Appalachian mountains and identified in the initial PCR screening. Isolates are identified by their clade (1a, 2c, 7a,7c, and 8a) (Yang, Tyler, & Hong, 2017). Other non-*Phytophthora* Oomycete species of interest are included at "Other Oomycetes".

County	Sites	Samples	Total Phytophthora	7c	8a	2c	7a	1a	Other Oomycetes
Alleghany	16	49	22	22					
Ashe	23	70	21	14	3	1	2	1	1
Avery	14	68	33	33					
Buncombe	2	11	7	7					
Carter	1	5							
Cherokee	1	2	2	2					
Clay	1	5	1					1	
Grayson	8	25	8	6		2			2
Haywood	1	6	1	1					
Henderson	1	2	1	1					
Jackson	5	25	18	17	1				
Macon	1	6	3	1	2				
Madison	2	9	4	4					
Mitchell	3	15	7	4	2		1		
Transylvania	1	6	4	4					
Watauga	9	28	19	18	1				1
Total	89	332	151	134	9	3	3	2	4

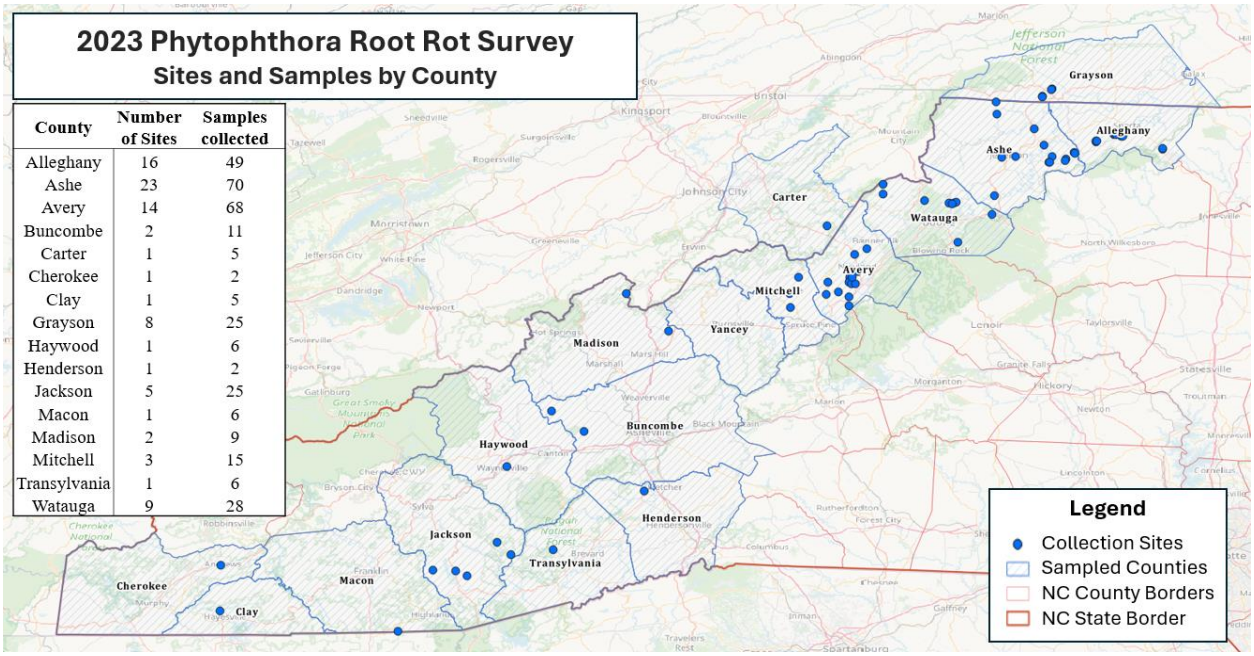
Table 2.6 *Phytophthora* isolates by species and county. Isolates were identified using sequences from 5 DNA gene sequences (β-tub, Ef-1α, ITS, Ypt1, and Cox1).

County	Sites	% of sites positive for *Phytophthora*	Number of isolates Recovered					
			P. cinnamomi	***P. cryptogea***	***P. europaea***	***P. cactorum***	***P. plurivora***	***P. citricola***
Alleghany	16	50%	14					
Ashe	23	57%	13	2	2	1	1	
Avery	14	43%	7					
Buncombe	2	0%						
Carter	1	0%						
Cherokee	1	100%	2					
Clay	1	100%				1		
Grayson	8	50%	5					2
Haywood	1	0%						
Henderson	1	0%						
Jackson	5	80%	4	1				
Macon	1	100%		2				
Madison	2	50%	1					
Mitchell	3	100%	4	2	1			
Transylvania	1	100%	2					
Watauga	9	22%	1	1				
Total	89	51%	53	8	3	2	1	2

Table 2.7 Population statistics calculated for *Phytophthora cinnamomi* isolate sequence alignments of target loci.

Loci	Alignment Length	Haplotype Diversity (Hd)	Nucleotide Diversity (π)	Tajima's D
βtub	965	0.710	0.00201	-2.986
Ef1a	1050	0.871	0.00190	-2.895
ITS	656	0.631	0.00241	-2.825
Ypt	353	0.688	0.00749	-1.114
Cox	750	0.996	0.01906	-2.417

Chapter 2 Figures:



County	Number of Sites	Samples collected
Alleghany	16	49
Ashe	23	70
Avery	14	68
Buncombe	2	11
Carter	1	5
Cherokee	1	2
Clay	1	5
Grayson	8	25
Haywood	1	6
Henderson	1	2
Jackson	5	25
Macon	1	6
Madison	2	9
Mitchell	3	15
Transylvania	1	6
Watauga	9	28

Figure 2.1 Map of Sites in each county where root tissue samples were collected from symptomatic Fraser fir trees. Inset table displays numbers of sites and trees sampled in each county.

Figure 2.2 Process for culture and isolation of Phytophthora from infected root tissue using PARP-v8 selective media (Jeffers & Martin, 1986).

Figure 2.3 Fir seedlings arranged on greenhouse benches into treatment blocks with 10 Fraser fir and 4 Momi fir seedlings per treatment block.

Figure 2.4 Fir seedlings being inoculated with colonized rice grains. A ¼ inch thick rod is used to make 2 small holes in the potting media approximately 2.5cm from the seedling stem. Sterilized forceps are used to place 2 colonized rice grains into each hole.

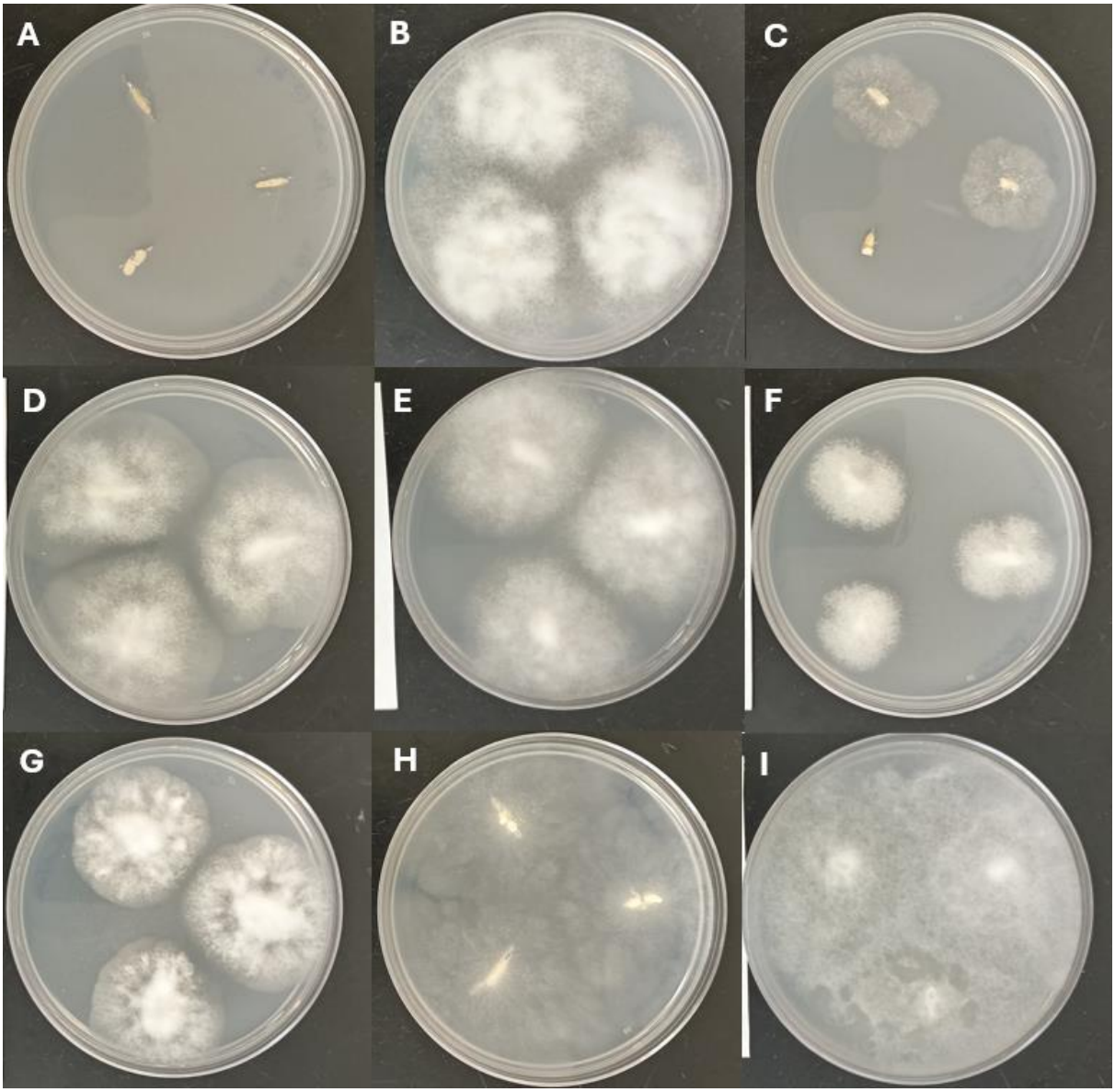


Figure 2.5 3 day-old cultures on PARP-v8 agar media inoculated with colonized rice grains used to inoculate fir seedlings. (A) non-colonized negative control, (B) 23ss04 *P. cinnamomi* positive control, (C) *P. cactorum*, (D) *P. cryptogea* isolate 1, (E) *P. cryptogea* isolate 2, (F) *P. europaea*, (G) *P. plurivora*, (H) *Elongisporangium Undulatum*, (I) *Globisporangium macrosporum*.

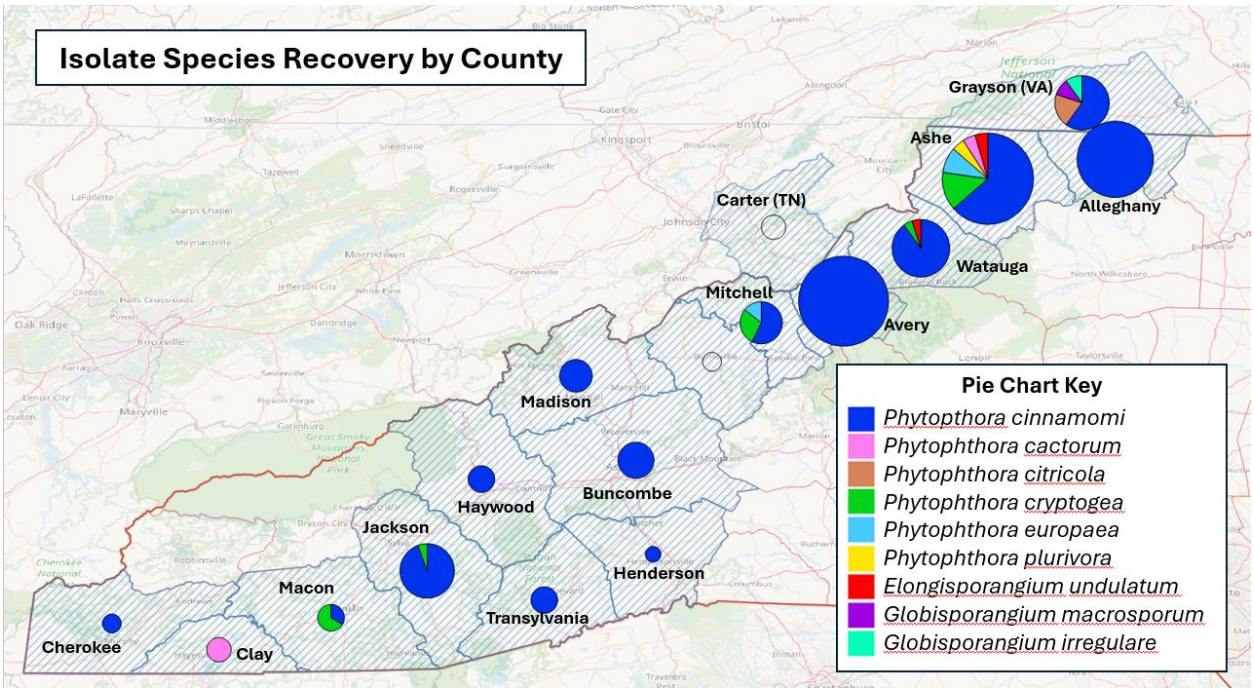


Figure 2.6 Visual representation of *Phytopthora* isolate recovery by county. Pie charts represent the diversity of species isolated from symptomatic Fraser fir in each county. The size of each pie chart is scaled to the number of trees sampled in each county (ranging from 2 trees, in Cherokee and Henderson Counties, to 70 trees in Ashe County). Also included are 3 non-*Phytophthora* oomycete species of interest.

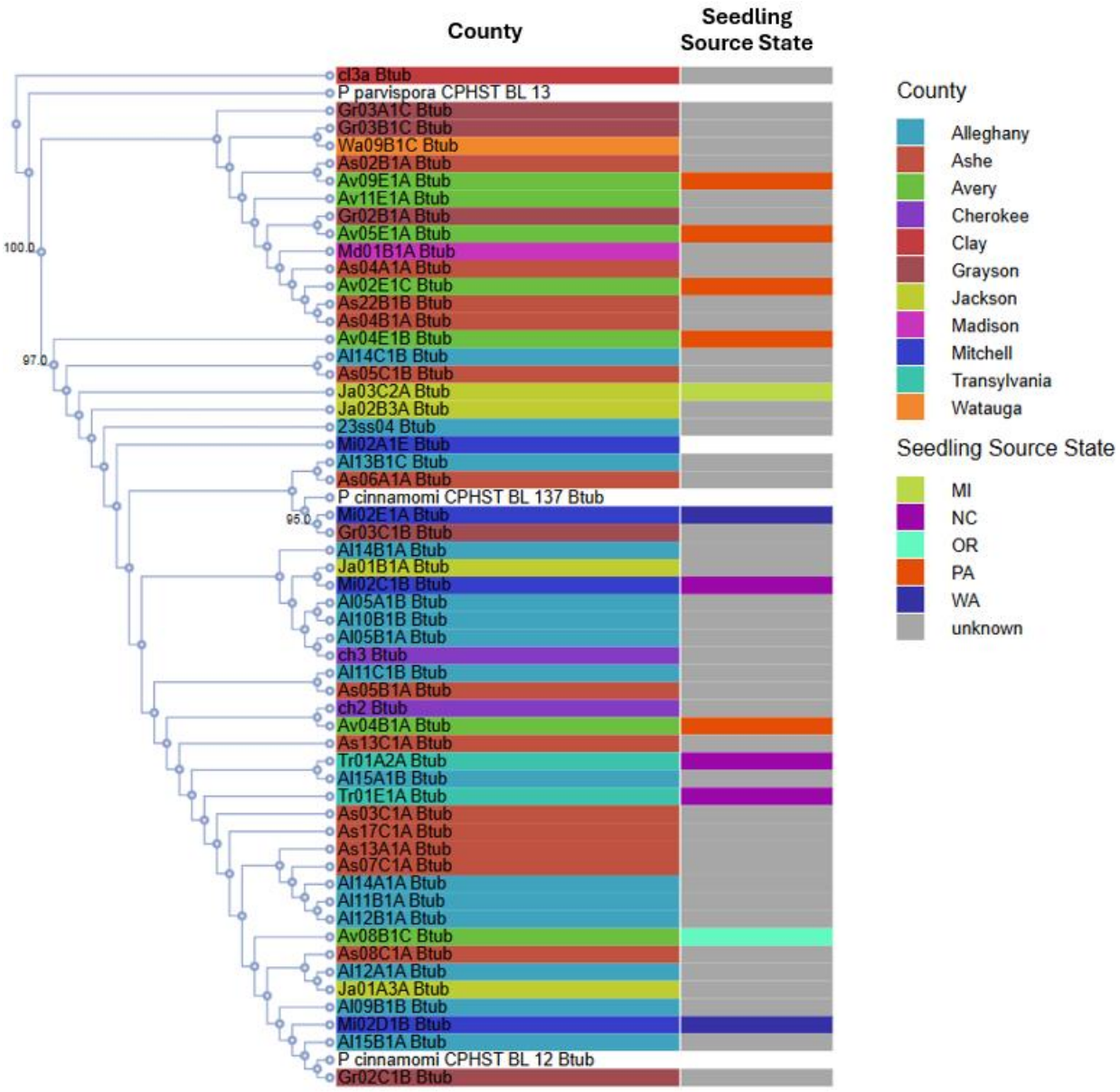


Figure 2.7 Maximum-Likelihood tree built from aligned *βtub* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types of *P. cinnamomi* and *P. parvispora* (a closely related species) were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state.

