ENHANCING THE ORNAMENTAL POTENTIAL OF RARE THEACEAE SPECIES THROUGH GENETIC DIVERSITY AND TISSUE CULTURE RESEARCH

by

HEATHER JEANNE GLADFELTER

(Under the Direction of Scott A. Merkle and H. Dayton Wilde)

ABSTRACT

Two genera of the Theaceae family, *Stewartia* and *Franklinia*, are valued for their exquisite ornamental characteristics. *Franklinia* (*F. alatamaha)* and two of the seven *Stewartia* species tested are native to the southeastern US; however *Franklinia* is extinct in the wild. The two North American species, *Stewartia malacodendron* and *Stewartia ovata* are rare due to loss of habitat. *Franklinia* was first recorded by the naturalists John and William Bartram in 1765 and seeds were collected on a second excursion in 1773. The last observation of *Franklinia* in the wild was in 1803, but *Franklinia* has been cultivated in gardens and arboreta for nearly 250 years. The potential for breeding *Franklinia* for traits such as disease resistance depends on the level of genetic diversity in this cultivated population. The genetic diversity of a collection of *Franklinia* leaves from international sources was examined using genotyping-by-sequencing. An analysis of single-nucleotide polymorphisms (SNPs) found 9605 high quality SNPs to evaluate polymorphic differences among the samples. Tissue culture systems for *Franklinia* and *Stewartia* species were developed for the propagation and cryopreservation of specific genotypes. Plant growth regulator experiments led to the development of a shoot

micropropagation system for *F. alatamaha* and somatic embryogenesis systems for seven *Stewartia* species. Embryogenic cultures of six *Stewartia* species could be recovered after cryopreservation at -196°C.

INDEX WORDS: Cultivated, ornamental, genotype-by-sequencing, GBS, single nucleotide polymorphisms, SNPs, genomic sequencing, genetic diversity, mutation breeding, gamma radiation, micropropagation, PGRs, cryopreservation

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DEDICATION

I would like to dedicate this thesis to my late parents, Ralph and Jeanne Gladfelter, sister Sharon Werick, brother Alan Gladfelter, to all my cats over time that put up with my late nights of studying, Big Kitty, Gray Kitty, Alexis, Shark, Krink, Mulder, Thor, Hannibal, Darth Vader, BJ, Gandalf, Obi-Wan Kenobi, Arwen, Snowflake, Stardust, and Margaux, my sweet bunny Belle, and 18 budgies including Cumulus, Kiwi, Nimbus, and Cirrus.

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES xiv

LIST OF FIGURES xv

CHAPTER

1 INTRODUCTION 1

Stewartia species 1

Franklinia alatamaha 2

2 LITERATURE REVIEW 4

Theaceae 4

Stewartia species 4

Franklinia alatamaha 7

3 SOMATIC EMBRYOGENESIS OF *STEWARTIA* SPECIES FOR COMMERCIAL PROPAGATION AND CONSERVATION 13

Abstract 14

Introduction 15

Materials and methods 17

Results and discussion 21

Conclusions 26

References 28

4 TISSUE CULTURE PROPAGATION OF *FRANKLINIA ALATAMAHA* FOR COMMERCIAL PROPAGATION AND CONSERVATION 43

Abstract 44

Introduction 45

Materials and methods 46

Results 50

Discussion and conclusions 52

References 54

5 GENETIC DIVERSITY AND POPULATION STRUCTURE OF *FRANKLINIA ALATAMAHA* USING GENOTYPE-BY-SEQUENCING 66

Abstract 67

Introduction 68

Materials and methods 72

Results 76

Discussion 79

Conclusions 81

References 83

6 CONCLUSION 97

REFERENCES 100

APPENDICES

A *PHYTOPHTHORA* ROOT ROT TRIAL #1 111

LIST OF TABLES

Page

Table 3-1: Comparison of the induction frequency of somatic embryogenesis of *S. rostrata* by media treatments 1-8 using Chi-Square analysis with continuity correction 30

Table 3-2: Percent survival of 17 genotypes representing six different *Stewartia* species following recovery from cryopreservation 31

Table 4-1: Details of experiments testing different plant growth regulators (PGRs).....56

Table 4-2: Plant growth regulator (PGR) treatments tested.....57

Table 5-1: Locations of 95 accessions of *F. alatamaha* used in GBS analysis.....87

Table 5-2: Genetic diversity statistics of STACKS data 89

Table A-1: Rating scale for disease symptoms.....116

Table A-2: Trial 1: Survival percentages.....117

LIST OF FIGURES

Page

Figure 3-1: *S. malacodendron* ‘Delmarva’ 32

Figure 3-2: *S. ovata* ‘Red Rose’.....33

Figure 3-3: Induction of somatic embryogenesis in three *Stewartia* species.....34

Figure 3-4: Induction of somatic embryogenesis in five *Stewartia* species 35

Figure 3-5: Induction of somatic embryogenesis in *S. rostrata*.....36

Figure 3-6: Induction of somatic embryogenesis in *S. ovata* ‘Otto, NC’ 37

Figure 3-7: Somatic embryos and embryogenic callus of *Stewartia* species.....38

Figure 3-8: Cold vs. no cold treatments on percentage of germination and conversion of *S. rostrata* somatic embryos 39

Figure 3-9: Cold vs. no cold treatment vernalization periods on percentage of germination and conversion of *S. rostrata* somatic embryos.....40

Figure 3-10: Cold vs. no cold treatment on percentage of germination and conversion of *S. sinensis* somatic embryos.....41

Figure 3-11: Somatic embryogenesis of *Stewartia* species from explant to plants 42

Figure 4-1: Flower of *Franklinia alatamaha*.....58

Figure 4-2: Explant source is immature zygotic embryo.....59

Figure 4-3: Results of Experiment 4.....60

Figure 4-4: Organogenesis from immature zygotic embryo explants 61

Figure 4-5: Effects of 3 PGR treatments on axillary shoot multiplication 62

Figure 4-6: Effects of 3 PGR treatments on axillary shoot elongation.....63
Figure 4-7: Comparison of flowering over time with trees of different genotypes derived from organogenesis.....64
Figure 4-8: Comparison of flowering among different genotypes derived from organogenesis.....65
Figure 5-1: Read depth of sequencing data.....90
Figure 5-2: Delta K.....91
Figure 5-3: STRUCTURE analysis with STACKS data.....92
Figure 5-4: Principal component analysis (PCA). A).....93
Figure 5-5: Principal component analysis (PCA). B).....94
Figure 5-6: Phylogenetic relationships of *Franklinia alatamaha* accessions.....95
Figure 5-7: A weighted neighbor-joining distance heatmap.....96
Figure A-1: Experimental design for Trial 1 of *P. cinnamomi* pot screen test.....118
Figure A-2: *P. cinnamomi* isolate UGA-Pc01.....120
Figure A-3: Beginning of disease symptoms: Rating 1.....121
Figure A-4: Progressing disease symptoms: Rating 3.....122
Figure A-5: Progressing disease symptoms: Rating 4.....123
Figure A-6: Progressing disease symptoms: Rating 5.....124
Figure A-7: Healthy root vs. root infected with *P. cinnamomi*.....125

CHAPTER 1

INTRODUCTION

Stewartia species

Stewartia is a genus of 8-20 species of flowering plants in the family Theaceae and is related to *Franklinia*. The genus was named in 1753 by Carl Linnaeus to honor John Stuart, 3rd Earl of Bute (Bean, 1980). Linnaeus was given the name as 'Stewart' and through a transcription error, all his publications spelled the name as "*Stewartia*" (Bean, 1980). Most of the species are native to Asia, specifically China, Japan, and Korea. Two species, *Stewartia malacodendron* and *Stewartia ovata*, are native to southeastern North America from Virginia and Kentucky to Georgia, South Florida, and Louisiana. The species are mostly deciduous shrubs and trees, but some are evergreen, and these are in a genetically distinct group. Most botanists retain the evergreen species within *Stewartia* (Royal Botanical Gardens, KEW). The Asian species are both shrubs and trees which grow from 3-20 m in height, while the North American species grow only 3-5 m and rarely become trees. The bark is very distinctive in that the color ranges from smooth orange to yellow-brown and flakes. The leaves are alternately arranged, simple, serrated, usually glossy, and 3-14 cm long. The flowers are large and showy, 3-11 cm in diameter, with five white petals but can occasionally have 6-8 (Rushforth, 1999). Flowering occurs mid to late summer for the Asian species and late April to mid-June for the North American species. The fruit is a dry five-valved capsule with one to four seeds in each

compartment. *Stewartia* spp. prefer acidic soils and do not thrive on chalk or other calcium-rich soils (Flora of China). They have a high rainfall requirement and will not tolerate drought (Bean, 1980). Several species of *Stewartia* are grown as ornamental plants for their decorative smooth orange bark and showy white flowers as well as beautiful reddish-orange fall foliage.

Franklinia alatamaha

Franklinia alatamaha Bartram ex Marshall is a monotypic genus from within the family Theaceae (Prince and Parks, 2001; Yang et al., 2004). In cultivation, *Franklinia* is grown as a woody ornamental, either as a large shrub or small tree. The species can grow to heights of nine meters or more and displays beautiful, fragrant, white flowers with delicate yellow-colored stamens. The plants bloom continually from July to early September in the Southeastern U.S. In the fall, the foliage turns a brilliant scarlet red, adding to its many desirable ornamental qualities. Following its original discovery by William Bartram in 1765, *Franklinia* has not been observed in the wild since 1803 (Del Tredici, 2005; Harper and Leeds, 1937; Plummer, 1977). However, specimens from Bartram's original collections still exist in Bartram's Garden in Philadelphia, PA, and 96 botanical gardens worldwide (BGCI, 2015). *Franklinia* is rarely used in ornamental plantings within its native range due to its susceptibility to a plant pathogen, *P. cinnamomi*, which causes root rot (Koslow and Peterson, 1980; Meyer et al., 2009a; Plummer, 1977). Eventually the disease leads to the demise of this unique and elegant tree species in cultivation, a tragic situation since *Franklinia* is already extinct in the wild. Research is needed to determine whether *Franklinia* has any genetic diversity within cultivated populations that can be exploited for breeding resistance to the

devastating root rot pathogen. If no genetic diversity exists, it will be valuable and necessary to introduce diversity through artificial means such as DNA mutations, doubling of chromosomes, or genetic modification by gene transfer. Upon successful efforts to produce genetic resistance to *P. cinnamomi*, *Franklinia* can once again become a beautiful, thriving tree species both in cultivation and in native plantings within its natural range in Georgia and the Southeastern U.S.

CHAPTER 2

LITERATURE REVIEW

Theaceae

Theaceae is a family of flowering trees and shrubs that includes the camellias. It is described as having seven to 40 genera. Two genera of interest for this research are *Franklinia* and *Stewartia*. Most of the genera have evergreen foliage, but *Franklinia* and *Stewartia* are deciduous. The flowers in this family range from white to pink, are large and showy, and are often scented (Luna & Ochoterena, 2004). Members of the Theaceae family are found in Southeast Asia and Malesia, tropical South America and the Southeastern US. Three genera, specifically *Franklinia*, Gordonia, and *Stewartia*, have species native only to the Southeastern US. While some members of the Theaceae family are used for tea production, such as Camellia, others are grown widely as ornamentals for their flowers and attractive foliage and bark characteristics, specifically *Franklinia* and *Stewartia*.

Stewartia species

Horticultural value

According to Yang (1997), there are 21 species of *Stewartia*. *Stewartias* are distributed globally and primarily dwell in the woodlands. *Stewartia* includes both temperate (deciduous) and tropical species (evergreen). In the horticultural trade,

Japanese *Stewartia* (*Stewartia pseudocamellia*) is one of the most valued *Stewartia* species. *S. pseudocamellia* was introduced into the United States in 1866 by Thomas Hogg, Jr. to a private greenhouse in New York (DeWolf, 1969). *Stewartia* grows well in U.S. Department of Agriculture hardiness zones 5 to 8 (Dirr, 1998). Most of the *Stewartias* are small trees reaching 30 to 40 ft. in height and are distinguished by their numerous ivory-colored camellia-like flowers and smooth mottled bark (Hohn, 1994). The bark flakes in patches to reveal buff, tan, cinnamon, and plum colors during winter (DeWolf, 1969). *Stewartia* species are valued for their camellia-like flowers, beautiful and intense fall color, low insect and disease problems, and exfoliating bark (Spongberg & Fordham, 1975). Despite having outstanding ornamental value and features, *Stewartias* are not readily available for landscaping in the horticultural trade due to challenges with propagation (Struve & Lagrimini, 1999) that would limit mass propagation and production (Nair & Zhang, 2010).

Conventional propagation

Extensive research has been conducted on various aspects of *Stewartia* propagation, specifically with seed germination, scarification, cutting type, nutrition, light, rooting medium, rooting hormone, cold acclimation, and tissue culture (Nair & Zhang, 2010). Reports indicate success in adventitious rooting of cuttings but the challenge to date is with overwinter survival following rooting (Nair & Zhang, 2010). Researchers have focused on using a variety of sexual and asexual propagation techniques to overcome the propagation problems associated with *Stewartias*. Some of the issues related to sexual and asexual propagation techniques that hinder *Stewartia* propagation are low seed viability, seedling variability, and poor overwinter survival

(Nair & Zhang, 2010). Therefore, tissue culture propagation may be the ideal method to circumvent the previously mentioned conventional propagation techniques. Also, a large number of plants could be produced and would be true to type.

Micropropagation

McGuigan et al. (1997) successfully propagated Japanese *Stewartia* by *in vitro* micropropagation. Single node explants of Japanese *Stewartia* were taken at the softwood stage (versus from mature stem tissue) because of less contamination and greater bud break, and also collected from softwood stock plants. The nutrient medium used was woody plant medium (WPM) (Lloyd & McCown, 1980) supplemented with two plant growth regulators (PGRs). The auxin 2iP and the cytokinin thidiazuron (TDZ) were tested singly and in combination at different concentrations. Results showed the highest shoot elongation was from softwood stock plants and the highest bud break was from explants cultured on WPM with 48.2 µM 2iP. Explants cultured on WPM medium containing TDZ exhibited reduced bud break, less vigorous growth, and appeared stunted. McGuigan et al. (1997) determined that the position of the axillary node of the explant and incorporation of GA_3 did not influence bud break frequency nor shoot elongation. Although Japanese *Stewartia* was propagated successfully by micropropagation, large numbers of plantlets were not achieved because explants produced only one micro-cutting per nodal segment. The protocol was inapplicable to commercial situations due to little axillary shoot proliferation.

Somatic embryogenesis

Employing other plant tissue culture methods such as somatic embryogenesis would allow for the production of numerous genotypes exponentially. Somatic or asexual

embryogenesis is the production of embryo-like structures from somatic cells without the fusion of gametes. Somatic embryos develop through stages similar to those observed in zygotic embryogenesis (Dodeman et al., 1997). Somatic embryos arise from *in vitro* cultured cells in the process known as indirect somatic embryogenesis. The common method to induce embryogenic competence requires exposure of explants to a high auxin concentration during a variable period and then transferring cells to an auxin-free medium. Indirect somatic embryogenesis is the most common method to generate somatic embryos and has been described in hundreds of plant species (reviewed by Redenbaugh, 1993; Bajaj, 1995). The rapid improvements in somatic embryogenic methods allow for a variety of practical and commercial applications, specifically for *in vitro* clonal micropropagation (Bornman, 1993; Vasil, 1994). Somatic embryogenesis has many applications in Agroforestry, including crop improvement (cell selection, genetic transformation, somatic hybrid and polyploidy plant production), synthetic seeds, germplasm preservation, virus elimination, *in vitro* metabolite production, *in vitro* mycorrhizal initiation, scaled-up production in bioreactors (Onishi et al., 1994), and plant conservation.

Franklinia alatamaha

Discovery, rarity, and genetic variation of *Franklinia alatamaha*

Specimens of *Franklinia alatamaha* were collected in 1765 by John and William Bartram from the only known population site in coastal Georgia along the banks of the Alatamaha (now Altamaha) River near Fort Barrington, Georgia. The original population discovered by the Bartrams was described as being only two to three acres in size (Harper and Leeds, 1937; Plummer, 1977). No other sites have been identified for this species

except for the original site surveyed by the Bartrams. The last known visual documentation of *Franklinia* was by plant collector John Lyon in 1803. He noted only six to eight trees covering less than half an acre in that approximate location where the Bartrams first collected *Franklinia* (Del Tredici, 2005; Harper and Leeds, 1937; Plummer, 1977).

To date, no reports exist in the literature describing an analysis of genetic variation in the existing cultivated specimens of *Franklinia*. It is believed that all existing specimens originated from only one location with a very small population size and that they were all derived from material collected by the Bartrams in the 1700s. However, there is some uncertainty concerning the possibility that others may have collected *Franklinia* from the original location for the nursery trade as well (Plummer, 1977). *Franklinia* specimens are in collections at Kew Gardens as well as in the French royal gardens in Trianon (Thomson, 1990). If the original seed were collected in 1773 Bartram, this would have left little time for its propagation and subsequent transport to Europe to be cataloged there in 1774 (Thomson, 1990). Records from 1785 show that the seed was not brought back to Pennsylvania until "about fifteen years after 1760", after which it took five more years for the plants to produce seed (Marshall, 1785). If Marshall, Lyon, or some other unknown collector did manage to collect material from the original population of *Franklinia*, it could have significantly increased the genetic diversity of the currently cultivated stock, and the intraspecific diversity of *Franklinia* might prove to be greater than many have assumed (Plummer, 1977; Thomson, 1990; Marshall, 1785). However, since *Franklinia* naturally reproduces asexually by layering, the prediction of genetic variation within the population would be low (Del Tredici, 2005). The question is

whether the plants at the original site were clones of one or a few individuals, rather than offspring of a sexually reproducing population (Del Tredici, 2005; Gresham and Lipscomb, 1985).

Susceptibility of *Franklinia* to *Phytophthora cinnamomi*

Cultivated *Franklinia* is highly susceptible to root rot resulting in a shortened life span. For this reason, it is less desirable in ornamental plantings. The pathogen causing this disorder is *Phytophthora cinnamomi* (Koslow and Peterson, 1980; Meyer et al., 2009a; Plummer, 1977). Differential resistance of Gordonieae trees to *P. cinnamomi* has been shown for *Schima wallichii* Choisy, *Schima khasiana* Dyer, *Gordonia lasianthus* (L.), and x *Gordlinia grandiflora* Ranney & Fantz (Meyer et al., 2009). Neither *S. khasiana* nor *S. wallichii* exhibited any root rot symptoms or mortality (Meyer et al., 2009). Symptoms in *Franklinia alatamaha* were visible before other taxa and mortality reached 100% by the end of the experiment (Meyer et al., 2009). There was no significant difference in resistance among the other more resistant taxa and intergeneric hybrids, indicating that resistance to *P. cinnamomi* is a partially dominant trait. These results indicate the potential to breed increased resistance to *P. cinnamomi* into *Franklinia* populations by intergeneric hybridization and potentially by molecular breeding.

Assessing natural genetic variation within *Franklinia*

Franklinia is now cultivated in more than 1000 sites worldwide (Bartramsgarden.org, 2015). Germplasm collections of *Franklinia alatamaha* can be found at 99 botanical gardens worldwide (BGCI, 2015). For example, two accessions, each with two individual plants, are cultivated in the Arnold Arboretum of Harvard University in Boston, MA, one accession is located in the Arboretum Wespelaar in

Belgium, one specimen is growing in the New York Botanical Garden, and seven accessions are in the living collection at the Royal Botanical Garden in Edinburgh, United Kingdom (BGCI, 2015).

Determination of within-species genetic variation using GBS

Genetic diversity can be determined using molecular methods such as genotyping by sequencing (GBS). GBS is a method to discover single-nucleotide polymorphisms (SNPs) to perform genotyping studies. A SNP is a variation in a single nucleotide that occurs at a specific position in the genome, where each variant is present to some appreciable degree within a population. SNP genotyping is the measurement of genetic variation of SNPs among members of a species, which is one of the most common types of genetic variation. Not only are SNPs ideal for assaying genetic diversity and genotyping, they are useful in linkage and association mapping and molecular marker-assisted selection (MAS) studies with such advantages as locus-specificity, co-dominant inheritance, high reproducibility, and detection by PCR (Varshney et al., 2005; Kalia et al., 2011). GBS of *Franklinia alatamaha* would be ideal since the SNPs would be specific to *Franklinia* and not a distantly related species.

Generation of new genetic variation within species

If it should be determined that no genetic variation exists within the cultivated specimens of *Franklinia alatamaha* assayed from the various germplasm collections, then methods to introduce genetic variation to the population will need to be considered. Radioactive cobalt-60 displays some mutagenic activity and can produce numerical and structural chromosome aberrations in plant cells (Léonard and Lauwerys, 1990). In sesame, gamma rays from 450 Gy and 600 Gy from a ^{60}Co source produced lines tolerant

to *Phytophthora* Blight caused by *Phytophthora nicotianae* var. *parasitica* (Pathirana, 1984). The use of induced mutations in sesame breeding has produced several useful morphological and physiological mutants (Kobayashi, 1958; Murthy et al., 1985; Ashri, 1982, 1985). Three mutants were released for cultivation (Micke et al., 1987). Al-Safadi & Arabi (2003) used gamma radiation in their mutation breeding program with potato to improve resistance to late blight disease caused by *Phytophthora infestans* (2003). *In vitro* cultivated nodal sections of three potato cultivars were exposed to gamma rays at 25, 30, and 35 Gy. Mutant explants were cultured on MS medium (Murashige & Skoog, 1962) inoculated with mycelia of *P. infestans*. Surviving plants were propagated and re-incubated with the pathogen for three consecutive generations. Resistant plants were acclimatized and transferred to pots in greenhouse conditions. When the resistant plants were in the adult stage, they were inoculated with a sporangial suspension of *P. infestans*. One cultivar produced 10 resistant plants while only one resistant plant was produced from each of the other two cultivars.

Germplasm conservation

By sampling specimens from these worldwide germplasm collections, we cannot only assay for genetic diversity but preserve the genetic diversity through methods such as *in vitro* culture and cryopreservation. These biotechnological methods are very useful in maintaining *ex situ* germplasm of plant species that primarily reproduce asexually and that of species that are impossible to keep as seeds or in field-based gene banks (Gonzales-Arnao et al., 2014). A method of *in vitro* culture has been developed for *Franklinia* using axillary shoot culture (Beleski, 2016), however no reports have been published on *de novo* morphogenesis in the species, such as adventitious shoots or

somatic embryogenesis. Different techniques have been developed for long-term storage of plant germplasm in liquid nitrogen. One method is the "vitrification technique" which involves treatment of samples with cryoprotective substances, dehydration with a highly concentrated plant vitrification solution, rapid cooling and rewarming, removal of cryoprotectants, and recovery of the plant tissue (Sakai and Engelmann, 2007; Sakai et al., 2008). The development of new vitrification-based procedures allows for the cryopreservation of valuable genetic plant material. The vitrification procedure has been used to cryopreserve *in vitro*-grown shoot-tips of tea (*Camellia sinensis*), which is in the family Theaceae (Kuranuki & Sakai, 1995). Other tree species where *in vitro*-grown shoot-tips were cryopreserved by vitrification include mulberry, apple, pear, cherry, and sweet cherry (Niino et al. 1992; Niino & Sakai, 1992b; & Niino et al., 1997). Cryopreservation improvements have made possible cyrobank storage for well over 200 plant species (Engelmann, 2014).

