SOMATIC EMBRYOGENESIS OF RARE AND THREATENED FOREST TREE SPECIES

by

NICOLE ALEXANDRIA LOCKE

(Under the Direction of Scott Arthur Merkle)

ABSTRACT

Redbay (*Persea borbonia*), swamp bay (*Persea palustris*), sassafras (*Sassafras albidum*), Georgia oak (*Quercus georgiana*), and Oglethorpe oak (*Quercus oglethorpensis*) are all forest tree species native to the southeastern United States that are rare in the landscape and/or facing a significant threat from introduced insects and pathogens. In an effort to aid in the conservation of these trees, we established somatic embryogenesis induction procedures for all five species, with this being the first report of somatic embryogenesis in redbay, swamp bay, sassafras, and Oglethorpe oak. Complete somatic plantlet regeneration of Georgia oak is also being reported for the first time. In addition, a method of re-inducing embryogenic cultures was explored to overcome the senescence of embryogenic culture lines.

INDEX WORDS: *Persea*, *Sassafras*, *Quercus*, Redbay, Swamp bay, Georgia oak, Oglethorpe oak, Somatic Embryogenesis

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DEDICATION

This thesis is dedicated to the conservation of the under-acknowledged tree species of the world. All species have value and merit and deserve the same concerns and efforts to protect and conserve them for the well-being of all of Earth's creatures.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Somatic embryogenesis (SE) in plants is the growth of structures resembling zygotic embryos, yet the result of a process occurring asexually (the absence of gamete fusion). The new embryo arises from a single somatic cell (Dodeman et al. 1997) and can be a powerful tool in tree breeding and conservation, as it has the potential to shorten an exceedingly long process (often decades), as well as providing optimum target material for genetic transformation. Somatic embryogenesis has been explored previously for tree species in the genera *Persea*, *Sassafras*, and *Quercus*, either for the purposes of improving an agricultural crop (*Persea americana*), increasing production of a forest product (*Quercus suber* and *Quercus rubra*), or for the purpose of improving genetics and conserving germplasm for species that are facing declining populations (*Sassafras randaiense*, *Quercus ilex*, and *Quercus arkanasasa*). Redbay (*Persea borbonia*), swamp bay (*Persea palustris*), sassafras (*Sassafras albidum*), Georgia oak (*Quercus georgiana*), and Oglethorpe oak (*Quercus oglethorpensis*) are all tree species native to southeastern United States that can benefit from having an established somatic embryogenesis system. They all are currently experiencing, to varying degrees, population decline from multiple causes including introduced insect pests and pathogens, population fragmentation, habitat loss, and human-caused climate change.

Redbay, Swampbay & Sassafras

Redbay (*Persea borbonia* (L.) Spreng) and swamp bay (*Persea palustris* (Raf.) Sarg.) are closely related species in the Lauraceae family. They are small to medium sized forest tree species found in the southeastern United States and have closely overlapping native ranges (Weakley, 2015). They are not of importance in commercial forestry, but they do have ornamental and ecological merit (Lederhouse et al., 1992; Gezon et al., 2019). Frequently, swamp bay is considered a subspecies of redbay, or not considered different at all (Shearman et al., 2022). This is due to the difficulties faced in differentiating them in field settings. Both species have lanceolate, glossy green leaves 4 -5 inches in length, with a prominent midrib vein, and dense ventral pubescence. They are mid-story trees with multi-stem growth habits. Flowers appear in May and June and are a creamy, white color. The bisexual flowers are grouped on peduncles and are heterodichogamous in nature (Chanderbali et al., 2006). For most of their native ranges, the two species are overlapping, however, redbay has a more coastal distribution, whereas the range of swamp bay extends more inland and northerly to the extent of coastal plain soils (Weakley, 2015). Efforts to differentiate the species have included using a scanning electron microscope to analyze the pubescence found on the undersides of the leaves (Wofford and Pearman, 1975) and studying the chemical compounds found in the wood and foliage (Wofford, 1975; Niogret et al., 2011). These studies found that the hairs of swamp bay are on average, more than 3 times as long as those of redbay, and that there are differences in the presence of certain flavonoids and volatile terpenoids.

In an effort to limit the effects of laurel wilt disease on redbay (and by extension, swamp bay), methods for vegetatively propagating redbay have been explored by researchers at the University of Florida. Their research found that the use of IBA (indole-3-butyric acid) as a

rooting aid, and a 3:1 perlite-vermiculate rooting medium increased establishment of rooted cuttings when compared to other types of rooting media and lack of a rooting aid (Hughes and Smith, 2014). Research has also been conducted to identify individual redbay trees that show putative resistance to laurel wilt disease. This research also found that trees showing resistance had reduced tylosis production and granular deposition in xylem lumina (Hughes et al., 2022).

Sassafras albidum (Nutt.) Nees, commonly referred to as sassafras, is another moderately sized tree species in the Lauraceae family native to the eastern United States, as well as parts of eastern Canada. The species can be found as far west as Oklahoma and Texas, and from southern Ontario to Florida (Weakley, 2015). Sassafras has a long history of being used in medicinal practices and as a flavoring in foods. The leaves of this species can be unlobed, mitten-shaped, or three-lobed (de Soyza et al., 1991). Trees will frequently have all leaf-shapes present at a given time. During the growing season, foliage are a bright green and change to deep oranges and reds during the fall. The species is dioecious, with flowers appearing shortly before foliage in spring. The flowers are not showy, yellow-green in color, and have five to six tepals. The fruits are small drupes that are produced on individual pedicels and change from green to dark purple as they mature (Little, 1980). In most of its range, sassafras shares the responsibility of being an important food source for the larval stages of the spicebush swallowtail (*Papilio troilus*) with spicebush (*Lindera benzoin*) (Nitao et al., 1991).

Redbay Ambrosia Beetle and Laurel Wilt

Members of Lauraceae in the southeastern United States are currently facing decline due to habitat loss and the introduction and spread of the redbay ambrosia beetle (*Xyleborus glabratus*) and the fungal pathogen *Raffaelea lauricola* which together cause the laurel wilt

disease complex. The redbay ambrosia beetle and the fungal pathogen *Raffaelea lauricola* are native to southeast Asia and were first documented in Georgia in the Port of Savannah region in 2002 (Rabaglia et al., 2006). Although the disease has remained concentrated in Georgia and South Carolina, there have been reports of it as far west at east Texas (Menard et al., 2016). It is thought that the insect was introduced, and continues to spread, through the movement of infested wooden packaging and wood products.

The beetle bores into the branches and trunks of trees in the Lauraceae family, creating galleries in which they mate and lay eggs, and the larvae develop (Rabaglia et al., 2006). The main threat to the trees, however, does not come from direct beetle damage to trees. The redbay ambrosia beetle and the *Raffaelea lauricola* fungal pathogen have a symbiotic relationship in which the beetle acts as a vector for the pathogen, and then the pathogen acts as food source for the beetle. The beetle carries spores of *Raffaelea lauricola* in a specialized portion of its mandibles, called mycangia. As the beetle creates galleries within the trunk and branches of host trees, it spreads the fungal pathogen's spores throughout the tree, which then infect the vascular system of the tree. As the pathogen grows throughout the galleries and vascular system, the redbay ambrosia beetle feeds on the fungus and re-inoculates its mycangia with spores (Spiegel et al., 2013). As a mechanism of protection, the tree walls off the infected areas, but unfortunately the infections tend to be so widespread that this action disrupts the vascular system of the tree to the point that it begins to decline, and eventually dies. There does not appear to be a preference among the beetles for trees already in decline over otherwise healthy trees (Hanula et al., 2008), which distinguishes this exotic pest from native North American ambrosia beetles. A continued spread of the beetle and pathogen through redbays to other related species could greatly affect southern forest compositions and eventually threaten the avocado industry.

Somatic embryogenesis in Lauraceae species

Among members of Lauraceae, somatic embryogenesis was first achieved in avocado (*Persea americana*) using a Murashige and Skoog (1962) medium (MS) supplemented with sucrose at 30g/1; thiamine HC1 at 0.4mg/l; i-inositol at 100 mg/1, solidified with 8% agar and picloram at 0.1 mg/L (Pliego-Alfaro and Murashige, 1988). Since then, a significant amount of work has been conducted to expand and improve somatic embryogenesis in avocado. Much of the work has focused on increasing the amount and quality of somatic embryos produced in one cycle. Picloram is a very common plant growth regulator (PGR) used to induce somatic embryogenesis in avocado, while media composition and culture conditions have been the main avenue explored to improve production of bipolar embryos and germination (Witjaksono and Litz, 1999; Peran-Quesada et al., 2004; Marquez-Martin et al., 2012; Lopez Encina et al., 2014). Genotype appears to have an impact on the amount and quality of somatic embryos produced (Witjaksono and Litz, 1999; Lopez Encina et al., 2014).

In 1978, researchers at The Institute of Botany, Academia Sinica induced somatic embryogenesis in *Sassafras randaiense* (Chen and Wang, 1985), with the highest rate of induction found in zygotic embryo explants that were cultured in liquid, ½-strength MS medium supplemented with 5 x 10^{-5} M 2, 4-D (2,4-dichlorophenoxyacetic acid). Varying concentrations of NAA (1-naphthaleneacetic acid) and 6-BA (6-benzylaminopurine) were tested as well. These experiments resulted in the production of embryos that arose directly from callus, with some that separated easily and others that were fused.

Oglethorpe oak and Georgia oak

Oglethorpe oak (*Quercus oglethorpensis* Duncan) is an oak species belonging to the subgenus *Quercus* and section *Quercus*, native to the southeastern United States, with disjointed populations in Louisiana, Mississippi, Alabama, Georgia, South Carolina and North Carolina (Lobdell and Thompson, 2016). This species is described by the Red List of Oaks 2020 as being endangered (Carrero et al., 2020). Much of the research concerning this species has involved determining where populations have historically been, and where they currently remain, as well as where the species is present *ex situ* (Lobdell and Thompson, 2016; Backs and Ashley, 2021; Spence et al, 2021).