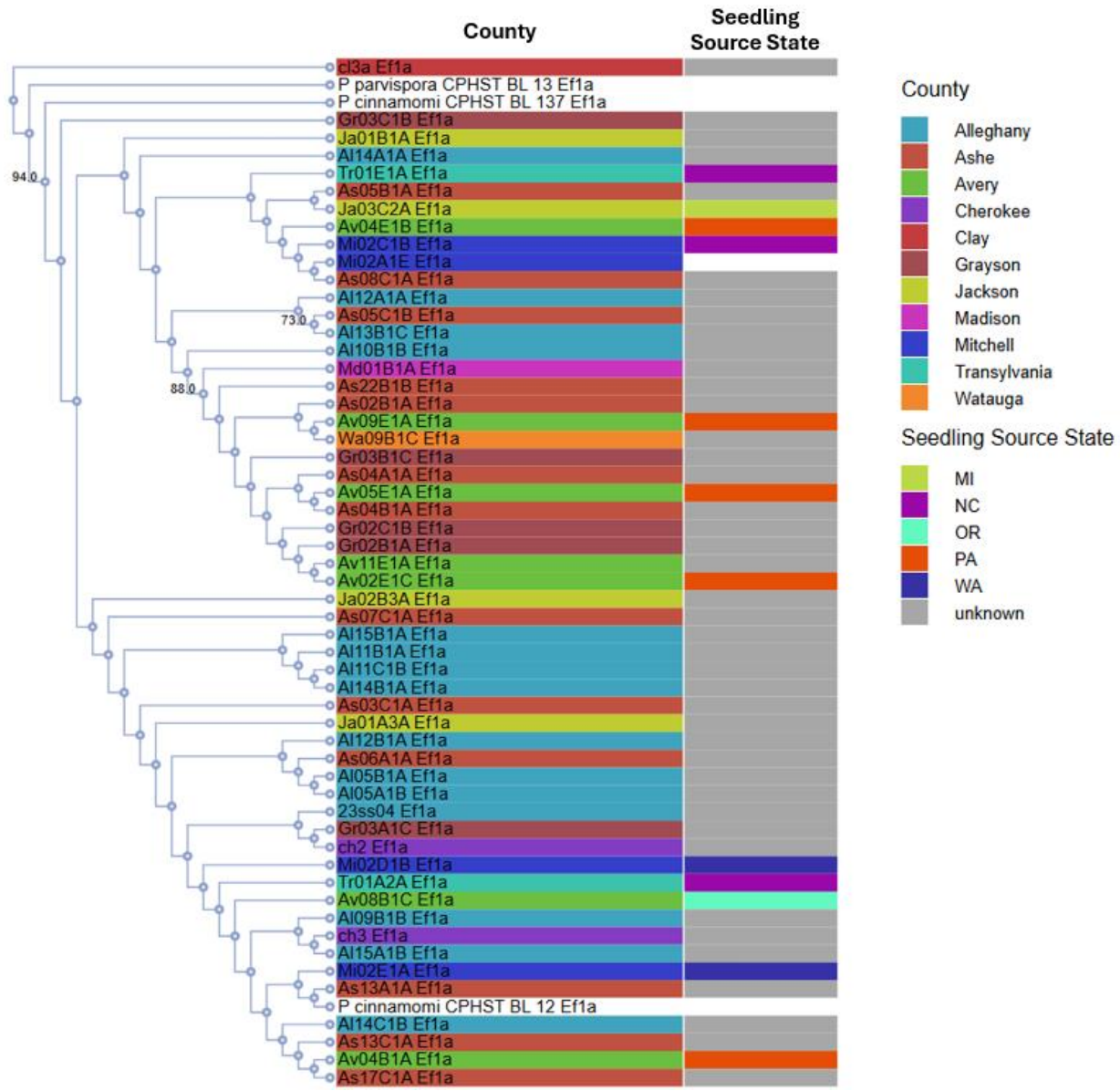


Figure 2.8 Maximum-Likelihood tree built from aligned *Ef1a* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types of *P. cinnamomi* and *P. parvispora* (a closely related species) were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state.

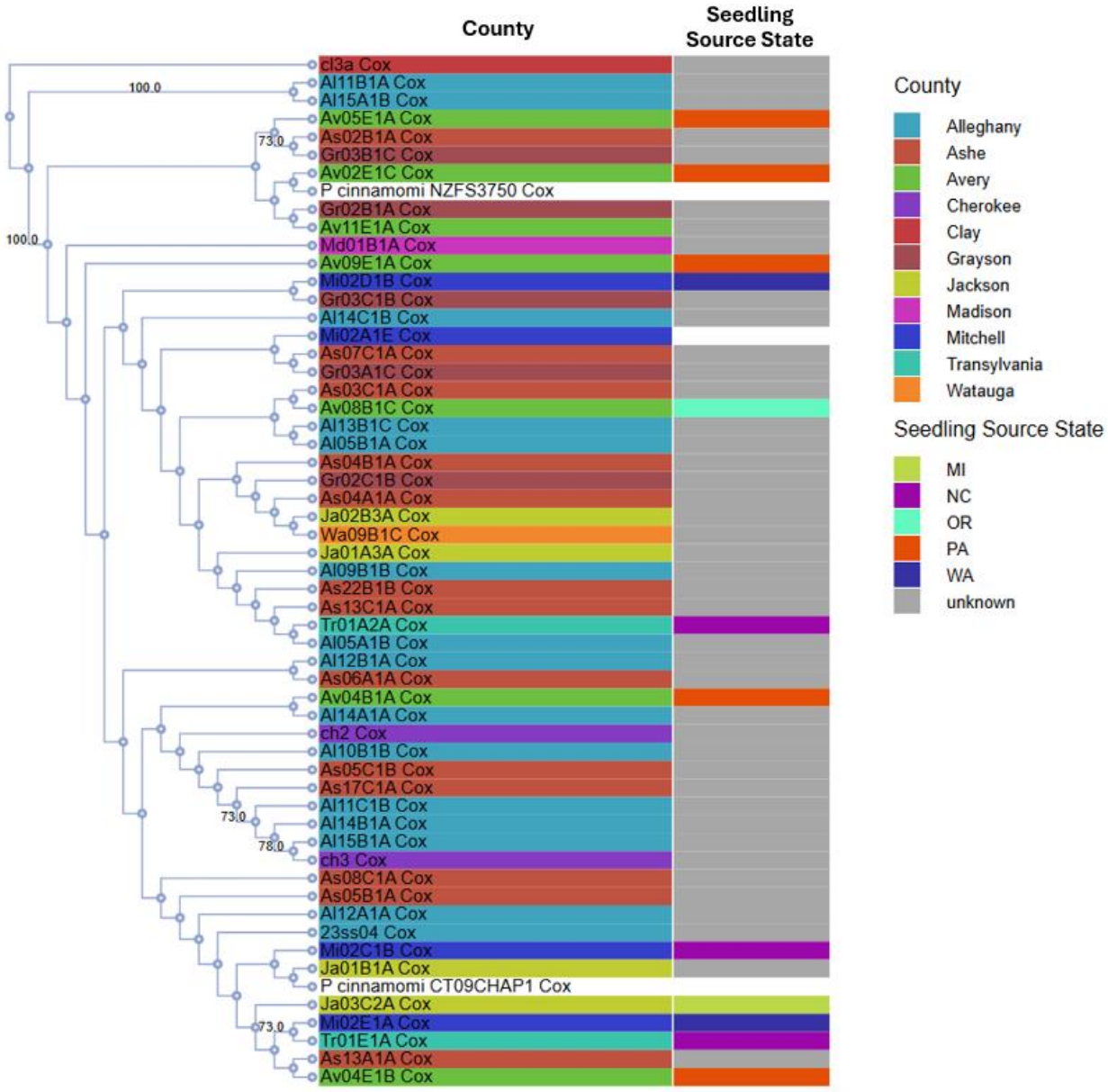


Figure 2.9 Maximum-Likelihood tree built from aligned *Cox1* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences obtained from published mitochondrial genomes of P. cinnamomi isolates were included in the Phylogeny (Winkworth, et al., 2022). Colored bands were added to display metadata, including county of collection and seedling source state.

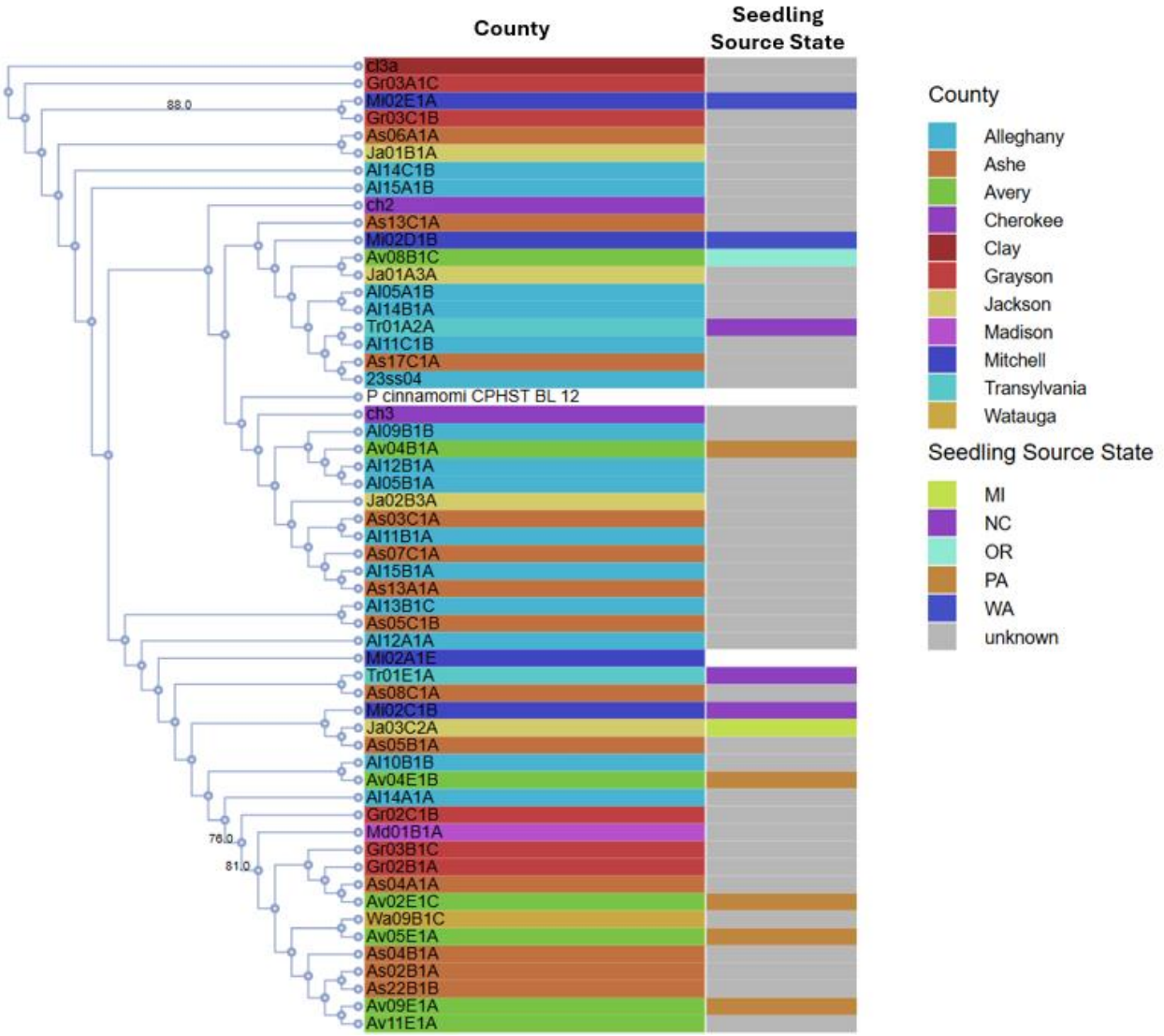


Figure 2.10 Maximum-Likelihood tree built from aligned and concatenated βtub and Ef1a sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types culture of *P. cinnamomi* were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state.

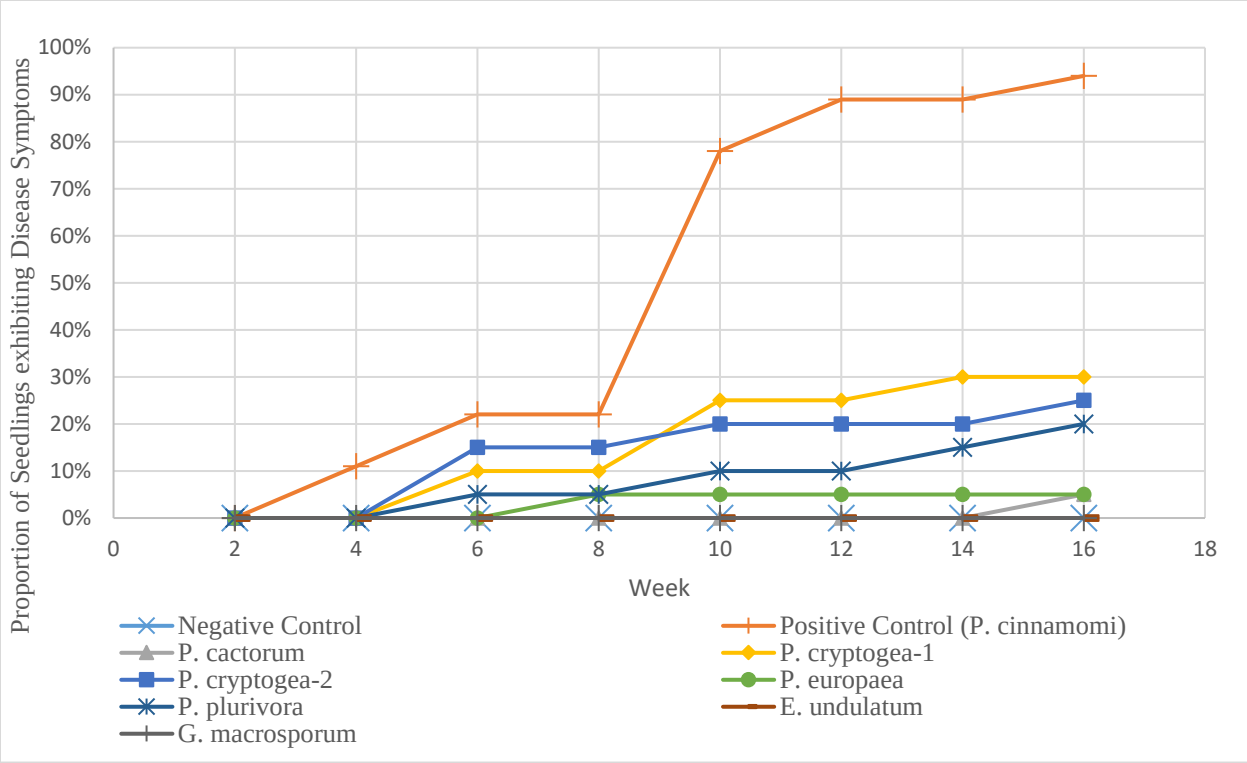


Figure 2.13 Disease symptom progression over the 16-week experimental period for Fraser fir seedlings in each treatment group. Seedlings were observed biweekly for either disease symptoms or mortality. The proportion of seedlings exhibiting disease symptoms (such as foliage chlorosis or stem canker) or mortality was plotted at each timepoint.

Chapter 3

Inoculation of Poplar (*Populus*) Clones with *Phytophthora* Isolates for Evaluation of Deployment on Phytophthora Root Rot Contaminated Land.

3.1 Introduction

Fraser fir (*Abies fraseri*) is one of the most economically important Christmas tree species in North America. It is especially important in North Carolina where it makes up 96% of all real Christmas trees produced (Owen, 2016). Fraser fir is highly favored by consumers thanks to various characteristics like its post-harvest needle retention, pleasant aroma, sturdy branches, and soft foliage. While this species is native to the southern Appalachian Mountains where it is heavily cultivated, the natural range is constrained to high elevation montane ecosystems that have unique ecological and climatic conditions. Fraser fir is especially adapted to these unique ecosystems and as a result is sensitive to environmental changes and the cascading impacts of climate change. The availability and productivity of land often means that Fraser fir is planted at much lower elevations compared to its natural range, increasing susceptibility to stress from both abiotic and biotic factors.

Commercial production of Fraser fir Christmas trees in North Carolina is already limited by several pests and pathogens. The largest contributor to loss in plantations is Phytophthora root rot (PRR) which is a disease caused primarily by the pathogen *Phytophthora cinnamomi*. This devastating disease kills 100% of trees that become infected (Kohlway, Whetten, Benson, & Frampton, 2017; Pettersson, Frampton, & Sidebottom, 2017). The disease is difficult to control due to *Phytophthora*'s ability to persist in the soil and resist treatments from fungicides. Once the pathogen is present in the soil of a site it is functionally impossible to eliminate. Phytophthora is also difficult to exclude from a site due to its ability to move through surface and soil water.