CHAPTER 3

SOMATIC EMBRYOGENESIS OF *STEWARTIA* SPECIES FOR COMMERCIAL PROPAGATION AND CONSERVATION[1]

[1] H.J. Gladfelter, J. Johnston, H.D. Wilde, and S.A. Merkle. Submitted to *Plant Cell, Tissue and Organ Culture*, 09.24.19.

Abstract

Stewartia is a genus of flowering shrubs and trees in the family Theaceae. *Stewartia* species are native to eastern Asia, except for *S. ovata* and *S. malacodendron*, which are indigenous to southeastern North America. Despite having outstanding ornamental value and features, *Stewartias* are not readily available for landscaping in the horticultural trade due to difficulty with mass propagation. In addition to commercial propagation, there is a need to develop propagation techniques for the conservation of the North American species that are rare (*S. ovata*) or endangered (*S. malacodendron*). This research examines somatic embryogenesis as a means to mass propagate Asian and North American *Stewartia* species and to provide material for cryopreservation of valuable *Stewartia* germplasm. By testing a variety of plant growth regulators (PGRs), somatic embryogenesis was induced from immature zygotic embryo explants of seven *Stewartia* species—five Asian and two North American. Additionally, embryogenic cultures were obtained from three North American cultivars, *S. malacodendron* 'Delmarva' and *S. ovata* 'Red Rose' and 'Royal Purple'. Picloram (0.05 or 0.1 mg/L) and 2,4-Dichlorophenoxyacetic (2,4-D; 2.0 or 4.0 mg/L) were the most effective PGRs for inducing embryogenic cultures of North American and Asian *Stewartia* species, respectively. All seven *Stewartia* species examined produced somatic embryos that converted into plants that could be transferred to the greenhouse. Embryogenic tissue from five *Stewartia* species was recovered from cryopreservation, providing a means of long-term germplasm storage. This is the first report of somatic embryogenesis from immature zygotic embryos in the genus *Stewartia*.

Keywords cryopreservation, embryogenic, maturation, germination, conversion

Key Message This is the first report of somatic embryogenesis from immature zygotic embryos in the genus *Stewartia* and it was accomplished with seven different species.

Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
2,4-D	2,4-Dichlorophenoxyacetic acid
Picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
meta-Topolin	6-(3-Hydroxybenzylamino)purine
NAA	1-Naphthaleneacetic acid
Kinetin	6-Furfurylaminopurine
Zeatin	6-(4-Hydroxy-3-methylbut-2-enylamino)purine
DMSO	Dimethyl sulfoxide
MS	Murashige and Skoog medium
WPM	Woody Plant Medium
EMM	Embryo maturation medium
PGRs	Plant Growth Regulators
PEMs	Pro-embryogenic masses

Introduction

Stewartia is a genus of flowering shrubs and trees in the family Theaceae. *Stewartia* species are native to eastern Asia, except for two species, Mountain camellia (*S. ovata)* and Silky camellia (*S. malacodendron),* which are indigenous to southeastern North America. All species have large and showy flowers that are 3-11 cm in diameter,

usually with 5 white petals (Fig 3-1, 3-2). The barks of the trees are very distinctive in that the colors range from smooth orange to yellow-brown and flakes. Despite having outstanding ornamental value and features, *Stewartias* are not readily available for landscaping in the horticultural trade due to difficulty with mass propagation (Struve and Lagrimini 1999; Nair and Zhang 2010). Japanese *Stewartia* (*S. pseudocamellia*) has been propagated *in vitro* from single node explants (McGuigan et al. 1997), but axillary shoots were not produced at a level applicable for commercial production.

In addition to the commercial propagation of *Stewartia*, there is a need for developing propagation techniques for the conservation of North American species. *S. malacodendron* and *S. ovata* are both considered rare, with limited distribution in the southeast USA. *S. malacodendron* ranks G4/S3 on the Georgia Natural Heritage Ranks (Chafin 2007) with only 40 known populations in Georgia, three of which are on state conservation lands and 10 on military bases (Patrick et al.1995). The habitat of *S. malacodendron* is rich ravine and slope forests of the coastal plain, often with beech, oak, basswood, and spruce pine. It also occurs on lower slopes of sand-hills above bogs and creek swamps (Chafin 2007). *S. ovata* occurs in the mountains and piedmont in moist hardwood forests and bluff forests. Preservation of these species through seed is limited by difficulty with collection, seed yield and viability, germination requirements, and the fact that seedlings do not transplant (Chafin 2007) well. The objective of this research was to develop somatic embryogenesis as a means to mass propagate Asian and North American *Stewartia* species and to provide material for cryopreservation of valuable *Stewartia* germplasm.

Materials and Methods

Explant sources

Immature fruit from cultivars *S. malacodendron* 'Delmarva', *S. ovata'* Red Rose', and *S. ovata* 'Royal Purple' was obtained in August 2015 from The Polly Hill Arboretum (Tisbury, Massachusetts). The immature fruit of *S. pseudocamellia* var. *koreana*, a cold-tolerant cultivar, was provided by the University of Maine in August 2016. The immature fruit of *S. sinensis, S. koreana, S. monadelpha*, and *S. pseudocamellia* was obtained from plants owned by Jack Johnston in August 2015 and 2016. Wild material of *S. malacodendron* was collected from two sites in Georgia and one in Alabama and wild material of *S. ovata* was collected from one site each in North Carolina and Georgia every August from 2012-2016.

Culture initiation

The disinfestation and culture initiation procedures for immature fruit of the *Stewartia* species employed the methods outlined for somatic embryogenesis of American chestnut (Merkle et al. 1991). Under sterile conditions and using a stereomicroscope, the immature fruits were dissected to retrieve the immature seeds containing the immature zygotic embryos, which were excised and placed on a semisolid nutrient medium with or without plant growth regulators (PGRs). The basal nutrient medium was Woody Plant Medium (WPM; Lloyd & McCown 1980) supplemented with 3% sucrose, 500 mg/L glutamine, and 3 g/L Gelrite, set at a pH of 5.65. The PGR treatments tested included picloram at concentrations ranging from 0.05 mg/L to 10 mg/L; indolebutyric acid (IBA) at concentrations of 0.1 and 1.0 mg/L; 2,4-Dichlorophenoxyacetic acid (2,4-D) at three concentrations, 1, 2, and 4 mg/L; 0.1 mg/L

IBA in combination with 0.5 or 1.0 mg/L 6-benzylaminopurine (BAP); 0.1 mg/L IBA in combination with 0.5 or 1.0 mg/L meta-Topolin; and 2 mg/L 2,4-D in combination with 0.2 mg/L meta-Topolin. The immature zygotic embryos were cultured in the dark at 25 ± 2 ºC.

Cell suspensions and embryo production

Somatic embryogenic tissues produced from explant cultures in the PGR study were placed in a 125 ml Erlenmeyer flask containing 30 ml of liquid WPM with or without the PGR(s) that produced the somatic embryogenic tissue. The liquid suspension cultures were grown at 25 ± 2 ºC on a platform shaker in the dark at 100 rpm for 45 days. The cultures were fed with fresh medium every two weeks. Once one gram of tissue was produced, the embryogenic tissue was collected and size-fractionated using 100 µm pore size metal sieves. The pro-embryogenic masses (PEMs) recovered from the sieves were thoroughly rinsed with 150 ml of embryo maturation medium (EMM), which was WPM devoid of PGRs. The PEMs collected on the sieves were transferred to a new sterile 125ml flask with 30 ml of EMM. The cultures were placed back onto the shaker in the dark for five days. Using sterile glass Büchner funnels and vacuum, the PEMs were collected by pipetting 10 ml of the suspension culture evenly onto rafts of nylon mesh (30 µm pore size; Sigma-Aldrich). The nylon rafts with PEMs were transferred to semisolid EMM and placed in the dark at 25 ± 2 ºC for one month.

Embryo maturation and pre-germination cold treatment

Once somatic embryos started to form on the nylon rafts, individual embryos were transferred to fresh EMM to allow for further maturation. The embryos were incubated in the dark for one month. In some cases, somatic embryos were exposed to a

cold period vernalization treatment whereby the plates were wrapped in foil and placed in a walk-in cooler at 4 ºC for 30, 45, or 90 days.

Germination and Conversion

Following maturation and cold treatment, somatic embryos were placed on germination medium (WPM without glutamine or PGRs, with 0.25 mg/L activated charcoal) in an incubator with a 16:8 hour photoperiod, fluorescent lighting (30 µmol m^{-2} s^{-1}), at 25 $\pm$ 2 ºC until the embryos produced roots from the radicle and primary shoots from the apical meristem.

Transfer to soil and acclimation

Each somatic seedling was carefully transferred to a 4 inch square pot containing 1 part Fafard 3B: 1 part vermiculite soil mix, misted with a dilute solution of Miracle-Gro (1/2 teaspoon/L tap water), and covered by an inverted GA7 vessel (Magenta Corp.) to create a humid micro-environment. The pots were placed in a dome-covered tray on moist perlite and incubated in a growth chamber with a 16:8 hour photoperiod, fluorescent lighting (30 µmol m^{-2} s^{-1}), at 25 $\pm$ 2 ºC. The somatic seedlings were gradually exposed to lower humidity by removing the GA7s after 2-3 weeks and gradually opening the vents in the lid covering the tray. Once the dome was removed, plantlets were transferred to the greenhouse and eventually potted up into 1-gal pots containing 3 parts mini nugget bark chips: 1 part peat: 2 parts perlite, and top-dressed with Osmocote slow-release fertilizer. The final step was the transfer of the plants to the shade house and monitoring of survival.

Cryopreservation

The cryopreservation method used with the *Stewartia* embryogenic cultures was described by Vendrame et al. (2001). Embryogenic tissue of each *Stewartia* species was grown in culture flasks on a shaker at 100 rpm in the dark containing liquid WPM maintenance medium with PGRs for at least one week. The day before freezing, the tissue was transferred to flasks containing the same medium with 0.4M sorbitol. The culture flasks were incubated on the shaker overnight, and the next day they were placed in the cold room at 4 ºC. After 30 minutes in the cold, the flasks were placed on ice until the cells were ready to transfer to cryovials. The medium was replaced with fresh WPM maintenance medium containing 0.4 M sorbitol and 10% DMSO. The cells were mixed thoroughly in the DMSO/sorbitol solution and 1.8 ml of cells were pipetted into the 2 ml cryovials and placed in a -1 ºC "Mr. Frosty" freezing container (Nalgene, Rochester, NY) The freezing container was placed in an ultralow freezer set at -80 ºC overnight. The next day the cryovials were put in a cryobox and placed in the liquid nitrogen freezer set at -196 ºC. Before removal from liquid nitrogen, the cryovials were stored for at least one year. Upon removal from liquid nitrogen, the cryovials were transferred from the cryobox to a pre-frozen "Mr. Frosty" container at -80 ºC for 1.5h. Cryovials were then placed in a floating tray in a 40 ºC water bath for 2 minutes to facilitate rapid thawing. Once thawed, the embryogenic cell clusters were collected on a sterile nylon mesh raft overlaying several layers of filter paper to soak up the DMSO solution. The nylon rafts were then transferred to a fresh WPM maintenance medium containing the specific PGRs the tissue grew on prior to cryopreservation. The transfer was repeated the next day for continued removal of remaining DMSO. The cultures were placed in the dark at 25 $\pm$ 2 ºC.

Recovery was measured by the growth of cells and by maintenance by these cultures of the ability to maintain embryogenicity.

Experimental design and statistical analysis

Because seed quantities were limiting for some species and cultivars, there was a range from five to 10 explants (immature zygotic embryos) tested per PGR treatment. For the pre-germination cold vs. no cold treatments, 30 somatic embryos were used per treatment per genotype. All statistical analyses were performed using the R statistical package, R-3.5.1.tar.gz (2018-07-02). The frequencies of somatic embryogenesis induction by the different PGR treatments were compared using a 2-sample test for equality of proportions with continuity correction. The test variables for the germination and conversion response were tested for significance using the ANOVA function following the transformation of data using the arcsine function. Differences among treatment means were determined using Tukey's HSD multiple comparison test at a significance of $\alpha=0.05$.

Results and Discussion

Induction of somatic embryogenesis

The North American species of *Stewartia* were more responsive to picloram than other PGRs in producing somatic embryos, although only at low concentrations. Figure 3-3 shows that picloram concentrations above 2 mg/L proved to be ineffective for inducing somatic embryos. In fact, upon exposure to high concentrations of picloram, the tissue turned brown and died within a very short time. Interestingly, somatic embryogenesis was induced from the North American native cultivar *S. malacodendron* 'Delmarva' when exposed to no PGRs. Thirty-three percent of the immature embryos of

this cultivar produced somatic embryos upon exposure to 0.1 mg/L picloram. Immature embryos of another North American native cultivar, *S. ovata* 'Red Rose', were only responsive at the lower concentrations of picloram, 0.05 and 0.1 mg/L, at frequencies of 10% and 20%, respectively (Fig 3-3). Immature embryos from the Asian species, *S. rostrata*, produced somatic embryos upon exposure to 0.1 and 2.0 mg/L picloram at frequencies of 65% and 25%, respectively (Fig 3-3).

Two additional PGRs, IBA and 2,4-D, were tested for induction of somatic embryos with several Asian species, including *S. monadelpha*, *S. pseudocamellia*, *S. rostrata*, and *S. sinensis*, along with the two North American native species, an Alabama cultivar of *S. malacodendron* and the Polly Hill Arboretum cultivar *S. ovata* 'Royal Purple'. In one experiment, two concentrations of 2,4-D (2.0 and 4.0 mg/L), one concentration of IBA, 0.1 mg/L, the control with no PGRs, and two concentrations of picloram (0.1 and 1.0 mg/L), were tested for induction of embryogenesis. The cultivar *S. ovata* 'Royal Purple' produced somatic embryos when treated with no PGRs as well as picloram (0.1 mg/L) and 2,4-D (4.0 mg/L), at frequencies of 33.3 percent, 14.3 percent, and 16.7 percent, respectively (Fig 3-4). Induction of embryogenesis was only observed on medium containing 2.0 mg/L 2,4-D for the Asian species *S. sinensis* and the Blount County Alabama cultivar of *S. malacodendron*, at frequencies of 30 percent and 20 percent, respectively (Fig 3-4).

Three *Stewartia* species produced somatic embryos upon exposure to IBA, *S. rostrata*, *S. ovata* 'Otto, NC' and *S. koreana*. Figure 4 shows the results of a PGR experiment with *S. rostrata* where IBA alone or in combination with the cytokinins meta-Topolin and BAP induced embryogenesis from immature embryo explants. In this

experiment, 2,4-D was also tested at two different levels, 2.0 and 4.0 mg/L, as well as a no-PGR treatment. Chi-Square analysis with continuity correction showed no significant differences at $\alpha = 0.05$ for treatment comparisons for the induction frequency of somatic embryogenesis (Table 3-1). Eighty percent of the explants exposed to 2.0 mg/L 2,4-D, 0.1mg IBA, and 0.1 IBA with 0.5 mg/L of BAP produced somatic embryos. No somatic embryos were produced on 4.0 mg/L 2,4-D, or when the medium was devoid of PGRs. Embryogenesis was only induced from 20 percent of the immature embryos when IBA at 0.1 mg/L was in used in combination with the cytokinin meta-Topolin at 0.5 or 1.0 mg/L. However, IBA at 1.0 mg/L induced embryogenesis at a frequency of 60 percent, while IBA at 0.1 mg/L in combination with 1.0 mg/L BAP induced embryogenesis from 40 percent of the explants. Vieitez and Barciela (1990) also induced somatic embryogenesis with *Camellia japonica* using immature cotyledon explants and the PGRs IBA and BAP but using Murashige and Skoog medium (MS) (1962) instead of WPM. Somatic embryos of *Camellia sinensis* were also induced from immature cotyledon explants using MS basal medium, but the PGR treatments which produced the highest induction frequency were phenylboronic acid (PBOA) in combination with BAP or combination with kinetin (Ponsamuel et al. 1996). Lü et al. (2013) reported the induction of somatic embryos with *Camellia nitidissima* using WPM basal medium but with different PGRs, zeatin in combination with NAA.

An unexpected and unique result was observed for the PGR study with *S. ovata* 'Otto, NC'. Somatic embryos were produced from the endosperm of some immature seeds as well as from the immature embryo itself. Somatic embryos from endosperm tissue were induced upon exposure to 0.1 mg/L picloram, 2.0 mg/L 2,4-D, 0.1 mg/L IBA,

and in the absence of PGRs (Fig 3-6). Induction of somatic embryos from the immature embryos occurred when exposed to the same media treatments as the endosperm tissue except for 0.1 mg/L IBA (Fig 3-6). Higher levels of picloram and 2,4-D did not induce somatic embryogenesis from the endosperm tissue. Figure 3-7 shows the embryogenic callus and/or somatic embryos induced from the seven *Stewartia* species and endosperm tissue of *S. ovata* 'Otto, NC'.

Germination and Conversion

A comparison of cold pre-germination treatment (4 °C) versus no cold (25 °C) treatment was conducted for several embryogenic lines of *S. rostrata*. Figure 3-8 shows the effect of cold on the germination and conversion of somatic embryos from six *S. rostrata* genotypes. Analysis of variance results revealed that the treatment effect for percent germination was significant ($p= 1.5e^{-11}$), and there was a significant difference among genotypes following Tukey's HSD multi-comparison test (p= 0.0309) which was only due to a difference between two genotypes, SR1 and SR10. Analysis of variance results also showed that the cold (4 °C) treatment gave significantly higher percent somatic embryo conversion than the no cold (25 °C) treatment ($p< 0.001$).

A subsequent experiment tested whether extending the length of the cold period from 30 days to 45 or 90 days improved the germination and conversion of somatic embryos from *S. rostrata* genotypes SRC1-4 and SR-15-1-6 (Fig 3-9). The analysis of variance revealed no significant difference in percent germination (p=0.1870) or conversion (p=0.5890) among genotypes. Cold storage duration treatment was significant for percent germination (p=0.0018) but not for percent conversion (p=0.283). For both percent germination (p=0.9836) and conversion (p=0.9999), there was no significant

genotype by cold storage duration interaction. Tukey's HSD multi-comparison test of differences between cold periods showed 90 days gave significantly higher germination than 30 days ($p=0.00005$) or 45 days ($p=0.0002$). There was no significant difference between the 30 and 45-day cold period treatments ($p=0.2376$). Tukey's HSD multi-comparison test for cold storage duration on percent conversion showed 90 days gave significantly higher conversion than 30 days ($p=0.0016$) or 45 days ($p=0.0082$). There was no significant difference in conversion between the 30 and 45-day cold periods ($p=0.2705$).

Another experiment tested a 30-day cold period vernalization treatment using somatic embryos from another *Stewartia* species, *S. sinensis*, to determine if there was an impact on percent germination and conversion. Figure 3-10 shows the cold treatment increased the percentage of germination and conversion of *S. sinensis* somatic embryos.

Figure 10 shows the entire process of somatic embryogenesis for all the *Stewartia* species tested beginning from induction of somatic embryogenesis to the germination and conversion of a somatic seedling-derived plant. Interestingly, Lü et al. (2013) reported that somatic embryos produced from *C. nitidissima* germinated on medium containing 0.9 M BAP and 0.1 M NAA while the germination medium in this study did not contain any PGRs. Ponsumel et al. (1996) showed that somatic embryos of *C. sinensis* developed into somatic seedlings when incubated on MS-based hormone-free medium but the *Stewartia* somatic embryos from each of the species and cultivars tested in this study germinated and converted to somatic seedlings using WPM based PGR-free medium. In our study, a cold period significantly increased the percent germination and conversion of somatic embryos into somatic seedlings, while Ponsumel et al. (1996) exposed somatic

embryos of *C. sinensis* to the brassinosteroid 2α, 3α, 22, 23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one to increase the conversion frequency by 50%.

Cryopreservation

Somatic embryogenic cultures of 17 genotypes across six species of *Stewartia* were put into cryopreservation: *S. rostrata*, *S. koreana*, *S. monadelpha*, *S. pseudocamellia*, *S. pseudocamellia* var. *koreana* (UMaine-cultivar), and *S. ovata* 'Red Rose'. Cultures of four out of the six tested species were recovered from cryopreservation and five genotypes were recovered at 100% (Table 3-2).

Conclusions

Somatic embryogenesis was successfully induced from immature zygotic embryo explants of seven *Stewartia* species—five Asian and two North American. Additionally, three North American cultivars, *S. ovata* 'Red Rose', *S. malacodendron* 'Delmarva', and *S. ovata* 'Royal Purple' from The Polly Hill Arboretum were propagated by somatic embryogenesis using a variety of PGRs or no PGR treatment at all. Picloram at low concentrations, such as 0.05 and 0.1 mg/L, was more successful at producing somatic embryos with the North American *Stewartia* species. Most of the Asian *Stewartia* species responded best to 2,4-D at concentrations of 2.0 or 4.0 mg/L with regard to producing somatic embryos

Somatic embryo conversion and somatic seedling acclimatization to the greenhouse were achieved with all seven tested *Stewartia* species and the three North American cultivars. Somatic embryos from all *Stewartia* species germinated and converted at a higher frequency following a cold treatment. This is the first report of somatic embryogenesis in the genus *Stewartia*, which will facilitate the mass propagation

of these species for the horticulture industry. Furthermore, embryogenic tissue from five *Stewartia* species was recovered from cryopreservation, suggesting the feasibility of long-term storage of valuable germplasm such as that of rare or endangered North American *Stewartia* species.

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Table 3-1. Comparison of the induction frequency of somatic embryogenesis of *S. rostrata* by media treatments 1-8 using Chi-Square analysis with continuity correction.

TREATMENT	**PGRS (MG/L)**
1	None
2	2.0 2, 4-D
3	0.1 KIBA
4	0.1 KIBA/0.5 meta-topolin
5	0.1 KIBA/1.0 meta-topolin
6	0.1 KIBA/0.5 BAP
7	0.1 KIBA/1.0 BAP
8	1.0 KIBA

TREATMENT COMPARISON	**X^2***	**DF**	**P-VALUE****
1 VS. 2, 3, 6	3.75	1	0.0528
1 VS. 4, 5	0.00	1	1.0000
1 VS. 7	0.63	1	0.4292
1 VS. 8	1.90	1	0.1675
2, 3, 6 VS. 4, 5	1.60	1	0.2059
2, 3, 6 VS. 7	0.41	1	0.5186
2, 3, 6 VS. 8	0.00	1	1.0000
4, 5 VS. 7	0.00	1	1.0000
4, 5 VS. 8	0.41	1	0.5186
7 VS. 8	0.00	1	1.0000

*Chi-Square (X^2) value probability with 1 degree of freedom.
** p-values significant at α=0.05

Table 3-2. Percent survival of 17 genotypes representing six different *Stewartia* species following recovery from cryopreservation.