The species was first described by Wilbur H. Duncan in 1940 (Duncan, 1940). In May of 1940, Duncan, along with Professors Bishop and McKellar visited a site in Oglethorpe County, Georgia along Buffalo Creek where they encountered a population of trees that was initially identified as shingle oak (*Quercus imbricaria*). On a return trip to the area in August of the same year, it was noticed that the first year twigs of these trees were bearing mature fruit, and Duncan concluded that the original identification was made in error. Upon further inspection, it was decided that the trees of this population were of a previously undescribed species. Duncan named the species for the county in which it was initially identified. Later, populations of Oglethorpe oak were discovered in Elbert, Greene, Jasper, and Wilkes counties within Georgia (Haehnle and Jones, 1985) as well as in other states.

Trees of the species are described as being roughly 25 meters tall, and as having dark green, glossy leaves that are 5 – 13 centimeters in length, and 2 – 4 centimeters in width, obovate in shape, with margins that can be variable in appearance (Duncan, 1940). Observation of specimens being grown in botanical collections outside of the species native range suggest that

the trees can be surprisingly cold-hardy if summertime temperatures are high enough for the new growth to harden off (Coombes and Coates, 1997; Lobdell and Thompson, 2016). Populations in Louisiana and Mississippi have been found in areas described as outcrops of clay beds (Wiseman, 1987), while in Georgia, populations have been documented in areas described as flatwoods, open woods, and bottomlands (Haehnle and Jones, 1985). In *Trees of the southeastern United States* (Duncan & Duncan, 1988), it is mentioned that Oglethorpe oaks are susceptible to chestnut blight (*Cryphonectria parasitica*). Oglethorpe oak trees showing symptoms of chestnut blight have been observed on trees planted at Thompson Mills Forest, near Braselton, Georgia.

Georgia oak (*Quercus georgiana*) M.A. Curtis, is a forest tree species native to portions of the southeastern United States. The species was first identified on Stone Mountain, near Atlanta, Georgia by M.A. Curtis. The leaves are a vivid green, with irregular, bristle-tipped lobes. The distribution of Georgia oak is very limited, with several small populations being reported in Georgia, and a few in South Carolina and Alabama (Spence et al., 2021). However, it has been noted that in the areas where the species is found in Georgia, it regularly appears as one of the most abundant species in that given location (Carter and Floyd, 2013). Some sources report that in native habitats, Georgia oak is primarily in a shrubby form, while others refute this and state that many populations show standard tree growth habits (Small, 1895; Palmer, 1932). The primary soil types and habitats mentioned for Georgia oak are sandy or clay soils on slopes, and granite outcrops (Small, 1895; Carter and Floyd, 2013). Many references to this species are in passing and quite old, with the sources being primarily focused on plant communities and other plant species (McVaugh, 1943; Schornherst, 1945; Lipps and De Selm, 1969). Select Trees nursery in Oglethorpe County, Georgia produces and sells a cultivar of Georgia oak named

'Jaybird'. Georgia oak is not an important species for timber production, but does have ornamental and ecological merit.

Oak somatic embryogenesis

Somatic embryogenesis (SE) and plantlet regeneration in *Quercus* has been reported since the 1980's (Chalupa, 1990; Sasaki et al., 1988), with somatic embryogenesis having been achieved with multiple species and across the Red Oak and White Oak sections. A majority of the work has focused largely on European species of oak such as holm oak (*Quercus ilex*), cork oak (*Quercus suber*) and pedunculate oak (*Quercus robur*). However, two North American species, northern red oak (*Quercus rubra*) and white oak (*Quercus alba*) are well represented in the literature (Rancillac et al. 1996; Vengadesan and Pijut, 2009; Corredoira et al., 2011; Corredoira et al., 2014), and SE has also been reported in some rare North American oak species such as *Quercus georgiana*, *Quercus arkansana*, and *Quercus boyntonii* (Kramer and Pence, 2012). A multitude of explant materials, such as immature zygotic embryos, young leaves, and shoot apices have been utilized successfully in the induction of SE in a variety of oak species (Sasaki et al., 1988; Bueno et al., 1992; Cuenca et al., 1999; Corredoira et al., 2011), using an assortment of induction media (Endemman and Wilhelm, 1999; Mauri and Manzanera, 2003; San-José et al., 2010; Martinez et al., 2017; Martinez and Corredoira, 2023). The report of SE in Georgia oak from Kramer and Pence (2012) used immature leaves as explants, and no plantlet production was reported. There are no previous reports of SE induction for Oglethorpe oak. The biggest struggle in SE of oak, like many other woody plant species, has been the conversion of somatic embryos to plantlets. Factors such as pre-germination cold treatments, addition of gibberellic acid and activated charcoal to media, and reduction of available water in

media have been investigated to produce higher conversion rates (García-Martín et al., 2001; Martinez et al., 2008; Vengadesan and Pijut, 2009).

Research Objectives

The experiments in this thesis were designed to aid in the conservation and restoration of redbay, swampbay, sassafras, Georgia oak, and Oglethorpe oak by establishing somatic embryogenesis (SE) propagation systems. Embryogenic cultures were successfully initiated for these rare and threatened forest tree species, which had not been previously reported with the exception of Georgia oak. Developing SE propagation systems for these species will further conservation and protection efforts. Progress was also made towards creating procedures to successfully convert Georgia oak and Oglethorpe oak somatic embryos to somatic seedlings. Finally, a method of revitalizing declining embryogenic lines was investigated in an effort to produce more robust somatic embryogenesis propagation systems.

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CHAPTER 2

SOMATIC EMBRYOGENESIS FOR CLONAL PROPAGATION OF REDBAY, SWAMP BAY AND SASSAFRAS

N. Locke, S.A. Merkle. To be submitted to *Forests*.

Abstract

Redbay (*Persea borbonia)* and swamp bay (*Persea palustris)* are two closely related tree species in the Lauraceae family native to the coastal plains of the southeastern United States. Sassafras (*Sassafras albidum*) is a third member of the Lauraceae family that grows throughout the eastern US. Populations of all three species are currently declining due to mortality caused by the introduced laurel wilt pathogen *Raffaelea lauricola* that is spread by the insect vector *Xyleborus glabratus.* There are no previous reports of somatic embryogenesis (SE) for these species. In this study, attempts were made over multiple years to induce SE from immature zygotic embryo explants of these species. Explants from different redbay and swamp bay source trees were utilized for these experiments. Embryogenic cultures were produced from one explant of each *Persea* species cultured on medium with 0.2 mg/L picloram. The established cultures continued to proliferate with regular transfer to fresh medium over two years via repetitive embryogenesis. Some *Persea* somatic embryos enlarged and appeared to mature, and some root germinated, but none produced whole plantlets. For sassafras, embryogenic cultures were produced from four explants in medium with 0.2 mg/L picloram. The cultures eventually produced PEMs and some embryos root germinated, but no plantlets were produced.

Introduction

Redbay (*Persea borbonia*) and swamp bay (*Persea palustris*) are two small tree species that are native to portions of the southeastern United States. *Sassafras albidum* is a small to medium sized tree species that is native to much of the eastern United States. All three species belong to the family Lauraceae that includes many commercially important food crops such as avocado (*Persea americana*), cinnamon (*Cinnamomum spp.*), and bay laurel (*Laurus nobilis*).

Persea borbonia, commonly known as redbay, is an evergreen tree native to the southeastern United States that grows in moist and regularly flooded soils having a high amount of organic matter (Gilman et al., 1994). The species is not commonly harvested for timber but is used as an ornamental within its native range. The range of this species hugs the coastal waters and inlets of the southern United States, from North Carolina to Texas, with disjunct populations in Louisiana. Both species have attracted attention from federal and state agencies as well as university researchers (Fraedrich et al., 2008; Hubbard n.d.; Hughes et al., 2015) due to their high susceptibility to the introduced redbay ambrosia beetle (*Xyleborus glabratus*) and its associated fungal pathogen *Raffaelea lauricola*, which is the causal agent of laurel wilt disease.

Sassafras albidum (Nutt.) is a small, to medium sized, deciduous tree species native to the eastern half of the United States. The species has a range that extends from the most southerly border of Ontario, Canada to Florida, and from the edges of Texas and Oklahoma to the Atlantic coast (Griggs, 1990). Frequently referred to solely by its generic name, it is known for its aromatic wood, leaves, roots, and extracted oils, which have historically been used in foods and drinks such as gumbo filé and tisanes (Segelman et al., 1976), folk medicine (Cavender, 2006) and perfumes (Magnaghi, 1997).

At present, all three species face population decline due to loss of habitat and die-off caused by introduced pathogens and insect pests (Bates et al., 2013). Of particular concern for these species is the laurel wilt pathogen that is spread by the redbay ambrosia beetle. The redbay ambrosia beetle is a wood-boring beetle native to multiple Asian countries (Harrington et al., 2011; Wood et al., 1992) that has a symbiotic relationship with *R. lauricola*. The fungal pathogen results in laurel wilt disease, which is fatal to the host tree (Fraedrich et al., 2008). Members of Lauraceae are susceptible to the disease and present with symptoms such as wilting, lack of vigor, and vascular streaking (Spiegel et al., 2013).

Reports on clonal propagation of redbay, swamp bay, and sassafras have been limited, and currently, there is no published research on somatic embryogenesis (SE) in these species. The available published research on the propagation of redbay focuses on rooted cuttings to test trees that are putatively resistant to laurel-wilt as part of a breeding program at the University of Florida (Hughes et al., 2014). Limited *in vitro* propagation of swamp bay has been reported (Kane et al., 1989) in which shoot cultures were produced in liquid suspension, and rooted micro-cuttings were produced *ex vitro*. A close relative of redbay and swamp bay is the commercially important avocado (*Persea americana*), for which over 30 years worth of research involving SE has been conducted (Cruz-Hernández et al., 1998; Encina et al., 2014; Márquez-Martín et al., 2011; Perán-Quesada et al., 2004; Pliego-Alfaro et al., 1988). This work provides a solid initial point for establishing a somatic embryogenesis propagation system in redbay. Somatic embryogenesis induction and plantlet regeneration has also been reported (Chen &Wang, 1985) in a *Sassafras* species native to Asia (*Sassafras randaiense*).

The goal of the studies described here is to increase the knowledge concerning propagation of redbay, swamp bay, and sassafras so as to help further conservation efforts of these three

species. Establishing SE protocols for these species provide an avenue by which specific genotypes of these species can be clonally propagated for resistance testing and potentially be used as target material for genetic transformation.