Contaminated irrigation water or flooding events have the potential to spread PRR. Moisture and soil temperature play a major role in limiting the range and success of the disease (Hardham, 2017).

Unfortunately, the effects of global climate change such as increased temperatures, changes in precipitation patterns, and more frequent extreme weather events create conditions that are more favorable to the spread of the PRR. Additionally, the use of out-of-state planting materials in the southern Appalachian mountains has increased in recent decades (Pettersson, Frampton, & Sidebottom, 2017) which has opened up opportunities for the introduction of other soil pathogens (McKeever & Chastagner, 2016). A survey conducted in 2014 identified six different phytophthora species causing disease on Fraser fir Christmas trees in the region (Pettersson, et al., 2017).

The limited availability of land for the production of Fraser fir Christmas trees is further limited by this destructive disease, and growers are left with few options. Growers could offset losses from PRR by growing a resistant alternative crop on contaminated lands that are no longer suitable for Fraser fir. One such alternative is poplar (*Populus* spp.) cultivated as a short rotation woody crop (SRWC) for high-value wood products such as veneer and bioenergy. The high growth rate of poplar allows for rotations as short as 12 years in intensively managed stands (Ghezehei, Ewald, Hazel, Zalesny, & Nichols, 2021). While it is likely not providing the same level of profit as Fraser fir Christmas trees, this option may help growers recover some lost income from contaminated acreage.

Previous studies in a temperature-controlled greenhouse, which are currently under review, seem to suggest that certain genotypes are less affected by *P. cinnamomi* than others. While no poplar genotypes exhibited mortality when inoculated with *P. cinnamomi*, pure *P.*

deltoides (DxD) clonal genotypes had reduced relative growth rate (RGR) compared to hybrid poplar clones [*P. trichocarpa x P. deltoides* (TxD)]. However, this is not necessarily indicative of performance against other *Phytophthora* species present on sites that poplar may be deployed on. There is, therefore, a need to evaluate the performance of these poplar genotypes when inoculated with other Phytophthora species currently affecting Fraser fir Christmas tree production in the region. This study aims to evaluate the effects of inoculation by 5 different Phytophthora species on eight different clonal genotypes of poplar to determine their suitability on PRR contaminated sites. We hypothesize that TxD hybrid poplar clones will be relatively unaffected by Phytophthora treatments and that DxD poplar clones will have limited reductions in relative growth rate, primarily when inoculated with *P. cinnamomi* while inoculation with other *Phytophthora* species will have a lesser effect.

3.2 Materials and Methods

Plant Materials

Eight clonal genotypes were selected based on their suitability as veneer (Ghezehei, Nichols, & Hazel, 2016; Ghezehei, Nichols, & Hazel, 2020) as well as consistency with previous experiments. Four of the clones were TxD crosses (185, 229, 339, 342) and four were DxD crosses (356, 437, 438, 441). Five of these clonal genotypes were identified in previous work as having elite characteristics for veneer (229, 356, 437, 438, 441).

Dormant Hardwood cuttings were collected in the spring of 2024. Approximately 70 cuttings were collected for each of the eight clones. All poplar clones used in this study were collected from four ongoing field trials located across North Carolina including: (1) Laurel Springs (36.398835, 123 -81.317767); (2) Salisbury (35.696517, -80.622733); (3) Fountain

(35.675478, - 124 77.6351045) and (4) Wallace (34.763866, -78.099345). Each cutting was ~30cm long and ~2cm in diameter (Figure 3.1). Poplar cuttings were stored at 4ºC immediately after field collection. Rooting of cuttings was initiated within 2-3 weeks of field collection. Initiation of rooting involved cutting 2cm of tissue from either end of the cuttings then placing the cuttings in water and storing them for 48 hours at 4ºC. After soaking in water, the bottom end of each cutting was dipped into Hormex rooting hormone before being planted into 1.94-gallon containers filled with potting media (1:1:1 vermiculte, perlite, and peat moss). Poplar cuttings were placed into the potting media so that as much of the cutting was below the surface as possible, leaving only the top-most node/bud visible (Figure 3.2).

Preparation of Inoculum

Five species of *Phytophthora* were chosen for the experiment representative of the diversity of species currently found in Christmas tree production sites in the Southern Appalachian Mountains. These species include *P. cinnamomi, P. cactorum, P. cryptogea, P. europaea,* and *P. plurivora*. Isolates used in this experiment were collected and identified in the survey detailed in Chapter 2 except for the *P. cinnamomi* isolate (isolate 23ss04), a strain that has been shown to be highly virulent against Fraser fir (Kohlway, Whetten, Benson, & Frampton, 2017). All isolates were cultured onto PARP v8 agar media (Jeffers & Martin, 1986). Agar plugs from actively growing cultures were placed into flasks of twice-autoclaved rice according to (Frampton & Benson, 2012). Flasks were incubated at 25ºC for two weeks and shaken daily to ensure even distribution of inoculum. A Flask of non-inoculated, twice-autoclaved rice was treated similarly, only using sterile PARP v8 agar plugs to be used as a negative control.

Inoculation

Rooted cuttings were inoculated by making two small holes, in the potting media, ~2cm deep and ~2cm from the stem using a sterile rod. Then two rice grains colonized by Phytophthora are placed into each hole using sterile forceps (Figure 3.3). Once inoculated the rooted cuttings were heavily watered so that the water level reached the top of the container. Rooted cuttings were watered in this manner three times per week. The cuttings were re-inoculated at the 8-week timepoint in the manner described above.

Experimental Design

The experiment was conducted at the NC State University Horticultural Field Laboratory in the summer of 2024 over the course of 16 weeks starting on 19 June and ending on 9 October 2024. A completely randomized design with blocking was used. Rooted cuttings were arranged into six blocks by treatment. These treatments included: mock-inoculated negative control (-), *P. cinnamomi, P. cactorum, P. cryptogea, P. europaea*, and *P. plurivora*. A total of 18 rooted cuttings were randomly selected from each clone for use in the experiment. Three rooted cuttings from each clone were randomly selected and placed in each block as biological replicates. Each block contained a total of 24 rooted cuttings randomly arranged. The treatment blocks were arranged on 2 adjacent benches within a greenhouse (Figure 3.4). Each block received its corresponding inoculation treatment on 19 June 2024. Starting height was recorded at the time of inoculation. Height measurements were recorded in centimeters by measuring the distance between the start of current season growth (or soil surface if the shoot was emerging from below the soil surface) to the tip of the terminal bud. In the case of multiple stems, the taller stem was recorded. Height was measured in the same manner every two weeks for 16 weeks.

Relative Growth Rate (RGR)

An ANOVA was performed comparing the starting heights of the treatment blocks, clones, and crosses. There were no significant differences between starting height of each treatment group ($F_{5,137}$ = 0.89; P = 0.49) (Figure 3.5). There was also no significant difference between starting heights of each cross ($F_{1,141}$ = 3.317; P = 0.0707) (Figure 3.6). However, there were significant differences between the starting heights of each clone ($F_{7,135}$ = 3.583; P = 0.00144). Fisher's LSD was used to do the post-hoc analysis (Figure 3.7). To standardize the differences in starting heights across the clones, relative growth rate (RGR) was used to compare growth over time. *Equation 1*, below, was used to calculate RGR:

Eqn 1: $RGR = \frac{\ln(Height_{Ti}) - \ln(Height_{T0})}{T_i - T_0}$ (Hunt 1982)

Where *RGR* is the relative growth rate; $Height_{Ti}$ is the stem length at the time of measurement; $Height_{T0}$ is the initial stem length; T_i-T_0 is the change in time in days, from the time of measurement to the time of initial measurement.

Statistical Analysis

Statistical analyses were performed using Rstudio software (R version 4.2.2; Rstudio Team, 2020). ANOVA was performed comparing the relative growth rates at each time point for each treatment by clone and cross. Normality was evaluated using a Shapiro-wilk test from the R package 'cat' (Schafer, Harding, & Tusell, 2023) which found significant differences between groups (Table 3.1). A Levene's test was used to evaluate the homogeneity of variances from the R package 'car' (Fox & Weisburg, 2019) which found significant differences between groups (Table 3.2). Therefore, the data was determined to not be normally distributed, even after attempting transformations. A Kruskal-Wallis test was chosen to evaluate RGR. The analysis

was performed for each of the 8 timepoints and the average across all timepoints. Because no significant differences were found between treatments using the Kruskal-Wallis test, no post-hoc tests were performed. Additionally, a Kruskal-Wallis test was used to separately compare the effect of treatment, clone, and cross on RGR (the average across all timepoints). When significant differences were identified, a Dunn's post-hoc analysis was performed using the R package 'fsa' (Ogle, Doll, Wheeler, & Dinno, 2025).

3.3 Results

General Observations of Poplar Responses to Inoculation Treatments

Observations and data from this experiment did not align with expectations. Slight RGR reductions were expected in, at least, the DxD crosses inoculated with P. cinnamomi, but no such trend is apparent in any of the treatments across crosses or clones (Figures 3.8). No presence of PRR symptoms was observed, either in the first 8 weeks or following the second inoculation at the 8-week timepoint.

Effect of Inoculation Treatments on RGR

While there is a general increase in RGR from week 6 to week 10 (Table 3.3), there are no discernible trends among treatments and clonal genotypes. Differences in RGR were statistically significant between clones without considering treatment (Table 3.4; Figure 3.9). Clone 356 had the highest mean RGR at 0.0478 cm day^{-1} while clone 438 had the lowest at 0.0166 cm day^{-1}. The mean RGRs of crosses were very close at 0.0263 and 0.0253 cm day^{-1} for DxD and TxD respectively and were not significantly different (Figure 3.10). No significant differences were found between treatments at any timepoint or when RGR was averaged across

all timepoints (Table 3.5; Figure 3.8). Likewise, no significant differences were detected between treatments, even when only comparing treatments without considering clone or cross (Figure 3.11).

3.4 Discussion

The results of this experiment were surprising. At the very least, previous inoculation tests using *P. cinnamomi* revealed some reduction in the relative growth rate of some clonal genotypes of poplar within four weeks of inoculation, but similar observations were not present here. None of the *Phytophthora* treatments were significantly different than the negative control, in fact, all but one treatment had a higher mean RGR compared to the negative control. Several factors could contribute to this difference. Firstly, the number of biological replicates of each clone in each treatment group (3) is low. More biological replicates would increase the statistical power and might resolve the issue of lack of normality in the data, allowing the use of more conventional analyses. Differences could also result from the growing conditions of the cuttings. In previous experiments, ~15cm long cuttings were planted in 0.1-gallon containers versus ~30cm long cuttings planted in 1.94-gallon containers. It is possible that rooted cuttings were under greater stress in the previous experiments due to the smaller containers. If *Phytophthoras* are only opportunistically pathogenic against these poplar genotypes, then perhaps the use of larger cuttings and containers meant that rooted cuttings were less stressed and therefore less susceptible to potential pathogens, though, such a conclusion could not be made by this experiment alone and more work would be necessary to fully investigate this possibility. One major flaw in this experiment is the lack of a suitable positive control. In previous experiments, Fraser fir seedlings were used to confirm effectiveness of inocula. With no infected plants and no recovery of Phytophthoras, this experiment cannot be used to make any concrete conclusions

about these clones' susceptibility to this pathogen. This experiment also does not account for long-term effects of inoculation (more than one growing season); however, previous experiments do indicate that Phytophthora inoculated individuals exhibited earlier budbreak compared to non-inoculated controls. If changes in timing of budbreak or growth in subsequent seasons are observed in individuals used in this experiment, it may suggest a delayed effect from inoculation.

In conclusion, there is a lack of statistical differences between Phytophthora treatments and negative control, meaning our original hypothesis cannot be supported using this experiment. This result could indicate further support for the use of poplar clonal and hybrid cross genotypes for deployment on PRR-contaminated marginal lands in the Southern Appalachian Mountains. However, further work should be performed to confirm this and focus on the long-term effects of Phytophthora inoculation on these genotypes as well as field experiments evaluating their performance in real-world PRR-contaminated settings.

Chapter 3 Tables:

Table 3.1 Shapiro-Wilk test of normality for RGR by treatment and clone at each timepoint. α = 0.05. All timepoints had significant differences between indicating that the data is not normally distributed.

Timepoint	W	P-value
Week 2	0.70626	1.47E-15*
Week 4	0.73916	1.22E-14*
Week 6	0.73612	9.94E-15*
Week 8	0.73164	7.39E-15*
Week 10	0.7313	7.23E-15*
Week 12	0.73362	8.42E-15*
Week 14	0.73261	7.88E-15*
Week 16	0.7332	9.31E-15*

Table 3.2 Levene's test for homogeneity of variance of RGR by treatment and clone at each timepoint. $\alpha = 0.05$. All but one timepoints had significant differences indicating that the variances between the groups were not equal.

Timepoint	Df	F-value	P-value
Week 2	7	1.6306	0.1319
Week 4	7	2.2007	0.03792*
Week 6	7	2.7617	0.0103*
Week 8	7	2.9483	0.006617*
Week 10	7	3.0531	0.005152*
Week 12	7	3.0174	0.005611*
Week 14	7	3.0897	0.00472*
Week 16	7	2.9995	0.005874*

Table 3.3 Summary Statistics for each variable (clone, cross, treatment, and timepoint).

Variable		mean of RGR	sd	se	n
Clone	185	0.03103	0.02831	0.01635	144
	229	0.03490	0.01756	0.01014	144
	339	0.01739	0.01236	0.00714	144
	342	0.01768	0.00890	0.00514	144
	356	0.04781	0.05499	0.03236	136
	437	0.01874	0.01179	0.00681	144
	438	0.01662	0.00846	0.00488	144
	441	0.02223	0.00900	0.00519	144
	All Clones	0.02580	0.01892	0.01100	1144
Cross	DxD	0.02635	0.02106	0.01231	568
	TxD	0.02525	0.01678	0.00969	576
	All crosses	0.02580	0.01892	0.01100	1144
Treament	(-)	0.02392	0.01749	0.01010	192
	P. cactorum	0.02786	0.01938	0.01119	192
	P. cinnamomi	0.02885	0.02170	0.01253	192
	P. cryptogea	0.02801	0.02151	0.01242	192
	P. europaea	0.02924	0.02438	0.01453	184
	P. plurivora	0.01692	0.00907	0.00523	192
	All treatments	0.02580	0.01892	0.01100	1144
Timepoint	Week 2	0.01475	0.00981	0.00568	143
	Week 4	0.02279	0.01644	0.00954	143
	Week 6	0.02604	0.01939	0.01127	143
	Week 8	0.02862	0.02080	0.01210	143
	Week 10	0.02886	0.02106	0.01225	143
	Week 12	0.02864	0.02123	0.01235	143
	Week 14	0.02835	0.02122	0.01235	143
	Week 16	0.02836	0.02141	0.01246	143
	All timepoints	0.02580	0.01892	0.01100	1144

Table 3.4 Kruskal-Wallis test of RGR by clone. α = 0.05. Clones were significantly different at all timepoints and across all timepoints

Timepoint	chi-squared (*H*)	Df	P-value
Week 2	29.557	7	0.0001145*
Week 4	23.777	7	0.001247*
Week 6	21.508	7	0.003086*
Week 8	19.959	7	0.00566*
Week 10	19.62	7	0.006452*
Week 12	21.123	7	0.003592*
Week 14	21.978	7	0.002563*
Week 16	20.038	7	0.005489*
All Timepoints	22.396	7	0.00217*

Table 3.5 Kruskal-wallis test of RGR by treatment and clone. There were no significant differences between groups ($\alpha = 0.05$) at any timepoint and across all timepoints.

Timepoint	chi-squared (*H*)	Df	P-value
Week 2	4.8261	5	0.4375
Week 4	5.3842	5	0.3708
Week 6	4.8108	5	0.4394
Week 8	4.8933	5	0.429
Week 10	4.3642	5	0.4983
Week 12	5.864	5	0.3197
Week 14	5.2025	5	0.3917
Week 16	3.9294	5	0.5596
All Timepoints	5.2752	5	0.3832

Chapter 3 Figures:

Figure 3.1 Dormant cuttings collected from the Wallace, NC field trial site.

Figure 3.2 Cuttings were planted as deep as possible with only the top-most node/bud above the surface of the potting media.

Figure 3.3 Inoculation of rooted cuttings with colonized rice grains. A sterile rod is used to make two ~2cm deep holes, ~2cm from the stem of the rooted cutting, then 2 rice grains are placed into each hole.