Species	Genotype	Percent Survival*	Percent Embryogenic**
S. koreana	SK2-2-46	0%	0%
S. monadelpha	SMDA-2	100.0%	100.0%
S. ovata	SO-12	100.0%	100.0%
S. pseudocamellia	SPC1.21	100.0%	100.0%
S. pseudocamellia var *koreana* (UMaine)	SU-1	0%	0%
	SU-2	0%	0%
	SU-2-2.0	0%	0%
	SU-4	0%	0%
	SU-5	0%	0%
	SU-5-2.0	0%	0%
	SU-7	0%	0%
	SU-8	0%	0%
	SU-9	0%	0%
S. rostrata	SR1	100.0%	100.0%
	SR2	0%	0%
	SR4	0%	0%
	SR-15-1-6	100.0%	100.0%

*based on number of plates out of 3 showing growth
**based on number of plates out of 3 showing proliferation of embryogenic callus and/or somatic embryos

Figure 3-1. *S. malacodendron* ‘Delmarva’ (Courtesy of The Polly Hill Arboretum, Tisbury, MA)

Figure 3-2. *S. ovata* ‘Red Rose’ (Courtesy of the The Polly Hill Arboretum, Tisbury, MA)

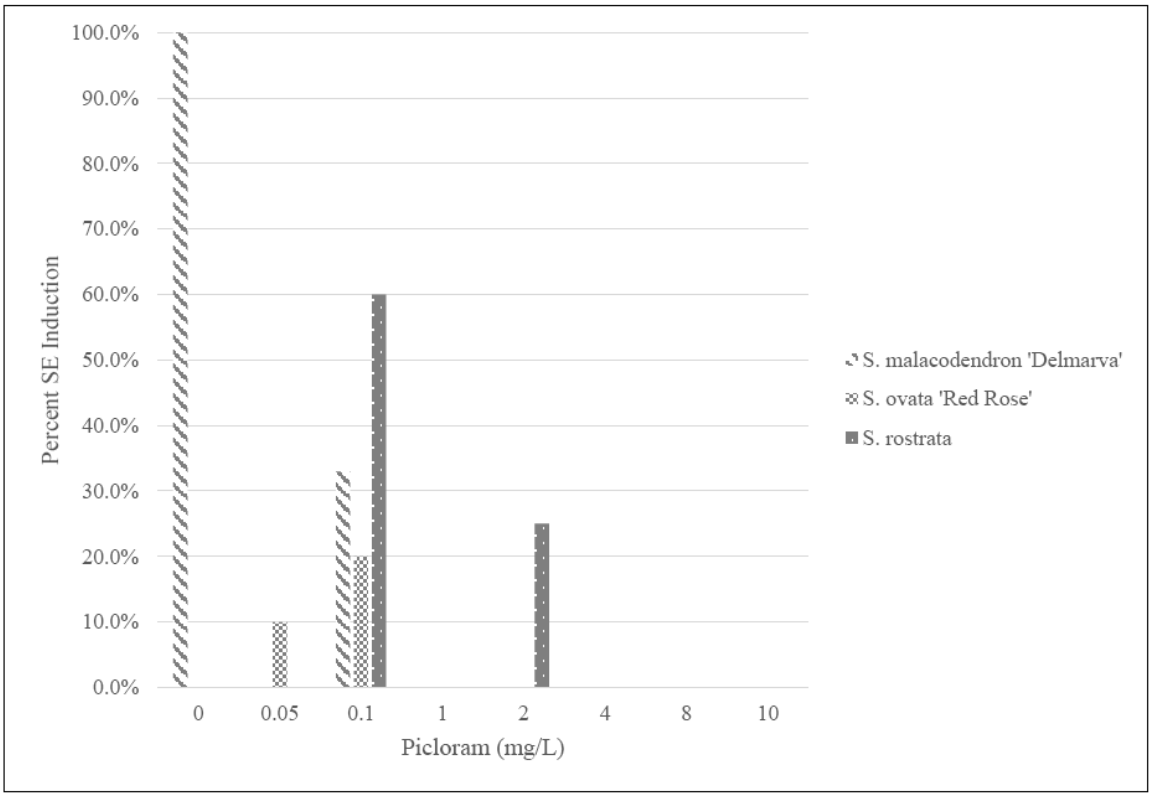

Figure 3-3. Induction of somatic embryogenesis in three *Stewartia* species, *S. malacodendron* ‘Delmarva’, *S. ovata* ‘Red Rose’, and *S. rostrata*, following exposure to picloram concentrations from 0 to 10mg/L in the nutrient medium. Percentages for *S. malacodendron* ‘Delmarva’ and *S. rostrata* are each based on five explants per PGR treatment while percentages for *S. ovata* ‘Red Rose’ were based on ten explants per PGR treatment.

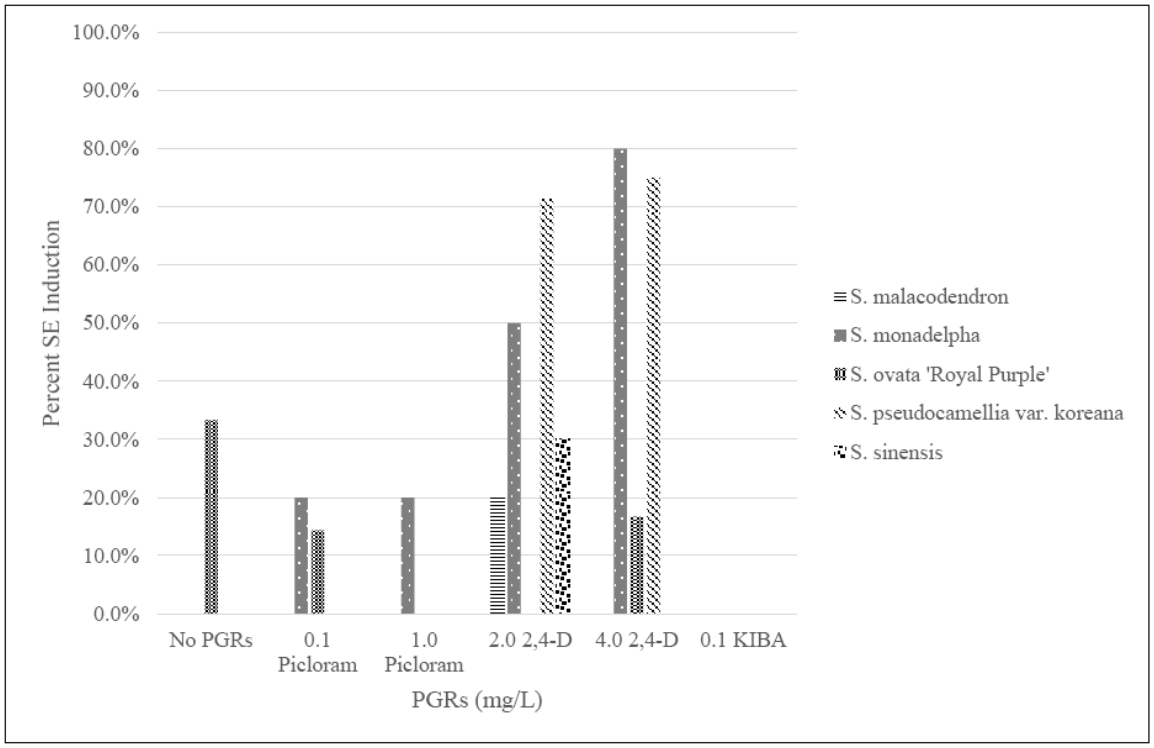

Figure 3-4. Induction of somatic embryogenesis in five *Stewartia* species, *S. malacodendron*, *S. monadelpha*, *S. ovata* 'Royal Purple', *S. pseudocamellia* var. *koreana* (UMaine), and *S. sinensis* following exposure to three different PGRs at various concentrations versus no PGRs in the nutrient medium. Percentages are based on ten explants per PGR treatment for *S. ovata* 'Royal Purple', *S. pseudocamellia* var. *koreana* (UMaine), and *S. sinensis.* For species *S. malacodendron* and *S. monadelpha* percentages were based on five explants.

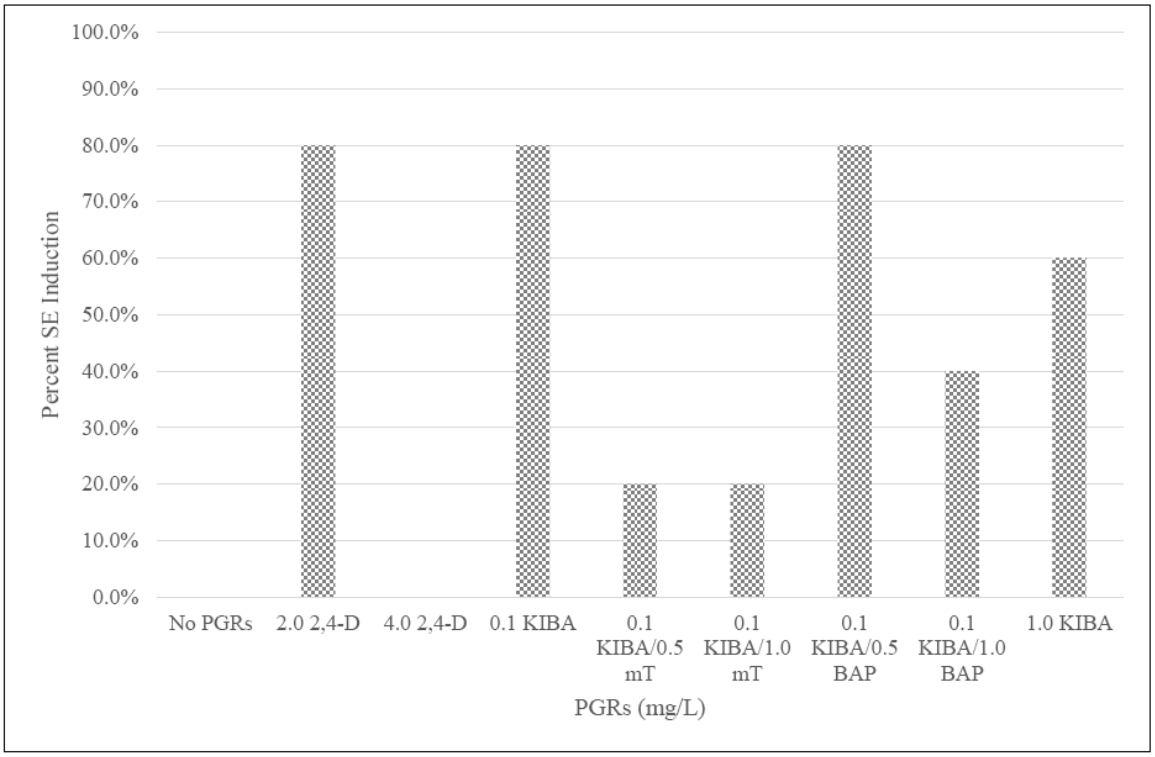

Figure 3-5. Induction of somatic embryogenesis of *S. rostrata* following exposure to varying concentrations and combinations of the auxin IBA with and without cytokinins meta-Topolin or BAP. The negative control is exposure to no PGRs and also previously tested levels of 2, 4-D, 2.0 mg/L or 4.0 mg/L. Percentages are based on five explants per PGR treatment.

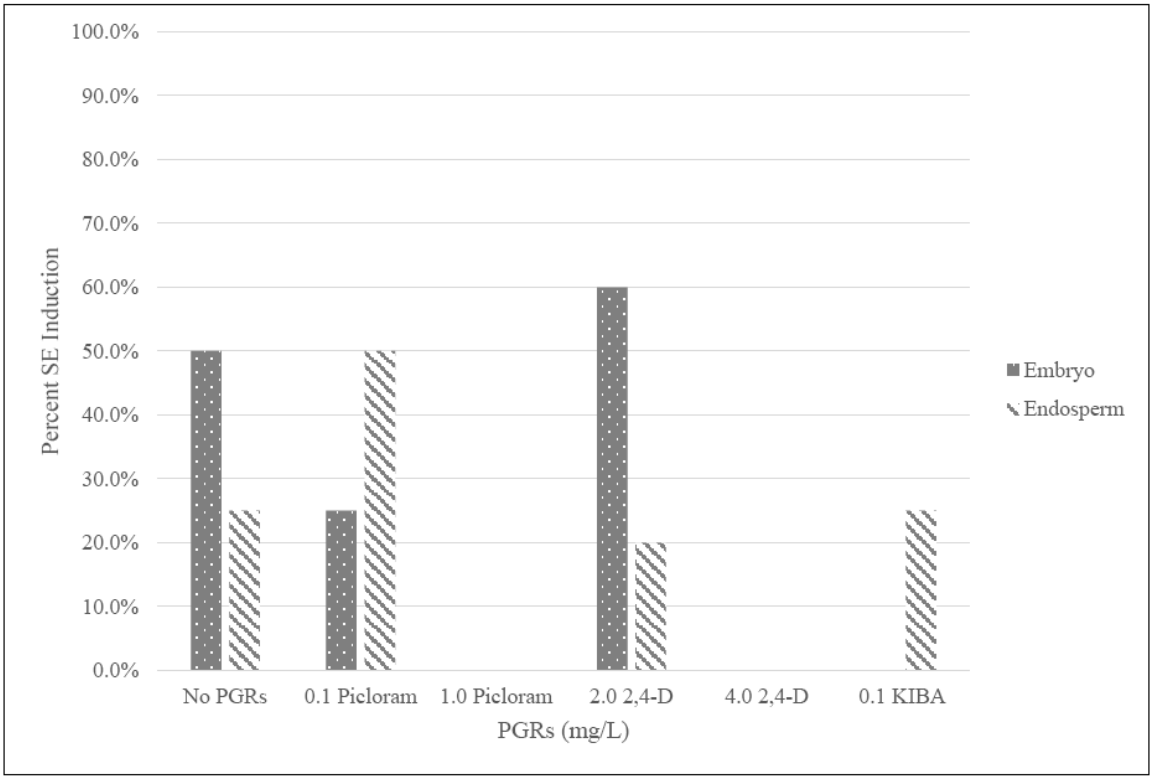

Figure 3-6. Induction of somatic embryogenesis of *S. ovata* 'Otto, NC' from both the endosperm and embryo following exposure to no PGRs, 0.1 mg/L picloram, and 2.0 mg/L 2, 4-D. Percentages are based on five explants per PGR treatment.

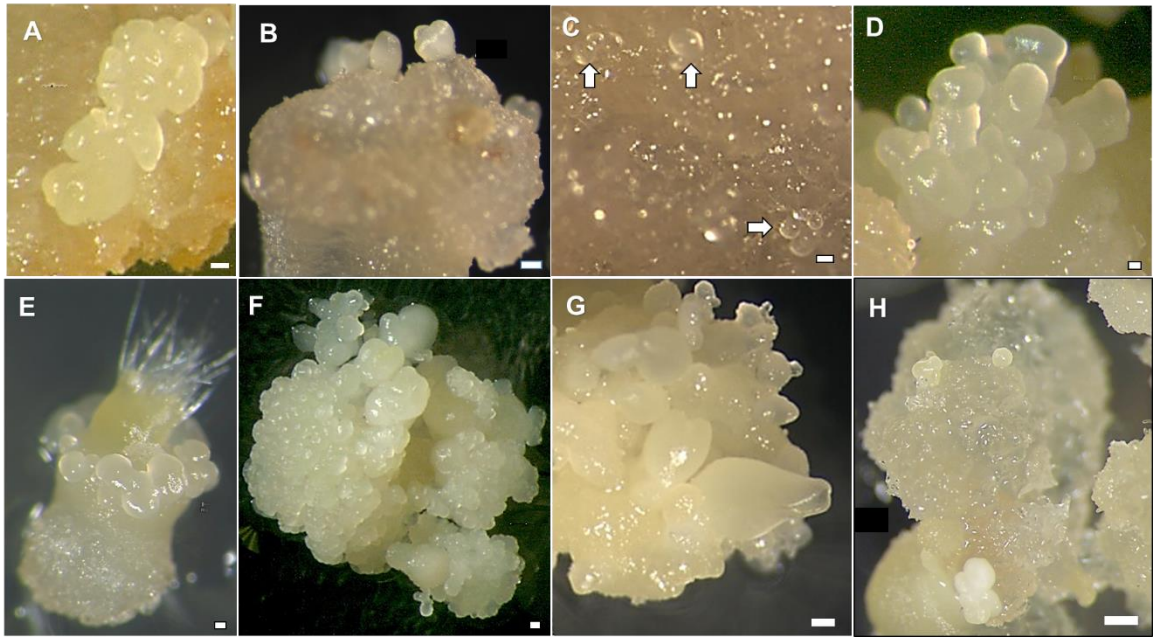

Figure 3-7. Somatic embryos and embryogenic callus of *Stewartia* species (A) *S. monadelpha* on WPM medium with 4.0 mg/L 2,4-D, (B) *S. malacodendron* 'Delmarva' with 0.1 mg/L picloram, (C) *S. rostrata* with 0.1mg/L picloram , (D) *S. pseudocamellia* var. *koreana* 'UMaine' with 2.0 mg/L 2,4-D, (E) *S. sinensis* with 2.0 mg/L 2,4-D, (F) *S. koreana* with 2.0 mg/L 2,4-D, (G) *S. ovata* 'Red Rose' and (H) endosperm of *S. ovata* 'Otto, NC' with 0.1 mg/L picloram. Arrows indicate globular somatic embryos. Scale bar length indicates 100 µm

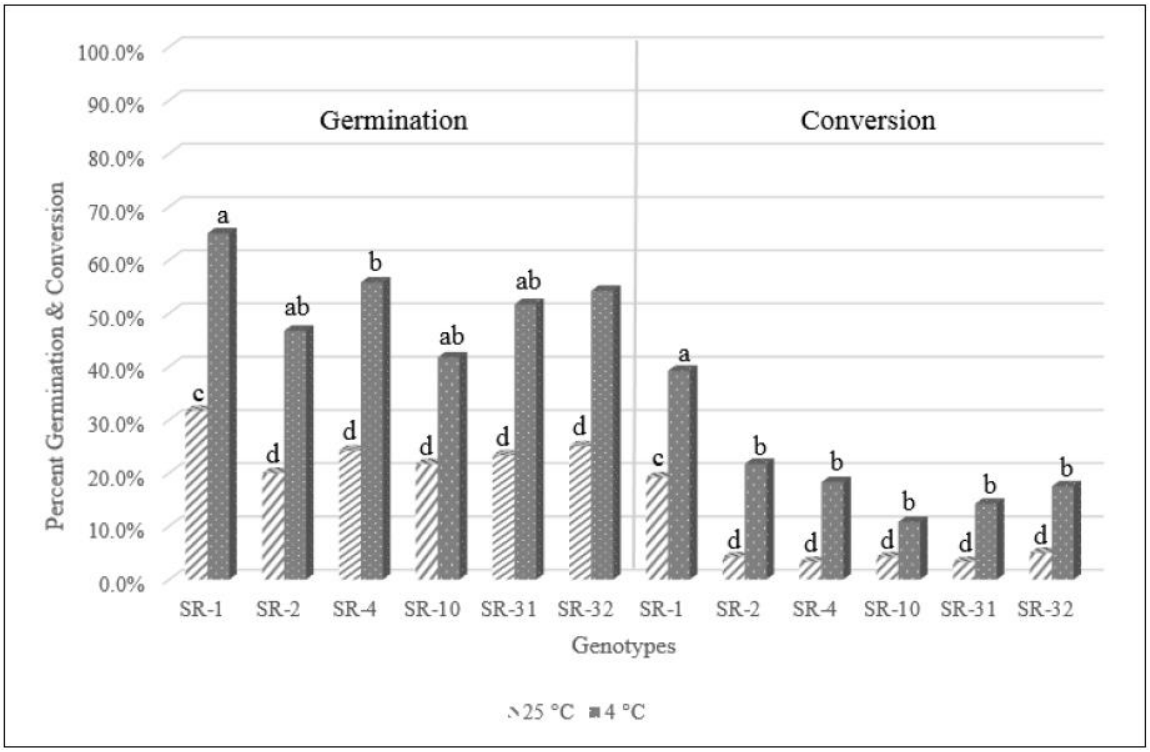

Figure 3-8. Comparison of no cold (25 °C) versus cold (4 °C) treatment on percentage of germination and conversion of somatic embryos for six *S. rostrata* genotypes, SR-1, SR-2, SR-4, SR-10, SR-31, SR-32. Tukey's HSD multi-comparison test indicated by letters a, ab, c, and d and those letters that differ are significant at α=0.05. Percentages are based on 30 explants per treatment per genotype.

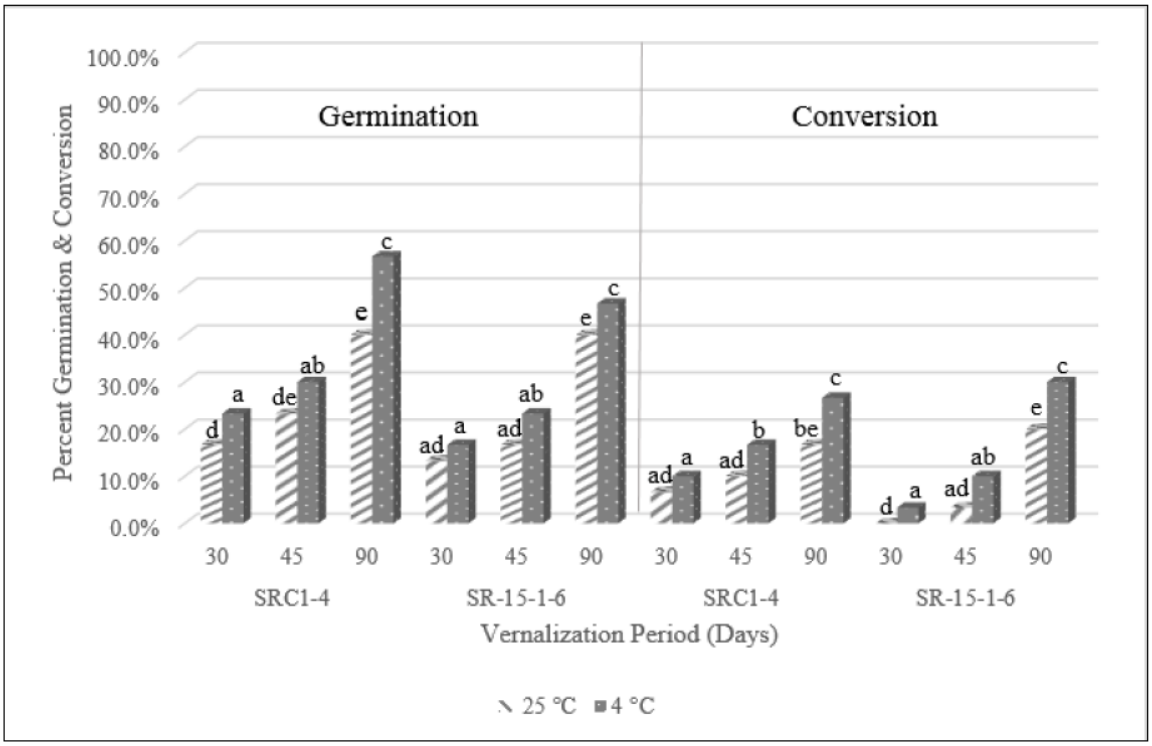

Figure 3-9. Cold (4°C) vs. no cold (25°C) treatment comparison for a period of 30 vs. 45 vs. 60 days in the dark at 4 °C for two *S. rostrata* genotypes, SRC1-4 and SR-15-1-6, and its effect on the percentage of germination and conversion of somatic embryos. Tukey's HSD multi-comparison test indicated by letters a, ab, ad, b, be, c, d, de, and e, and those letters that differ are significant at α=0.05. Percentages are based on 30 explants per treatment per genotype.