Materials and Methods

Plant Material

Immature redbay drupes were supplied by Dr. Jason Smith's lab at the University of Florida. The source trees for the drupes were part of a *P. borbonia* disease-resistance breeding program conducted by Dr. Smith. Over the course of the 2-year study, drupes were collected from seven source trees. Following collection, redbay drupes were stored overnight in a refrigerator, then shipped on cold packs via overnight delivery to Athens, GA. The collection dates for summer of 2020 were July 13th and August 27th. For the summer of 2021, the drupes were collected on July 19th and August 4th.

Immature swamp bay drupes were collected from trees (Fig. 2.1a) located on the University of Georgia campus in Athens, GA in summers of 2020 and 2021. Four individuals were used as source trees. In 2020 experiment, drupes were collected on two dates (July 8th and July 24th). In 2021, the number of source trees was reduced to two of the same trees used the previous year due to the decline in health of the other two trees. Fruits were collected on July 13th and July 27th.

Sassafras drupes were collected from four source trees (Figs. 2.1b, 2.1c) growing in a US Forest Service test planting in raised beds at UGA's Whitehall Forest in Athens, GA on June 1, 2022. The source trees were designated FS-1, FS-2, FS-3 and FS-4.

Explant preparation and culturing

Once collected or received, drupes were stored at 4^{o} C until used for culturing. The surface sterilization protocol for *Persea* and *Sassafras* drupes was modified from a protocol developed for use with dogwood (*Cornus florida*) drupes (Gladfelter and Wilde, 2018). Drupes were removed from their pedicels, placed into a stainless steel mesh sieve and sprayed with 95% ethanol, then rinsed under running tap water for two minutes. Then, they were placed into a 500 ml bottle containing superpure water with 2-3 drops of Dawn® dish soap and the bottle was thoroughly agitated for 10 minutes. Following washing, the drupes were collected on a stainless steel mesh sieve, with no further rinsing. The process was continued inside of a laminar flow hood utilizing aseptic manipulation, with the drupes being placed into a 200 ml glass beaker along with 140 ml of 70% ethanol and agitated for one minute using a magnetic stir bar and plate. Drupes were removed from the ethanol and placed into a second sterilized glass beaker containing 140 ml of 50% Clorox (8.25% NaOCl) and 2 drops of Tween 20, and agitated for 15 minutes. Then, drupes were removed from the liquid and placed into another glass beaker with 140 ml of sterile deionized water, and agitated for four minutes. This final step was repeated three times before the drupes were stored in a 100 mm sterile glass Petri dish containing two pieces of sterile filter paper and sealed with Parafilm until used (within 24 hours).

Each drupe was transferred to another sterile glass Petri dish containing two 55 mm filter papers for dissection. There, lengthwise incisions were made in the exocarp and seed coat of each drupe with a sterile scalpel. Then, one half was carefully peeled away using sterilized forceps to reveal the embryo and endosperm. A different set of sterilized forceps was used to remove the embryo and endosperm and place them on semi-solid induction media (detailed below) in 60 x 15 mm plastic Petri dishes.

In the 2020 *Persea* experiments, a single explant was cultured on each plate, and in 2021 experiments, two explants were cultured per plate. This method was used for both *Persea* species. For initiations performed in 2020, five explants from each of the source trees were cultured on each of the five tested media, with two plates per source tree × medium combination, for a total of 125 explants. For the 2021 experiment, 6-8 explants were cultured from each source tree on each of the three tested media, with three plates per source tree × medium combination, for a total of 168 explants. In the 2022 sassafras experiment, two or three explants were cultured per Petri plate from each of the four source trees on each of the 2 tested media, with ten plates per source tree × medium combination, for a total of 90 explants.

Tissue Culture Media, Culture Maintenance, and Culture Conditions

Two semi-solid basal media were tested for effects on SE induction in *Persea* in 2020. These were modified Woody Plant Medium (WPM); (Lloyd and McCown, 1980) and Murashige and Skoog's (1961) medium (MS). Both basal media were supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol, 0.5 g/L filter-sterilized L-glutamine. Picloram was tested at 1 mg/L and 0.1 mg/L, while naphthaleneacetic acid (NAA) was tested at 0.025 mg/L and 0.25 mg/L, based on the concentrations of these PGRs reported to induce SE in avocado (Pleiago-Alfarro & Murashige, 1988). For *Persea* culture initiations performed in 2021, only modified WPM basal medium was used. Picloram was tested at concentrations of 0.1 and 0.2 mg/L and 2, 4-D was tested at a concentration of 2 mg/L. Sassafras explants were cultured on the modified WPM described above with either 0.2 mg/L picloram or 2 mg/L 2,4-D. All cultures were incubated in the dark at 25±1 °C and transferred to fresh medium of the same composition at 3-4 week

intervals for six months. Culture plates were checked every two days for formation of embryogenic tissue or other evidence of morphogenesis during those six months.

Suspension cultures

In March 2022, liquid suspension cultures of both redbay and swamp bay were initiated using tissues from established SE cultures started in summer 2021 to test effects of liquid culture on repetitive somatic embryo production. The same modified WPM used for inductions was employed, minus the gelling agent. Liquid media contained either 0.2 mg/L picloram or no PGRs. Approximately 0.5 g of embryogenic tissue was inoculated into 25 ml of liquid medium in 125 ml Erlenmeyer flasks that were incubated on a gyratory shaker at 100 rpm in dark conditions at 25±1 C °. The cultures were allowed to grow for four weeks. Then the liquid medium was decanted and the flask was refilled with basal liquid WPM and returned to the shaker. After five days, embryogenic material was removed from liquid medium and collected on sterile filter papers overlayed on semisolid embryo development medium (EDM) containing basal WPM with no PGRs in 100 x 15 mm plastic Petri dishes. Embryos that were grown in suspension were monitored for maturation, but embryos darkened and continued to produce more repetitive embryos, so these cultures were discarded after three weeks.

Results

Persea somatic embryogenesis

Persea culture initiation experiments in 2020 resulted in cultures that consisted of non-morphogenic callus, or explants that root-germinated. No evidence of morphogenesis was observed in that experiment for either tested species. Embryogenesis induction was achieved in

the 2021 experiment, but only from one explant per species, which made statistical assessment for treatment or source tree effects on induction unfeasible. The two explants that resulted in embryogenesis induction were both cultured on WPM with 0.2 mg/L picloram. The redbay explant that produced embryogenic material was from the first collection date (July 19) and the swamp bay explant that produced embryogenic material was also derived a from an explant collected on the first collection date (July 13). The embryogenic culture of each species continued to proliferate via repetitive embryogenesis with monthly transfer to fresh medium with 0.2 mg/L picloram, producing many repetitive embryos (Figs. 2.2a, 2.2b). The appearance of the repetitive embryos from the two *Persea* species was very similar. Somatic embryo development strongly resembled that of zygotic embryo counterparts. Initially SEs were translucent before becoming opaque and a white/off-white color, then rapidly transitioning to a bright pink-red color as enlarging. As SEs matured, a slow transition to a dull reddish color (Fig. 2.3) was observed. The two main differences between appearances of the zygotic embryos and the somatic embryos were that the SEs almost always retained some red pigmentation at maturity, while zygotic embryos returned to a cream color, and the somatic embryos were usually observed to be much smaller than the zygotic embryos (1 cm or less for somatic embryos, versus 2-3 cm in diameter for zygotic embryos). Callus produced by zygotic embryo explants and associated with repetitive somatic embryos was a dark brown/black color and tended to be friable. New somatic embryos arose from dark colored callus and separated from it and each other easily. Callus darkened the media quickly, likely due to the presence of phenolics exuded by the callus. Some mature redbay embryos transferred to basal medium root germinated, but the embryos and root darkened rapidly, and no complete plantlets were produced (Fig. 2.4). The redbay somatic embryos that were grown in liquid suspension did not proliferate or mature,

and thus somatic embryo production was not enhanced over that from repetitive embryogenesis on semi-solid medium.

Sassafras somatic embryogenesis

Of the 90 cultured sassafras zygotic embryo explants, four produced embryogenic cultures, all of which were induced on WPM with 0.2 mg/L picloram. Three of the embryogenic cultures were derived from seeds of source tree FS-1 and one was derived from a source tree FS-3. Seeds from the other two source trees produced no embryogenic cultures, although some produced non-morphogenic callus. Sassafras cultures initially proliferated as repetitive embryos (Fig. 2.5a) yet after a few months of serial transfer to fresh medium, proliferation mode shifted to structures resembling proembryogenic masses (PEMs; Fig. 2.5b). The PEMs initially turned dark brown, followed by production of new, pale-yellow PEMs from the browned PEMs during each culture cycle. After several months of serial culture, all four culture lines continuously proliferated as yellow PEMs with little browning, as long as they were transferred to fresh medium on a strict 3-week schedule. Somatic embryos developed sporadically from the PEMs while still on the medium with picloram (Fig. 2.5c), but these did not develop beyond the production of small cotyledons, which varied in number from one to four, as long as they were maintained on medium with picloram. Some of these embryos root germinated and produced rosette-like structures at the shoot poles (Fig. 2.6), but no shoots elongated past 2 mm.

Discussion

In this study, somatic embryogenesis was induced in three members of the Lauraceae family: redbay, swamp bay, and sassafras. Due to the threats that these species face from

introduced insects and pathogens, such as the redbay ambrosia beetle and *Rafaella lauricola*, having a means for rapidly propagating new plants, either from resistant genotypes or for clonal testing of the products from a breeding program, is imperative.

Among members of the Lauraceae family, somatic embryogenesis has only been reported in avocado and Chinese sassafras. The PGR utilized in the single report of SE in Chinese sassafras was picloram (Chen and Wang, 1984) and, overwhelmingly, picloram has been the PGR used successfully in induction of SE in avocado (Pliego-Alfara and Murashige, 1988; Witjaksono and Litz, 1999; Perán-Quesada et al., 2003; Márquez-Martín et al., 2011; Márquez-Martín et al., 2012; Encina et al., 2014; Guzman-Garcia et al., 2013; Olivares-Garcia et al., 2020). Therefore, it is unsurprising that for all of the successful induction attempts made in this study, picloram was the PGR present in the media.