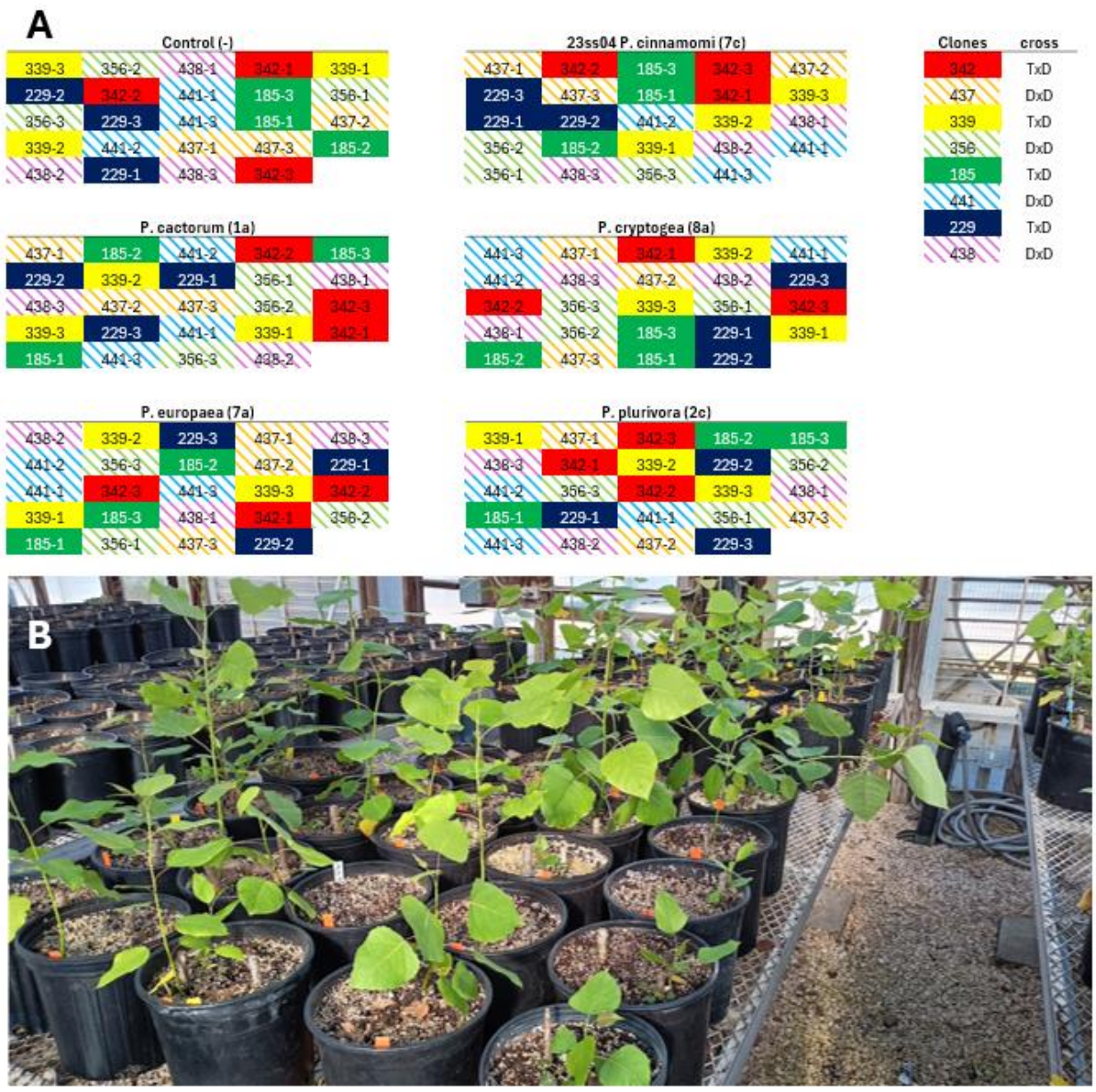


Figure 3.4 (A) Arrangement of rooted cuttings in the treatment blocks. Clones and biological replicates were arranged randomly within the treatment block. (B) Rooted cuttings arranged on greenhouse benches according to experimental design.

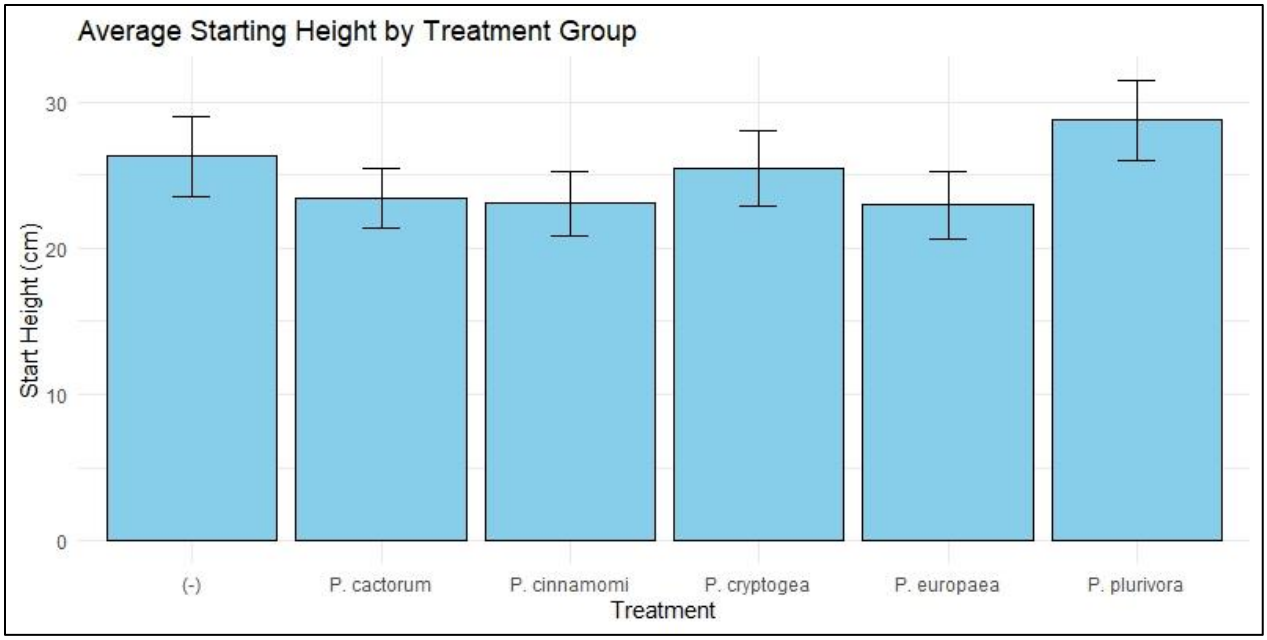


Figure 3.5 There were no significant differences in starting heights between treatment blocks. α = 0.05 ($F_{5,137}$ = 0.89; P = 0.49).

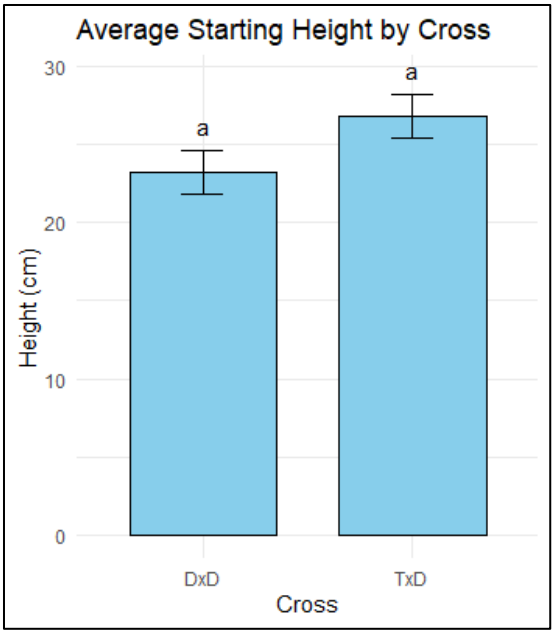


Figure 3.6 There were no significant differences in starting heights between crosses. α = 0.05 ($F_{1,141}$ = 3.317; P = 0.0707).

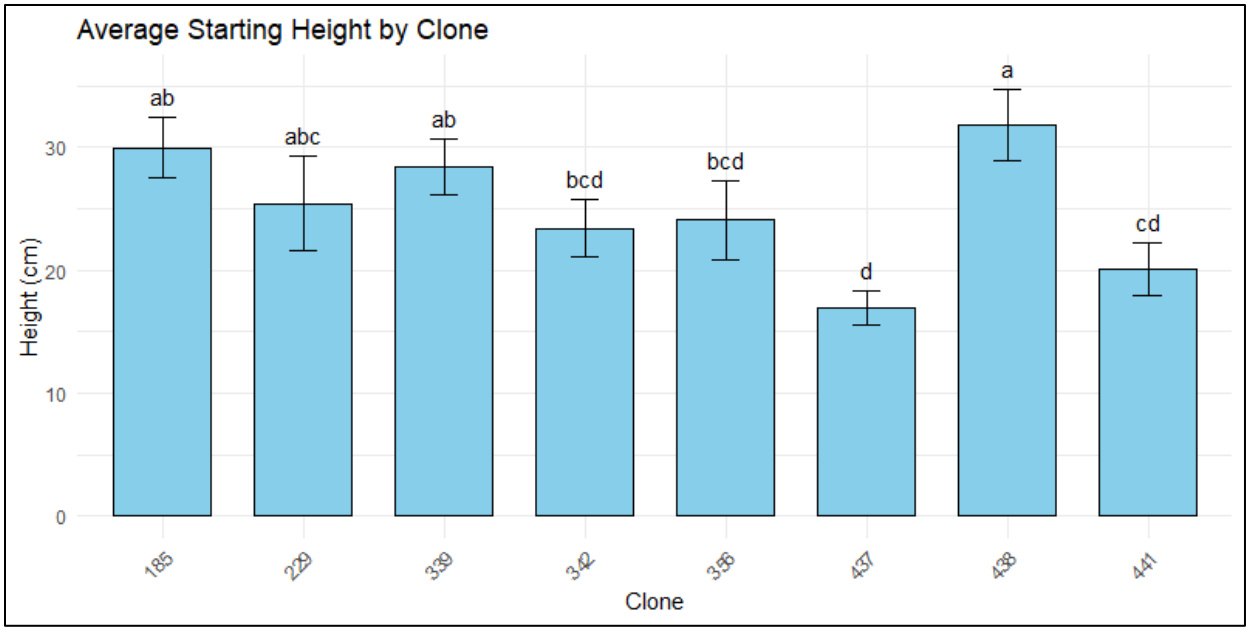


Figure 3.7 There were significant differences in starting heights between clones. α = 0.05 ($F_{7,135}$ = 3.583; P = 0.00144). Clone 438 had the highest value at 31.77cm while clone 437 had the lowest at 16.93cm.

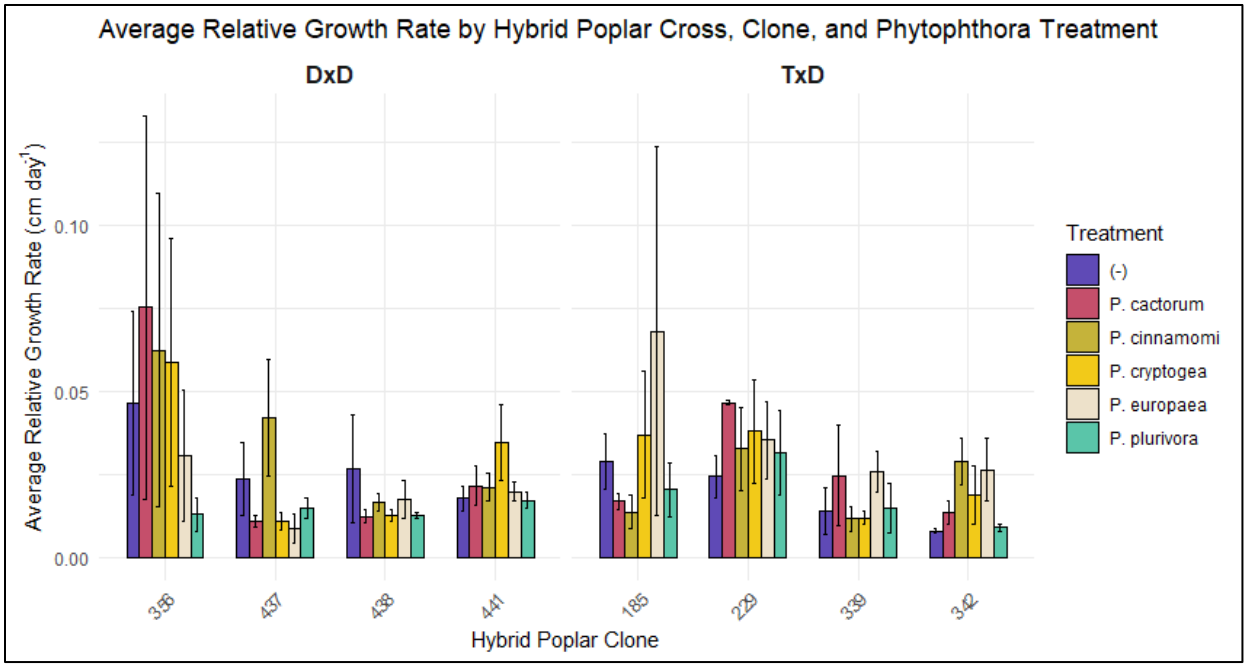


Figure 3.8 Average relative growth rate across timepoints by clone and treatment. Kruskal-Wallis test did not find significant differences between groups. $\alpha = 0.05$ [$H(5) = 5.2752$, $P = 0.3832$].

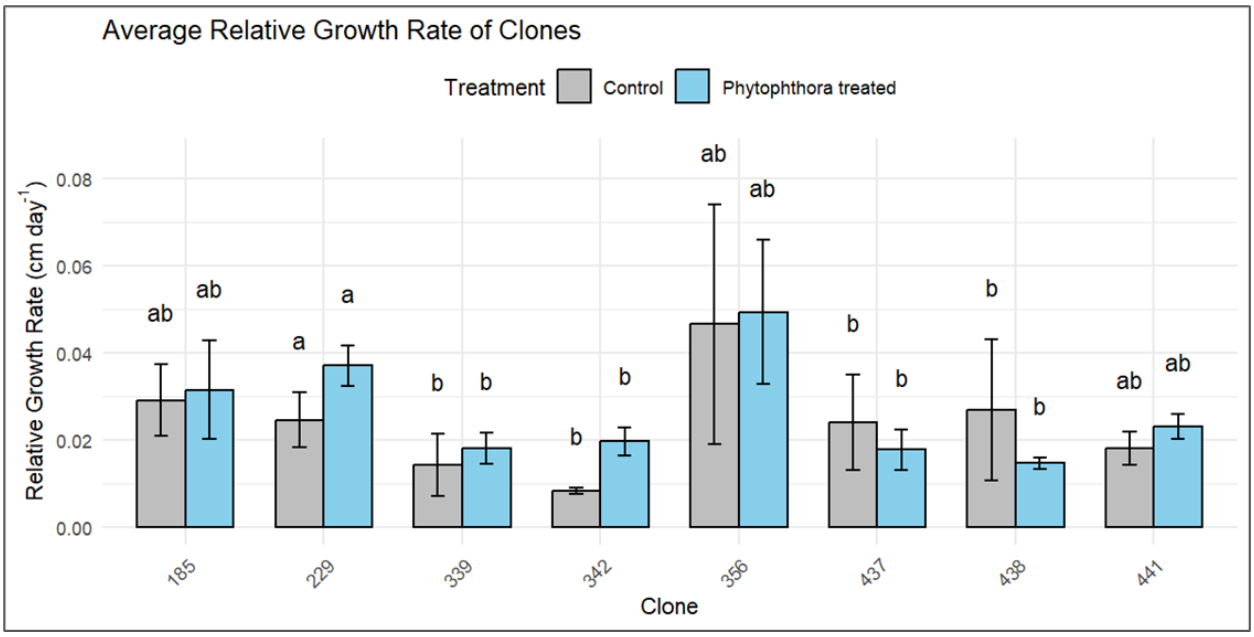


Figure 3.9 Average relative growth rate of clones. A Kruskal-wallis test found significant differences between clones. α = 0.05 [H(7) = 22.396, P = 0.00217].

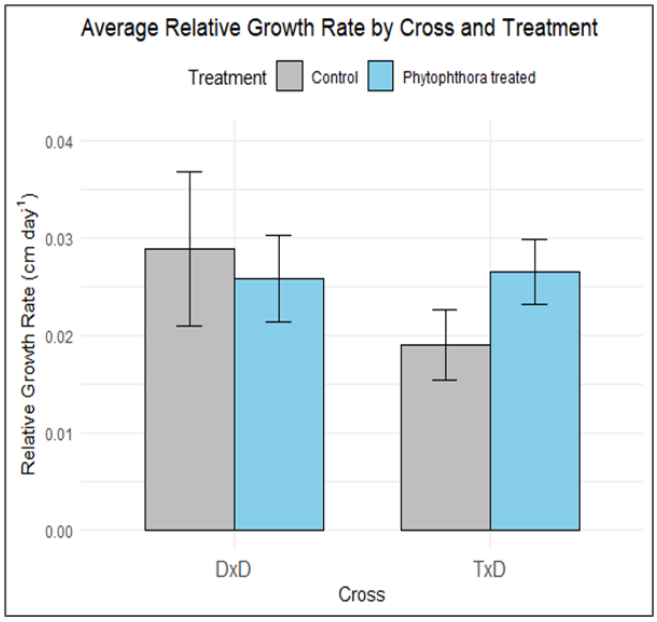


Figure 3.10 Average relative growth rate across timepoints by cross. Kruskal-Wallis test did not find significant differences between groups. $\alpha = 0.05$ [$H(1) = 0.33335$, $P = 0.5637$].