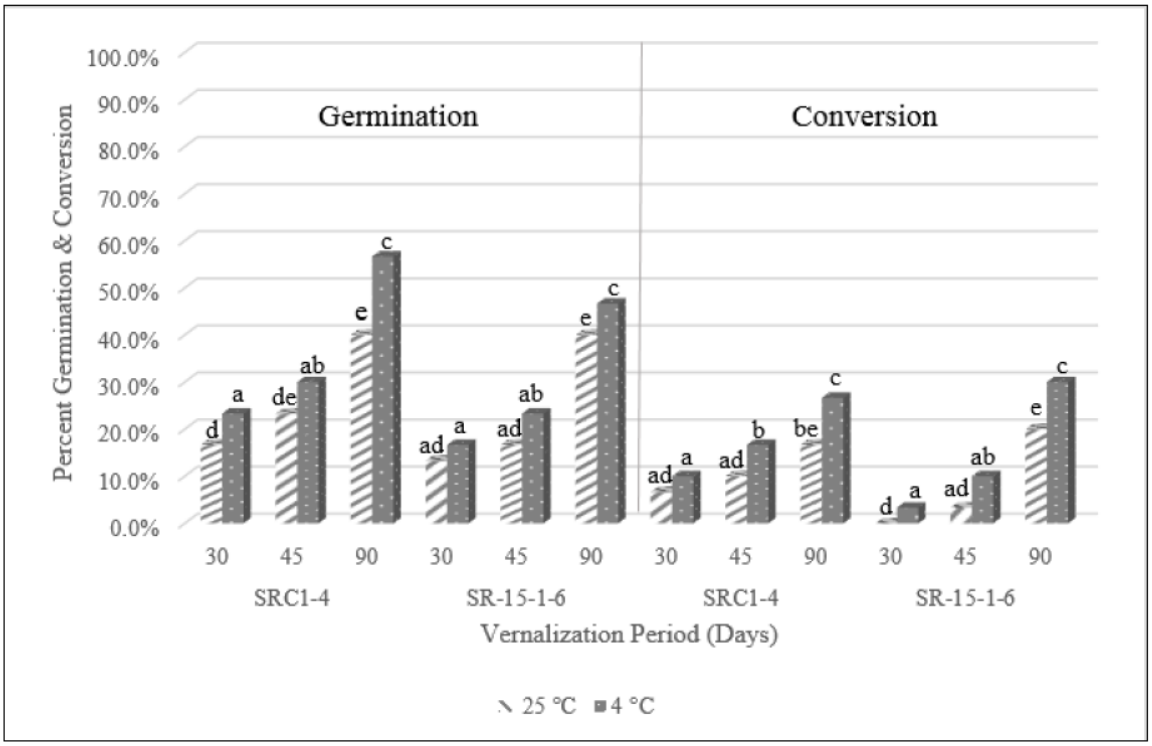

Figure 3-10. A 30 day cold (4°C) vs. no cold (25°C) vernalization treatment comparison with *S. sinensis* genotype SS-5 and its effect on the percentage of germination and conversion of somatic embryos. Tukey's HSD multi-comparison test indicated by letters a, b, and those letters that differ are significant at α=0.05. Percentages are based on 39 explants per treatment.

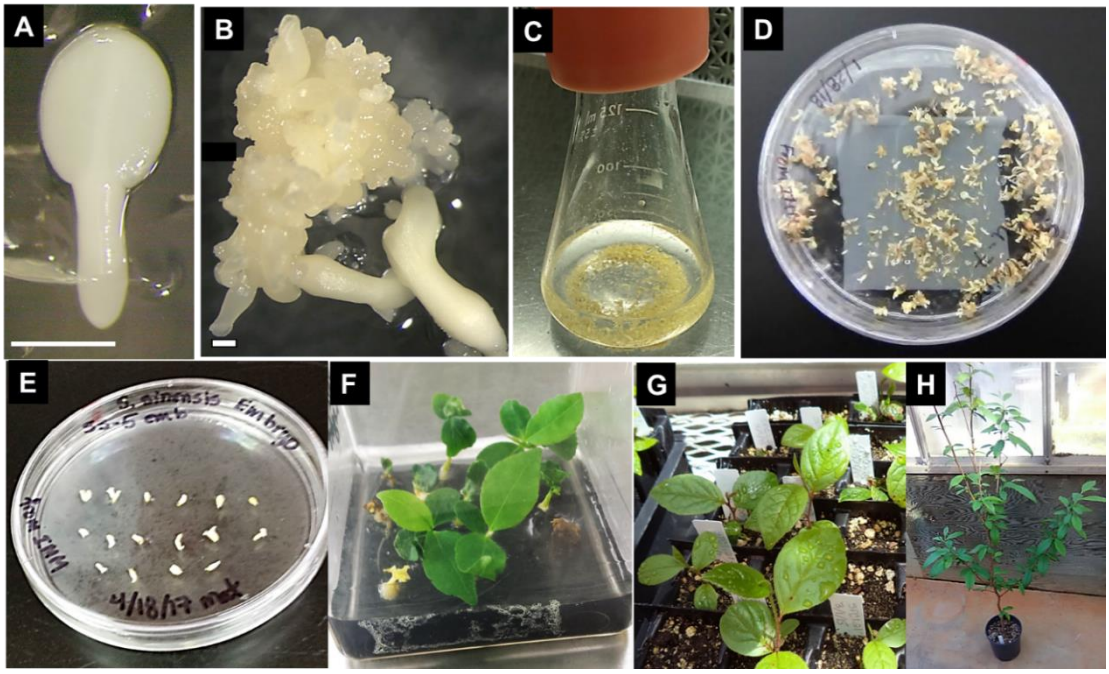

Figure 3-11. Somatic embryogenesis of *Stewartia* species from explant to plants. (A) Explant source is the immature zygotic embryo, (B) embryogenic callus proliferation, (C) liquid cell suspension, (D) embryo development, (E) embryo maturation, (F) conversion and germination, (G) *ex-vitro* acclimation, (F) 2 year-old tree derived from somatic embryogenesis. Scale bar for (A) is 1 mm in length and scale bar for (B) represents 0.5 mm in length

CHAPTER 4

TISSUE CULTURE PROPAGATION OF *FRANKLINIA ALATAMAHA* FOR COMMERCIAL PROPAGATION AND CONSERVATION[1]

[1] H.J. Gladfelter, J. Johnston, H.D. Wilde, and S.A. Merkle. To be submitted to *In vitro Cellular and Developmental Biology-Plant*.

Abstract

Franklinia alatamaha Bartram ex Marshall is a monotypic genus of the family Theaceae that is now extinct in the wild. It has been maintained in cultivation for nearly 250 years because of its exquisite ornamental qualities such as beautiful, delicate fragrant white flowers with golden stamens protruding from the center of the flower and outstanding fall colors with leaves ranging from orange to crimson red. The goal of this research was to develop an *in vitro* propagation for commercial propagation and conservation of this rare tree. Immature zygotic embryos were cultured on semi-solid Woody Plant Medium (WPM) with and without plant growth regulators (PGRs) in various concentrations and combinations. Shoot organogenesis was achieved using thidiazuron (TDZ), 4-CPPU, indole-3-butyric acid (IBA) in combination with 6-benzylaminopurine (BAP), or meta-Topolin. Adventitious buds were produced on the epidermal surfaces of immature zygotic embryos. Shoots elongated from both adventitious buds and axillary shoot cultures were rooted in WPM without PGRs but supplemented with 0.25 g/L activated charcoal. The resulting plantlets were transferred to soil and acclimated to the greenhouse and outdoor environments. Within one and a half years, the tissue culture-derived trees flowered under an extended photoperiod with LED supplemental lighting in the greenhouse.

Keywords propagation · adventitious · axillary · immature fruit · organogenesis

Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
2,4-D	2,4-Dichlorophenoxyacetic acid
Picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
meta-Topolin	6-(3-Hydroxybenzylamino)purine
NAA	1-Naphthaleneacetic acid
Zeatin	6-(4-Hydroxy-3-methylbut-2-enylamino)purine
4-CPPU	*N*-(2-Chloro-4-pyridyl)-*N*′-phenylurea
TDZ	1-phenyl-3- (1,2,3-thiadiazol-5-yl) urea
TIBA	tri-iodobenzoic acid
MS	Murashige and Skoog's medium (1962)
WPM	Woody Plant Medium (1980)
PGRs	Plant Growth Regulators

Introduction

Franklinia alatamaha Bartram ex Marshall is a monotypic genus of the family Theaceae (Prince and Parks, 2001; Yang et al., 2004). In cultivation, *F. alatamaha* can grow to heights of 6 m or more and displays beautiful, fragrant, white flowers with delicate orange-colored stamens (Figure 4-1). The plants bloom continually from July to early September in the Southeastern U.S. In the fall, the foliage turns a brilliant scarlet red, adding to its many desirable ornamental qualities.

Specimens of *F. alatamaha* were collected in 1773 by William Bartram from the only known population, which was on the coastal plain of Georgia. *F. alatamaha* is now extinct in the wild, although cultivated specimens from Bartram's original collection still exist in over 1000 sites worldwide (Bartramsgarden.org, 2019). Being able to restore this valuable, rare tree to the wild successfully would be of significant importance, not only in conservation of the species, but in its contribution to the habitat from which it originated hundreds of years ago.

Although a tissue culture propagation system for *F. alatamaha* was developed by Beleski (2014) that involved axillary shoot proliferation, the goal of this research was to develop an *in vitro* propagation system via organogenesis and/or somatic embryogenesis. This would allow for the testing of candidate genes that could confer resistance to the soil organism *Phytophthora cinnamomi*, which kills *F. alatamaha* in any soils that have this devastating pathogen. Introducing genes through biotechnology methods would require an *in vitro* propagation system either through organogenesis or somatic embryogenesis that can produce plants from individual cells.

Materials and Methods

Explant sources

Immature fruit of *F. alatamaha* was collected from a single source mother tree in Lakemont, GA every August from 2013 to 2017; from a single source tree growing at Whitehall Forest, Warnell School of Forestry and Natural Resources, Athens, GA, in August 2014 to 2016; and from two different source mother trees located at Bartram's Garden in Philadelphia, PA in August 2019.

Experimental design of PGR treatments

A total of six experiments were designed to test different types of PGRs alone or in combination with other PGRs for their effects on morphogenic responses *in vitro*. Immature zygotic embryo explants were randomly assigned to different PGR treatments with an equal number of explants per treatment, which depended on the number of immature zygotic embryos collected from the available immature fruits. The numbers of explants per treatment for a given experiment ranged from a low of five to a high of 21. Most of the experiments were replicated over multiple years. The only experiment not replicated twice was Experiment 6 since it was the most recent experiment. Parameters for each experiment are detailed in Table 4-1.

Culture initiation

The disinfestation and culture initiation procedures used on immature fruit of *F. alatamaha* were the methods outlined for the *in vitro* culture of American chestnut (Merkle et al., 1991). Briefly, under sterile conditions using a stereomicroscope, the surface-disinfested immature fruits were dissected to retrieve the immature seeds containing the immature zygotic embryos, which were then excised from the seed and placed on a semisolid nutrient medium in 60x15 mm plastic Petri dishes with or without PGRs (Figure 4-2). The basal nutrient medium was Woody Plant Medium (WPM) (Lloyd & McCown 1980) supplemented with 3% sucrose, 500 mg/L glutamine (filter-sterilized and added to cooled medium following autoclaving), and 3 g/L Gelrite, set at a pH of 5.65. The PGR treatments tested for organogenesis, either singly or in combinations, included different concentrations of picloram, thidiazuron (TDZ), meta-Topolin, zeatin, *N*-(2-Chloro-4-pyridyl)-*N′*-phenylurea (4-CPPU), indolebutyric acid (IBA),

dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), and 6-benzylaminopurine (BAP). A total of 34 different PGR treatments were tested in six different experiments replicated over several years, except for Experiment 6 (Tables 4-1 and 4-2). Immature zygotic embryos were cultured in the dark at 25 ± 2 ºC. Cultures producing adventitious buds were then transferred to a lighted growth chamber with a 16:8 hour photoperiod under fluorescent lighting (30 µmol m^{-2} s^{-1}) at 25 ± 2 ºC. Explants were transferred to the fresh medium of the same composition monthly.

Axillary shoot proliferation from adventitious buds

Adventitious buds produced on WPM supplemented with 0.1 mg/L IBA and 1.0 mg/L BAP were multiplied and also elongated into shoots on the same nutrient medium. Shoot clusters were grown on 80 ml of semisolid medium in GA7 (Magenta Corp.) vessels. To initiate axillary shoot cultures, elongated shoots were cut from the base of proliferating adventitious shoot clusters and cut into nodal segments, which were cultured on fresh nutrient medium in GA7 vessels. This medium was also WPM supplemented with 0.1 mg/L IBA with 1.0 mg/L BAP. The shoots were grown in an environmentally controlled incubator with a 16:8 hour photoperiod under fluorescent lighting (30 µmol m−2 s −1), at 25 ± 2 ºC. Shoots from both adventitious and axillary shoot cultures were transferred to the fresh medium of the same composition monthly.

Axillary shoot proliferation from axillary buds

An experiment was conducted to test different PGRs treatments to improve the quality and production of the axillary shoots. The PGR treatment that consistently induced adventitious buds, 0.1 mg/L IBA in combination with 1.0 mg/L BAP, was compared to two other PGR treatments: 0.5 mg/L meta-Topolin with 0.1 or 0.05 mg/L

4CPPU. The experimental design tested the three PGR treatments on three *F. alatamaha* genotypes in a factorial layout with three replicates and a total of 27 nodal sections for each PGR x genotype combination. There were nine nodal sections per GA7 vessel and the data collected was the number of shoots and the shoot lengths in cm from each nodal section. One-Way analysis of variance (ANOVA) was used to analyze the data.

Rooting of axillary shoots

Elongated *in vitro* shoots were excised from both adventitious and axillary shoot cultures when they were 6 mm in length, and the cut basal ends of the shoots were dipped in a 1 mg/ml solution of IBA and placed on a semisolid WPM-based rooting medium containing 0.25 mg/L activated charcoal and no PGRs. The shoots were incubated in a dark chamber at 25 $\pm$ 2 ºC for seven days for the induction of roots. The cultures were then transferred to a lighted growth chamber with a 16:8 hour photoperiod under fluorescent lighting (30 µmol m^{-2} s^{-1}) at 25 $\pm$ 2 ºC until roots developed from the bases of the *in vitro* shoots.

Transfer to *ex vitro* conditions and acclimation

Each rooted *in vitro* shoot was transferred to a 4 in square pot containing 1 part Fafard 3B: 1 part vermiculite soil mix, misted with a dilute solution of 'Miracle-Gro' (1/2 teaspoon/L tap water), and covered by an empty inverted GA7 vessel to create a humid micro-environment. The pots were placed in a clear plastic dome-covered tray on moist perlite and incubated in a growth chamber with a 16:8 hour photoperiod under fluorescent lighting (30 µmol m^{-2} s^{-1}) at 25 $\pm$ 2 ºC. The plantlets were gradually exposed to lower humidity by removing the GA7 vessels after 2-3 weeks and gradually opening the vents in the dome covering the tray. Once the dome was removed, plantlets were

transferred to the greenhouse and eventually potted up into 1-gal pots containing 3 parts mini nugget bark chips: 1 part peat: 2 parts perlite, and top-dressed with the slow-release fertilizer Scottsbrand 'Osmocote-Plus' 19-6-12 formula at a rate of 1 teaspoon every four months. The final step was the transfer of the plants to the shade house and monitoring of survival.

Statistical analysis

All statistical analyses were performed using the R statistical package, R-3.5.1.tar.gz (2018-07-02). Because of the large number of PGRs treatments evaluated between 2013-2019, it was impossible to compare all the treatments at one time since the PGR treatments were tested in different experiments over several years and the source of explants varied from one experiment to another. Any statistical comparison of PGR treatments was made within an experiment and between repeats of the same experiment in successive years. The frequencies of adventitious bud induction by the different PGR treatments were compared using a 2-sample test for equality of proportions with the continuity correction only when adventitious buds were observed in an experiment. The test variables for the axillary shoot proliferation and elongation responses were tested for significance using the ANOVA function. Differences among treatment effects were determined using Tukey's HSD multiple comparison test at a significance of $\alpha=0.05$.

Results

Induction of organogenesis

Of the 34 PGR treatments tested for *in vitro* response, six treatments induced adventitious shoot production: 0.1 mg/L and 0.5 mg/L of IBA, both in combination with 1.0 mg/L BAP, 0.05 mg/L IBA in combination with 2.0 mg/L meta-Topolin, 0.1 mg/L 4-

CPPU, and 0.05 and 0.1 mg/L thidiazuron (Table 4-2). Due to a large number of PGR experiments conducted between 2013 to 2019 (Experiments #1-6 and their respective replicated experiments), only results from Experiment 4 for 2014 are presented here since adventitious buds were observed in all three experiments in 2014, 2015, and 2016. Experiment 4 in 2014 compared seven different PGR treatments, and the only treatment that produced adventitious shoots was PGR treatment #19 (0.1 mg/L IBA in combination with 1.0 mg/L BAP) (Figure 4-3). However, PGR treatment #19 again produced adventitious buds when Experiment 4 was repeated in 2015 and 2016 (data not shown). Figures 4-4A and 4-4B show the induction and elongation of these adventitious buds from the epidermal surface immature zygotic embryos on this PGR treatment #19. The other PGR treatments tested either resulted in elongation and/or swelling of the explant, growth of undifferentiated callus, indeterminate morphogenic structures, and/or production of adventitious roots (Table 4-2). None of the PGR treatments produced somatic embryos.

Axillary shoot proliferation from axillary buds

One-Way ANOVA results showed a significant difference among the three tested PGR treatments for the number of shoots produced per nodal section (p-value = 2.34e-13). Neither genotype nor genotype by treatment interaction had a significant effect on the number of shoots. However, shoot length differed significantly among both genotypes (p-value = 3.54e-07) and treatments (p-value = 5.66e-10), and the genotype by treatment interaction was not significant (p-value = 0.0709) for this variable. Tukey's HSD multiple comparison test showed that the PGR treatment 0.5 mg/L meta-Topolin with 0.05 mg/L 4-CPPU produced more shoots and longer shoots compared to the other two PGR

treatments (Figures 4-5, 4-6). Also, genotype FWH-C1-4 produced longer shoots than genotypes FWH-C2-1 and FWH-C3-5 at $\alpha = 0.05$ (Figure 4-6).

Flowering

In the greenhouse environment, 1.5 year-old trees of eight *in vitro* genotypes of *F. alatamaha* produced by organogenesis were exposed to supplemental LED lighting and an extended photoperiod of 16 h daylight with 8 h dark. All eight genotypes produced flowers over the course of eight months, February 1st to September 30th (Figure 4-7). One-way analysis of variance results showed that flower numbers per plant were affected by both genotype (p-value = 8.69e-05) and month (p-value = 5.21e-06), but there was no genotype by month interaction (Table 4-2). The genotype FWH-C1-4 produced the most flowers based on Tukey's HSD multi-comparison test (Figure 4-8).

Discussion and Conclusion

In this study, thirty-four different PGR treatments were tested for the development of an *in vitro* plant propagation system for *F. alatamaha*. Organogenesis was the only morphogenic process observed, and it was induced by five PGR treatments that included TDZ or 4-CPPU alone or IBA in combination with BAP or meta-Topolin. Kato (1986) showed that BAP combined with IBA was also successful for the induction of organogenesis with *C. japonica* L. and *C. sinensis* L.; however the explant source used was cotyledons while that of *F. alatamaha* was immature zygotic embryos. In another study with *C. japonica* L., organogenic callus was derived from leaf explants of micropropagated shoots when cultured on a modified MS medium followed by immersion in MS liquid medium supplemented with 1 g/L of IBA (Pedroso and Pais, 1993). With the cultivar *Camellia* x *williamsii* cv. Debbie, organogenic callus was

obtained from internode and leaf explants from using IBA in combination with BAP, TDZ, or tri-iodobenzoic acid (TIBA) (Tosca, 1996; Tosca et al., 1991). The most common nutrient formula used for Camellia micropropagation was MS; however other nutrient media were tested, such as WPM, Heller's medium, and Nitsch and Nitsch medium (Mondal, 2004B). WPM was the only basal nutrient medium tested for initiation of organogenesis of *F. alatamaha* from immature zygotic embryo explants. There are reports of both direct and indirect somatic embryogenesis occurring simultaneously with organogenesis on the same explants in both *C. japonica* L. and *C. sinensis* L. (Pedroso and Pais, 1993; Akula and Dodd, 1998). However, this is the first report of plants being produced from *F. alatamaha* via organogenesis.

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Table 4-1. Details of experiments testing different plant growth regulator (PGR) treatments and the years the experiments were initiated and/or repeated between years 2013 through 2019.

* Experiments 1, 2, and 4 used the same source mother tree from Lakemont, GA each year.

Experiment #	Treatment #	PGRs (mg/L)										Source of Mother Tree	# of explants/ treatment/year					
		none	picloram	2, 4-D	IBA	BAP	mT	zeatin	NAA	TDZ	4-CPPU		2013	2014	2015	2016	2017	2019
1*	1	0.00										Lakemont, GA*	10			10	10	
	3		0.10										10			10	10	
	5		1.00										10			10	10	
2*	1	0.00										Lakemont, GA*		10	10			
	2		0.05											10	10			
	3		0.10											10	10			
	4		0.50											10	10			
	5		1.00											10	10			
	6		2.00											10	10			
	7		2.50											10	10			
	8		4.00											10	10			
	9		5.00											10	10			
	10		8.00											10	10			
3**	1	0.00										Whitehall Forest Athens, GA**			10	10		
	11			2.00											10	10		
	12			2.00		0.10	0.10								10	10		
	13			4.00											10	10		
	14			6.00											10	10		
4*	1	0.00										Lakemont, GA*		21	21	21		
	15				0.05									21	21	21		
	17				0.10									21	21	21		
	19				0.10	1.00								21	21	21		
	20				0.50									21	21	21		
	22				1.00									21	21	21		
	29				0.10				0.05					21	21	21		
5**	1	0.00										Whitehall Forest Athens, GA**		10	10			
	17				0.10									10	10			
	19				0.10	1.00								10	10			
	20				0.50									10	10			
	21				0.50	1.00								10	10			
6***	1	0.00										Bartram's Garden Philadephia, PA*** Accessions: (JBA#86005 A and JBA#112018)						10
	2		0.05															10
	3		0.10															10
	5		1.00															10
	11			2.00														10
	15				0.05													10
	16				0.05		2.00											10
	17				0.10													10
	19				0.10	1.00												10
	20				0.50													10
	22				1.00													10
	23					0.05												10
	24					0.10												10
	26						2.00											10
	27							0.10										10
	28							2.00	0.05									10
	30									0.05								10
	31									0.10								10
	34										0.10							10

**Experiments 3 and 5 used the same mother source tree from Whitehall Forest in Athens, GA.
***Experiment 6 used two different source mother trees from Bartram's Garden, JBA#86005A & JBA#11.2018. There were 5 explants per treatment per mother tree.