Similarities among SE in avocado and Chinese sassafras and the species used in this study can also be seen in the physical qualities of the cultures. Non-fused, repetitive SEs arising from a darkened callus which resembled their zygotic counterparts but remaining smaller at maturity (Fig. 2.3), were observed in the redbay and swamp bay cultures. This observation is consistent with reports of avocado culture proliferation (Witjaksono and Litz, 1999; Márquez-Martín et al., 2012) The embryogenic cultures of sassafras differed in appearance from the redbay and swamp bay cultures. These cultures also changed throughout serial culturing from repetitive embryos to PEMs. They were not unlike some types reported in avocado, but in those instances, embryos arising from those types of cultures could not mature and germinate (Márquez-Martín et al., 2012; Guzman-Garcia et al., 2013; Olivares-Garcia et al., 2020). It has been noted in several studies involving avocado that maturing and germinating somatic embryos is a difficulty that has not been fully resolved, though different methods have been explored to

overcome those difficulties. Approaches tested to improve maturation, germination, and conversion of avocado somatic embryos have included liquid suspension, the addition of proline and glutamine to the media, various types of media and subculturing methods, different concentrations of gelling agent and sucrose in the media, different forms of available nitrogen, and the addition of coconut water to the medium (Witjaksono and Litz, 1999; Perán-Quesada et al., 2003; Márquez-Martín et al., 2011; Encina et al., 2013). It is plausible that the findings of studies aimed at improving maturation and germination of avocado somatic embryos can be applied to future experiments involving redbay, swamp bay, and sassafras.

Given the close relation of the tested species to avocado and the similarities between embryogenic cultures of avocado and the species in this study, it is not unreasonable to assume that much of the knowledge gained from experiments regarding avocado SE can be applied to experiments with redbay, swamp bay, and sassafras. However, avocado is a tropical South American species while redbay, swamp bay, and sassafras are subtropical or temperate North American species. These physiographic differences may point to differing nutritional, environmental, and/or hormonal needs relating to embryo maturation, germination, and seedling growing conditions. The most obvious difference is the requirement of cold stratification for seed germination of the many North American species. It is known that the zygotic embryos of sassafras require 120 days of cold at 2-4° C to germinate (Bonner et al., 2008). While there is very little information regarding the requirements for germination of redbay and swamp bay seeds, the small amount of research conducted (Huff, 2012) suggests that they require 60 days of cold at 5° C to germinate.

Conclusion

This study provides the first noted successful production of embryogenic cultures of redbay, swamp bay, and sassafras. Results indicate the potential exists for a new means by which to propagate trees that may have resistance to laurel wilt or that have other desirable characteristics, as well as creating material that can be used genetic modification or germplasm conservation via cryopreservation. Due to the very low percentage of successful somatic embryogenesis inductions from redbay, swamp bay, and sassafras zygotic embryos in this study, it is impossible to draw conclusions regarding the impacts of variables such as genotype or seed collection time. However, it is worth noting that picloram was the PGR involved in all six successful embryogenesis inductions of the species studied. As with all research, this provides an important stepping-stone for future studies to continue enhancing the efficiency of protocols related to SE in these vitally important species.

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Figure 2.1 : Swamp bay tree on the University of Georgia campus from which drupes were collected (a). Sassafras source trees growing at the University of Georgia's Whitehall Forest from which drupes were collected for culture initiation (b), and clusters of immature drupes (c).

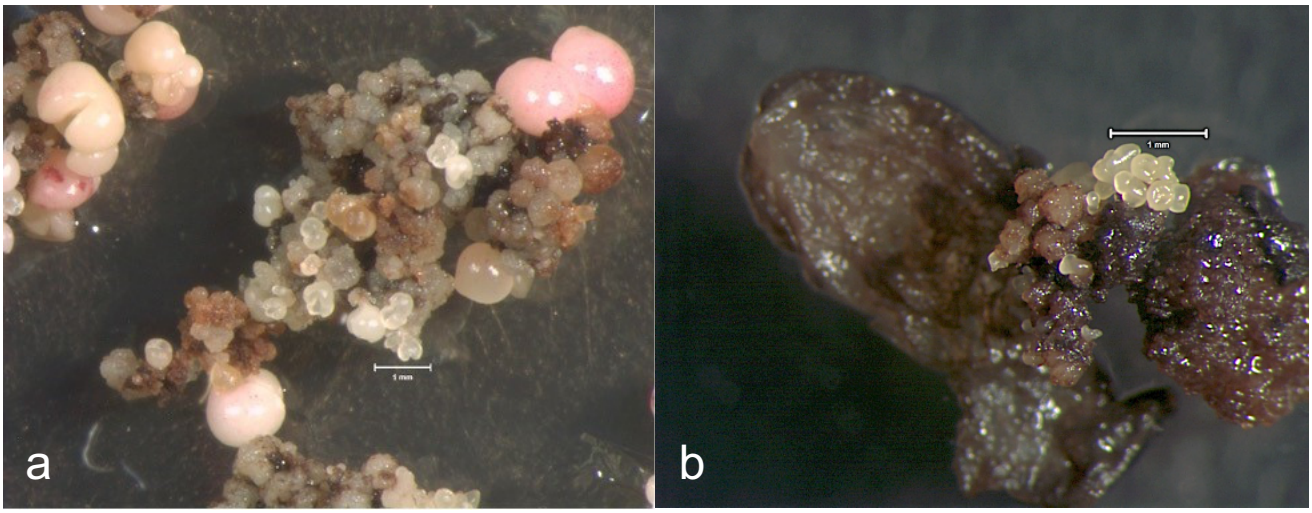

Figure 2.2: Redbay (a) and swamp bay (b) embryogenic cultures proliferating by production of repetitive somatic embryos. Bar in both photos = 1mm.

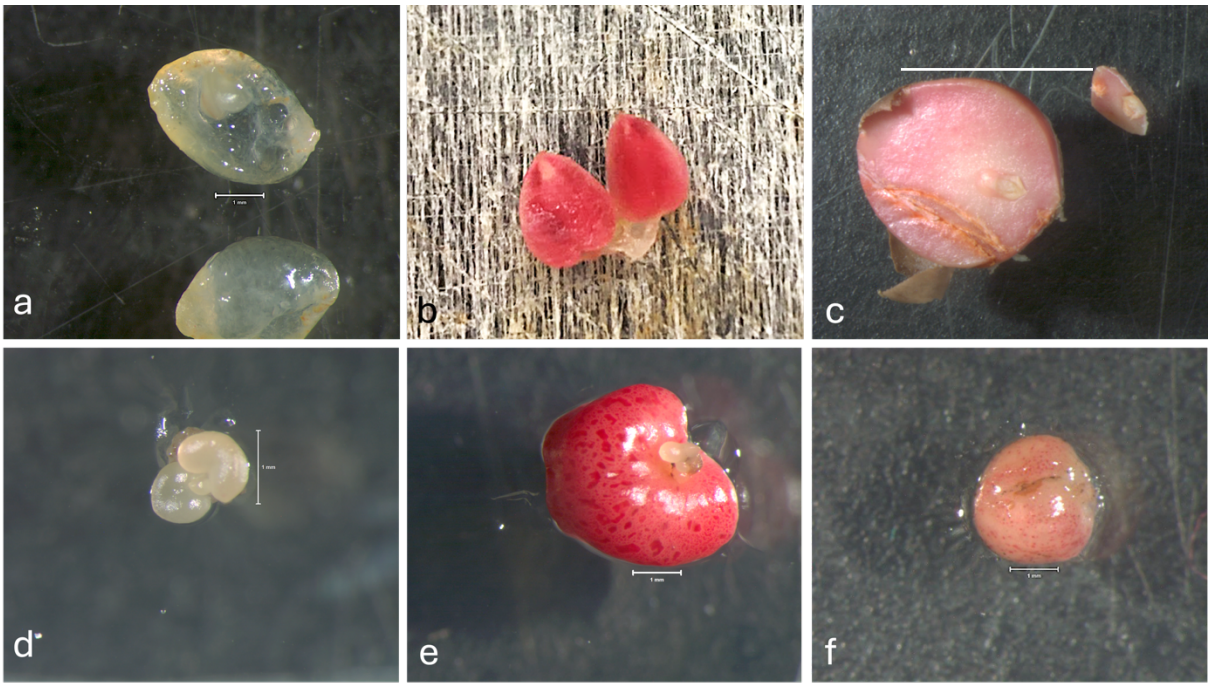

Figure 2.3: Series of images showing the similarities between redbay zygotic embryos and somatic embryos. Zygotic embryos are represented by the images in the upper row (a, b, & c) and somatic embryos are represented by images in the lower row (d, e, & f). Bar in all photos = 1mm.

Figure 2.4: Root germination of some redbay somatic embryos. Petri plate is 100 mm in diameter.