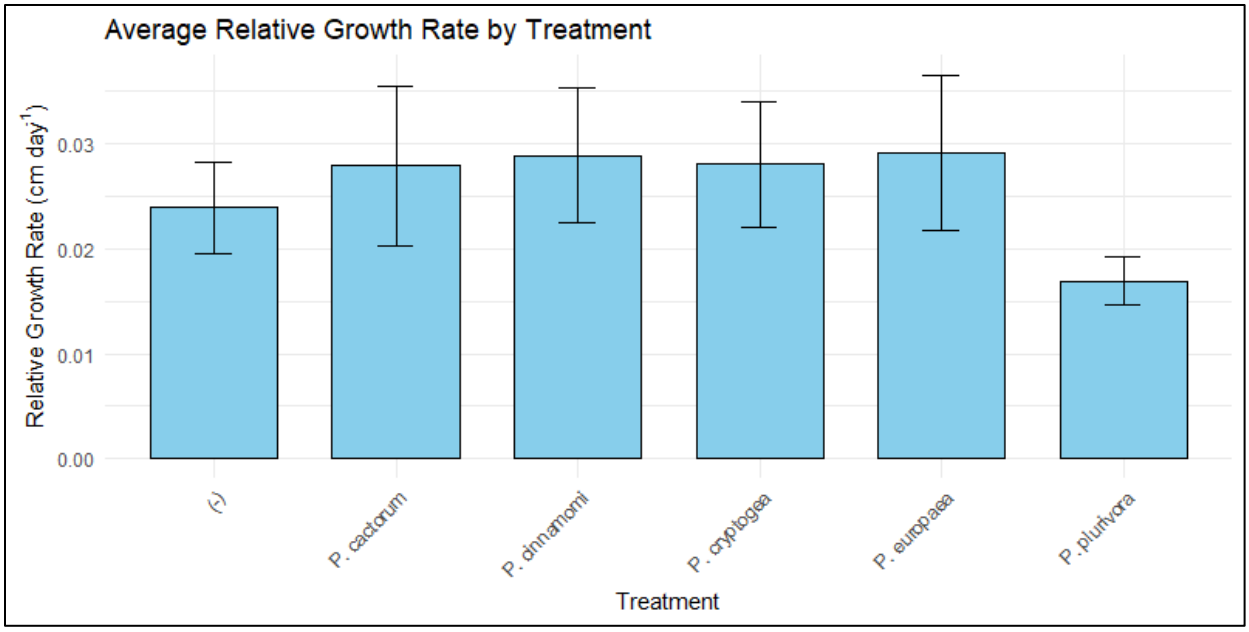


Figure 3.11 Average relative growth rate across timepoints by treatment. Kruskal-Wallis test did not find significant differences between groups. $\alpha = 0.05$ [$H(5) = 5.2752$, $P = 0.3832$].

Chapter 4

Conclusions and Future Opportunities

4.1 Summary and Future Goals

The *Phytophthora* survey revealed six species of *Phytophthora* isolated from symptomatic Fraser fir as well as at least one other oomycete species that has some reports of causing disease in conifers (Weber, Sulzer, & Haarhaus, 2004; Shafizadeh & Kavanagh, 2005). Overall, many of the species isolated were previously found in the region except for *Phytophthora plurivora*. This detection may be new or, more likely, may have previously been detected as *P. citricola*, a closely related species which has been in the region for at least 4 decades (Shew & Benson, 1981). The survey likely underrepresents the actual incidence of Phytophthora across the region, using a combination of detection methods would significantly improve monitoring for this disease. This information is nonetheless important as it provides updated data on the current diversity of species affecting Fraser fir cultivation and a collection of isolates which can be used for downstream projects. This study revealed how unknown the sources of planting material are. Future monitoring and survey projects should focus on identifying origins of planting material and monitoring of incoming out-of-state seedlings. Furthermore, extension and outreach efforts should encourage growers to keep detailed records of their planting material sourcing to aid researchers in identifying future disease introductions.

The phylogenetic analysis of *Phytophthora cinnamomi* isolates revealed varied genetic diversity within the population and weak bootstrap support across the target genes, limiting the ability to draw conclusions about the population structure and lineages of isolates but the results do seem to suggest that the population is structured and likely consistent with the hypothesis that there are few clonal lineages represented in the population. While a well-supported clade was

observed in the *Ef1a* and *Btub* trees, which included several isolates from Avery County, other isolates from the same county and seedling supplier were found outside of this grouping. This could be due to the introduction of multiple strains, but the available data is insufficient to confirm this hypothesis. Future work should focus on identifying DNA regions with higher variation that can be used to improve phylogenetic resolution. Additionally, disease monitoring efforts should incorporate detailed metadata collection, including seedling source and planting year, to aid tracking of pathogen spread and new introductions.

The Fraser fir inoculation experiment demonstrated that *Phytophthora cinnamomi* remains the most aggressive pathogen affecting Fraser fir in the Southern Appalachian region, as the positive control group exhibited the highest mortality and most rapid disease progression. While other *Phytophthora* species induced some disease symptoms and, in some cases, mortality, their effects were less severe compared to *P. cinnamomi*. No symptoms or mortality were observed in the negative control or non-*Phytophthora* treatment groups. However, re-isolation of the pathogens from inoculated plants is still necessary to fulfill Koch's postulates and confirm pathogenicity. This step is currently underway and will be essential for verifying the pathogenicity of the tested isolates.

The poplar inoculation experiment found no statistical differences in relative growth rate between any of the *Phytophthora* inoculation treatments and the negative control group. No disease symptoms were observed in any treatment, which was unexpected given previous studies suggesting that DxD clonal genotypes exhibit reduced growth rates when inoculated with *Phytophthora,* while TxD hybrid crosses are less affected. These results could suggest that all evaluated poplar clones were unaffected by *Phytophthora* inoculation, regardless of pathogen species. The likely explanation for this outcome is the experimental conditions—since plants

were grown from larger cuttings and in larger containers, they may have experienced reduced stress, making them less susceptible to opportunistic pathogens. Unfortunately, this experiment did not have a susceptible positive control which severely weakens any conclusions which might be made from it. Future work should focus on evaluating elite poplar genotypes in a *Phytophthora*-contaminated field site to determine their response under natural infection conditions.

The results of this work draw more questions than answers, but it creates several interesting opportunities for investigation of Phytophthora root rot. It provides a snapshot of the *phytophthora* species currently affecting Christmas tree growers, evaluates their relatedness and aggressiveness, and explores the potential use of alternative woody crops on contaminated land that can no longer be used for Fraser fir cultivation. The knowledge gained from these studies and the collection of isolates will be important in shaping future research and management of this destructive disease.

References

Abad, G. Z., & Abad, J. A. (2007). Another Look at the Origin of Late Blight of Potatoes, Tomatoes, and Pear Melon in the Andes of South America. *Plant Disease, 81*(6), 682-688. doi:https://doi.org/10.1094/PDIS.1997.81.6.682

Abad, G. Z., Burgess, T. I., Redford, A. J., Bienapfl, J. C., Srivastava, S., Matthew, R., & Jennings, K. (2023). IDphy: An International Online Resource for Molecular and Morphological Identification of Phytophthora. *Plant Disease, 107*(4), 987-1252. doi:https://doi.org/10.1094/PDIS-02-22-0448-FE

Abad, G. Z., Burgess, T., Bourret, T., Bensch, K., Cacciola, S., Scanu, B., . . . Redford, A. (2023). Phytophthora: taxonomic and phylogenetic revision of the genus. *Studies in Mycology, 106*(1), 259-348. doi:https://doi.org/10.3114/sim.2023.106.05

Agdia, Inc. (2024). ImmunoStrip® for Phytophthora. Retrieved from https://www.agdia.com

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular Biology, 215*(3), 403-410. doi:https://doi.org/10.1016/S0022-2836(05)80360-2

Azenta Life Sciences. (2024). GENEWIZ sanger Sequencing Service. Research Triangle Park, NC, USA. Retrieved from https://www.genewiz.com/en

Benson, D. M., & Grand, L. F. (2000). Incidence of Phytophthora root rot of Fraser fir in North. *Plant Disease, 84*(6), 661-664.

Blair, J. E., Coffey, M. D., Park, S., Geiser, D. M., & Kang, S. (2008). A multi-locus phylogeny for Phytophthora utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology, 45*(3), 266-277. doi:https://doi.org/10.1016/j.fgb.2007.10.010

Brasier, C. M. (2008). The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology, 57*(5), 792-808. doi: https://doi.org/10.1111/j.1365-3059.2008.01886.x

Busing, R., White, P. S., & MacKenzie, M. D. (1993). Gradient analysis of old spruce — fir forests of the Great Smoky Mountains circa 1935. *Canadian Journal of Botany, 71*, 951-958.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics, 10*, 421. doi:https://doi.org/10.1186/1471-2105-10-421

Carbone, I., White, J. B., Miadlikowska, J., Arnold, A. E., Miller, M. A., Magain, N., . . . Lutzoni, F. (2019). T-BAS version 2.1: Tree-Based Alignment Selector toolkit for evolutionary placement of DNA sequences and viewing alignments and specimen

metadata on curated and custom trees. *Microbiology Resource Announcements, 8*. doi:https://doi.org/10.1128/mra.00328-19

Cerny, M., Berka, M., Dvorak, M., Milenkovic, I., Saiz-Fernandez, I., Brzobohaty, B., & Durkovic, J. (2022). Defense mechanisms promoting tolerance to aggressive Phytophthora species in hybrid poplar. *Frontiers in Plant Science, 13*. doi: https://doi.org/10.3389/fpls.2022.1018272

Charif, D., & Lobry, J. R. (2007). SeqinR 1.0-2: A Contributed Package to the R Project for Statistical Computing Devoted to Biological Sequences Retrieval and Analysis. In U. Bastolla, M. Porto, H. Roman, & M. Vendruscolo, *Structural Approaches to Sequence Evolution. Biological and Medical Physics, Biomedical Engineering* (pp. 207-232). Berlin: Springer. doi:https://doi.org/10.1007/978-3-540-35306-5_10

Chastagner, G., & Benson, D. (2000). The Christmas tree: Traditions, production, and. *Plant Health Progress, 1*(1). doi:https://doi.org/10.1094/PHP-2000-1013-01-RV

Choudhuri, S. (2014). Chapter 6 - Sequence Alignment and Similarity Searching in Genomic Databases: BLAST and FASTA. In S. Choudhuri, *Bioinformatics for Beginners* (pp. 133-155). Academic Press. doi:https://doi.org/10.1016/B978-0-12-410471-6.00006-2.

Churco, J. M. (1938). CHRISTMAS AND THE ROMAN SATURNALIA. *The Classical Outlook, 16*(3), 25-26.

Coomber , A., Saville, A., Carbone, I., & Ristaino, J. B. (2023). An open-access T-BAS phylogeny for emerging Phytophthora species. PLOS ONE, 18(4), e0283540

Contreras-Cornejo, H. A., Larsen, J., Fernandez-Pavia, S. P., & Oyama, K. (2023). Climate change, a booster of disease outbreaks by the plant pathogen Phytophthora in oak forests. *Rhizosphere, 27*. doi:https://doi.org/10.1016/j.rhisph.2023.100719

Darriba, D., Toboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods, 9*(8), 772. doi:https://doi.org/10.1038/nmeth.2109

Delcourt, H., & Delcourt, P. (1984). Late-Quaternary history of the spruce–fir ecosystem in the Southern Appalachian region. In H. Delcourt, P. Delcourt, & P. White (Ed.), *The Southern Appalachian spruce–fir ecosystem: its biology and threats* (pp. 22-35). Atlanta, GA: U.S. Department of the Interior.

Dhingra, O. D., & Sinclair, J. B. (1995). Sterilization of Apparatus and Culture Media. In *Basic Plant Pathology Methods* (pp. 1-9). CRC Press.

Dyke, H. V. (1897). *The First Christmas Tree.*

Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics, 5*, 113. doi:https://doi.org/10.1186/1471-2105-5-113

Engelbrecht, J., Duong, T. A., & Berg, N. V. (2017). New microsatellite markers for population studies of Phytophthora cinnamomi, an important global pathogen. *Scientific Reports, 7*(1), 17631. doi:https://doi.org/10.1038/s41598-017-17799-9

Eskalen, A., Bender, G., Faber, B., & Rios, S. (2017). Phytophthora Root Rot of Avocado. *From the Grove, 7*(2), 31-32.

Fonseca, T. F., Abreu, C. G., & Parresol, B. R. (2004). Soil compaction and chestnut ink disease. *Forest Pathology, 34*(4), 273-283. doi: https://doi.org/10.1111/j.1439-0329.2004.00371.x

Fox, J., & Weisburg, S. (2019). *An R Companion to Applied Regression* (third ed.). Sage. doi:https://doi.org/10.32614/CRAN.package.car

Frampton, J., & Benson, D. M. (2012). Seedling resistance to Phytophthora cinnamomi. *Annals of Forest Science, 69*, 805-812. doi:10.1007/s13595-012-0205-4

Frampton, J., & Benson, D. M. (2012). Seedling resistance to Phytophthora cinnamomi in the genus Abies. *Annals of Forest Science, 69*, 805-812.

Fry, W. E., & Grünwald, N. J. (2010). Introduction to Oomycetes. *Plant Health Instructor, 10*. doi:10.1094/PHI-I-2010-1207-01

Ghezehei, S. B., Ewald, A. L., Hazel, D. W., Zalesny, R. S., & Nichols, E. G. (2021). Productivity and Profitability of Poplars on Fertile and Fertilization Treatments. *Forests, 12*(7), 869. doi:https://doi.org/10.3390/f12070869

Ghezehei, S. B., Nichols, E. G., & Hazel, D. W. (2016). Early Clonal Survival and Growth of Poplars Grown on North Carolina Piedmont and Mountain Marginal Lands. *Bioenergy Res., 9*, 548-558. doi:https://doi.org/10.1007/s12155-015-9707-x

Ghezehei, S. B., Nichols, E. G., & Hazel, D. W. (2020). Productivity and cost-effectiveness of short-rotation hardwoods on various land types in the southeastern USA. *Int. J. Phytoremediation, 22*, 98-110. doi:https://doi.org/10.1080/15226514.2019.1647404

Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology, 52*, 696-701. doi:https://doi.org/10.1080/10635150390235520

Hardham, A. R. (2017). Phytophthora cinnamomi. *Molecular Plant Pathology, 19*(2), 260-285. doi:https://doi.org/10.1111/mpp.12568

Hibbert-Frey, H., Frampton, J., Blazich, F. A., & Hinesley, L. E. (2010). Grafting Fraser Fir (Abies fraseri): Effect of Grafting Date, Shade, and Irrigation. *HortScience, 45*(4), 617-620. doi:https://doi.org/10.21273/HORTSCI.45.4.617

Ho, H. H. (2018). The taxonomy and biology of Phytophthora and Pythium. *Journal of Bacteriology & Mycology, 6*(1), 40-45. doi:DOI: 10.15406/jbmoa.2018.06.00174

Holmes, K. A., & Benson, D. M. (1994). Evaluation of Phytophthora parasitica var. nicotianae for Biocontrol of Phytophthora parasitica on Catharanthus roseus. *Plant Disease, 78*(2), 193-199.

Hunt, R. (1982). *Plant Growth Curves: The Functional Approach to Plant Growth Analysis.* London: Edward Arnold.

Jeffers, S. N., & Martin, S. B. (1986). Comparison of Two Media Selective for Phytophthora and Pythium Species. *Plant Disease, 70*(11), 1038-1043. doi:10.1094/PD-70-1038.

Jung, T., & Burgess, T. I. (2009). Re-evaluation of Phytophthora citricola isolates from multiple woody hosts in Europe and North America reveals a new species, Phytophthora plurivora sp. nov. *Persoonia, 22*, 95-110. doi:https://doi.org/10.3767/003158509X442612

Jung, T., Colquhoun, I. J., & Hardy, G. E. (2013). New insights into the survival strategy of the invasive soilborne pathogen Phytophthora cinnamomi in different natural ecosystems in Western Australia. *Forest Pathology, 43*(4), 266-288. doi:https://doi.org/10.1111/efp.12025

Kohlway, W. H., Cothron, C. P., & Whitehill, J. G. (2019). Management of Phytophthora Root Rot in Fraser Fir Christmas Trees. *Christmas Tree Notes*. Retrieved from https://content.ces.ncsu.edu/management-of-phytophthora-root-rot-in-fraser-fir-christmas-trees#:~:text=Use%20Subdue%20MAXX%20fungicide%20in,another%20fungicide%20such%20as%20Aliette.