Table 4-2. Plant growth regulator (PGR) treatments tested on *Franklinia* immature zygotic embryo explants and the respective explant responses over 6 experiments.

Treatment	Plant Growth Regulators (PGRs) mg/L										Explant response
	none	picloram	2,4-D	IBA	BAP	mT	zeatin	NAA	TDZ	4-CPPU	
1	0.00										elongation of zygotic embryo; callus; roots
2		0.05									callus; roots
3		0.10									callus; roots
4		0.50									callus
5		1.00									callus; roots
6		2.00									callus
7		2.50									callus
8		4.00									callus
9		5.00									callus
10		8.00									callus
11			2.00								callus
12			2.00		0.10	0.10					callus
13			4.00								callus
14			6.00								callus
15				0.05							elongation of zygotic embryo; callus; roots
16				0.05		2.00					adventitious buds
17				0.10							elongation of zygotic embryo; callus; roots
18				0.10		0.50					elongation of zygotic embryo; callus; roots
19				0.10	1.00						adventitious buds
20				0.50							callus; roots
21				0.50	1.00						adventitious buds
22				1.00							callus; roots
23					0.05						elongation of zygotic embryo; roots
24					0.10						elongation of zygotic embryo; callus; roots
25					1.00						callus; roots
26						2.00					enlarged zygotic embryo, browning tissue
27							0.10				elongation of zygotic embryo; callus; roots
28							2.00	0.05			enlarged zygotic embryo, browning tissue
29					0.10			0.05			elongation of zygotic embryo; callus; roots
30									0.05		adventitious buds
31									0.10		adventitious buds
32						0.50				0.05	shoot multiplication
33						0.50				0.10	shoot multiplication
34										0.10	adventitious buds

Figure 4-1. Flower of *Franklinia alatamaha*.

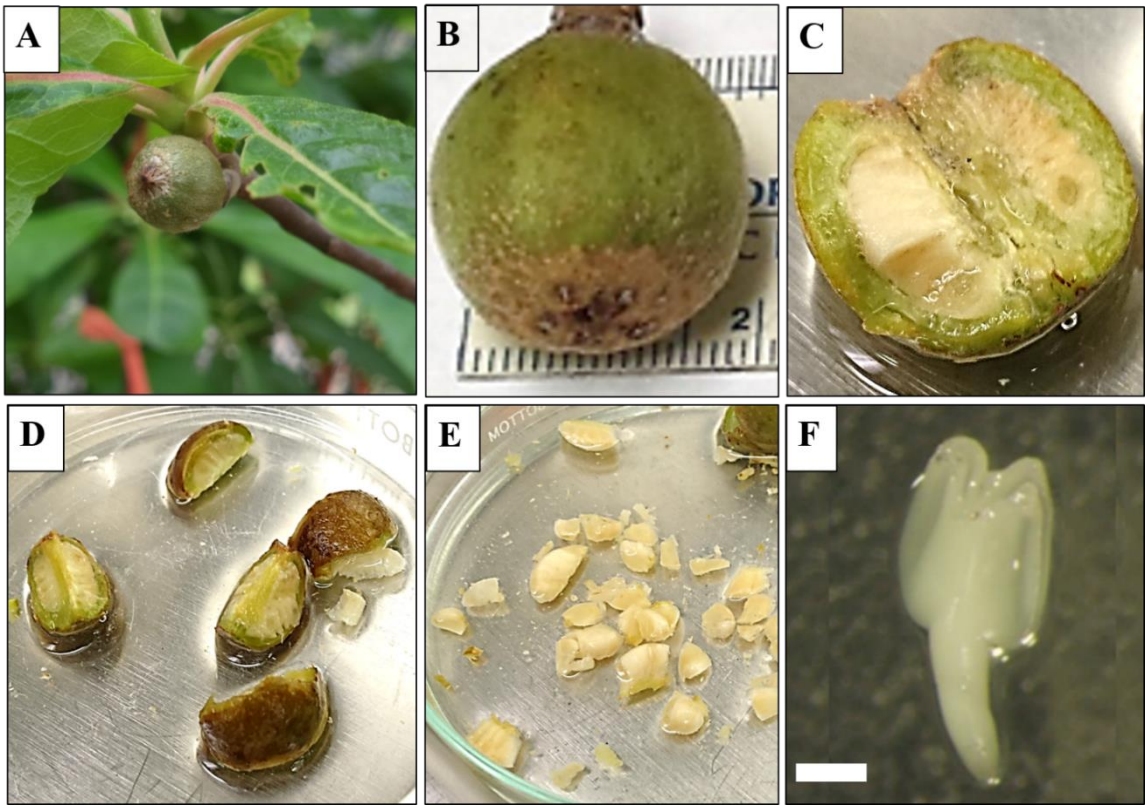

Figure 4-2. Steps to obtain immature zygotic embryo explants. A) immature fruit on stem. B) size of immature fruit is 2 cm in diameter. C) cross-section of immature capsule. D) locules of capsule. E) immature seed, and F) immature zygotic embryo. Bar = 1mm.

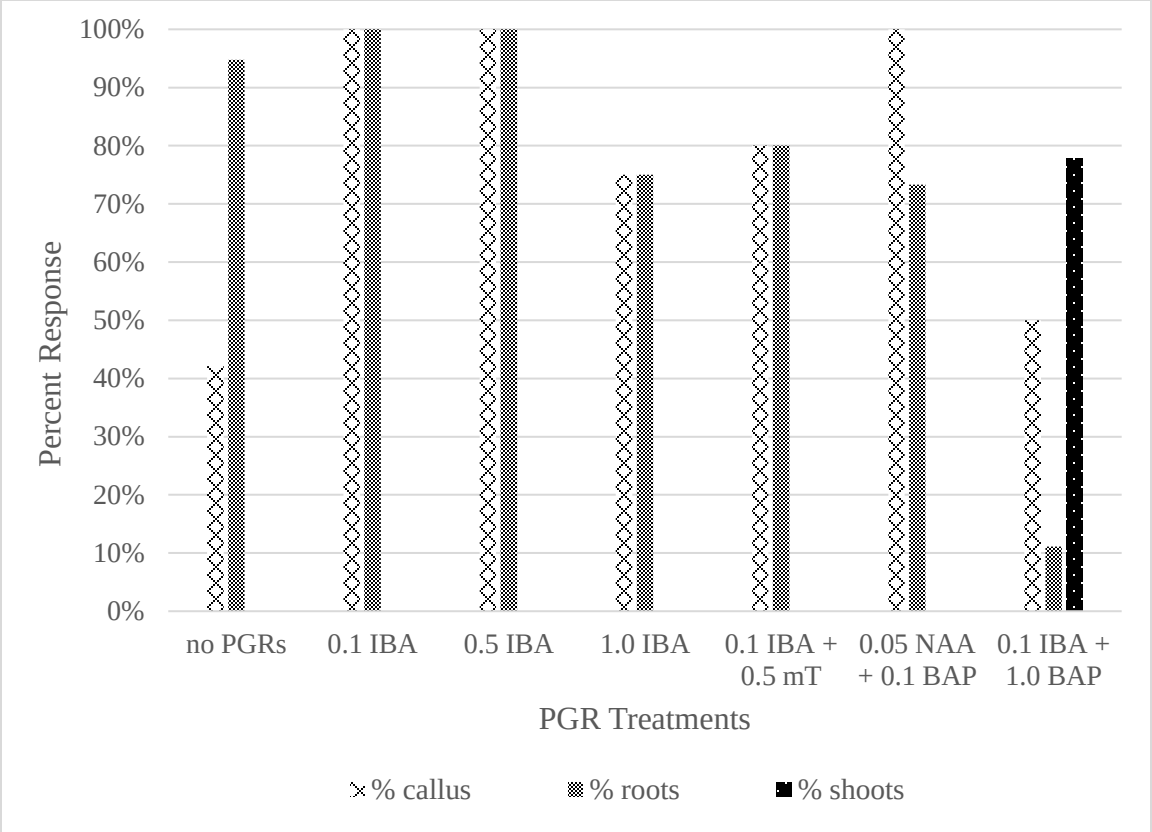

Figure 4-3. Results of Experiment 4. Developmental responses of immature zygotic embryo explants from exposure to 7 different plant growth regulator (PGR) treatments: 0– 1.0 mg/L IBA, 0.1 mg/L IBA in combination with 0.5 mg/L meta-Topolin or with 1.0 mg/L BAP, and 0.05 mg/L NAA in combination with 0.1 mg/L BAP (Table 1): 147 immature zygotic embryos were collected from 9 immature fruits from a single mother tree from Lakemont, GA in August 2014. The immature zygotic embryos for each year were combined together before being randomly assigned to the seven treatments. Each treatment had 21 immature zygotic embryos. This experiment was replicated three times (i.e. each year for 3 years 2014-2016)

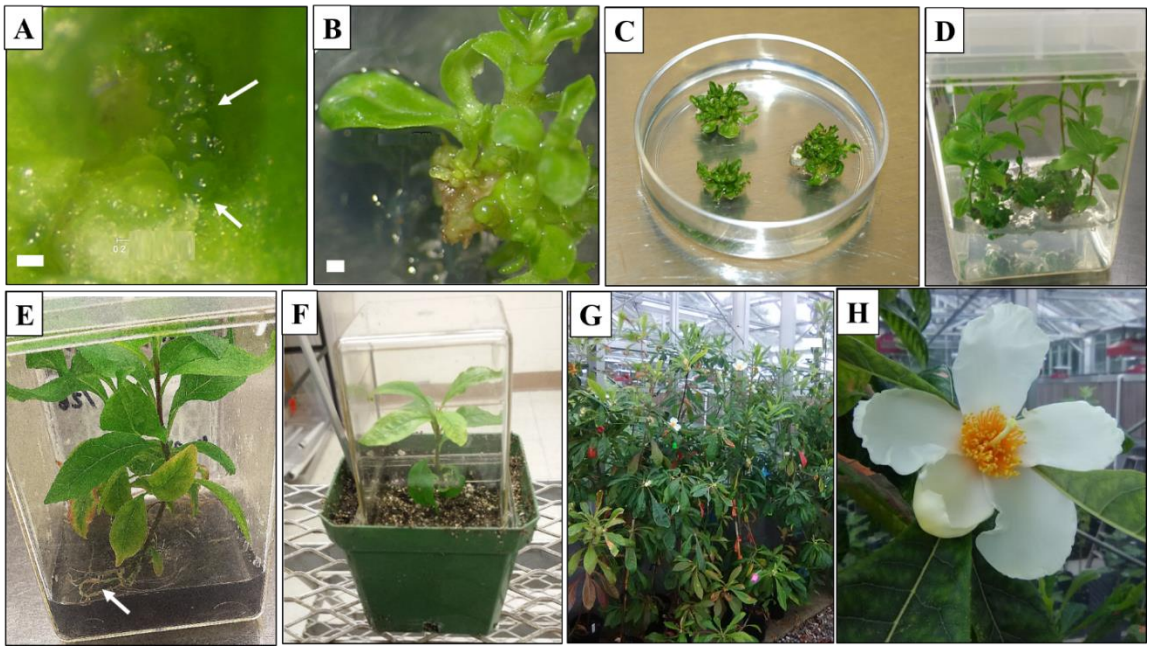

Figure 4-4. Organogenesis from zygotic embryo explants of *Franklinia alatamaha* from the induction of adventitious buds to plants in the greenhouse. A) adventitious buds induced on WPM with 0.1 mg/L IBA and 1.0 mg/L BAP. Bar = 0.2 mm. B) elongation of adventitious shoots. Bar = 0.5 mm. C) multiplication of adventitious buds and shoots. D) Axillary shoot multiplication cultures originated from nodal segments of adventitious shoots, maintained on 0.5 mg/L meta-Topolin and 0.05 mg/L 4CPPU. E) elongated axillary shoot transferred to rooting medium containing 0.25 mg/L activated charcoal and no PGRs; white arrow indicates emerging adventitious roots. F) plantlet following transfer to potting mix and acclimation. G) micropropagated trees growing in the greenhouse, and H) flower produced by tree derived from micropropagated shoot.

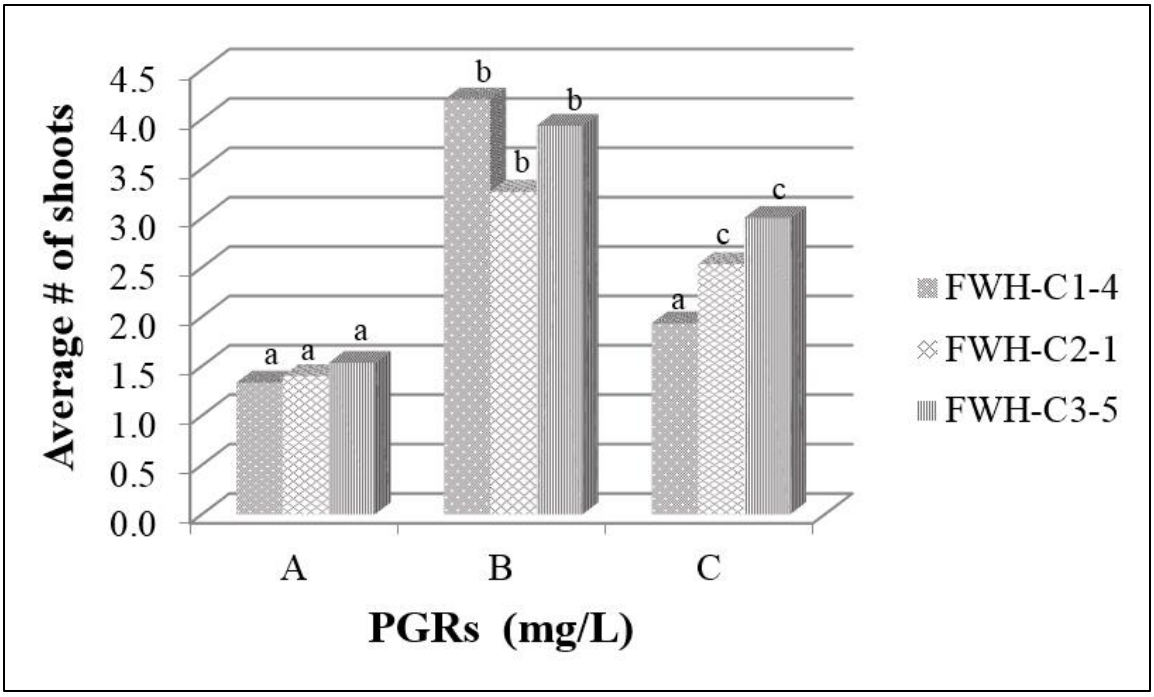

Figure 4-5. Effects of three plant growth regulator (PGR) treatments on axillary shoot multiplication from nodal segments of three *Franklinia* genotypes. A) bud induction medium 0.1 mg/L IBA with 1.0 mg/L BAP. B) 0.5 mg/L meta-Topolin with 0.05 mg/L 4-CPPU, and C) 0.5 mg/L meta-Topolin with 0.1 mg/L 4-CPPU. Tukey's HSD multi-comparison test indicated by letters a, b, c, and those letters that differ are significant at α=0.05. Averages are based on 15 explants per treatment per genotype.

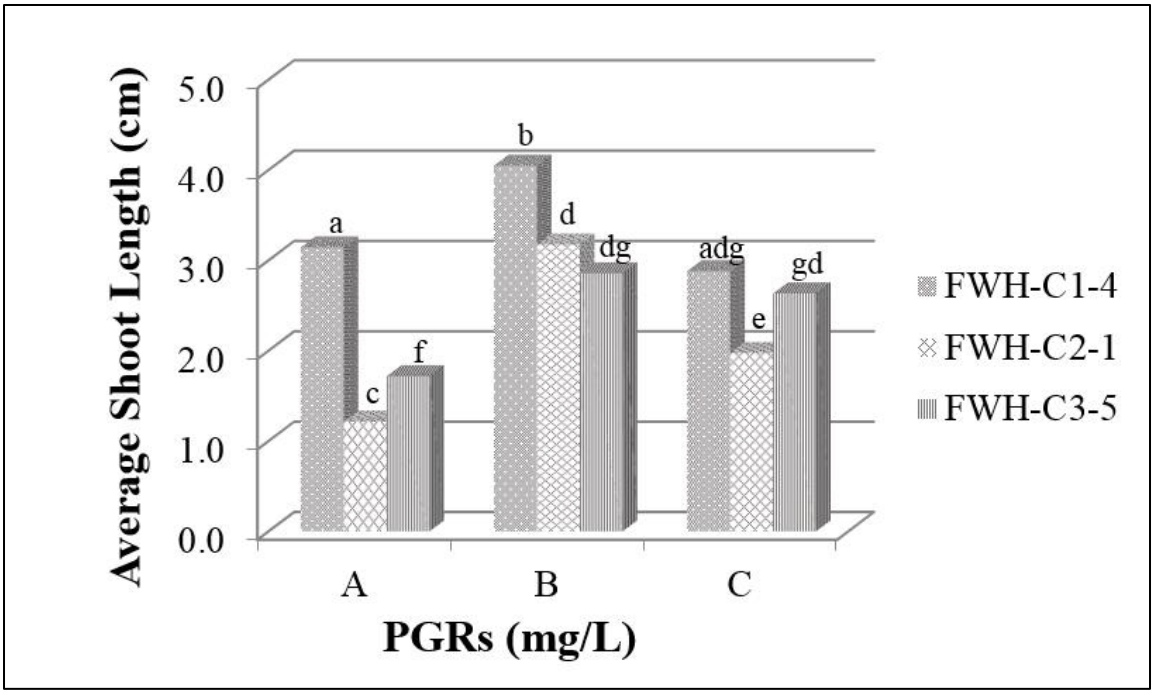

Figure 4-6. Effects of three plant growth regulator (PGR) treatments on axillary shoot elongation from nodal segments of three *Franklinia* genotypes. A) bud induction medium 0.1 mg/L IBA with 1.0 mg/L BAP. B) 0.5 mg/L meta-Topolin with 0.05 mg/L 4-CPPU, and C) 0.5 mg/L meta-Topolin with 0.1 mg/L 4-CPPU. Tukey's HSD multi-comparison test indicated by letters a, adg, b, c, d, dg e, f, gd, and those letters that differ are significant at α=0.05. Averages are based on 15 explants per treatment per genotype.

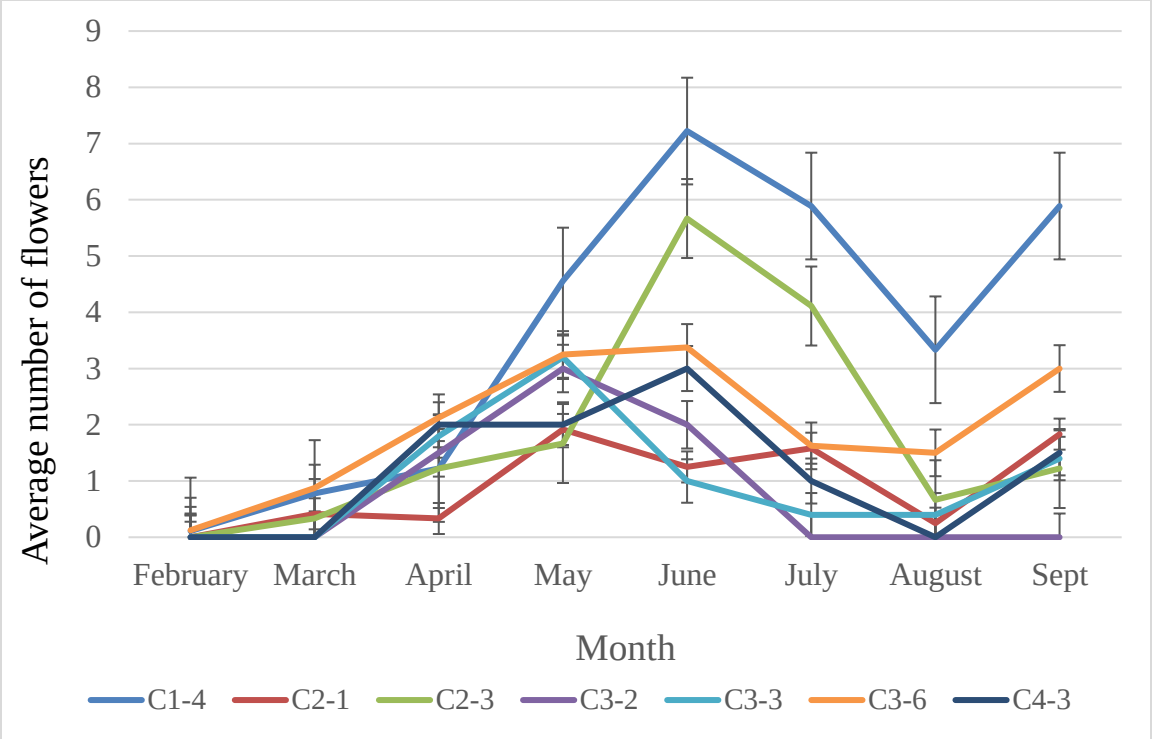

Figure 4-7. Comparison of monthly flowering on trees of different genotypes derived from organogenesis. Bars represent standard error.