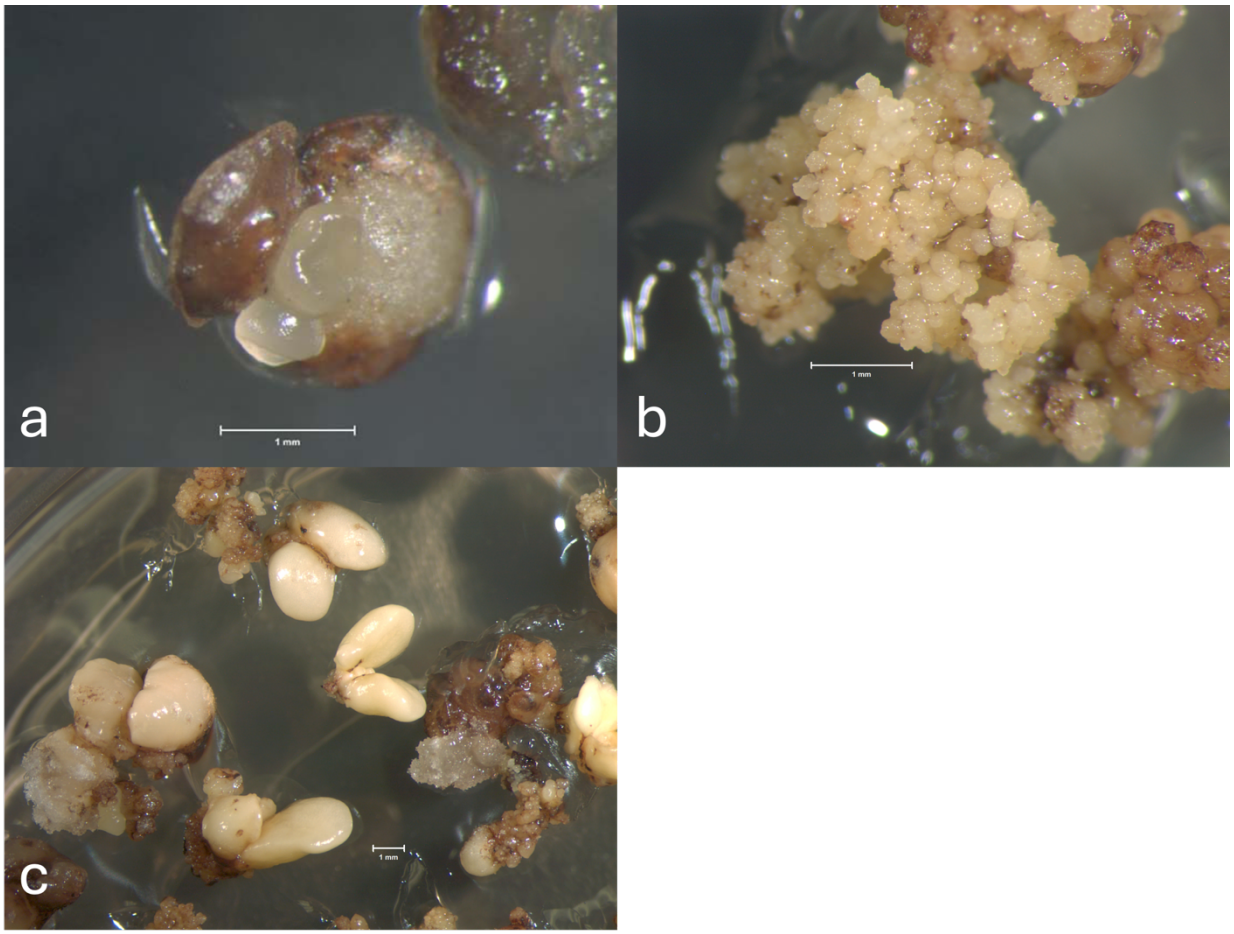

Figure 2.5: Newly-initiated embryogenic sassafras culture showing production of first repetitive somatic embryos (a), sassafras PEM production (b), and sassafras somatic embryo production from PEMs (c). Bar = 1mm.

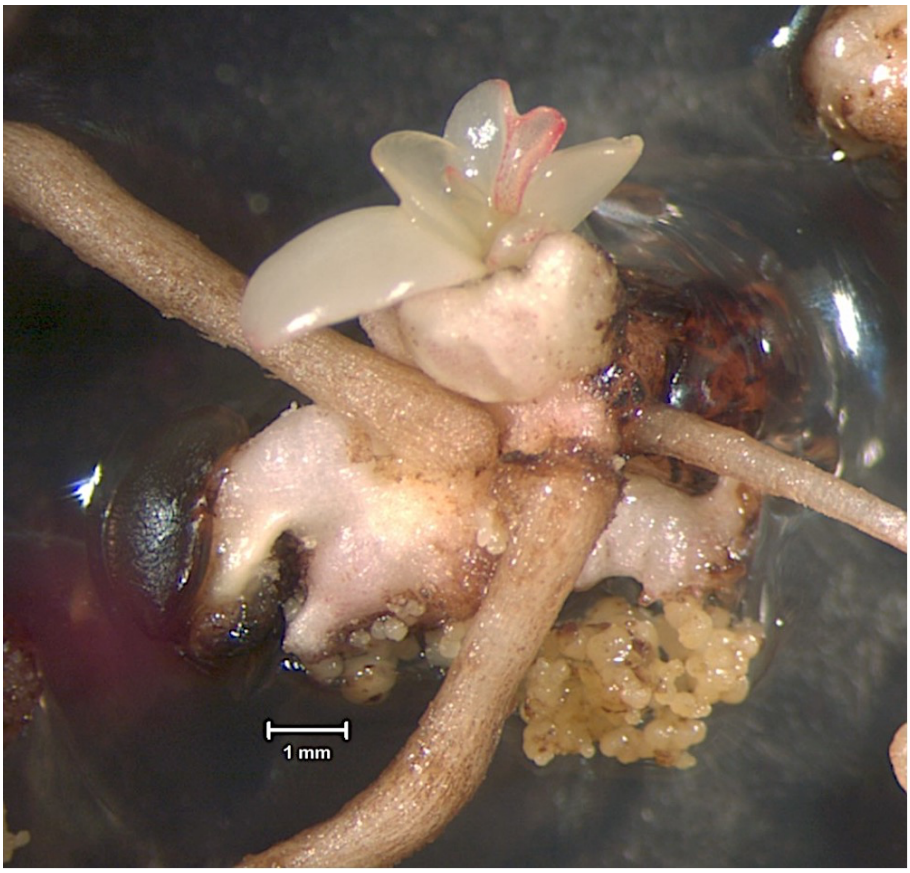

Figure 2.6: Germinated sassafras somatic embryo showing rosette structure at shoot pole. Bar = 1 mm

CHAPTER 3

SOMATIC EMBRYOGENESIS IN *QUERCUS GEORGIANA* AND *QUERCUS OGLETHORPENSIS*

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Abstract

Georgia oak (*Quercus georgiana*) and Oglethorpe oak (*Quercus oglethorpensis*) are two rare forest tree species native to the southeastern United States. They are documented as having small, widely dispersed populations throughout the southeast, with the majority of distribution within Georgia and surrounding states. These species face threats from introduced pathogens such as chestnut blight (*Cryphonectria parasitica*), habitat loss and fragmentation, as well as human caused climate change. This has led to both species being listed as endangered by the IUCN Red List. Most of the conservation efforts regarding these species have focused on *ex situ* collections, as the recalcitrant acorns of oak species are incompatible with long-term seed banking. This fact makes these species prime candidates for propagation via somatic embryogenesis. In this study, somatic embryogenesis was induced from immature seed explants of both species cultured on a modified Woody Plant Medium supplemented with either 2 mg/L 2, 4-D or 0.2 mg/L picloram. Somatic embryos of Georgia oak were induced to germinate following a pre-germination cold treatment, and converted to plantlets that survived transfer to *ex vitro* conditions and acclimatization. Georgia oak embryogenic cultures were re-induced in an effort to preserve an embryogenic line that began declining, and those experiments showed that PEM and somatic embryo production can be re-invigorated using this approach with Georgia oak.

Introduction

Georgia oak (*Quercus georgiana* M. A. Curtis), is a rare forest tree species found in Piedmont regions in the southeastern United States. Populations of the tree are small, few in number, and widely dispersed. Data on the exact geographic distribution of the tree is limited and possibly outdated, but it is known that populations remain in Georgia and Alabama. One of the most widely known populations is located on Stone Mountain in Georgia. In natural settings, such as on Stone Mountain, Georgia oaks have a shrubby growth habit, likely due to poor soils that do not readily retain water. However, specimens cultivated in arboretums have form and stature typical of other red oak species (personal observations). The species is not widely utilized commercially, but there is at least one instance of a commercial nursery establishing a cultivar of the species (Select Trees, Crawford, GA, 'Jaybird'). Studies have been conducted to assess the genetic diversity of both *in situ* and *ex situ* populations of the species (Morton Arboretum and Chicago Botanic Garden.) The IUCN Red List classified Georgia oak as endangered in March 2015. The number of mature individuals is currently decreasing, with causes for this ranging from human-caused climate change to destruction of habitat (Beckman et al. 2019). Continued fragmentation of habitat is impacting the species by increasing the likelihood that isolated stands of Georgia oak trees will hybridize with other, more common reproductively compatible oak species near them (Rushton, 1993).

Oglethorpe oak (*Quercus oglethorpensis*) W.H. Duncan, is native to the southeastern United States and is found in small, widely dispersed populations. The identification and naming of the species are relatively recent, as it was first named by W.H. Duncan in 1940 and received the specific epithet *oglethorpensis* due to being discovered in Oglethorpe County, Georgia (Duncan 1940). Its appearance is common for species in the white oak subsection of the genus,

with an upright habit and typical height being between 50 and 80 feet (Duncan, 1940). It has been reported that trees of this species are susceptible to the pathogen *Cryphonectria parasitica*, which causes chestnut blight (Duncan and Duncan, 1988). Infection of Oglethorpe oaks with the pathogen was observed by members of this research team in July of 2021 on trees planted at Thompson Mills Forest near Braselton, Georgia (Fig. 3.1). Oglethorpe Oak trees are reportedly poor producers of acorns, which has led to difficulties in preserving the species in ex situ collections (Lobdell and Thompson, 2016). As with all oak species, the acorns of Oglethorpe oaks are recalcitrant and not suitable for seed banking (Spence et al., 2021; Kramer and Pence, 2012; Toppila, 2012). This fact, in combination with the tree's infrequent acorn production, makes the availability of vegetative propagation systems desirable for this species.

Somatic embryogenesis (SE), an in vitro propagation system characterized by the production of germinable embryo-like structures called somatic embryos, has been achieved with multiple *Quercus* species across the Red Oak and White Oak sections. There have been reports of successful somatic embryogenesis induction from different types of explant material, including leaves and zygotic embryos. Over the past three decades, somatic embryogenesis has been reported for white oak (*Quercus alba*), cork oak (*Quercus suber*), Holm oak (*Quercus ilex*), pedunculate oak (*Quercus robur*), and northern red oak (*Quercus rubra*) (Chalupa, 1990; García-Martín et al., 2001; Corredoira et al., 2011; Martínez et al., 2017). A frequently noted bottleneck in the production of trees from the embryogenic cultures is the low rate of somatic embryo conversion to somatic seedlings. The effects of stratification and plant growth regulators on the maturation and conversion potential of somatic embryos have frequently been investigated to increase production of somatic seedlings and bring greater viability to the *Quercus* somatic embryogenesis propagation method (Endemann and Wilhelm, 1991; Martínez et al., 2008).

Production of somatic embryos from young leaf tissue of Georgia oak has been reported, although no somatic seedlings have been produced (Kramer & Pence 2012).