Kohlway, W. H., Whetten, R. W., Benson, D. M., & Frampton, J. M. (2017). Response of Turkish and Trojan fir to Phytophthora cinnamomi and P. cryptogea. *Scandinavian Journal of Forest Research, 32*(5), 406-411. doi:https://doi.org/10.1080/02827581.2017.1280076

Kroon, L., Bakker, F. T., Van Den Bosch, G. B., Bonants, P. J., & Flier, W. G. (2004). Phylogenetic analysis of Phytophthora species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology, 41*(8), 766-782. doi:https://doi.org/10.1016/j.fgb.2004.03.007

Kuhlman, E. G., & Hendrix, F. F. (1963). Phytophthora root rot of Fraser Fir. *Plant Disease Reporter, 47*(6), 552-553.

Levene, H. (1960). Robust Tests for Equality of Variances. In I. Olkin, *Contributions to Probability and Statistics* (pp. 278-292). Palo Alto: Stanford University Press.

McKeever, K. M., & Chastagner, G. A. (2016). A survey of Phytophthora spp. associated with. *Plant Disease, 100*(6), 1161-1169. doi:https://doi.org/10.1094/PDIS-08-15-0939-RE

McKinley, C., Hazel, D., & Kohlway, W. (2018). Christmas Tree Species. *Christmas Tree Notes*. Retrieved from https://content.ces.ncsu.edu/christmas-tree-species

McKinley, C., Hazel, D., & Kohlway, W. (2019). Christmas Tree Species. *Christmas Tree Notes*. Retrieved from https://content.ces.ncsu.edu/christmas-tree-species

Milenković, I., Keča, N., Karadžić, D., Z. R., Nowakowska, J. A., Oszako, T., . . . Jung, T. (2018). Isolation and Pathogenicity of Phytophthora Species from Poplar Plantations in Serbia. *Forests, 9*(6), 330. doi:https://doi.org/10.3390/f9060330

Mistretta, P. A. (1984). Littleleaf Disease. *Forest Insect & Disease Leaflet, 20*.

Nguyen, L.-T., Schmidt, H. A., von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular Biology and Evolution, 32*(1), 268-274. doi:https://doi.org/10.1093/molbev/msu300

Ogle, D. H., Doll, J. C., Wheeler, A. P., & Dinno, A. (2025). FSA: Simple Fisheries Stock Assessment Methods. *R package version 0.9.6*. Retrieved from https://CRAN.R-project.org/package=FSA

Otloh of Sankt Emmeram. (11th cent.). *Life of Saint Boniface.*

Owen, J. (2011). Selecting the Right Tree. Retrieved 12 10, 2024, from https://christmastrees.ces.ncsu.edu/christmastrees-selecting-the-right-tree/

Owen, J. (2016). North Carolina Christmas Trees by the Numbers. Retrieved 12 10, 2024, from https://christmastrees.ces.ncsu.edu/christmastrees-nc-christmas-trees-by-the-numbers/

Paradis, E. (2010). An R package for population genetics with an integrated-modular approach. *Bioinformatics, 26*(3), 419-420. doi:https://doi.org/10.1093/bioinformatics/btp696

Paradis, E., & Schliep, K. (2019). ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics, 35*(3), 526-528. doi:https://doi.org/10.1093/bioinformatics/bty633

Perry, J. (2010). In J. Perry, *Christmas in Germany: A Cultural History* (p. 32). University of North Carolina Press.

Pettersson, M., Frampton, J., & Sidebottom, J. (2017). Influence of Phytophthora Root Rot on Planting Trends of Fraser Fir Christmas Trees in the Southern Appalachian Mountains. *Tree Planters Notes*, 4-11.

Pettersson, M., Frampton, J., Ronnberg, J., Ronnberg, J., Shew, H. D., Benson, D. M., . . . Cubeta, M. A. (2017). Increased diversity of Phytophthora species. *Scandinavian Journal of Forest Research*, *32*(5), 412-420. doi:https://doi.org/10.1080/02827581.2016.1265144

Qiagen. (2024). DNeasy Plant Pro DNA extraction kit handbook.

QIAGEN. (2024). DNeasy Plant Pro Kit. Retrieved from https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-plant-pro-kits

Qiagen Digital Insights. (2023). CLC Main Workbench (Version 23.0.4). QIAGEN. Retrieved from https://digitalinsights.qiagen.com/

Rhoades, C. C., Brosi, S. L., Dattilo, A. J., & Vincelli, P. (2003). Effect of soil compaction and moisture on incidence of phytophthora root rot on American chestnut (Castanea dentata) seedlings. *Forest Ecology and Management*, *184*(1-3), 47-54. doi:https://doi.org/10.1016/S0378-1127(03)00147-6

Rizzo, D. M., & Garbelotto, M. (2003). Sudden oak death: endangering California and Oregon forest ecosystems. *Frontiers in Ecology and the Environment*, *1*(4), 197-204. doi:https://doi.org/10.1890/1540-9295(2003)001[0197:SODECA]2.0.CO;2

Sanchez-Cuesta, R., Gonzalez-Moreno, P., Cortez-Marquez, A., Navarro-Cerrillo, R. M., & Ruiz-Gomez, F. J. (2022). Soil distribution of Phytophthora cinnamomi inoculum in oak afforestation depends on site characteristics rather than host availability. *New Forests*, *54*, 1037-1059. doi:https://doi.org/10.1007/s11056-022-09951-9

Schafer, J. L., Harding, T., & Tusell, F. (2023, 09 02). Package 'cat' version 0.0-9. *Analysis and Imputation of Categorical-Variable Datasets with Missing Values*.

Schena, L., Hughes, K. J., & Cooke, D. E. (2006). Detection and quantification of Phytophthora ramorum, P. kernoviae, P. citricola and P. quercina in symptomatic leaves by multiplex real-time PCR. *Molecular Plant Pathology*, *7*(5), 365-379. doi: https://doi.org/10.1111/j.1364-3703.2006.00345.x

Shafizadeh, S., & Kavanagh, J. A. (2005). Pathogenicity of Phytophthora species and Pythium undulatum isolated from Abies procera Christmas trees in Ireland. *Forest Pathology*, *35*(6), 444-450. doi:https://doi.org/10.1111/j.1439-0329.2005.00430.x

Shakya, S. K., Grunwald, N. J., Fieland, V. J., Knaus, B. J., Weiland, J. E., Maia, C., . . . Jung, T. (2021). Phylogeography of the wide-host range panglobal plantpathogen Phytophthora

cinnamomi. *Molecular Biology, 30*(20), 5164-5178. doi:https://doi.org/10.1111/mec.16109

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika, 52*(3-4), 591-611. doi:https://doi.org/10.1093/biomet/52.3-4.591

Shew, H. D., & Benson, D. M. (1981). Fraser Fir Root Rot Induced by Phytophthora citricola. *Plant Disease, 65*(8), 688-689.

Sidebottom, J., & Bookewalter, J. (2023). Minor Pests of Fraser Fir Christmas Trees. *Christmas Tree Notes*. Retrieved from https://content.ces.ncsu.edu/minor-pests-of-fraser-fir-christmas-trees#:~:text=Most%20growers%20of%20Fraser%20fir,aphids%20and%20balsam%20woolly%20adelgids.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics, 22*, 2688-2690. doi:https://doi.org/10.1093/bioinformatics/btl446

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution, 38*(7), 3022-3027. doi:https://doi.org/10.1093/molbev/msab120

Thermo Fisher Scientific. (2024). Phire Plant Direct PCR Kit. Retrieved from https://www.thermofisher.com/order/catalog/product/F130WH

Turkensteen, L. J., Flier, W. G., Wanningen, R., & Mulder, A. (2008). Production, survival and infectivity of oospores of Phytophthora infestans. *Plant Pathology, 49*(6), 688-696. doi:https://doi.org/10.1046/j.1365-3059.2000.00515.x

USDA National Agricultural Statistics Service. (2022). *2022 Census of Agriculture.* United States Department of Agriculture. Retrieved from https://www.nass.usda.gov/Publications/AgCensus/2022/Full_Report/Volume_1,_Chapter_2_US_State_Level/

Uzuhasi, S., Tojo, M., & Kakishima, M. (2010). Phylogeny of the Genus Pythium and description of new genera. *Mycoscience, 51*(5), 337-365. doi:https://doi.org/10.1007/S10267-010-0046-7

Weber, R. W., Sulzer, F., & Haarhaus, M. (2004). Pythium undulatum, cause of root rot of Abies procera Christmas trees and Pseudotsuga menziesii in Northern Germany. *Mycological Progress, 3*, 179-188.

Werner, E. E. (2006). In Pursuit of Liberty: Coming of Age in the American Revolution. Greenwood Publishing Group.

White, T. J., Bruns, T. D., Lee, S. B., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White, *PCR Protocols: a guide to methods and applica-* (pp. 315-322). San Diego, California: Academic Press.

Winkworth, R. C., Neal, G., Ogas, R. A., Nelson, B. C., McLenachan, P. A., Bellegard, S. E., & Lockhart, P. J. (2022). Comparative Analyses of Complete Peronosporaceae (Oomycota) Mitogenome Sequences—Insights into Structural Evolution and Phylogeny. *Genome Biology and Evolution, 14*(4). doi:https://doi.org/10.1093/gbe/evac049

Yachdav, G., Wilzback, S., Rauscher, B., Sheridan, R., Sillitoe, I., Procter, J., . . . Goldberg, T. (2016). MSAViewer: interactive JavaScript visualization of multiple sequence alignments. *Bioinformatics, 32*(22), 3501-3503. doi:https://doi.org/10.1093/bioinformatics/btw474

Yang, X., Tyler, B. M., & Hong, C. (2017). An expanded phylogeny for the genus Phytophthora. *IMA Fungus, 8*, 355-384. doi:https://doi.org/10.5598/imafungus.2017.08.02.09

APPENDICES

Appendix A supplemental material for chapter 2

Table A.1 BLAST search results for five gene loci (ITS, β-tubulin, Cox1, Ypt1, and Ef1a) from *Phytophthora* isolates. For each isolate, the closest matching species, along with the corresponding GenBank accession number, is listed for each gene. The final column provides the consensus species identity based on the best match across all loci.

Sample	ITS	accession	βtub	accession	Cox1 species	accession	Ypt1	accession	Ef1α	accession	Consensus Identity
23ss04	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al05A1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al05B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al09B1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al10B1B	*P. cinnamomi*	MN755747.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Al11B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cryptogea*	AY659574.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al11C1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al12A1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al12B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al13B1C	*P. cinnamomi*	MT355399.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al14A1A	*P. cinnamomi*	MT355399.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Al14B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al14C1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al15A1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cryptogea*	PQ655243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Al15B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*

Table A.1 (continued)

As02B1A	*P. cinnamomi*	OM734765.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
As03C1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
As04A1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
As04B1A	*P. cinnamomi*	MN497236.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
As05B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
As05C1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
As06A1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
As07C1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
As08C1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
As13A1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
As13C1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
As17C1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
As18C1C	*P. pluvivora*	MG561734.1	*P. pluvivora*	KX250832.1	*P. pluvivora*	NC_063811.1	*P. plurivora*	OR620395.1	*P. pluvivora*	KX250826.1	*P. pluvivora*
As20B1A	*P. europaea*	OM984724.1	*P. europaea*	ON008132.1	*P. abietivora*	MK164270.1	*P. europaea*	MH988442.1	*P. abietivora*	MK507885.1	*P. europaea*
As20C1A	*P. europaea*	OM984724.1	*P. europaea*	ON008132.1	*P. abietivora*	MK164270.1	*P. europaea*	MH988442.1	*P. abietivora*	MK507885.1	*P. europaea*
As22B1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
As23B1A	*P. cryptogea*	PP783498.1	*P. cryptogea*	HQ455655.1	*P. cryptogea*	AY659562.1	*P. pseudocryptogea*	MK058424.1	*P. kelmanii*	KX251988.1	*P. cryptogea*
As23B1C	*P. cryptogea*	PP783498.1	*P. cryptogea*	HQ455655.1	*P. cryptogea*	AY659574.1	*P. pseudocryptogea*	MK058424.1	*P. kelmanii*	KX251988.1	*P. cryptogea*

Table A.1 (continued)

Av02E1C	*P. cinnamomi*	OM734765.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Av04B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Av04E1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Av05E1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Av08B1C	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Av09E1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Av11E1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
ch2	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
ch3	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
cl3a	*P. cactorum*	EU045775.1	*P. cactorum*	LC595851.1	*P. cactorum*	OK555490.1	*P. cactorum*	MH975000.1	*P. hedraiandra*	KX250392.1	*P. cactorum*
ctf_gh1	*P. cactorum*	EU662221.1	*P. cactorum*	LC595851.1	*P. cactorum*	OK555490.1	*P. cactorum*	MH975000.1	*P. hedraiandra*	KX250392.1	*P. cactorum*
Gr01C1A	*P. citricola*	MT647267.1	*P. citricola*	EU079526.1	*P. caryae*	MW927567.1	*P. pluriv ora*	OR620395.1	*P. curvata*	OR594728.1	*P. citricola*
Gr02B1A	*P. cinnamomi*	OM734765.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Gr02C1B	*P. cinnamomi*	OM734765.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Gr03A1C	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Gr03B1C	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Gr03C1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Gr05B1A	*P. citricola*	MT647267.1	*P. citricola*	EU079526.1	*P. caryae*	MW927567.1	*P. acerina*	MH209241.1	*P. curvata*	OR594728.1	*P. citricola*

Table A.1 (continued)

Ja01A3A	*P. cinnamomi*	OR577325.0	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Ja01B1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Ja02B3A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Ja03C2A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Ja04A1A	*P. cryptogea*	KP070721.1	*P. cryptogea*	HQ455655.1	*P. cryptogea*	AY659574.1	*P. pseudocryptogea*	MK058424.1	*P. kelmanii*	KX251988.1	*P. cryptogea*
Ma01A1Ac	*P. cryptogea*	AB366735.1	*P. cryptogea*	HQ455654.1	*P. cryptogea*	AY659566.1	*P. kelmanii*	LC596340.1	*P. kelmanii*	KX251988.1	*P. cryptogea*
Ma01A1Ak	*P. cryptogea*	MK386566.1	*P. cryptogea*	HQ455654.1	*P. cryptogea*	MW927571.1	*P. kelmanii*	LC596340.1	*P. kelmanii*	KX251988.1	*P. cryptogea*
Md01B1A	*P. cinnamomi*	MN497236.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Mi01E1C	*P. europaea*	OM984724.1	*P. europaea*	ON008132.1	*P. abietivora*	MK164270.1	*P. europaea*	MH988442.1	*P. abietivora*	MK507885.1	*P. europaea*
Mi02A1E	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Mi02C1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Mi02D1B	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Mi02E1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Mi03C1A	*P. cryptogea*	KU942412.1	*P. cryptogea*	HQ455654.1	*P. cryptogea*	AY659566.1	*P. kelmanii*	LC596340.1	*P. kelmanii*	KX251988.1	*P. cryptogea*
Mi03E1B	*P. cryptogea*	KP070721.1	*P. cryptogea*	HQ455655.1	*P. cryptogea*	AY659574.1	*P. pseudocryptogea*	MK058424.1	*P. kelmanii*	KX251988.1	*P. cryptogea*
Tr01A2A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067938810.1	*P. cinnamomi*
Tr01E1A	*P. cinnamomi*	OR577325.1	*P. cinnamomi*	EU079770.1	*P. cinnamomi*	PP108243.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*
Wa08B1A	*P. cryptogea*	KP070721.1	*P. cryptogea*	HQ455655.1	*P. cryptogea*	AY659562.1	*P. pseudocryptogea*	MK058424.1	*P. kelmanii*	KX251988.1	*P. cryptogea*

Table A.1 (continued)

Wa09B1C	*P. cinnamomi*	OM734765.1	*P. cinnamomi*	KX251805.1	*P. cinnamomi*	PP108235.1	*P. cinnamomi*	MH975009.1	*P. cinnamomi*	XM_067931756.1	*P. cinnamomi*

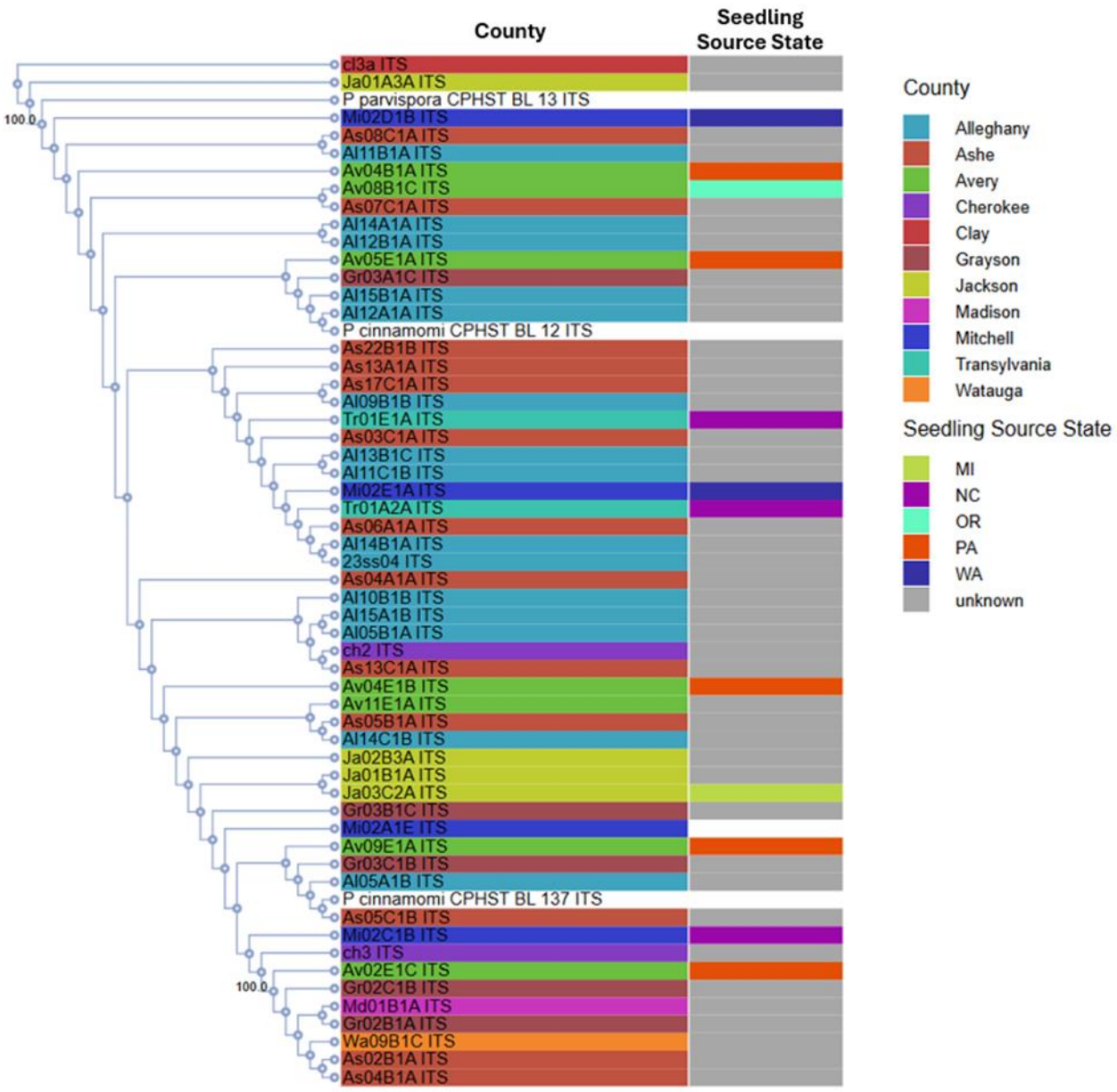


Figure A.1 Maximum-Likelihood tree built from aligned *ITS* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-types of *P. cinnamomi* and *P. parvispora* (a closely related species) were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state.