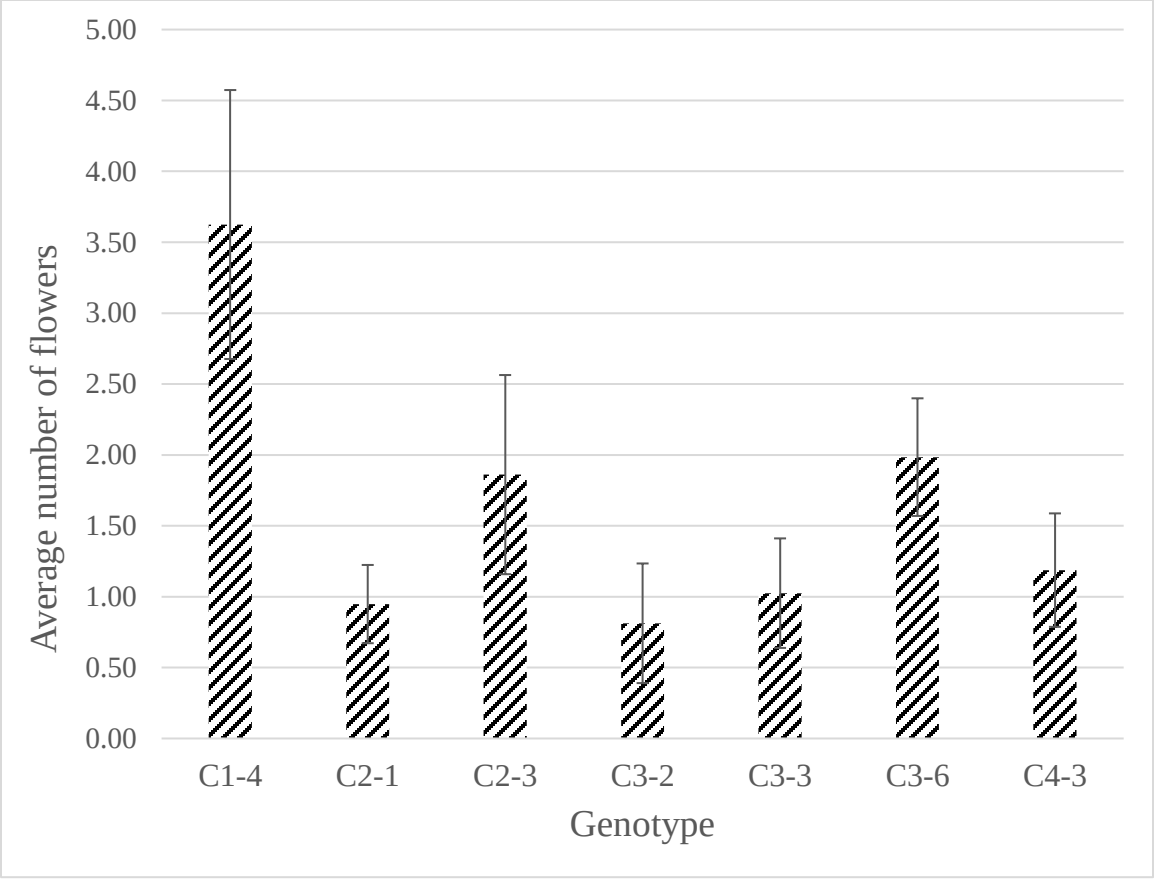

Figure 4-8. Comparison of flowering among different genotypes derived from organogenesis. Bars represent standard error.

CHAPTER 5

GENETIC DIVERSITY AND POPULATION STRUCTURE ANALYSIS OF *FRANKLINIA ALATAMAHA* USING GENOTYPE-BY-SEQUENCING[1]

[1]H.J. Gladfelter, L. Yadav, S.A. Merkle, and H.D. Wilde. To be submitted to *Tree Genetics & Genomes*.

Abstract

Franklinia (*F. alatamaha*) is a woody shrub in the Theaceae family that is of great ornamental interest. It was last observed in the wild in 1804 along the Altamaha River on the coastal plain of Georgia. Seeds and cuttings of *Franklinia* were collected by William Bartram in the 1770s. Although it is extinct in the wild, *Franklinia* has been cultivated in gardens and arboreta for nearly 250 years. The potential for breeding *Franklinia* for traits such as disease resistance depends on the level of genetic diversity in this cultivated population. The genetic diversity of a collection of *Franklinia* leaves from international sources was examined using genotyping-by-sequencing (GBS). Analysis of 9604 high-quality single-nucleotide polymorphisms (SNPs) identified by GBS found that the 76 *F. alatamaha* accessions were clustered into two subpopulations. The larger cluster consisted of 43 accessions, and the smaller cluster contained 33 accessions. These findings were supported by the Principal Component Analysis (PCA) and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), a simple agglomerative hierarchical clustering method that produces a tree or dendrogram showing the population structure. Statistical analysis showed a moderate amount of genetic diversity present in the population, as indicated by the F_{ST} value of 0.09. The genetic diversity was high among individuals of the two subpopulations, as indicated by 94.9% ($1-G_{ST}$) when G_{ST} (H_S/H_T) was 5.1%. The inbreeding co-efficient of the population was negative (F_{IS} = -0.49), indicating *F. alatamaha* is a highly cross-pollinating species. This study showed genetic diversity does exist in the cultivated population of *F. alatamaha* contrary to the hypothesis of minimal or no genetic diversity due to the bottleneck created by one small collection from the original native population.

Introduction

Franklinia alatamaha.

Franklinia alatamaha Bartram ex Marshall is a monotypic genus in the family Theaceae and is now extinct in the wild (Prince and Parks, 2001; Yang et al., 2004). *F. alatamaha* is a botanical mystery, unique in its origin and location, rare in cultivation, and possesses extremely desirable ornamental traits such as its fragrant, white blooms and crimson fall foliage. In cultivation, *F. alatamaha* is grown as a woody ornamental, either as a large shrub or small tree. The species can grow to heights of 6 m or more. The plants bloom continually from July to early September in the Southeastern U.S. Being able to successfully restore this valuable, rare tree to the wild would be of significant importance, not only in conservation of the species, but in its contribution to the habitat from which it originated hundreds of years ago.

Specimens of *F. alatamaha* were collected in 1773 by William Bartram from the only known population site in coastal Georgia, along the banks of the Altamaha River near Fort Barrington, Georgia. The original population discovered by John and William Bartram in 1765 was described as being only two to three acres in size (Harper and Leeds, 1937; Plummer, 1977). No other sites have been identified for this species except for the original site surveyed by the Bartrams. The last known visual documentation of *F. alatamaha* was by plant collector John Lyon in 1803. He noted only six to eight trees covering less than half an acre in the approximate location where the Bartrams first collected *F. alatamaha* (Del Tredici, 2005; Harper and Leeds, 1937; Plummer, 1977). The small population size and geographic isolation of *F. alatamaha* prevented recruitment of new individuals to the population to increase the genetic diversity of the

species. However, cultivated specimens from Bartram's original collection still exist in over 1000 sites worldwide (Bartramsgarden.org, 2019). Two of the oldest living accessions are located at Arnold Arboretum of Harvard University in Boston, MA.

To date, no reports exist in the literature describing an analysis of genetic variation in the existing cultivated specimens of *F. alatamaha*. It is believed that all of these specimens originated from only one location with a very small population size and that they were all derived from material collected by William Bartram in 1773. There is some uncertainty concerning the possibility that others may have collected *F. alatamaha* from the original location for the nursery trade as well (Plummer, 1977). *F. alatamaha* specimens are in collections at Kew Gardens as well as in the French royal gardens in Trianon (Thomson, 1990). If the original seed was collected in 1773 by Bartram, this would have left little time for its propagation and subsequent transport to Europe, in order to be cataloged there in 1774 (Thomson, 1990). Records from 1785 show that the seed was not brought back to Pennsylvania until 1775, after which it took five more years for the plants to produce seed (Marshall, 1785). If Marshall, Lyon, or some other collector(s) did manage to collect material from the original population of *F. alatamaha*, it could have significantly increased the genetic diversity of the currently cultivated stock, and the intraspecific diversity of *F. alatamaha* might prove to be greater than many have assumed (Plummer, 1977; Thomson, 1990; Marshall, 1785). However, *F. alatamaha* naturally can reproduce asexually by layering, which would tend to reduce genetic variation within the population (Del Tredici, 2005). The question is whether the plants at the original site were clones of one or a few individuals, rather than offspring of a sexually reproducing population (Del Tredici, 2005; Gresham and Lipscomb, 1985).

Phytophthora cinnamomi

A very serious problem for commercial production of *F. alatamaha* is crown and root disorders of container-grown trees. The most common symptom of these disorders is root rot. Death can occur in a few days, weeks, months, or linger through the summer and then the plant becomes susceptible to winter injury (dieback) or winter kill (Peterson et al., 1975). The affected root system will show numerously rotted and decaying roots while healthier ones can have small brown lesions (Peterson et al., 1975). Crown canker is less prevalent and the trees appear to be healthy until the canker girdles the trunk causing the same wilt symptoms observed with root rot infection (Peterson et al., 1975). Cankers and root rot can occur simultaneously on the same plant. Koslow and Peterson (1980) were able to show that it was *P. cinnamomi* which produced the disease symptoms following growth *in vitro* of *P. cinnamomi* from infected roots and morphological identification. *F. alatamaha*, originally native to Georgia, is now unable to grow and survive for any length of time in the soil where *P. cinnamomi* is present, primarily in the southeastern US.

Genotype-by-sequencing

Genetic diversity can be determined using molecular methods such as genotyping-by-sequencing (GBS). It is a useful tool to explore genetic diversity in the absence of a reference genome. GBS allows the discovery of single-nucleotide polymorphisms (SNPs) to perform genotyping studies. A SNP is a variation in a single nucleotide that occurs at a specific position in the genome, where each variation is present to some appreciable degree within a population. SNP genotyping is the measurement of genetic variation of SNPs between and among members of a species, which is of the most common types of

genetic variation. Not only are SNPs ideal for assaying genetic diversity and genotyping, they are useful in linkage and association mapping, and molecular marker-assisted selection (MAS) studies based on its features, which include locus-specificity, co-dominant inheritance, high reproducibility, and detection by PCR (Varshney et al., 2005; Kalia et al., 2011).

Today's cultivated population of *F. alatamaha* has undergone significant artificial selection over time. The individuals of this population are either progeny of the original Bartram collection, progeny developed through cross- or self-pollination, or a result of vegetative propagation. There is speculation that the individuals of this cultivated population may be clones since *F. alatamaha* readily propagates by asexual propagation. In this case, no natural variation would be present in the cultivated population of *F. alatamaha*. All of these scenarios would create a serious genetic bottleneck and further narrow the genetic base in the cultivated population of *F. alatamaha*. Genetic analysis using GBS would identify any genetic diversity that remains in the cultivated population of *F. alatamaha* that originated from the wild population collected by Bartram in 1773. This is the first study to identify genetic diversity *F. alatamaha* and to use GBS to determine the existence and amount of genetic diversity that exists in today's cultivated population. The goal is to identify SNPs at the genomic level for examining genetic diversity and population structure. Any diverse genotypes identified could be used for future breeding programs. Also, molecular markers identified through this GBS study can be used for breeding and to survey the population for natural resistance to the devastating root rot pathogen, *P. cinnamomi*. If no genetic variation exists among the cultivated specimens, mutations will need to be introduced to add genetic variation. Without

resistance to the *P. cinnamomi*, *F. alatamaha* will never be able to be restored to the wild nor survive any length of time in soils infected with the *Phytophthora* pathogen.

Materials and Methods

Plant material collection

Leaf material of *F. alatamaha* was collected from private collectors, botanical gardens, university collections, commercial sources, and germplasm banks (Table 5-1). Requests were made to obtain the necessary permits and/or permissions to collect live plant material (leaves and/or seeds) and herbarium specimens from germplasm collections, specifically the Royal Botanical Garden (RBG) of Edinburgh, Scotland. Leaf tissue was acquired from two accessions cultivated in the Arnold Arboretum of Harvard University in Boston, MA; three accessions at Bartram's Garden in Philadelphia, PA, one accession at Awbury Arboretum, Philadelphia, PA; two accessions at Morris Arboretum in Philadelphia, PA; one accession located in the Arboretum Wespelaar in Belgium; two accessions at Botanischer Garten in Frankfurt, Germany; one accession from the New York Botanical Garden; one accession found in the living collection at the Royal Botanical Garden in Edinburgh, United Kingdom; and three accessions at a nursery in Boskoop, Holland. Other known sources come from private landowners such as Jack Johnston of Lakemont, GA; Marshall Adams of Gwinnett, GA; and Richard Dailey in Huntsville, AL. Examples of university sources of *F. alatamaha* include the University of Connecticut, Smith College in Northampton, MA; Scott Arboretum at Swarthmore College in Philadelphia, PA, the University of Georgia in Athens, GA; and the University of British Columbia in Canada. Examples of commercial vendors include Transplant Nursery (Lavonia, GA) and Heritage Seedlings and Liners (Salem, OR).

Genomic DNA extraction

High-quality genomic DNA for GBS was extracted from 150 mg of leaf tissue that was either fresh or dry, flash-frozen in liquid nitrogen, ground to a powder using tissue-lyser II (Qiagen), and processed using the E.Z.N.A. HP DNA kit (Omega Bio-tek, Norcross, GA) following the manufacturer's protocols. The quality and quantity of the genomic DNA was analyzed using Qubit 2.0 fluorometer dsDNA HS assay kit (Invitrogen).

Library preparation

The genomic DNA was sent to the Georgia Genomics and Bioinformatic Core (GGBC) for the GBS library preparation and sequencing. GGBC's internal protocol was adapted from a lab protocol by Jin Hee Shin of Dr. Scott Jackson's research lab Center for Applied Genetics and Technology (CAGT), University of Georgia (2.27.2017). The genomic DNA was quality-checked using SYBR fluorometry and a Fragment Analyzer to determine the concentration and size distribution of the DNA. Samples were normalized to 200 ng in 34 µl before starting the library prep. Samples were fragmented by restriction digestion with enzymes *Taq*αI and *Mse*I (New England Biolabs (NEB), Cat# R0149S and Cat# R0525L, respectively). A common adapter and barcoded adapters (custom-designed oligos from Integrated DNA Technologies (IDT)) were added by ligation with T4 ligase (NEB, Cat # M0202L) before libraries were purified with SPRI beads. Libraries were amplified by PCR (NEB 5X PCR Master Mix, Cat# M0285L and custom-designed oligos from IDT) and again purified with SPRI beads for size selection. Individual libraries were quantified by SYBR fluorometry and qPCR before pooling in equimolar amounts. Final

pooled libraries were quality-checked by Qubit, qPCR, and Fragment Analyzer to determine an accurate concentration.

Sequencing specifications

GBS libraries were sequenced with 5% PhiX and diluted to 2.2 pM for loading. The runs were sequenced on NextSeq high output flow cells with 150 read 1 cycle and without i5 and i7 cycles since the barcoding was in-line. De-mulitiplexing was performed locally using a custom python script developed for inline de-multiplexing.

Sequence analysis

The sequence data were analyzed using FASTQC for quality. The sequences obtained were 150 bp in length. The quality checked sequence was further analyzed using the bioinformatics software program STACKS (Catchen et al., 2013) and the reads were trimmed to 115 bp in length. Further analysis was carried out for diploid species. During STACKS analysis, ragtags were processed in that sequences were de-multiplexed and cleaned up. The cleaned data for each accession was used to create USTACKS with a minimum coverage of 7. Once USTACKS were created for all individuals, loci were grouped across individuals using CSTACK. The allelic state of loci was determined in SSTACK which were converted into mappable genotypes that were used for population genetic statistics in the downstream pipeline.

SNP identification and analysis

STACKS helped identify SNPs across 76 *F. alatamaha* accessions. The identified SNPs were further filtered using VCFtools (Danecek et al., 2011), keeping variants that have been successfully genotyped in 50% of individuals, a minimum quality score of 30 and a minor allele count of 3. Once filtered SNPs were obtained, the vcf file was used to

determine the read depth of the sequences using the 'adegenet' package in R. The data was used to determine the ancestral population cluster of *F. alatamaha* using STRUCTURE (Flaush et al., 2003). STRUCTURE uses admixture model to determine the genetic clusters called K based on the maximum likelihood method. STRUCTURE was initiated with a K value of 10 and same number of iterations. The maximum burning period was set to 100000. STRUCTURE harvester was used to determine natural logarithms of probability data (LnP(K)) and the ΔK. STRUCTURE PLOT version 2.0 was used to create visual structure charts (Ramasamy et al., 2014). Using the same data, the principal component analysis (PCA) was conducted in R version 3.5.1 using the PCAdapt package (Dufroret-Frebourg et al., 2014). The PCA estimation was based on the SNP frequency. A UPGMA tree showing sample relatedness was created in R version 3.5. 1 using the 'adegenet' package (Jombart, 2008).

The SNPs variant call file was used to estimate Nei's genetic distance among the individuals (Nei, 1978). F statistics were also calculated for various population parameters including gene diversity (D_{ST}) among individuals and the corrected gene diversity (D_{STP}); the overall gene diversity (H_T) among populations and the corrected gene diversity (H_{TP}); the fixation index (F_{ST}) and corrected fixation index (F_{STP}) based on population; the inbreeding coefficient (F_{IS}); observed heterozygosity (H_O) and genetic diversity (H_S) within population. G_{ST}, the proportion of species genetic diversity attributed to among-population variation, was calculated as $1 - (H_S / H_T)$. All of these calculations were done using HierFstat (De Meeûs & Goudet, 2007) and the 'adegenet' package (Jombart, 2008) in R version 3.5.1.

Results

SNP identification and genetic analysis

Genetic diversity analysis of *F. alatamaha* accessions collected from around the world (Europe, Canada, New Zealand, and the United States) from botanical gardens, arboreta, and private collectors (Table 5-1) was performed using Nextseq 500 high output flow cells. STACKS was used to create genome assembly in the absence of a reference genome. Following genome assembly and SNP calling, a total of 84,000 SNP markers were obtained across 95 *F. alatamaha* accessions. The SNPs identified were further filtered keeping variants that were successfully genotyped in 50% of individuals, with a minimum quality score of 30 and a minor allele count of 3. The filtering yielded 9604 high-quality SNPs. Twenty out of 95 accessions were gamma irradiated mutants from the same genotype source. The first GBS analysis of the 95 accessions indicated the mutants were not providing an accurate representation of the genetic diversity of the cultivated population of *F. alatamaha* since they originated from one genotype source. The final number of accessions following the removal of the gamma irradiated mutants was 76 and these were used to conduct a new genetic diversity analysis. The average read depth of the filtered SNPs was ≥ 9 (Figure 5-1).

Population structure and genetic relationship

The genetic structure of the 76 *F. alatamaha* accessions was examined through a STRUCTURE analysis of the SNP data. STRUCTURE was run with K = 1 to K = 10 and subpopulations were determined by STRUCTURE Harvester to be K = 2, which indicated two subpopulations (P1 and P2) as the genetic structure estimated for the 76 accessions of *F. alatamaha*. The results were supported by LnP (K) variance and the largest delta K value

(Figure 5-2). Subpopulation P1 consisted of 33 genotypes, and P2 contained 43 genotypes (Figure 5-3). The analysis helped to identify two ancestral subpopulation that consisted of both pure and admixture accessions. Subpopulation P1 was comprised of 8 pure and 25 admixture accessions, while subpopulation P2 had 18 pure and 25 admixture accessions. Of the 76 accessions of *F. alatamaha* evaluated in this study, 32% were pure type, and 44% were admixtures.

PCA was carried out with the genotypic data, and the findings were consistent with the STRUCTURE analysis. PCA revealed two ancestral *F. alatamaha* subpopulations and two components explained 17% of the total variance (Figure 5-5). Pure accessions were more scattered apart, while admixtures were scattered between the two clusters (Figure 5-4). The larger cluster P2 included accessions mostly from the United States but also two accessions from Frankfurt, Germany; three accessions from Warsaw, Poland; two from British Columbia, Canada; two from the Royal Botanical Garden-Kew; and one living accession along with a 61 year-old herbarium accession from the Royal Botanical Garden-Edinburgh. The smaller cluster P1 also included accessions mostly from the United States; one accession from Warsaw, Poland; one accession from Raglan, New Zealand; one accession from the Royal Botanical Garden-Kew; one accession from Boskoop, Netherlands; one accession from British Columbia, Canada; and a 177 year-old (1842) herbarium accession from the Royal Botanical Garden-Edinburgh.

The UPGMA tree also supported the findings of STRUCTURE and PCA; however there were a few discrepancies (Figure 5-6). These disputed accessions were admixture types which consisted of almost 50% contribution from both ancestral subpopulations. The UPGMA tree also identified admixture accessions that were genetically distant from other

accessions in both subpopulations. This result was supported by PCA which identified the genetically most diverse individuals (A07, E08, and H01) in the population (Figure 5-6).

Population statistics

Genetic diversity was analyzed within and between the *F. alatamaha* populations. The average heterozygosity for the species (H_T) was 0.4038, and the average heterozygosity within populations (H_S) was 0.4085 (Table 5-2). The proportion of species genetic diversity attributed to variation among populations (G_{ST}) was calculated to be 5.1%, a low value indicating significant gene flow within the species. Low values were also observed for gene diversity among individuals (D_{ST}) 0.0222 and the fixation index (F_{ST}) 0.0994 (Table 5-2). The low value of F_{ST} is consistent with G_{ST} indicating a higher degree of similarity between the two subpopulations. The inbreeding coefficient F_{IS} of the *F. alatamaha* population under study was -0.4902, a negative value indicative of a highly outcrossing population (Table 5-2). The high negative value suggests that the population under study is not under Hardy-Weinberg equilibrium. Nei genetic diversity and genetic gain were also estimated among the 76 *F. alatamaha* accessions and between the subpopulation clusters identified by PCA. Genetic distance between individuals is shown in the heatmap (Figure 5-7). The genetic distance between individuals ranged from 0.2-1.2 (Figure 5-7). The highest genetic distance was identified between accessions D11 and E11, the latter being the 177 year-old herbarium accession from the Royal Botanical Garden-Edinburgh. These analyses also found more variation among individuals than between the two subpopulation clusters (Table 5-2).

Discussion

Genetic diversity analysis

A GBS approach was used to characterize the genetic structure and diversity of a *F. alatamaha* germplasm collection from a cultivated population since this species is extinct in the wild. High-quality genomic DNA was successfully isolated from two very old herbarium samples, 177 and 61 years-old. All the living trees of *F. alatamaha* in the world today are believed to be the progenies of the collection made by Bartram in 1773 from a single population found growing in the coastal piedmont region of Georgia along the Altamaha River. To our knowledge, no other GBS studies with other plant species have successfully used DNA from herbarium specimens as old as 177 years, and which produced high-quality sequence reads for GBS analysis.

Controlled breeding can reduce genetic diversity. With Bartram's single collection of *F. alatamaha*, any controlled breeding from this small sample population would reduce the genetic diversity even further. The hypothesis before this GBS study was that there would be low or no genetic diversity present in the cultivated population, specifically among the 76 accessions. In the wild, *F. alatamaha* was only ever found in one isolated location. This in itself would contribute to reduced genetic diversity, and the individuals in this isolated population could only breed among each other. Surprisingly, significant genetic diversity was observed in the cultivated population of *F. alatamaha* represented by the 76 accessions in this study using GBS analysis. One explanation for this observation is that Bartram did not limit his collection to one tree but collected from several different trees. The trees could have been near to each other or very far apart. Another explanation

consistent with the GBS results is that other plant collectors may have collected seed from the original location for the nursery trade.