Methods and Materials

Initial induction of somatic embryogenesis of Georgia oak and Oglethorpe oak

Plant material

Immature Georgia oak acorns were collected in summer 2021 from trees maintained at the Thompson Mills Forest and State Arboretum near Braselton in Jackson County, Georgia. In the planting, two specimen trees are planted directly adjacent to one another. The first collection of acorns from these trees occurred on July 14, 2021. It was discovered during this collection that acorns on one of the trees lagged slightly behind the other regarding developmental stage. The embryos from the first acorn collection from this tree were not cultured as we could not be certain that fertilization had occurred. The second collection of acorns from the same two source trees was made on August 26, 2021. The tree that had the more advanced acorn development was designated GA-2, while the tree with the more delayed acorn development was designated GA-1.

Immature Oglethorpe oak acorns were collected from a single source tree growing near the entrance to Oconee Forest Park on the University of Georgia campus in Athens, GA on July 29, 2021. The source tree was growing within 10 m of another Oglethorpe oak tree, which had produced staminate inflorescences but no acorns, so there is a high probability that the second tree was the pollen parent for the acorns collected from the source tree. Acorn production on the source tree was very sparse and a bucket lift was required to collect even a few dozen acorns.

Other mature Oglethorpe oaks growing at Thompson Mills Arboretum were checked for acorn production, but without success.

Culture initiation

Acorns of both species were disinfested following the protocol established for American chestnut by Andrade and Merkle (2005), with the addition of supplemental rinsing and washing prior to being manipulated in a sterile environment. Briefly, the cupule of the acorns were an impediment to the removal of the immature zygotic embryos of the Georgia Oaks, as they could not be removed prior to disinfestation without damaging pericarp of the acorn, potentially exposing the embryo to surface disinfestation chemicals. This required the cupule to be left on during surface disinfestation and removed later during the dissection of the acorn and removal of the immature zygotic embryo. Prior to the portion of the disinfestation protocol that was performed in the sterile environment, acorns were sprayed thoroughly with 90% ethanol, rinsed for 5 minutes under warm, running water, agitated vigorously for 5 minutes in 500 ml of water containing a few drops of Tween® 20 (Sigma-Aldrich), and then rinsed again until no suds remained. The surface disinfestation protocol for the acorns in a sterile environment was: Agitate for 20 seconds in 70% ethanol, agitate for 3 minutes in 20% Roccal-D Plus (9.2% didecyl dimethyl ammonium chloride, 13.8% alkyl dimethyl benzyl ammonium chloride, 1% bis-n-tributyltin oxide; Pfizer). Thereafter the first two steps were repeated followed by agitating for 5 minutes in 50% Clorox (8.25% sodium hypochlorite), rinsing for 3 minutes with sterile water, agitating 3 minutes in 0.01 N HCl, and rinsing for 3 minutes with sterile water (rinsing repeated three times). Following surface disinfestation, the cupule was cut away from the rest of the acorn using a scalpel and discarded. The pericarp was then cut longitudinally with a fresh

sterilized scalpel and pried apart with forceps to reveal the developing zygotic embryo. The zygotic embryo was removed and placed onto semisolid medium in 60 mm plastic Petri dishes. Explant extraction for Oglethorpe oak acorns followed the same procedure as that for Georgia oak. The embryogenesis induction-maintenance medium (IMM) used was a modified Woody Plant Medium (WPM; Lloyd and McCown, 1980) with 30 g/L sucrose, and 0.5 g/L filter sterilized L-glutamine, gelled with 3 mg/L Gellex gellan gum (Caisson) and supplemented with either 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) or 0.2 mg/L picloram, or without plant growth regulators (basal medium). For Georgia oak, zygotic embryo explants from each source tree were cultured, four to a Petri dish, on four plates of each of the three media, for a total of 48 explants per source tree. Oglethorpe oak acorns, all of which came from a single source tree, were much more limited, so that only nine embryos total were cultured on each medium. They were placed three to a Petri dish, with three dishes per medium. Cultures were maintained in darkness at 22° C. Explants were transferred to fresh medium of the same composition after one month and were checked for evidence of embryogenesis induction at 21-day intervals for at least two more months. Following the appearance of embryogenic material, individual explants along with any repetitive embryos or proembryogenic masses (PEMs) derived from explants were transferred from the initiation plates to individual plates of the same medium and given their own culture line designations. These cultures were maintained in the dark and transferred to fresh medium of the same composition every three weeks.

Liquid Suspension Cultures

Liquid suspension cultures of Georgia oaks were initiated by inoculating 0.5 g of embryogenic tissue from each of the 5 embryogenic culture lines produced into 125 ml

Erlenmeyer flasks containing 30 ml of liquid IMM supplemented with either 0.2 mg/L picloram or 2 mg/L 2, 4-D. Flasks were placed onto a gyratory shaker set at 100 rpm for a total of 33 days. Fourteen days after the initiation of suspension cultures, the liquid media was poured off and replaced with 32 mL of fresh media of the same type. After another 14 days, the suspension cultures were size fractionated using two nested stainless steel CELLECTOR® sieves. The mesh in the top sieve had a pore size of 860 μm and the bottom sieve had a pore size of 140 μm. Liquid IMM was poured through the nested sieves to wash smaller cell clumps through the top sieve to the lower sieve. Material collected in the bottom sieve was re-inoculated into fresh flasks containing 30 mL of liquid IMM with the same PGR supplement that they had previously been maintained with, and the larger cell clumps from the top sieve were discarded. Once the suspension cultures had been maintained on the gyratory shakers for 28 days, liquid IMM containing PGRs was near completely removed from the flask by pipetting and replaced with liquid embryo development medium (EDM) that was the same composition as IMM, but lacking PGRs. Then cultures were placed back onto gyratory shakers. After five days, the cultures were removed from liquid medium by pouring the contents of the flasks into a glass funnel and fritted glass stopper base (Wheaton filtration assembly) attached to a 500 mL sidearm flask while applying mild house vacuum to collect embryogenic cell clumps onto pieces of 30 μm pore size nylon mesh. The nylon mesh with cell clumps was transferred to the surface of semi-solid EDM in 100 mm plastic Petri dishes.

Maturation and Germination of Somatic Embryos

Three to four weeks after the nylon mesh with the fractionated material was transferred to semi-solid medium, plated culture material was examined to determine embryo development.

Embryos that were starting to show formation of cotyledons and were 2 mm – 4 mm in diameter were selected under sterile conditions and transferred to fresh EDM in 100 mm Petri dishes. Embryos were allowed to enlarge for 3 – 4 weeks in the dark at 25 °C. Embryos that had enlarged to at least 5 mm in diameter were selected and selectively transferred to new Petri dishes containing semi-solid EDM, and given zero, two, four, or eight weeks of pre-germination cold treatment at 4° C in the dark. At the end of the cold treatment period, embryos were transferred to Petri dishes containing germination medium (GM; same as EDM but lacking glutamine) for two weeks before being transferred to Petri dishes containing M1 medium [GM supplemented with 1mg/L of 6-benzylaminopurine (BAP)]. After one week on M1 medium, embryos were transferred to GA7 vessels (Magenta) containing 100 ml of semi-solid GM and placed in an incubator under cool white fluorescent lights (100 μmols m^{-2} sec^{-1}) with 16 hours of light per day at 25±1°C for germination.

Acclimatization

Only Georgia oak somatic embryo produced somatic seedlings with sufficiently developed shoots to attempt acclimatization (about 2.5 – 4 cm in height). These were removed from in vitro conditions and potted in 4 inch plastic pots containing a potting mixture (GreenThumb Premium Enriched potting mix in a 1:1:1 with HarborLite perlite and Hoffman Horticultural vermiculite), and with Osmocote® fertilizer pellets (NPK of 18-6-12). Empty GA-7 vessels were placed upside down over the potted somatic seedlings in order to provide additional control over the relative humidity for each somatic seedling. The pots with GA7 covers were placed in a plastic seedling tray with a humidity dome to maintain 100% relative humidity and incubated in a Plexiglas® acclimatization chamber at 25±1°C and 16 hours of

fluorescent light (40 μmol m^{-2} sec^{-1}) per day. Ninety days after potting, one edge of the GA7 vessels were lifted up one centimeter to allow greater airflow to the seedlings, and after another three months, they were removed entirely. Over the next six months, air vents in the humidity dome were opened incrementally. Due to the slow growth of the somatic seedlings, height was not used to determine readiness of the seedlings for removal from the growth chamber; rather overall vigor of the plants (color, number, and size of leaves and root growth) were utilized.

Re-induction of somatic embryogenesis of Georgia oak

Plant material

Of the embryogenic lines created during the initial induction experiment, proliferation of one particularly productive Georgia oak embryogenic culture line (GA-2-C) began dramatically slowing in November of 2021. In an attempt to revive the line, plans for an experiment to test re-induction using immature somatic embryos as "explants" were made. PEMs from two Georgia oak embryogenic culture lines (GA-2-C and GA-1-A) were grown in suspension culture and size fractionated following the aforementioned procedures. Globular somatic embryos of the two lines produced from the suspension cultures collected on nylon mesh and plated on semi-solid EDM were selected as target material to test for re-initiation of proliferating cultures.

Culture re-initiation and maintenance

Embryos at the globular stage were selected from populations of developing embryos on nylon mesh and gridded out, 16 per plate (Fig. 3.2) in 60 x 15 mm plastic Petri dishes containing IMM supplemented with the same three treatments as were used for the original inductions using zygotic embryos (no PGRs, 0.2 mg/L picloram or 2 mg/L 2, 4-D). The immature embryos

remained on the same media until evidence of repetitive embryogenesis or production of PEMs was observed.

Once PEMs were observed to proliferate from the re-induced somatic embryos, they were inoculated into liquid suspension cultures in IMM with the either 0.2 mg/L picloram or 2 mg/L 2, 4-D. Globular embryos were later size fractionated and used to re-establish cultures on semi-solid IMM in 60 mm Petri dishes.

Experimental design and data analysis

Three replicates of the experiment were performed for the GA-2-C line and one replicate of the experiment was performed for the GA-1-A line. One month after the globular somatic embryos (GSEs) had been re-induced, they were scored on the type of material or outcome produced by each GSE. The classifications were: dead explant, embryo maturation, repetitive embryo production, embryogenic callus, non-embryogenic callus, PEMs, or root-germinated explant. Many of the explants in this experiment were scored as having more than one outcome or type of material produced.