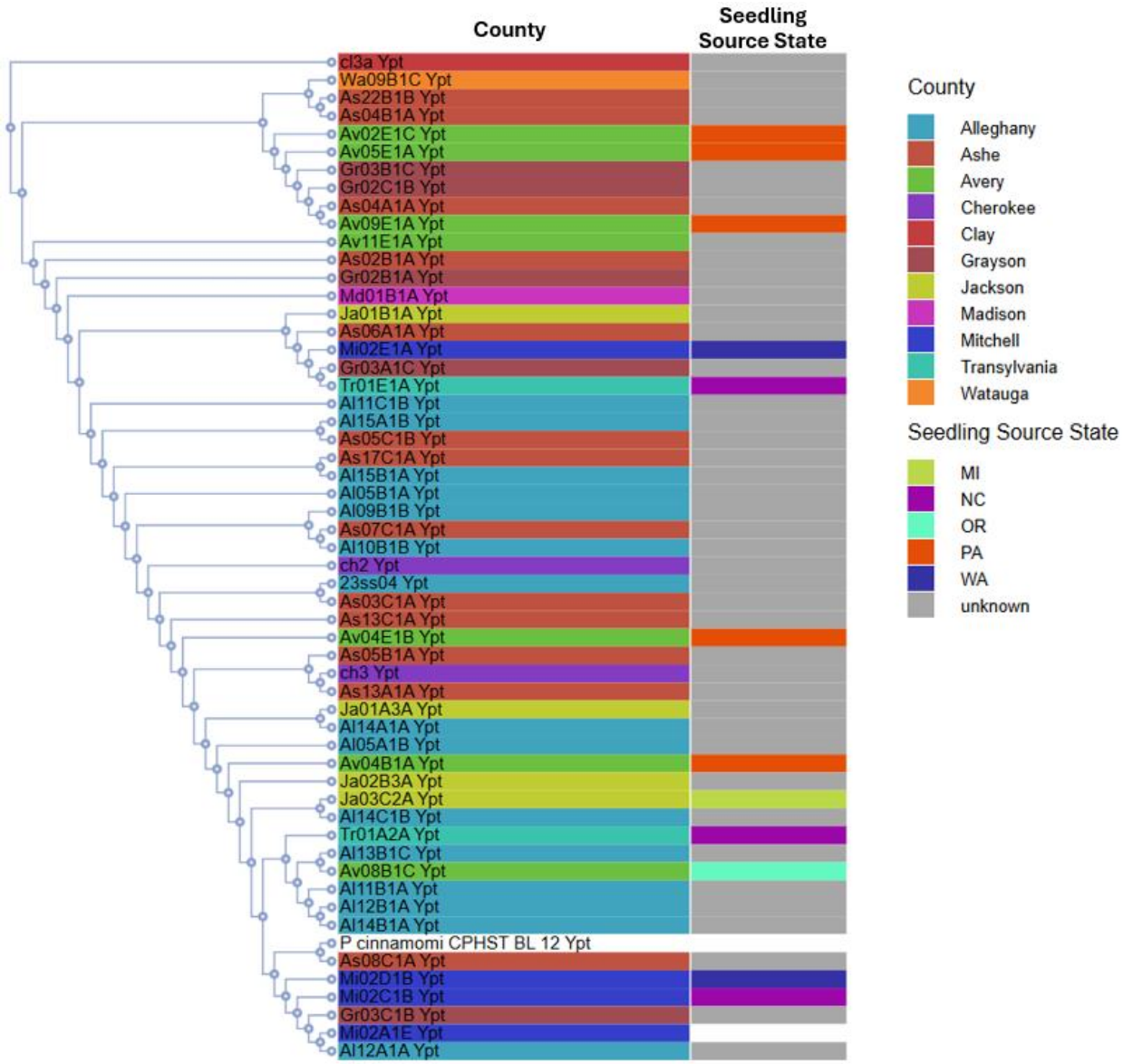


Figure A.2 Maximum-Likelihood tree built from aligned *Ypt1* sequences from *P. cinnamomi* isolates using RAxML (Stamatakis, 2006) and visualized using T-BAS v2.3 (Carbone, et al., 2019). Sequences from an ex-type of *P. cinnamomi* were included in the phylogeny (Abad G. Z., et al., 2023). Colored bands were added to display metadata, including county of collection and seedling source state.

Figure A.3 R script for performing chi-square test and combining duplicate experiment data for Fir inoculation.

```
  # Filter data for Species = "FF"
df_filtered <- df %>% filter(Species == "FF")

# Define the correct order of treatments
treatment_order <- c("Negative Control",
                     "Positive Control (P. cinnamomi)",
                     "P. cactorum",
                     "P. cryptogea-1",
                     "P. cryptogea-2",
                     "P. europaea",
                     "P. plurivora",
                     "E. undulatum",
                     "G. macrosporum")

# Initialize a dataframe to store chi-square test results
chi_square_results <- data.frame(Treatment = character(),
                                 Timepoint = character(),
                                 P_Value = numeric(),
                                 Significant = logical(),
                                 stringsAsFactors = FALSE)

# Perform chi-square tests for each treatment and timepoint
for (treatment in unique(df_filtered$Treatment)) {
  for (timepoint in paste0("w", seq(2, 16, by = 2))) {

    # Subset data for this treatment and timepoint
    temp <- df_filtered %>% filter(Treatment == treatment) %>%
select(Replicate, !!sym(timepoint))

    # Create a contingency table of symptom counts by replicate
    contingency_table <- table(temp$Replicate, temp[[timepoint]])

    # Ensure there are at least two rows and two columns to perform chi-
square test
    if (nrow(contingency_table) > 1 && ncol(contingency_table) > 1) {
      test <- chisq.test(contingency_table)
      chi_square_results <- rbind(chi_square_results,
                                  data.frame(Treatment = treatment,
                                             Timepoint = timepoint,
                                             P_Value = test$p.value,
                                             Significant = test$p.value <
0.05))
    }
  }
}

# Print chi-square test summary
print("Summary of Chi-Square Tests:")
print(chi_square_results)

# Check for significant differences
if (any(chi_square_results$Significant, na.rm = TRUE)) {
  print("Significant differences found between replicates. Analyze replicates
separately.")
```

```
} else {
  print("No significant differences found. Combining replicates for
analysis.")

  # Combine replicates
  df_combined <- df_filtered %>%
    select(-Replicate, -Species) %>%
    pivot_longer(cols = starts_with("w"), names_to = "Timepoint", values_to =
"Symptom") %>%
    group_by(Treatment, Timepoint, Symptom) %>%
    summarise(Count = n(), .groups = "drop") %>%
    mutate(Timepoint = factor(Timepoint, levels = paste0("w", seq(2, 16, by =
2))))  # Ensure timepoints are ordered
```

Figure A.4 R script for calculating haplotype diversity, nucleotide diversity, and Tajima's D for *P. cinnamomi* alignments.

```
# Get a list of all FASTA files in the directory
fasta_files <- list.files(alignment_dir, pattern = "\\.fas$", full.names =
TRUE)

# Open a results file
output_file <- file.path(alignment_dir, "Population_Genetics_Results.txt")
sink(output_file)

cat("Population Genetics Analysis\n")
cat("=====================================\n\n")

# Loop through each FASTA file and analyze
for (file in fasta_files) {

  # Extract gene name from file name
  gene_name <- tools::file_path_sans_ext(basename(file))
  cat("Analyzing:", gene_name, "\n")

  # Load alignment
  alignment <- read.dna(file, format = "fasta")

  # Compute summary statistics
  num_sequences <- length(alignment)
  alignment_length <- ncol(alignment)
  cat("Number of sequences:", num_sequences, "\n")
  cat("Alignment length:", alignment_length, "bp\n")

  # Compute haplotype diversity
  haps <- haplotype(alignment)
  hap_div <- hap.div(alignment)
  cat("Haplotype diversity:", hap_div, "\n")

  # Compute nucleotide diversity (π)
  pi_value <- nuc.div(alignment)
  cat("Nucleotide diversity (π):", pi_value, "\n\n")

  # Save results in output file
  cat(gene_name, "\t", num_sequences, "\t", alignment_length, "\t", hap_div,
"\t", pi_value, "\n", file = output_file, append = TRUE, sep = "")

}

# Close output file
sink()

cat("Analysis complete! Results saved in
'Population_Genetics_Results.txt'.\n")

# Define which locus is mitochondrial
mito_loci <- c("CoxCinnOnly")

# Open results file
output_file <- file.path(alignment_dir, "Neutrality_Test_Results.txt")
sink(output_file)
```

```
cat("Neutrality Test Results (Tajima's D)\n")
cat("========================================\n\n")

# Loop through each file
for (file in fasta_files) {

  # Extract gene name from file name
  gene_name <- tools::file_path_sans_ext(basename(file))
  cat("Analyzing:", gene_name, "\n")

  # Load alignment
  alignment <- read.dna(file, format = "fasta")

  # Compute Tajima’s D
  tajima_result <- tajima.test(alignment)

  # Interpret results differently for mitochondrial vs nuclear loci
  if (gene_name %in% mito_loci) {
    cat("(Mitochondrial locus)\n")
    cat("Tajima's D:", tajima_result$D, "\n")
    if (tajima_result$D < -2) {
      cat("Possible purifying selection or population expansion.\n")
    } else if (tajima_result$D > 2) {
      cat("Possible balancing selection or population bottleneck.\n")
    } else {
      cat("Neutral evolution likely.\n")
    }
  } else {
    cat("(Nuclear locus)\n")
    cat("Tajima's D:", tajima_result$D, "\n")
    if (tajima_result$D < -2) {
      cat("Strong purifying selection or recent population expansion.\n")
    } else if (tajima_result$D > 2) {
      cat("Balancing selection or structured populations.\n")
    } else {
      cat("Likely neutral evolution.\n")
    }
  }

  cat("\n")

  # Save results in output file
  cat(gene_name, "\t", tajima_result$D, "\n", file = output_file, append =
TRUE, sep = "")

}

# Close output file
sink()

cat("Tajima’s D analysis complete! Results saved in
'Neutrality_Test_Results.txt'.\n")
```

Appendix B supplemental material for chapter 3

Figure B.1 R script for ANOVA of start heights of treatment groups, clones, and crosses.

```
# Load necessary libraries
library(tidyverse)
library(ggplot2)
library(agricolae)  # For LSD test
library(car)        # For Levene's test

# Load the data
data <- read.csv("WorkingPoplar.csv")

# Perform the ANOVA
anova_result <- aov(w0 ~ Treatment, data = data)

# Extract residuals for normality checks
residuals <- residuals(anova_result)

# Check normality using Shapiro-Wilk test
shapiro_test <- shapiro.test(residuals)
cat("\nShapiro-Wilk Normality Test:\n")
print(shapiro_test)

# Check homogeneity of variances (Levene's Test)
levene_test <- leveneTest(w0 ~ Treatment, data = data)
cat("\nLevene's Test for Homogeneity of Variance:\n")
print(levene_test)

# Plot histogram of residuals
ggplot(data.frame(residuals), aes(x = residuals)) +
  geom_histogram(binwidth = 0.5, fill = "skyblue", color = "black") +
  labs(title = "Histogram of Residuals", x = "Residuals", y = "Frequency")

# Q-Q plot for normality
qqnorm(residuals)
qqline(residuals, col = "red")

# Display the ANOVA summary
summary(anova_result)

# Extract p-value
p_value <- summary(anova_result)[[1]]["Treatment", "Pr(>F)"]

# Perform LSD post-hoc test if significant differences are found
if (p_value < 0.05) {
  lsd_result <- LSD.test(anova_result, "Treatment", console = TRUE)

  # Extract LSD group labels
  lsd_groups <- lsd_result$groups
  lsd_groups$Treatment <- rownames(lsd_groups)  # Convert rownames to column

  # Plot bar graph with error bars and post-hoc comparison letters
  ggplot(data, aes(x = Treatment, y = w0)) +
    stat_summary(fun = mean, geom = "bar", fill = "skyblue", color = "black")
+
```

```
    stat_summary(fun.data = mean_se, geom = "errorbar", width = 0.2) +
    geom_text(data = lsd_groups, aes(x = Treatment, y = max(data$w0) + 1,
label = groups),
              position = position_dodge(width = 0.8)) +
    labs(title = "Average Starting Height by Treatment Group",
         x = "Treatment",
         y = "Start Height (cm)") +
    theme_minimal()
} else {
  # Plot bar graph without post-hoc comparison letters if no significant
differences
  ggplot(data, aes(x = Treatment, y = w0)) +
    stat_summary(fun = mean, geom = "bar", fill = "skyblue", color = "black")
+
    stat_summary(fun.data = mean_se, geom = "errorbar", width = 0.2) +
    labs(title = "Average Starting Height by Treatment Group",
         x = "Treatment",
         y = "Start Height (cm)") +
    theme_minimal()
}

# Perform ANOVA
anova_result <- aov(w0 ~ clone, data = data)

# Output ANOVA summary to the console
cat("\nANOVA Summary:\n")
print(summary(anova_result))

# Check normality of residuals
shapiro_test <- shapiro.test(residuals(anova_result))
cat("\nShapiro-Wilk Normality Test:\n")
print(shapiro_test)

# Plot residuals
par(mfrow = c(1, 2))  # Set up side-by-side plots
qqnorm(residuals(anova_result))
qqline(residuals(anova_result), col = "red")
hist(residuals(anova_result), main = "Histogram of Residuals", xlab =
"Residuals", col = "skyblue", border = "black")
par(mfrow = c(1, 1))  # Reset plot layout

# Perform Fisher's LSD test
lsd_result <- LSD.test(anova_result, "clone", group = TRUE)

# Output Fisher's LSD results to the console
cat("\nFisher's LSD Post-Hoc Analysis:\n")
print(lsd_result)

# Extract post-hoc comparison letters
letters <- lsd_result$groups
letters <- letters %>%
  rownames_to_column("clone") %>%
  rename(Letter = groups)

# Prepare data for plotting
summary_data <- data %>%
  group_by(clone) %>%
```

```
  summarise(
    mean_w0 = mean(w0, na.rm = TRUE),
    se_w0 = sd(w0, na.rm = TRUE) / sqrt(n()),
    .groups = "drop"
  ) %>%
  left_join(letters, by = "clone")

# Create the bar graph
ggplot(summary_data, aes(x = clone, y = mean_w0, fill = clone)) +
  geom_bar(stat = "identity", color = "black", fill = "skyblue", width = 0.7)
+
  geom_errorbar(aes(ymin = mean_w0 - se_w0, ymax = mean_w0 + se_w0), width =
0.2) +
  geom_text(aes(y = mean_w0 + se_w0 + 1, label = Letter), size = 4, vjust =
0) +
  labs(
    title = "Average Starting Height by Clone",
    x = "Clone",
    y = "Height (cm)"
  ) +
  theme_minimal() +
  theme(
    axis.text.x = element_text(angle = 45, hjust = 1),
    legend.position = "none"
  )

# Ensure relevant columns are factors
data$cross <- as.factor(data$cross)

# Perform ANOVA
anova_result <- aov(w0 ~ cross, data = data)

# Output ANOVA summary to the console
cat("\nANOVA Summary:\n")
print(summary(anova_result))

# Check normality of residuals
shapiro_test <- shapiro.test(residuals(anova_result))
cat("\nShapiro-Wilk Normality Test:\n")
print(shapiro_test)

# Plot residuals
par(mfrow = c(1, 2))  # Set up side-by-side plots
qqnorm(residuals(anova_result))
qqline(residuals(anova_result), col = "red")
hist(residuals(anova_result), main = "Histogram of Residuals", xlab =
"Residuals", col = "skyblue", border = "black")
par(mfrow = c(1, 1))  # Reset plot layout

# Perform Fisher's LSD test
lsd_result <- LSD.test(anova_result, "cross", group = TRUE)

# Output Fisher's LSD results to the console
cat("\nFisher's LSD Post-Hoc Analysis:\n")
print(lsd_result)

# Extract post-hoc comparison letters
```

```
letters <- lsd_result$groups
letters <- letters %>%
  rownames_to_column("cross") %>%
  rename(Letter = groups)

# Prepare data for plotting
summary_data <- data %>%
  group_by(cross) %>%
  summarise(
    mean_w0 = mean(w0, na.rm = TRUE),
    se_w0 = sd(w0, na.rm = TRUE) / sqrt(n()),
    .groups = "drop"
  ) %>%
  left_join(letters, by = "cross")

# Create the bar graph
ggplot(summary_data, aes(x = cross, y = mean_w0, fill = cross)) +
  geom_bar(stat = "identity", color = "black", fill = "skyblue", width = 0.7)
+
  geom_errorbar(aes(ymin = mean_w0 - se_w0, ymax = mean_w0 + se_w0), width =
0.2) +
  geom_text(aes(y = mean_w0 + se_w0 + 1, label = Letter), size = 4, vjust =
0) +
  labs(
    title = "Average Starting Height by Cross",
    x = "Cross",
    y = "Height (cm)"
  ) +
  theme_minimal() +
  theme(
    axis.text.x = element_text(angle = 0, hjust = 0.5),
    legend.position = "none"
  )
```