Population Structure

A cultivated population of 76 accessions of *F. alatamaha* was analyzed using STRUCTURE, which identified two distinct subpopulations based on the maximum likelihood parameter. Subpopulation P1 and P2 consisted of 33 and 43 accessions respectively. The results from the STRUCTURE analysis was confirmed by both PCA and the UPGMA tree. The accessions from a cultivated population of *F. alatamaha* used in this study are very diverse. Accessions included a herbarium sample from 1842 (177 years-old) to live trees growing in arboreta, botanical gardens, and private gardens. The herbarium specimens from 1842 (E11) and 1957 (E12) belonged to subpopulation P1 and subpopulation P2 respectively. Both accessions are admixtures. The pure accessions based on STRUCTURE analysis included the living and youngest (age) accessions. One explanation is that the seeds collected by Bartram in 1773 were grown in isolation. In the absence of cross-pollination, the trees would self, and over the years of growing in isolation and self-pollination, admixtures collected by Bartram would become pure types. PCA and UPGMA analysis identified diverse accessions such as A07 and H08 in the subpopulations which are the results of admixture of two pure types from P1 and P2. These two diverse accessions may contain unique and valuable traits to exploit for future breeding among the cultivated population of *F. alatamaha* trees. An example would be to breed for *Phytophthora* resistance.

Statistical Characterization

GBS analysis of *F. alatamaha* showed significant genetic diversity present within each subpopulation but low genetic diversity among the two subpopulations. The G_{ST} value was 5.1% indicating low genetic diversity among the populations. The calculate F_{ST} of the population was 0.09 which indicates there is moderate differentiation between the populations (Holsinger et al., 2009). The cultivated population of *F. alatamaha* was hypothesized to be more uniform, but the F_{ST} value indicated otherwise, which is of significant interest from both a historical and genetic perspective since the only collection known was from Bartram. These two subpopulations share more alleles with each other which is expected since they are the progeny from the same original Bartram collection. Further, the two subpopulations can cross-pollinate with each other and share alleles. The inbreeding coefficient F_{IS} of the cultivated population of *F. alatamaha* is negative (-0.49) suggesting a high amount of genetic diversity in the individuals of the subpopulations. A negative F_{IS} value indicates very low inbreeding which has also been documented in a GBS study with *Camellia sinensis*, also a member of the Theaceae family (Niu, 2019).

Conclusions

A GBS approach was used to characterize the genetic structure and diversity of a *F. alatamaha* germplasm collection from a cultivated population since this species is extinct in the wild. High-quality genomic DNA was successfully extracted from 76 accessions including two herbarium samples (177 and 61 years-old). SNPs were identified by the STACKS platform and used to develop a genome-wide genetic variant file. The genetic variation present in the germplasm collection was examined by STRUCTURE, a model-based Bayesian analysis, UPGMA tree, and PCA, a distance-based method.

Together these analyses showed two subpopulation clusters present among the 76 accessions of *F. alatamaha*. The population statistics yielded a low G_{ST} value, indicating that the proportion of genetic diversity between the two subpopulations was low, while the proportion of genetic diversity within the subpopulations was high. Minimal differentiation between the two *F. alatamaha* subpopulations was also demonstrated by the low F_{ST} value. These results show there is genetic diversity present in the 76 accessions of *F. alatamaha* in this study. The SNPs identified can be used to identify markers in the genome for breeding purposes. Seed from living accessions of *F. alatamaha* found to be diverse or of any interest could be collected and stored for future genetic studies. Also, plant material from these and any of the living accessions could be cryo-preserved for future genetic studies and preservation using plant tissue culture methods. The genetic diversity found will be useful to develop new genetic populations and to map useful traits important for marker-assisted breeding programs with *F. alatamaha*. To our knowledge this is the first population genetic analysis of the cultivated germplasm *F. alatamaha* and using GBS as an approach to studying the genetic diversity. The population structure and genetic differentiation revealed by this method will benefit further genetic studies, germplasm protection, and breeding to develop root-rot resistance to a devastating plant pathogen *P. cinnamomi*. Genetic resistance to *P. cinnamomi* will allow *F. alatamaha* to be grown successfully in soils infested with this organism, such as the Southeastern US, specifically in coastal Georgia where this tree was once native growing along the banks of the Altamaha River.

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Table 5-1. Locations of 95 accessions of *F. alatamaha* used in GBS analysis

Accession No.	Source	Location	Latitude	Longitude
42-Spruce-100	42nd & Spruce St- Philadelphia, PA	Philadelphia, PA, USA	39.952482	-75.206964
AW-00305	Arboretum Wespelaar (Belgium)	Wespelaar, BE	50.958635	4.633985
AA-2428-3*A	Arnold Arboretum of Harvard Univ.	Boston, MA, USA	42.307875	-71.120754
AA-254-81*C	Arnold Arboretum of Harvard Univ.	Boston, MA, USA	42.307875	-71.120754
AWA	Awbury Arboretum	Philadelphia, PA, USA	40.050870	-75.168080
BG-CrG-2013	Bartram's Garden (Carr Garden)	Philadelphia, PA, USA	39.932805	-75.212204
BG-CmG	Bartram's Garden (Common Garden)	Philadelphia, PA, USA	39.932805	-75.212204
BG-RT	Bartram's Garden (Resurrection Tree)	Philadelphia, PA, USA	39.932805	-75.212204
XX-0-FRT-2012/682	Botanischer Garten Frankfurt am Main	Frankfurt am Main, GE	50.128731	8.657313
XX-0-FRT-2000/545	Botanischer Garten Frankfurt am Main	Frankfurt am Main, GE	50.128731	8.657313
CCP-T2	Clayton Conservation Park (Tree 2)	Clayton, GA, USA	34.883642	-83.396641
CFM-T1	Concord Friends Meeting (Tree 1)	Concordville, PA, USA	39.885685	-75.519878
CFM-T2	Concord Friends Meeting (Tree 2)	Concordville, PA, USA	39.885685	-75.519878
CFM-T3	Concord Friends Meeting (Tree 3)	Concordville, PA, USA	39.885685	-75.519878
HS	Transplant Nursery	Lavonia,, GA, USA	44.892143	-122.914453
HHS	Highlands Historical Society	Highlands, NC, USA	35.055735	-83.196829
2005-32C	Holden Arboretum	Kirkland, OH, USA	41.609019	-81.299959
2005-32A	Holden Arboretum	Kirkland, OH, USA	41.609019	-81.299959
94-230B	Holden Arboretum	Kirkland, OH, USA	41.609019	-81.299959
2005-32B	Holden Arboretum	Kirkland, OH, USA	41.609019	-81.299959
1983-0644*C	Longwood Gardens	Kennett, PA, USA	39.871255	-75.674677
2016-0161*D	Longwood Gardens	Kennett, PA, USA	39.871255	-75.674677
1976-0424*E	Longwood Gardens (Bicentennial tree)	Kennett, PA, USA	39.871255	-75.674677
MA-WT2	Marshall Adams	Gwinnett County, GA, USA	33.885943	-83.357701
MA-var1	Marshall Adams Variant #1	Gwinnett County, GA, USA	33.885943	-83.357701
MA-var2	Marshall Adams Variant #2	Gwinnett County, GA, USA	33.885943	-83.357701
MA-var3	Marshall Adams Variant #3	Gwinnett County, GA, USA	33.885943	-83.357701
2017-057*A	Morris Arboretum of the U of PA	Philadelphia, PA, USA	40.091618	-75.224881
MA-1948	Morris Arboretum of the U of PA	Philadelphia, PA, USA	40.091618	-75.224881
437/2011*B	New York Botanical Garden	Bronx, NY, USA	40.862429	-73.877301
292/2016*A	New York Botanical Garden	Bronx, NY, USA	40.862429	-73.877301
PCC-1957 #1	Peggy Crosby Center	Highlands, NC, USA	35.049430	-83.195705
PCN-NZ-1	Peter Cave Nursery	Raglan, NZ	-37.821821	174.833599
RMB-NL	Rein en Mark Bulk	Boskoop, Holland, NTL	52.084952	4.682554
RD-AL	Richard Daily	Huntsville, AL, USA	34.631132	-86.570748
11390-4/44-II	Rogów Arboretum of Warsaw Univ. of Life Sci.	Rogów, PO	52.162219	21.046606
11390-4/44-IV	Rogów Arboretum of Warsaw Univ. of Life Sci.	Rogów, PO	52.162219	21.046606
11390-4/59-IIa	Rogów Arboretum of Warsaw Univ. of Life Sci.	Rogów, PO	52.162219	21.046606
11390-4/59-IIb	Rogów Arboretum of Warsaw Univ. of Life Sci.	Rogów, PO	52.162219	21.046606
2090*1	Royal Botanic Gardens Edinburgh (herbarium)	Edinburgh, SL	55.963493	-3.210347
2090*7	Royal Botanic Gardens Edinburgh (herbarium)	Edinburgh, SL	55.963493	-3.210347

RBGE-2016-0272	Royal Botanic Gardens Edinburgh	Edinburgh, SL	55.963493	-3.210347
2015-2143	Royal Botanic Gardens KEW	Wakehurst Place, UK	51.478269	-0.290541
2016-561	Royal Botanic Gardens KEW	Wakehurst Place, UK	51.478269	-0.290541
2013-52	Royal Botanic Gardens KEW	Wakehurst Place, UK	51.478269	-0.290541
91-334*B	Scott Arboretum of Swarthmore College	Swarthmore, PA, USA	39.906406	-75.352451
91-243*F	Scott Arboretum of Swarthmore College	Swarthmore, PA, USA	39.906406	-75.352451
93-007*C	Scott Arboretum of Swarthmore College	Swarthmore, PA, USA	39.906406	-75.352451
large tree 37A	Scott Arboretum of Swarthmore College	Swarthmore, PA, USA	39.906406	-75.352451
91-243*D	Scott Arboretum of Swarthmore College	Swarthmore, PA, USA	39.906406	-75.352451
SW-5PEL	Scott Wade (5 Prince Eugene Lane)	Media, PA, USA	39.903055	-75.419018
SW-KR	Scott Wade (Kuntz residence)	Media, PA, USA	39.899744	-75.418448
SW-RVP	Scott Wade (Rose Valley Park, Manchester Rd)	Media, PA, USA	39.905101	-75.388371
SW-SLT-1	Scott Wade (Stoneleigh Tree1)	Villanova, PA, USA	40.042261	-75.340521
SW-SLT-2	Scott Wade (Stoneleigh Tree2)	Villanova, PA, USA	40.042261	-75.340521
SMG-1931	Sister Mary Grace Burns Arb of Georgian Court	Lakewood, NJ, USA	40.098895	-74.226512
SMG-1898	Sister Mary Grace Burns Arb of Georgian Court	Lakewood, NJ, USA	40.098895	-74.226512
37717-A	Smith College	Northampton, MAs, USA	42.318775	-72.639687
36804-A	Smith College	Northampton, MA, USA	42.318775	-72.639687
493PA-C	Smith College	Northampton, MA, USA	42.318775	-72.639687
493PA-A	Smith College	Northampton, MA, USA	42.318775	-72.639687
Bartram-FWH-C3-6	Tissue Culture Genotype (FWH-C3-6)	Athens, GA, USA	33.885943	-83.357701
94-001	Tyler Arboretum	Lima, PA, USA	39.934897	-75.441629
2006-049-003	Tyler Arboretum	Lima, PA, USA	39.934897	-75.441629
2009-00029	UCONN Arboretum, University of CT	Storrs, CT, USA	41.810286	-72.255971
USBG-2012-0876	United States Botanical Garden	Washington D.C., USA	38.888095	-77.012969
USBG-Barth	United States Botanical Garden (BP)	Washington D.C., USA	38.886835	-77.012095
NA-62595-2	United States National Arboretum	Washington D.C., USA	38.912147	-76.965885
UBCBG-B	University of British Columbia Botanical Garden	Vancouver, BC, CA	49.254968	-123.248160
UBCBG-A	University of British Columbia Botanical Garden	Vancouver, BC, CA	49.254968	-123.248160
UBCBG-C	University of British Columbia Botanical Garden	Vancouver, BC, CA	49.254968	-123.248160
WTS-T1	Westtown School (Tree 1)	West Chester, PA, USA	39.948432	-75.538317
WTS-T2	Westtown School (Tree 2)	West Chester, PA, USA	39.948432	-75.538317
WTS-T3	Westtown School (Tree 3)	West Chester, PA, USA	39.948432	-75.538317
WHF-52	Whitehall Forest #52	Athens, GA, USA	33.885943	-83.357701
CHC	Cave Hill Cemetery, KY	Crestwood, KY, USA	38.249873	-85.714916
11390-4/59-I	Rogów Arboretum of Warsaw Univ. of Life Sci.	Rogów, PO	52.162219	21.046606

Table 5-2. Genetic diversity statistics of STACKS data.

H_O	H_S	H_T	D_{ST}	H_{TP}	D_{STP}	D_{EST}	F_{ST}	F_{IS}
0.6066	0.4085	0.4308	0.0222	0.4530	0.0445	0.0752	0.0994	-0.4912

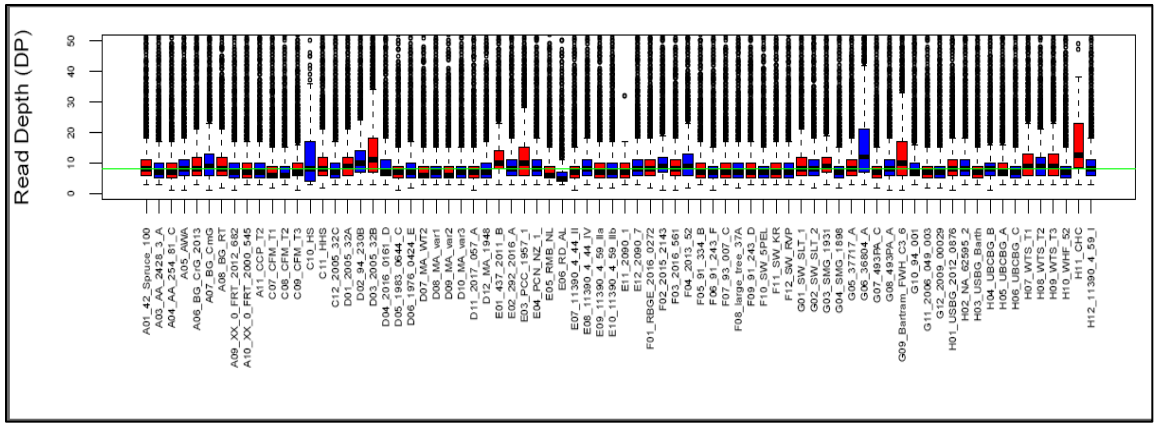

Figure 5-1. Read depth of sequencing data.

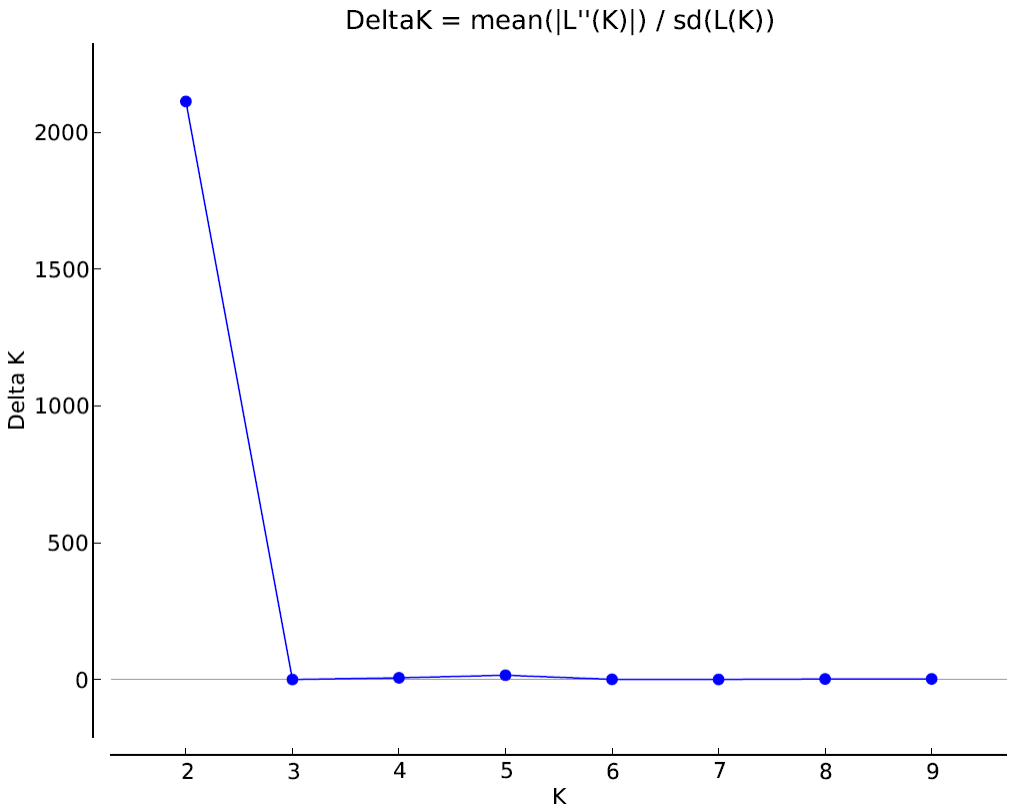

Figure 5-2. Delta K. Support for two optimal clusters based on delta K estimates from GBS data.

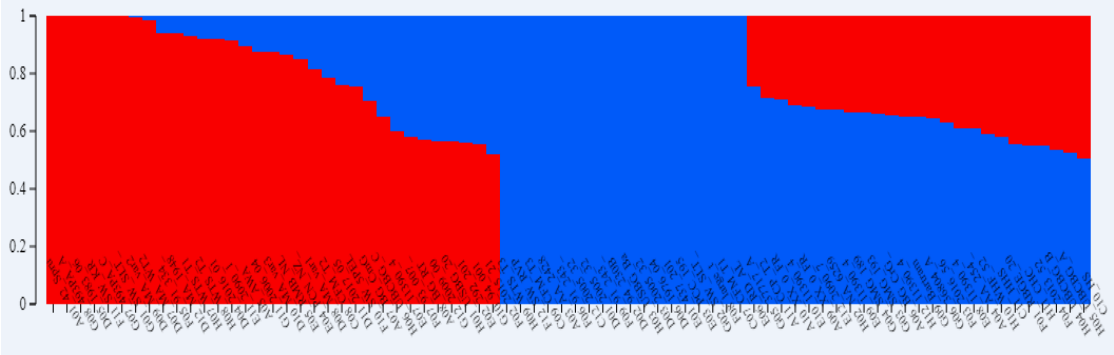

Figure 5-3. STRUCTURE analysis results with STACKS data.

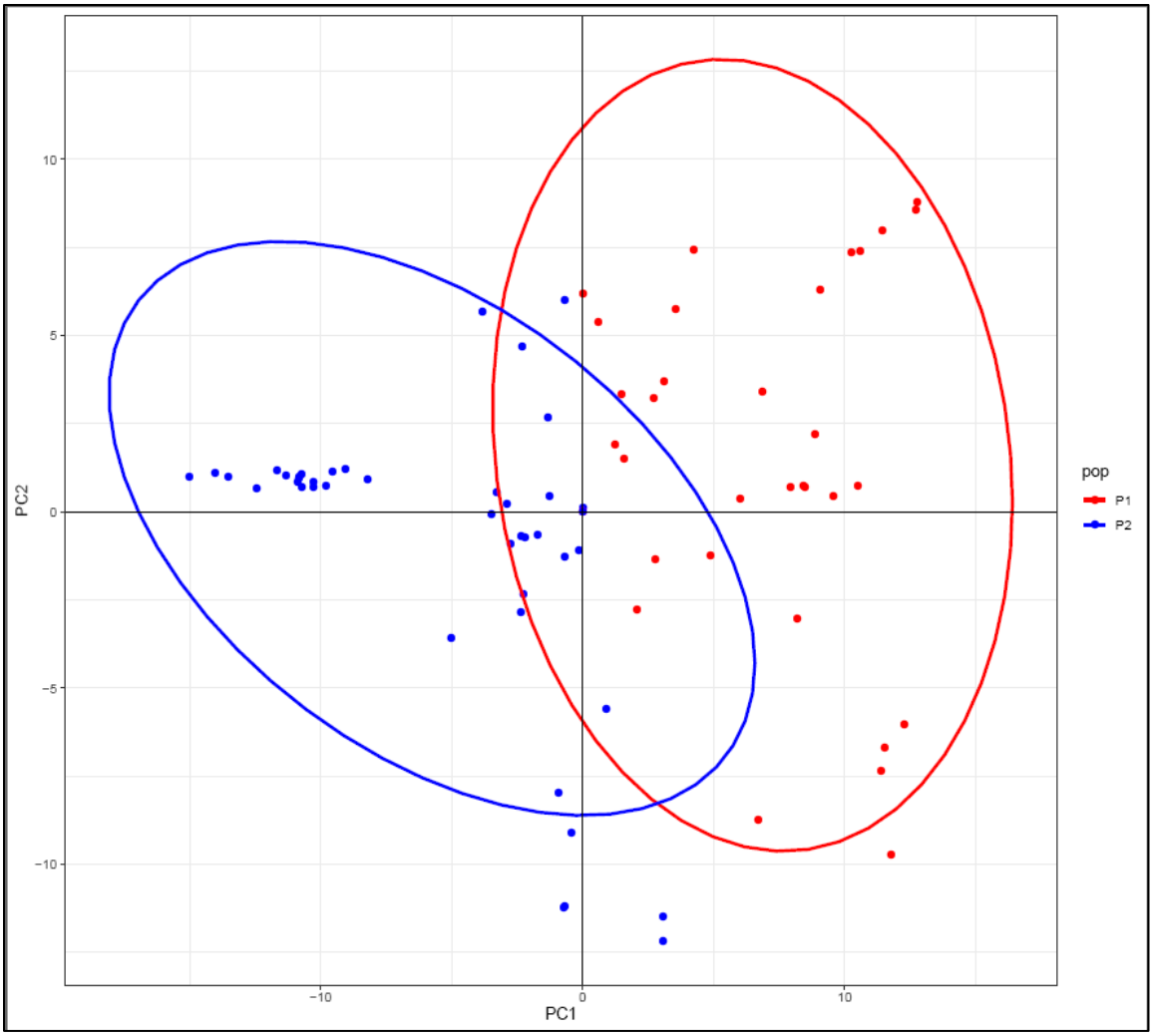

Figure 5-4. Principal component analysis (PCA). A) PCA with single nucleotide polymorphism data from the STACKS pipeline. Populations P1 and P2 as defined by STACKS analysis.