Results

Initial induction of somatic embryogenesis of Georgia oak and Oglethorpe oak

Induction

Due to the difficulties separating the cupule from acorn, rates of contamination were high, and some entire treatments were contaminated. Basal treatments for explants from the first collection that were procured from Georgia oak source tree GA-2 were all contaminated, and thus could not be evaluated for induction of somatic embryogenesis. Treatments containing

explants from the Georgia oak source tree GA-1 from the first collection had a contamination rate of 58.33%. Treatments containing explants from the first collection of GA-2 had a contamination rate of 12.5%; similar to the contamination rate of 13.33% containing explants from GA-2 from the second collection date.

Two Oglethorpe oak embryogenic cultures were induced, both on IMM supplemented with 0.2 mg/L picloram. Eight total Georgia oak embryogenic cultures were induced, with four inductions occurring on IMM supplemented with 0.2 mg/L picloram and four occurring on IMM supplemented with 2 mg/L 2, 4-D. Four of the inductions came from acorns collected from source tree GA-1 and four came from source tree GA-2. Overall, there was a 7.4% induction rate for Oglethorpe oak, a 16.66% induction rate for source tree GA-1, and an 8.8% induction rate for source tree GA-2. Oglethorpe oak embryogenic cultures mostly produced repetitive embryos that were easily separated from one another (Fig. 3.3). The newly initiated Georgia oak cultures produced a mixture of repetitive embryos and PEMs (Figs. 3.4a and 3.4b). The different Georgia oak culture lines varied in how rapidly they proliferated.

Somatic embryo production and germination

All Georgia oak suspension cultures produced embryos after fractionation and plating, and the embryos produced were all normal in appearance (having two, non-fused cotyledons and a radicle). However, because culture proliferation among the different embryogenic lines was unequal, some produced a greater number of embryos than others. The Oglethorpe oak embryogenic lines never produced PEMs and could not be manipulated using suspension culture and fractionation. Many of the somatic embryos (both Georgia oak and Oglethorpe oak) root germinated of their own accord (Fig. 3.5a and 3.5b), but cold treatment increased the number of

embryos that germinated in Georgia oak (Fig. 3.6a, 3.6b, and 3.6c), and exposure to M1 medium was required for complete somatic plantlet production in Georgia oaks. With exposure to M1 medium, some Oglethorpe oak somatic embryos produced shoots, but leaves did not expand from them (Fig 3.7). No complete Oglethorpe oak somatic seedlings were produced from either embryogenic culture line.

The Georgia oak somatic seedlings were slow (3-4 weeks) to produce shoots with expanded leaves. Once removed from in vitro conditions approximately a year was needed for acclimatization, and the majority of the potted somatic seedlings did not survive the full acclimatization process. Based upon observations, this seemed to be due to the slow development of a sufficient cuticle on the leaves of the somatic seedlings. Only four of the eighteen somatic seedlings that were potted survived to the point of being able to be removed from the growth chamber, and three of those only survived a few weeks after that. One somatic seedling from the GA-1-A line that received two weeks of cold stratification and one week of exposure to M1 medium survived acclimatization and continues to thrive today (Fig. 3.8)

Re-induction of somatic embryogenesis of Georgia oak

Of the three treatments tested as part of the re-induction experiment (0.2 mg/L picloram, 2 mg/l 2, 4-D, and basal medium), globular stage somatic embryos cultured on basal medium produced the least amount of new embryogenic material. Explants cultured on IMM with either 0.2 mg/L picloram or 2 g/L 2, 4-D mostly produced repetitive embryos, embryogenic callus, or non-embryogenic callus (Figs 3.9, 3.10, and 3.11a). When PEMs from re-induced explants were placed into suspension culture (IMM with either 0.2 mg/L picloram or 2 mg/L 2, 4-D) on gyratory shakers, PEMs readily produced material that was round, even, and pale yellow, and

which produced globular somatic embryos following plating in semi-solid EDM (Figs. 3.11b and 3.11c).

Discussion

The successful induction of somatic embryogenesis in Oglethorpe oak and the production of the first, complete Georgia oak somatic seedlings are important steps in the conservation of these two species. This new knowledge provides an approach by which Georgia oak and Oglethorpe oak germplasm can be conserved, and for Georgia oak, a protocol by which new trees can be propagated. Embryogenic cultures provide excellent material for cryopreservation for future use (Valladares et al., 2004). However, there are still some difficulties which must be addressed before somatic seedlings from these species can be reliably produced. Kramer and Pence (2012) produced Georgia oak embryogenic culture lines from leaf explants, but did not report the production of any plantlets. They reported the induction of embryogenic culture lines from five explants (5% of the total explants used). In comparison, in this study eight embryogenic culture lines were initiated from two source trees for an overall induction rate of 11.6%. No information was given regarding the proliferation of the leaf explant embryogenic cultures and it is unclear if plantlet production was attempted using embryos produced from those culture lines.

High contamination rates experienced in the initial inductions are a significant hurdle to be overcome, especially given the lack of readily available acorns, particularly for Oglethorpe oak. Potential solutions to high contamination rates would include development of a more refined disinfestation protocol and improved dissection techniques. The need for the cupule to remain attached to the acorn during disinfestation is likely a major cause of the contamination

rates seen in this study. Further exploration of methods in which surface disinfestation could better cope with the extremely small crevices and areas that harbor contaminants on intact cupules would be highly beneficial.

The issue of poor bipolar germination rates in both Georgia oak and Oglethorpe oak suggests that more in depth examination of the developmental stages and processes would be useful to improve taproot and shoot emergence from the somatic embryos. The inability of the Oglethorpe oak embryos to produce proper shoots and leaves leads to the question of what differing requirements the species may have and how those might best be met to improve plantlet production. Also, approaches need to be identified to reduce the long acclimatization period of Georgia oaks to make the mass production of somatic seedlings a more viable prospect. The current, nearly year-long period is impractical and, combined with the poor post-acclimatization survival rates, hinders the use of embryogenic cultures as a long-term conservation method for the species.

The successful re-induction of embryogenesis from individual globular somatic embryos shows promise as a way of prolonging the productivity of established SE cultures. It may also provide a way to quickly scale up selected culture lines. Further work needs to be undertaken to assess the quality of the embryos produced by a re-induced line to assure that they are capable of properly germinating and producing plantlets (Bradaï et al., 2016). It may also be beneficial to study long-term how many consecutive re-inductions can be performed without compromising the quality of the SE lines.

Conclusion

Successful production (for the first time) of embryogenic cultures of Oglethorpe oak, and the successful regeneration of complete Georgia oak somatic seedlings provides the potential for a new means by which to propagate these trees that are rare in natural habitats and have fragmented habitats, leaving them vulnerable to a multitude of threats. The successful re-induction of the Georgia oak embryogenic cultures offers more possibilities for the long-term maintenance of embryogenic cultures. Due to the lack of available acorns from Oglethorpe oak and the high percentage of induction culture contaminations of zygotic embryos in this study, it was impossible to draw conclusions regarding the impacts of variables such as genotype or seed collection time. However, it is worth taking notice that 2, 4-D and picloram both were effective in inducing SE in Georgia oak.

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Figure 3.1: An Oglethorpe oak at Thompson Mills Forest and State Arboretum near Braselton, Georgia showing infection with chestnut blight on lower trunk.

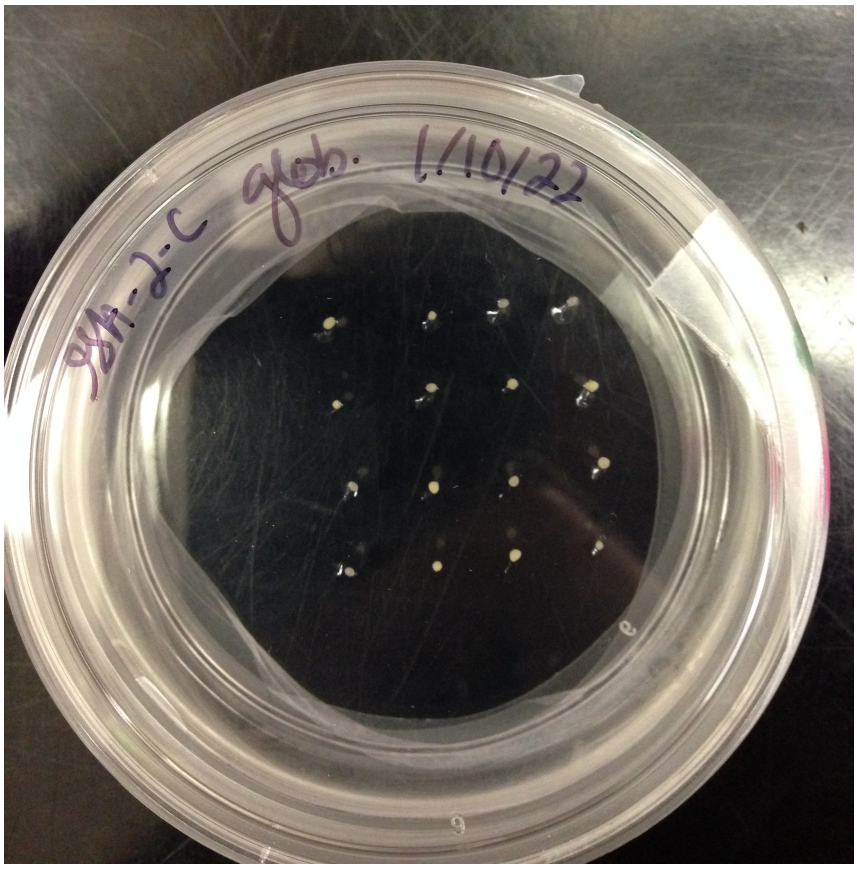

Figure 3.2: Re-induction of embryogenesis from newly plated globular somatic embryos of Georgia oak culture line GA-2-C following plating on IMM with either 0.2 mg/L picloram or 2 mg/L 2, 4-D. Petri dish is 55 mm in diameter.