Figure B.2 R script for testing normality of RGR data.

```
# Load required libraries
library(dplyr)
library(ggplot2)
library(car)  # For Levene's test
library(ggpubr)  # For Q-Q plots

# Set working directory and load data
setwd("C:/Users/cpcothro/OneDrive - North Carolina State
University/PoplarChapter")
data <- read.csv("WorkingPoplar.csv")

# Ensure Clone is a factor
data$clone <- as.factor(data$clone)

# Define RGR columns
rgr_columns <- c("RGR2", "RGR4", "RGR6", "RGR8", "RGR10", "RGR12", "RGR14",
"RGR16")

# Loop through each RGR timepoint
for (rgr in rgr_columns) {
  cat(paste("\n### ANOVA for", rgr, "by Clone ###\n"))

  # Perform ANOVA
  anova_result <- aov(as.formula(paste(rgr, "~ clone")), data = data)
  print(summary(anova_result))

  # Extract residuals
  residuals_anova <- residuals(anova_result)

  # Check normality using Shapiro-Wilk test
  shapiro_test <- shapiro.test(residuals_anova)
  cat("\nShapiro-Wilk Normality Test:\n")
  print(shapiro_test)

  # Check homogeneity of variances (Levene's Test)
  levene_test <- leveneTest(as.formula(paste(rgr, "~ clone")), data = data)
  cat("\nLevene's Test for Homogeneity of Variance:\n")
  print(levene_test)

  # Generate Q-Q plot
  qq_plot <- ggqqplot(residuals_anova, title = paste("Q-Q Plot for", rgr))
  print(qq_plot)

  # Generate histogram of residuals
  hist_plot <- ggplot(data.frame(residuals_anova), aes(x = residuals_anova))
+
    geom_histogram(binwidth = 0.02, color = "black", fill = "gray") +
    labs(title = paste("Residuals Histogram for", rgr), x = "Residuals", y =
"Frequency") +
    theme_minimal()
  print(hist_plot)
}
```

Figure B.3 R script for kruska-wallis test of RGR by treatment, clone, and cross.

```
# Load required libraries
library(dplyr)
library(ggplot2)
library(FSA)       # For Dunn's test
library(tibble)    # For rownames_to_column

# Set working directory and load data
setwd("C:/Users/cpcothro/OneDrive - North Carolina State University/PoplarChapter")
data <- read.csv("WorkingPoplar.csv")

# Ensure relevant columns are factors
data$Treatment <- as.factor(data$Treatment)
data$clone <- as.factor(data$clone)
data$cross <- as.factor(data$cross)

# Define the RGR columns to analyze
rgr_columns <- c("RGR2", "RGR4", "RGR6", "RGR8", "RGR10", "RGR12", "RGR14",
"RGR16")

# Calculate average RGR across all timepoints
data <- data %>%
  rowwise() %>%
  mutate(avg_RGR = mean(c_across(all_of(rgr_columns)), na.rm = TRUE)) %>%
  ungroup()

# Define the order of clones
clone_order <- c("185", "229", "339", "342", "356", "437", "438", "441")

# Create custom high-contrast colors for the treatments
treatment_colors <- c(
  "control" = "#5F4AB6",  # Purple
  "P. cactorum" = "#C54F6B",  # Pink
  "P. cinnamomi" = "#C5B33A",  # Yellow
  "P. cryptogea" = "#F2CA19",  # Beige
  "P. europaea" = "#EDE1CA",  # Light Beige
  "P. plurivora" = "#5AC5A9"   # Green
)

# Calculate summary statistics by cross, clone, and treatment
summary_data <- data %>%
  group_by(cross, clone, Treatment) %>%
  summarise(
    mean_avg_RGR = mean(avg_RGR, na.rm = TRUE),
    se_avg_RGR = sd(avg_RGR, na.rm = TRUE) / sqrt(n()),
    .groups = "drop"
```

```
  ) %>%
  mutate(
    clone = factor(clone, levels = clone_order),
    cross_section = ifelse(cross == "TxD", "TxD", "DxD")
  )

# Determine y-axis limits for consistent plotting
y_min <- min(summary_data$mean_avg_RGR - summary_data$se_avg_RGR, na.rm = TRUE)
y_max <- max(summary_data$mean_avg_RGR + summary_data$se_avg_RGR, na.rm = TRUE)

# Kruskal-Wallis Test for avg_RGR
cat("Kruskal-Wallis Analysis of Average Relative Growth Rates (avg_RGR) by Treatment\n")
cat("============================================================
===\n\n")
kw_test <- kruskal.test(avg_RGR ~ Treatment, data = data)
print(kw_test)

# Perform Dunn's post-hoc test if significant
if (kw_test$p.value < 0.05) {
  cat("\nSignificant difference found, performing Dunn's post-hoc test:\n")
  dunn_result <- dunnTest(avg_RGR ~ Treatment, data = data, method = "bh")
  print(dunn_result)
} else {
  cat("\nNo significant differences found between treatments.\n")
}

# Plot average RGR by clone, cross, and treatment
plot <- ggplot(summary_data, aes(x = clone, y = mean_avg_RGR, fill = Treatment)) +
  geom_bar(stat = "identity", position = position_dodge(width = 0.7), color = "black", width =
0.7) +
  geom_errorbar(aes(ymin = mean_avg_RGR - se_avg_RGR, ymax = mean_avg_RGR +
se_avg_RGR),
                position = position_dodge(width = 0.7), width = 0.2) +
  labs(
    title = "Average Relative Growth Rate by Hybrid Poplar Cross, Clone, and Phytophthora
Treatment",
    x = "Hybrid Poplar Clone",
    y = expression("Average Relative Growth Rate (cm day"^"-1"*")")
  ) +
  facet_wrap(~ cross_section, scales = "free_x", labeller = label_value) +
  theme_minimal() +
  theme(
    strip.text = element_text(size = 12, face = "bold"),
    axis.text.x = element_text(angle = 45, hjust = 1),
    legend.position = "right"
  ) +
```

```
  scale_fill_manual(values = treatment_colors, name = "Treatment") +
  expand_limits(y = c(y_min, y_max))  # Use consistent y-axis limits across plots

# Print the plot
print(plot)

# Ensure relevant columns are factors
data$Treatment <- as.factor(data$Treatment)

# Define the RGR columns to analyze
rgr_columns <- c("RGR2", "RGR4", "RGR6", "RGR8", "RGR10", "RGR12", "RGR14",
"RGR16")

# Calculate average RGR across all timepoints
data <- data %>%
  rowwise() %>%
  mutate(avg_RGR = mean(c_across(all_of(rgr_columns)), na.rm = TRUE)) %>%
  ungroup()

# Calculate summary statistics by treatment
summary_data <- data %>%
  group_by(Treatment) %>%
  summarise(
    mean_avg_RGR = mean(avg_RGR, na.rm = TRUE),
    se_avg_RGR = sd(avg_RGR, na.rm = TRUE) / sqrt(n()),
    .groups = "drop"
  )

# Kruskal-Wallis Test
cat("Kruskal-Wallis Analysis of Average Relative Growth Rates (avg_RGR) by Treatment\n")
cat("============================================================
===\n\n")
kw_test <- kruskal.test(avg_RGR ~ Treatment, data = data)
print(kw_test)

# Perform Dunn's post-hoc test if significant
if (kw_test$p.value < 0.05) {
  cat("\nSignificant difference found, performing Dunn's post-hoc test:\n")
  dunn_result <- dunnTest(avg_RGR ~ Treatment, data = data, method = "bh")
  print(dunn_result)
} else {
  cat("\nNo significant differences found between treatments.\n")
}

# Create bar graph for average RGR by treatment with skyblue color
plot <- ggplot(summary_data, aes(x = Treatment, y = mean_avg_RGR)) +
```

```
  geom_bar(stat = "identity", color = "black", fill = "skyblue", width = 0.7) +
  geom_errorbar(aes(ymin = mean_avg_RGR - se_avg_RGR, ymax = mean_avg_RGR +
se_avg_RGR),
                width = 0.2) +
  labs(
    title = "Average Relative Growth Rate by Treatment",
    x = "Treatment",
    y = expression("Relative Growth Rate (cm day"^"-1"*")")
  ) +
  theme_minimal() +
  theme(
    axis.text.x = element_text(angle = 45, hjust = 1),
    legend.position = "none"
  )

# Print the plot
print(plot)

# Ensure 'Cross' and 'Treatment' are factors
data$cross <- as.factor(data$cross)
data$Treatment <- as.factor(data$Treatment)

# Define RGR columns
rgr_columns <- c("RGR2", "RGR4", "RGR6", "RGR8", "RGR10", "RGR12", "RGR14",
"RGR16")

# Calculate average RGR across all timepoints
data$avg_RGR <- rowMeans(data[, rgr_columns], na.rm = TRUE)

# Specify the correct negative control treatment label
negative_control <- "(-)"

# Create a new column categorizing treatments
data <- data %>%
  mutate(Treatment_Group = ifelse(Treatment == negative_control, "Control", "Phytophthora
treated"))

# Set factor levels to ensure negative control appears first
data$Treatment_Group <- factor(data$Treatment_Group, levels = c("Control", "Phytophthora
treated"))

# Summarize data for plotting
summary_data <- data %>%
  group_by(cross, Treatment_Group) %>%
  summarise(
    mean_RGR = mean(avg_RGR, na.rm = TRUE),
```

```
    se_RGR = sd(avg_RGR, na.rm = TRUE) / sqrt(n()),
    .groups = "drop"
  )

# Create the plot
plot <- ggplot(summary_data, aes(x = cross, y = mean_RGR, fill = Treatment_Group)) +
  geom_bar(stat = "identity", position = position_dodge(width = 0.7), color = "black", width =
0.7) +
  geom_errorbar(aes(ymin = mean_RGR - se_RGR, ymax = mean_RGR + se_RGR),
                position = position_dodge(width = 0.7), width = 0.2) +
  scale_fill_manual(values = c("Control" = "gray", "Phytophthora treated" = "skyblue")) +  #
Custom colors
  labs(
    title = "Average Relative Growth Rate by Cross and Treatment",
    x = "Cross",
    y = expression("Relative Growth Rate (cm day"^"-1"*")"),
    fill = "Treatment"
  ) +
  theme_minimal() +
  theme(
    axis.text.x = element_text(size = 12),
    legend.position = "top"
  ) +
  ylim(0.00, 0.04)  # Adjust if needed

# Print the plot
print(plot)

# Ensure 'Clone', 'Cross', and 'Treatment' are factors
data$clone <- factor(data$clone)
data$cross <- factor(data$cross)
data$Treatment <- factor(data$Treatment)

# Define RGR columns
rgr_columns <- c("RGR2", "RGR4", "RGR6", "RGR8", "RGR10", "RGR12", "RGR14",
"RGR16")

# Calculate average RGR across all timepoints
data$avg_RGR <- rowMeans(data[, rgr_columns], na.rm = TRUE)

# Define the order of clones for proper grouping
clone_order <- c("185", "229", "339", "342", "356", "437", "438", "441")  # Adjust as needed

# Specify the correct negative control treatment label
negative_control <- "(-)"
```

```
# Create a new column categorizing treatments
data <- data %>%
  mutate(Treatment_Group = ifelse(Treatment == negative_control, "Control", "Phytophthora
treated"))

# Set factor levels to ensure negative control appears first
data$Treatment_Group <- factor(data$Treatment_Group, levels = c("Control", "Phytophthora
treated"))

# Perform Kruskal-Wallis Test
cat("\n### Kruskal-Wallis Test for Average RGR by Clone ###\n")
kw_test <- kruskal.test(avg_RGR ~ clone, data = data)
print(kw_test)

# Initialize an empty data frame for post-hoc letters (prevents missing column errors)
dunn_letters <- data.frame(clone = factor(clone_order, levels = clone_order), PostHoc = "")

# Perform Dunn's post-hoc test if Kruskal-Wallis is significant (p < 0.05)
if (kw_test$p.value < 0.05) {
  dunn_result <- dunnTest(avg_RGR ~ clone, data = data, method = "bh")
  print(dunn_result)

  if (nrow(dunn_result$res) > 0) {
    # Generate compact letter display using multcompView
    generate_cld <- function(dunn_result) {
      sig_matrix <- matrix(1, nrow = length(clone_order), ncol = length(clone_order))
      rownames(sig_matrix) <- clone_order
      colnames(sig_matrix) <- clone_order

      for (i in seq_len(nrow(dunn_result$res))) {
        comp <- unlist(strsplit(dunn_result$res$Comparison[i], " - "))
        if (dunn_result$res$P.adj[i] < 0.05) {
          sig_matrix[comp[1], comp[2]] <- 0
          sig_matrix[comp[2], comp[1]] <- 0
        }
      }

      return(multcompLetters(sig_matrix)$Letters)
    }

    posthoc_letters <- generate_cld(dunn_result)
    dunn_letters <- data.frame(clone = names(posthoc_letters), PostHoc = posthoc_letters)
    dunn_letters$clone <- factor(dunn_letters$clone, levels = clone_order)
  }
}
```

```
# Summarize data for plotting
summary_data <- data %>%
  group_by(clone, Treatment_Group) %>%
  summarise(
    mean_RGR = mean(avg_RGR, na.rm = TRUE),
    se_RGR = sd(avg_RGR, na.rm = TRUE) / sqrt(n()),
    .groups = "drop"
  ) %>%
  mutate(clone = factor(clone, levels = clone_order)) %>%
  left_join(dunn_letters, by = "clone")  # Ensures 'PostHoc' is always present

# Create bar plot ensuring negative control appears to the left
plot <- ggplot(summary_data, aes(x = clone, y = mean_RGR, fill = Treatment_Group)) +
  geom_bar(stat = "identity", position = position_dodge(width = 0.7), color = "black", width =
0.7) +
  geom_errorbar(aes(ymin = mean_RGR - se_RGR, ymax = mean_RGR + se_RGR),
                position = position_dodge(width = 0.7), width = 0.2) +
  scale_fill_manual(values = c("Control" = "gray", "Phytophthora treated" = "skyblue")) +  #
Custom colors
  geom_text(aes(y = mean_RGR + se_RGR + 0.01, label = PostHoc),
            size = 4, vjust = 0, position = position_dodge(width = 0.7)) +  # Ensure text aligns with
bars
  labs(
    title = "Average Relative Growth Rate of Clones",
    x = "Clone",
    y = expression("Relative Growth Rate (cm day"^"-1"*")"),
    fill = "Treatment"
  ) +
  theme_minimal() +
  theme(
    axis.text.x = element_text(angle = 45, hjust = 1),
    legend.position = "top"
  ) +
  ylim(0.00, 0.085)  # Fixed y-axis range

# Print the plot
print(plot)
```