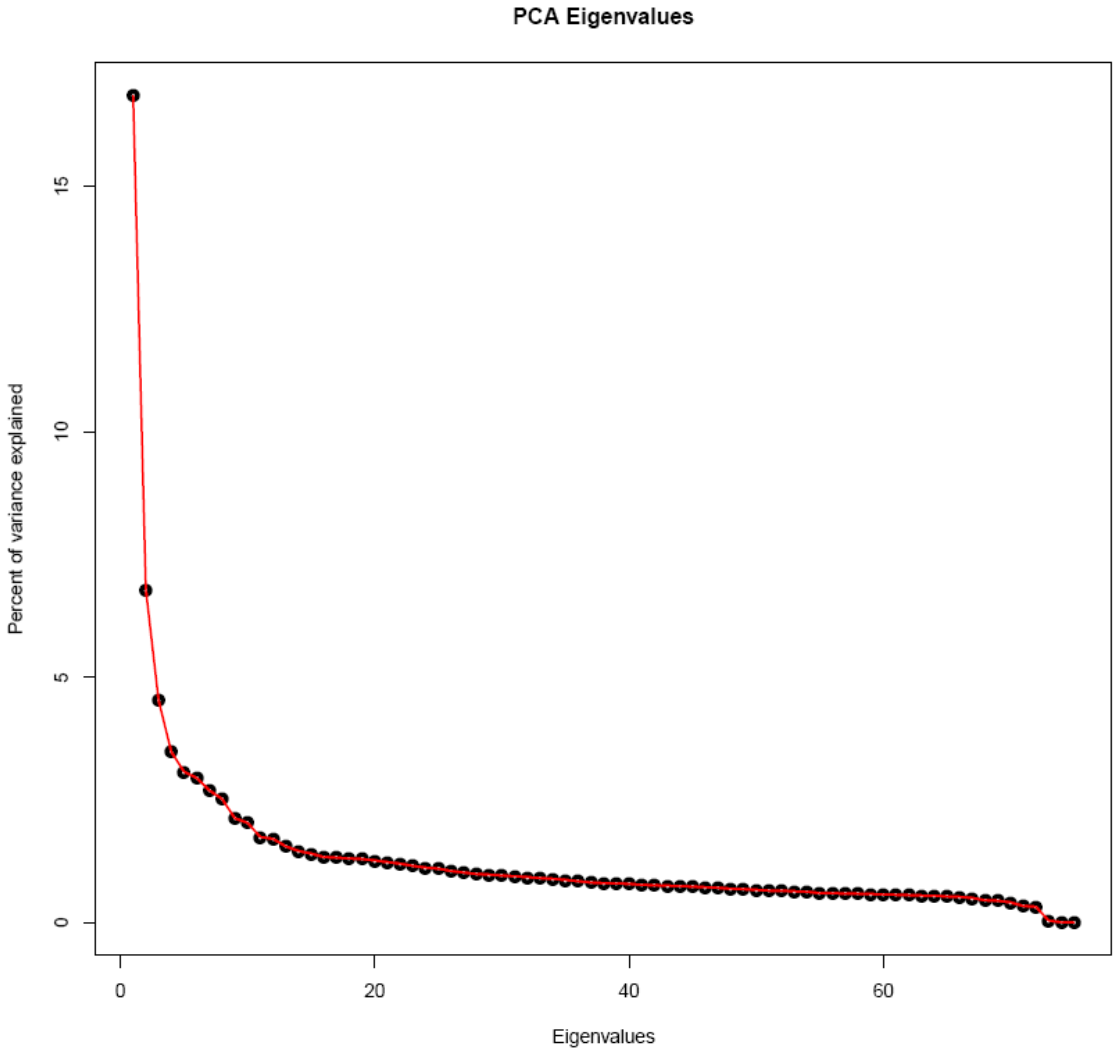

Figure 5-5. Principal component analysis (PCA). B) Proportion of variance explained by principal components

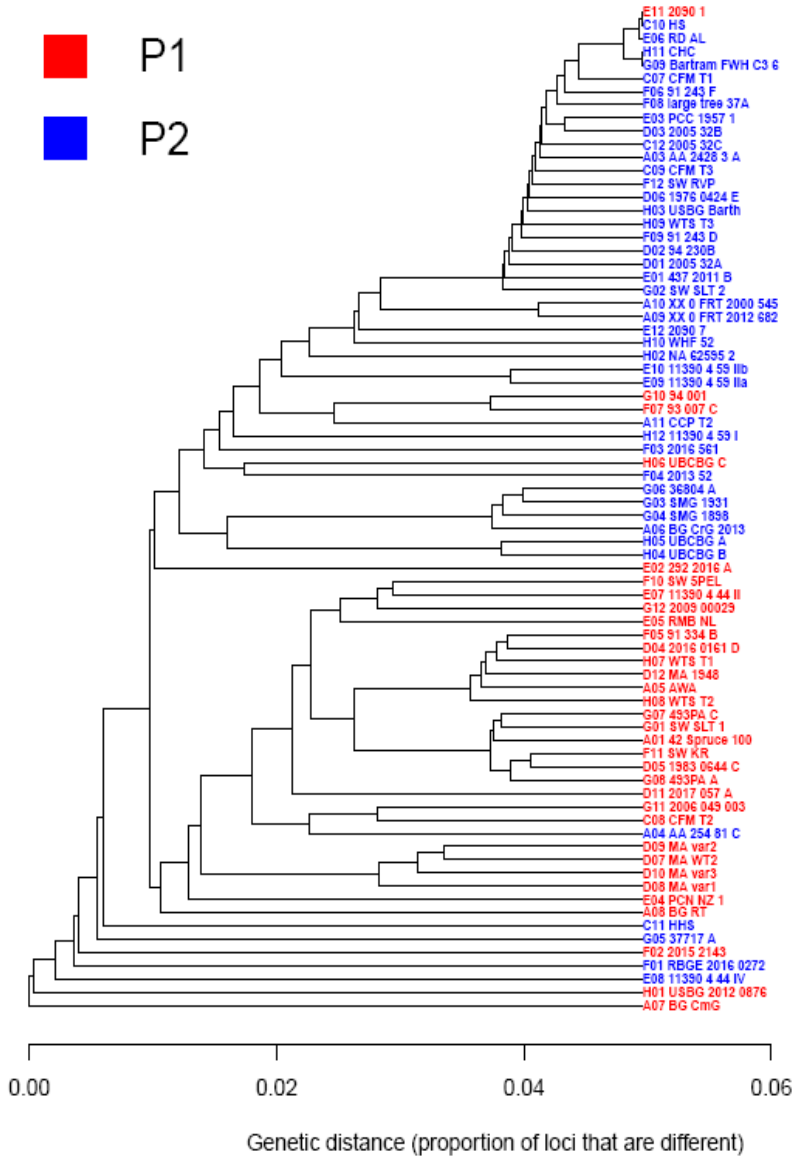

Figure 5-6. Phylogenetic relationships of *Franklinia alatamaha* accessions.

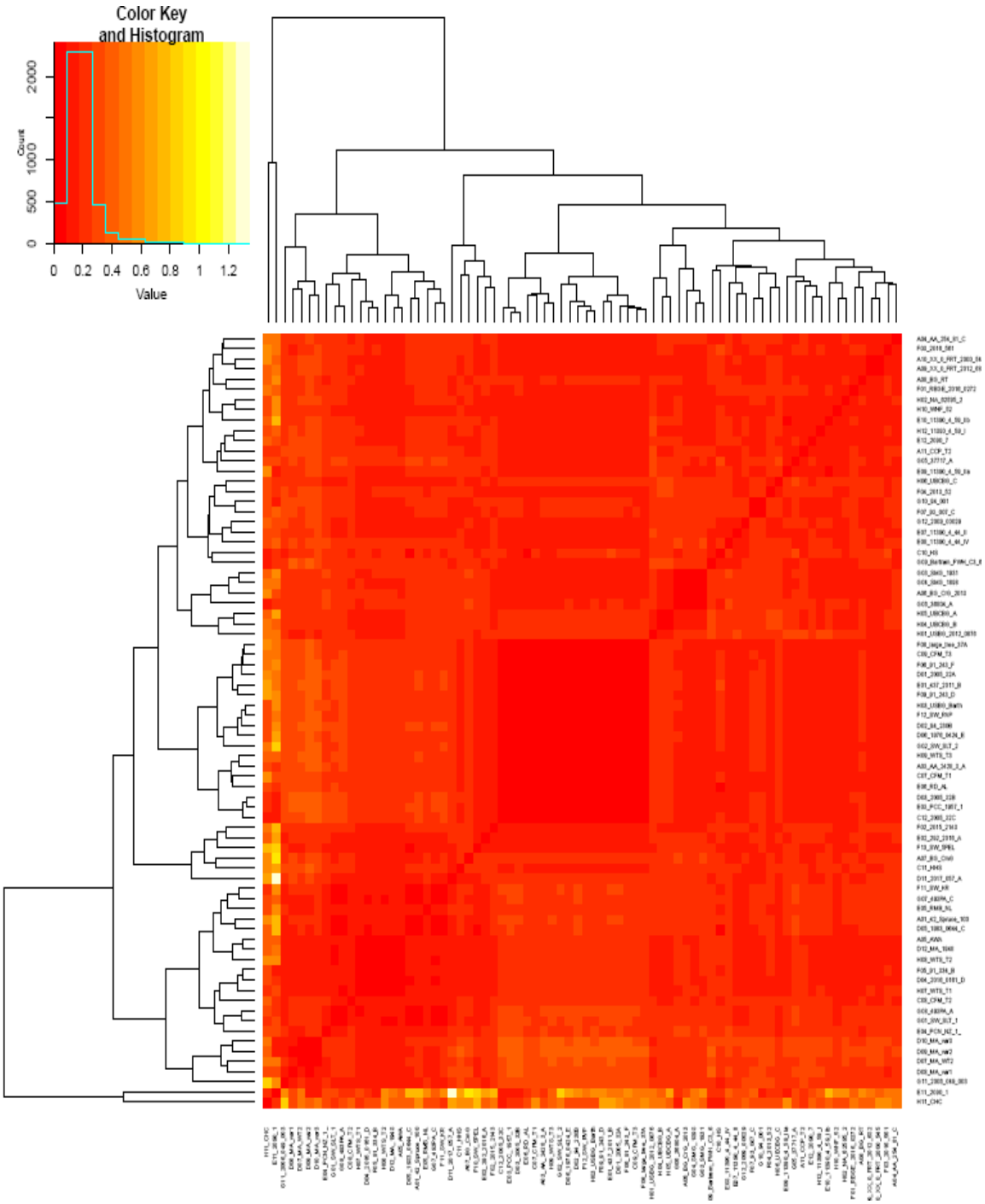

Figure 5-7. A weighted neighbor-joining distance heat-map of *Franklinia alatamaha* accessions.

CHAPTER 6

CONCLUSION

Stewartia somatic embryogenesis

Somatic embryogenesis was successfully induced from immature zygotic embryo explants of seven *Stewartia* species—five Asian and two North American. Additionally, three North American cultivars, *S. ovata* 'Red Rose', *S. malacodendron* 'Delmarva', and *S. ovata* 'Royal Purple' from The Polly Hill Arboretum were propagated by somatic embryogenesis using a variety of PGRs or no PGR treatment at all. Picloram at low concentrations, such as 0.05 and 0.1 mg/L, was more successful at producing somatic embryos with the North American *Stewartia* species. Most of the Asian *Stewartia* species responded best to 2,4-D at concentrations of 2.0 or 4.0 mg/L with regard to producing somatic embryos Somatic embryo conversion and somatic seedling acclimatization to the greenhouse was achieved with all seven tested *Stewartia* species and the three North American cultivars. Somatic embryos from all *Stewartia* species germinated and converted at a higher frequency following a cold treatment. This is the first report of somatic embryogenesis in the genus *Stewartia*, which will facilitate the mass propagation of these species for the horticulture industry. Furthermore, embryogenic tissue from five *Stewartia* species was recovered from cryopreservation, suggesting the feasibility of long-term storage of valuable germplasm such as that of rare or endangered North American *Stewartia* species.

***Franklinia alatamaha* micropropagation via organogenesis**

Immature zygotic embryos were cultured on semi-solid Woody Plant Medium (WPM) with and without plant growth regulators (PGRs) in various concentrations and combinations. Thirty-four different PGR treatments were tested for the development of an *in vitro* plant propagation system for *F. alatamaha*. Organogenesis was the only morphogenic process observed, and it was induced by five PGR treatments that included TDZ or 4-CPPU alone or IBA in combination with BAP or meta-Topolin. Adventitious buds were produced on the epidermal surfaces of the immature zygotic embryos. Shoots elongated from both adventitious buds and axillary shoot cultures were rooted in WPM without PGRs but supplemented with 0.25 g/L activated charcoal. The resulting plantlets were transferred to soil and acclimated to the greenhouse and outdoor environments. Within one and a half years, the tissue culture-derived trees flowered under an extended photoperiod with LED supplemental lighting in the greenhouse. This is the first report of plants being produced from *F. alatamaha* via organogenesis.

Genetic diversity analysis of *Franklinia alatamaha*

A genotype-by-sequencing (GBS) approach was used to characterize the genetic structure and diversity of a *F. alatamaha* germplasm collection from a cultivated population since this species is extinct in the wild. High-quality genomic DNA was successfully extracted from 76 accessions including two herbarium samples that were 177 and 61 years-old. SNPs were identified by the STACKS platform and used to develop a genome-wide genetic variant file. The genetic variation present in the germplasm collection was examined by STRUCTURE, a model-based Bayesian analysis, UPGMA tree, and PCA, a distance-based method. Together these analyses showed two

subpopulation clusters present among the 76 accessions of *F. alatamaha*. The population statistics yielded a low G_{ST} value, indicating that the proportion of genetic diversity between the two subpopulations was low, while the proportion of genetic diversity within the subpopulations was high. Minimal differentiation between the two *F. alatamaha* subpopulations was also demonstrated by the low F_{ST} value. These results show there is genetic diversity present in the 76 accessions of *F. alatamaha* in this study. The SNPs identified can be used to identify markers important for marker-assisted breeding programs with *F. alatamaha*. The genetic diversity found will be useful to develop new genetic populations and to map. To our knowledge this is the first population genetic analysis of the cultivated germplasm *F. alatamaha* as well as the first to use GBS as an approach to studying the genetic diversity. The population structure and genetic differentiation revealed by this method will benefit further genetic studies, germplasm protection, and breeding to develop root-rot resistance to the devastating plant pathogen *P. cinnamomi.* Genetic resistance to *P. cinnamomi* will allow *F. alatamaha* to be grown successfully in soils infested with this organism such as those in the Southeastern US, specifically in coastal Georgia where this tree was once native, growing along the banks of the Altamaha River.

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APPENDICES

A. *PHYTOPHTHORA* ROOT ROT TRIAL #1

Experimental procedure for assaying disease resistance of *Franklinia alatamaha* to *Phytophthora cinnamomi*

Collaborators: Dr. Steve Jeffers, Garner Powell, and Linus Schmitz, Dept. of Plant and Environmental Sciences, Clemson University.

Introduction

Crown and root disorders of container-grown *Franklinia alatamaha* Bartram ex Marshall is a serious problem for the commercial production of this highly attractive and desirable ornamental species. The most common symptom of this disorder is root rot. Symptoms of wilting in the infected plants occur after the first flush of growth in the spring and during the summer under hot temperatures. The symptoms begin with wilting of the top leaves, followed by a progressive yellowing of lower leaves. In some cases the lower leaves defoliate before the wilting starts at the apex of the plant (Peterson et al., 1975). Death can occur in a few days, weeks, months, or linger through the summer and then the plant becomes susceptible to winter injury (dieback) or winter kill (Peterson et al., 1975). The affected root system will show numerously rotted and decaying roots while healthier ones can have small brown lesions (Peterson et al., 1975). Crown canker is less prevalent, and the trees appear to be healthy until the canker girdles the trunk causing the same wilt symptoms observed with root rot infection (Peterson et al., 1975). Cankers and root rot can occur simultaneously on the same plant. Koslow and Peterson (1980) were able to consistently isolate *Phytophthora* from diseased *Franklinia* plants and also from healthy plants that were inoculated with *Phytophthora* and produced the

same disease symptoms. The general morphology of the *Phytophthora* isolates were similar to known isolates of *P. cinnamomi*. Currently, *Franklinia* is unable to grow and survive for any length of time in the soil where *Phytophthora* is present, primarily in the southeastern US. Interestingly, rhododendron isolates of *Phytophthora* have infected the roots of *Franklinia* indicating there is no host-specific isolate for *Franklinia* (Koslow and Peterson, 1980).

Materials and Methods

Seed source

F. alatamaha seed capsules were provided by Marshall Adams of Gwinnett County, Georgia. The seeds harvested from the capsules were combined prior to the selection of seed for the gamma radiation treatments.

Gamma irradiation of seed

Two thousand seeds were randomly chosen from a combined seed lot of *F. alatamaha* and randomly assigned to each of the test gamma irradiation treatments: 0 Gy (control), 50 gy, 100 gy, and 200 gy. The amount of radiation measured in gy determined the exposure time to gamma rays produced from a cobalt-60 source. The irradiation was performed at the Center for Applied Isotope Studies (CAIS) on the University of Georgia campus in Athens, GA.

Stratification and germination of seed

Following the gamma irradiation treatments, seeds from each treatment were added to their soil mix of 1 part vermiculite to 1 part Fafard 3B potting mix, placed in a Ziplock bag, and stratified at 4 °C in the dark for 30 days. After the stratification period, the seed was placed on the surface of the potting mix in a tray and carefully covered with 1.5 cm of moist vermiculite.

Transplanting of seedlings

Seedlings at least 6” in height were transferred to 4” plastic nursery pots in substrate mix containing 1 part perlite to 1 part Fafard 3B potting mix and allowed to grow to 1 ft before transfer to a 1-gal plastic pot. The soil mix for 1-gal pots was 1 part peat: 2 parts perlite: 3 parts mini nugget bark chips. The soil was top-dressed with 1 tsp of the slow-release fertilizer Osmocote.

Propagation, transplanting, and growth of cuttings

Once plants reached 3-4 ft, cuttings were taken from new shoot growth that was 6’’ in length. Cuttings were stuck in a soil mix of 1 part perlite: 1 part Fafard 3B in 4’’ pots and placed under mist under shade cloth. The mist setting was 1 min every 15 min. Cuttings were kept under mist until roots were observed and then transferred to full sun on the greenhouse bench and hand-watered as needed to keep cuttings moist during the transition from the shaded mist conditions. Once the cuttings were root bound, the plants were transferred to 1-gal pots in a substrate of 1 part peat: 2 parts perlite: 3 parts mini nugget bark chips and top-dressed with 1 tsp of the slow-release fertilizer Osmocote.

Experimental design

Completely randomized design:

2 treatments: Non-inoculated and Inoculated

16 genotypes: gamma irradiated lines of *F. alatamaha* from seed lot in Paulding Co, GA

Expt’l units: 2-8 clones (plants) of each genotype per treatment, non-inoculated and inoculated

(Table A-3 p. 115)

Preparing *P. cinnamomi* isolates

The isolate of *P. cinnamomi* used in this experiment were provided by Dr. Steve Jeffers of Clemson University in SC. One was SC-3061 (UGA-Pc01) isolated from Sandy Creek Nature Park near Athens, GA. The isolate was recovered from long-term storage and subcultured on PAR-V8 medium at 20°C in the dark. Once the culture grew, hyphae was transferred to cV8A to check for purity. The inoculation rate was calculated at 0.3-0.4% using 10ml inoculum/pot for trade gallon size pots. Therefore, 1200 ml of inoculum was made to inoculate 120 pots. A total 1600 ml of non-infested V8V was prepared for the non-inoculated controls.

Inoculation of plants with *P. cinnamomi* isolate

On the day of inoculation, 10 ml (2 teaspoons) of *P. cinnamomi* inoculum was applied to the surface of each pot, mixed gently into the top layer of soil and covered with fine propagation mix. All the pots were carefully watered to incorporate the inoculum and to prevent desiccation if the inoculum. Inoculation date was 7.9.2019

Scoring of infection symptoms

Plants were scored each week for foliage symptom severity and/or mortality using a 6-point rating scale (Table A-1). The dates of the first symptoms and mortality was recorded for each plant showing infection symptoms. From this data, the time to first symptoms and survival were calculated in terms of days or weeks. If a stem lesion was visible, the presence of the lesion was documented as well as the height (cm) from the soil surface to the lesion.

Harvesting of infected plants

When a plant died, the soil was carefully removed around the root system by gentle soaking and running under tap water. Root rot severity was recorded using a scale from 0 to 100% in increments of 10% with 0% being healthy and 100% being dead. The top part of the plant was cut away from the root system and fresh weights (g) were recorded for both the top and bottom of the plant. The root systems were then wrapped in wet paper towels, put in Ziplock (GLAD) bags, and given to Dr. Jeffers lab at Clemson to isolate *P. cinnamomi* from the root systems to prove infection by the *P. cinnamomi* isolate SC-3061 (UGA-Pc01) used to inoculate the plants. Samples of the stem tissue was harvested from the base of the stem up and including the stem lesion if present. The stem tissue was stored at -20 °C until PCR verification could be performed for additional confirmation of *P. cinnamomi* infection of the dead plant.

Results

(See Table A-2 for survival percentages of plants for Trial 1 inoculated with *P. cinnamomi*)

Only two non-inoculated plants died and it was confirmed to be caused by *Pythium* and not *Phytophthora*

Trial 1 is still in progress

Table A-1. Rating scale for disease symptoms.

Rating Scale for Disease Symptoms		
Score	Rating	% foliage showing chlorosis, wilt, or necrosis
0	Healthy	none
1	First	1-10%
2	Moderate	11-15%
3	Extensive	51-90%
4	Severe	91-99%
5	Dead	100%

Table A-2. Trial 1: Survival percentages of inoculated gamma irradiated genotypes of *F. alatamaha* inoculated with *Phytophthora cinnamomi*.

Plants inoculated with *P. cinnamomi*					
Genotype	#clones	#dead	# alive	% dead	% survival
50-1	6	1	5	16.7%	83.3%
50-3	2	1	1	50.0%	50.0%
50-4	8	0	8	0.0%	100.0%
50-5	6	3	3	50.0%	50.0%
50-6	3	1	2	33.3%	66.7%
50-7	6	0	6	0.0%	100.0%
50-8	8	2	6	25.0%	75.0%
50-10	4	0	4	0.0%	100.0%
50-11	5	1	4	20.0%	80.0%
50-12	3	1	2	33.3%	66.7%
50-13	5	2	3	40.0%	60.0%
50-14	8	1	7	12.5%	87.5%
50-15	4	3	1	75.0%	25.0%
50-16	3	1	2	33.3%	66.7%
50-17	5	2	3	40.0%	60.0%
50-18	4	1	3	25.0%	75.0%
WT1	4	1	3	25.0%	75.0%
WT3	4	1	3	25.0%	75.0%
WT18	3	0	3	0.0%	100.0%
WT38	4	2	2	50.0%	50.0%
WT40	3	2	1	66.7%	33.3%
Gordonia	7	0	7	0.0%	100.0%

Figure A-1. Experimental design for Trial 1 of *P. cinnamomi* pot screen test

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Fig A-2. *P. cinnamomi* isolate UGA-Pc01

Fig A-3. Beginning of disease symptoms. Rating 1= 1-10% of plant showing symptoms of wilt, chlorosis, or necrosis

Figure A-4. Progressing disease symptoms. Rating 3= 51-90% of plant showing symptoms of wilt, chlorosis, or necrosis

Figure A-5. Progressing disease symptoms. Rating 4= 91-99% of plant showing symptoms of wilt, chlorosis, or necrosis

Figure A-6. Progressing disease symptoms. Rating 5= 100% of plant showing symptoms of wilt, chlorosis, or necrosis

Figure A-7. Healthy root (left) vs. root infected with *P. cinnamomi* (right).