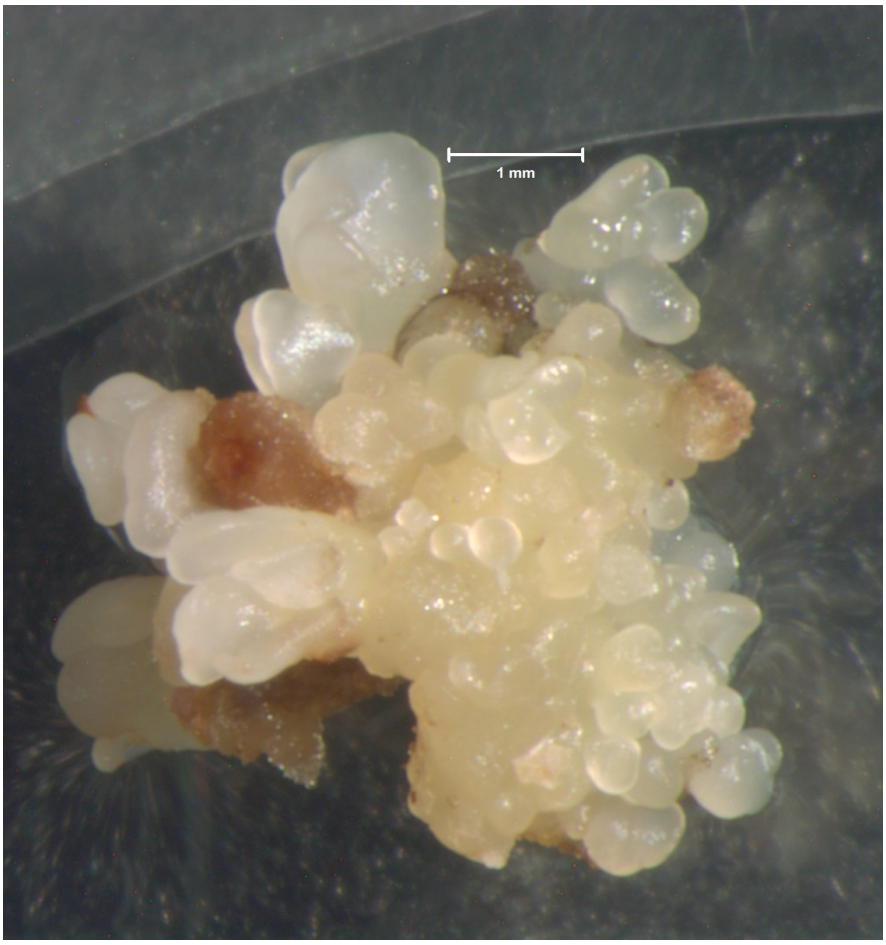

Figure 3.3: Repetitive embryos growing on Oglethorpe explant induced on IMM supplemented with 2 mg/L 2, 4-D. Bar = 1 mm.

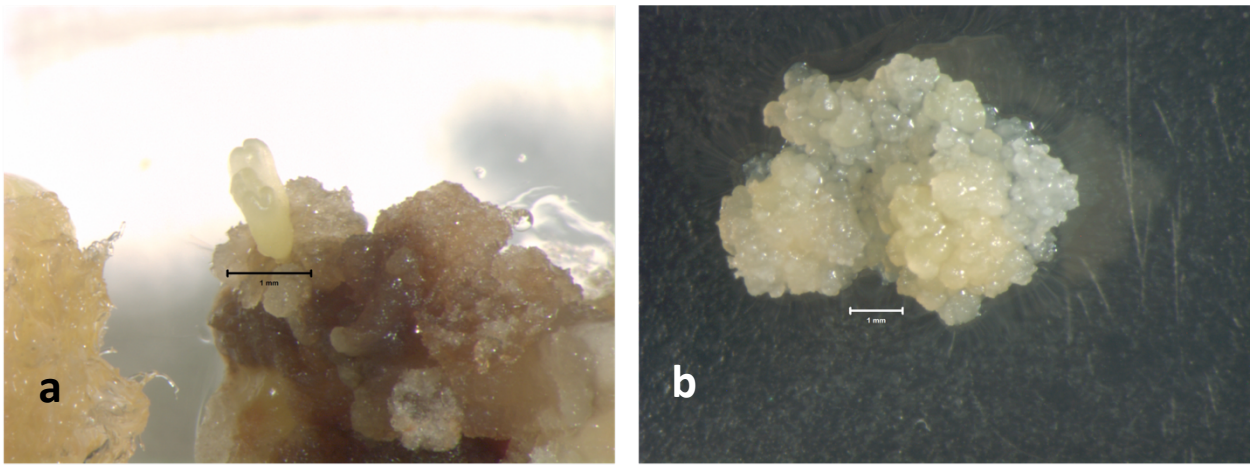

Figure 3.4: Proliferation of embryogenic material from Georgia oak explants during the initial induction experiment. Repetitive embryos forming (a) and PEMs proliferating (b). Bar = 1 mm in both photos.

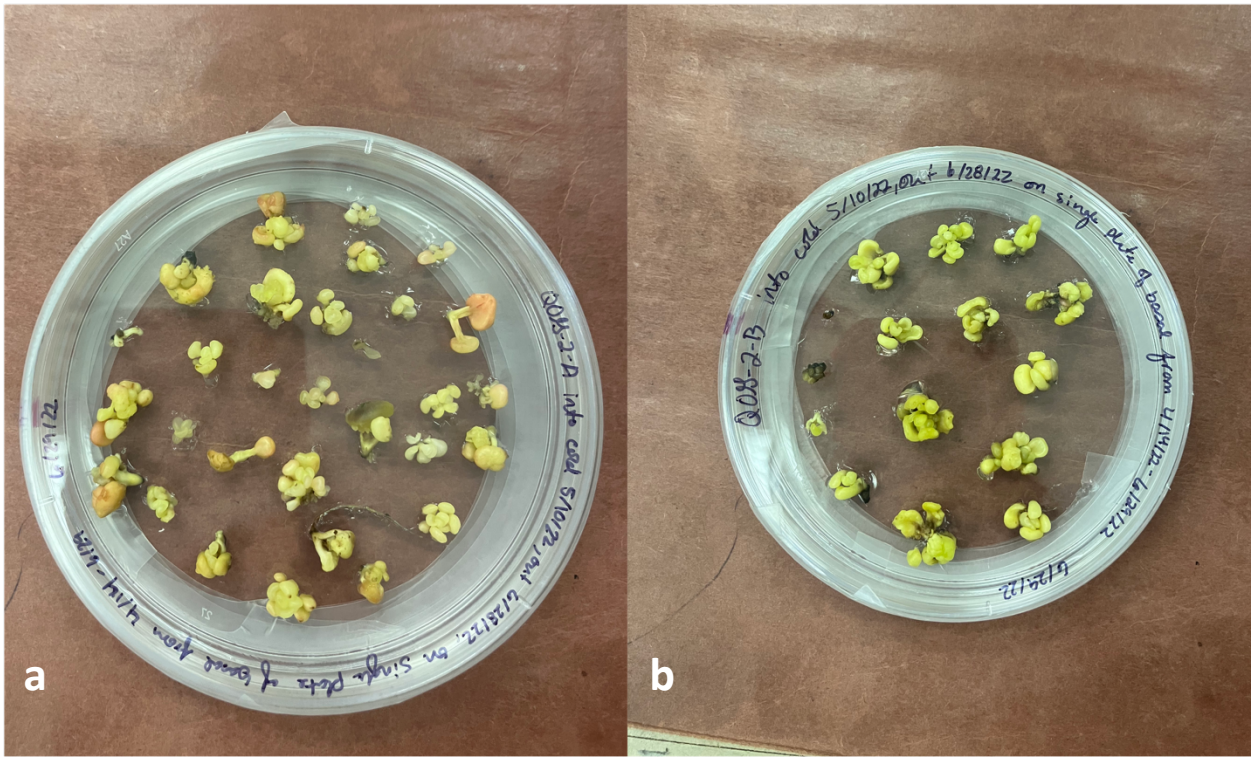

Figure 3.5: Oglethorpe oak somatic embryos from two culture lines root germinating after receiving ~ seven weeks of cold treatment at 4° C Petri dishes are 100 mm in diameter.

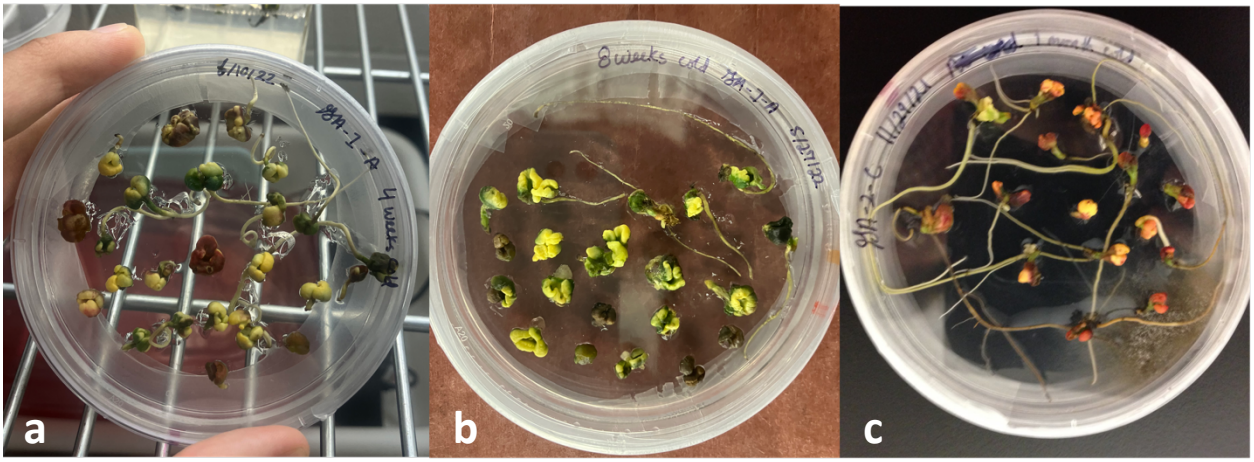

Figure 3.6: Georgia oak somatic embryos from three culture lines root germinating after receiving four (a and c) or eight (b) weeks of cold treatment at 4° C. Petri dishes are 100 mm diameter.

Figure 3.7: Oglethorpe oak somatic embryos showing abnormal shoot and leaf development during germination in a GA7 vessel.

Figure 3.8: Georgia oak somatic seedling from GA-1-A culture line, ~ 2 years after being initially potted.

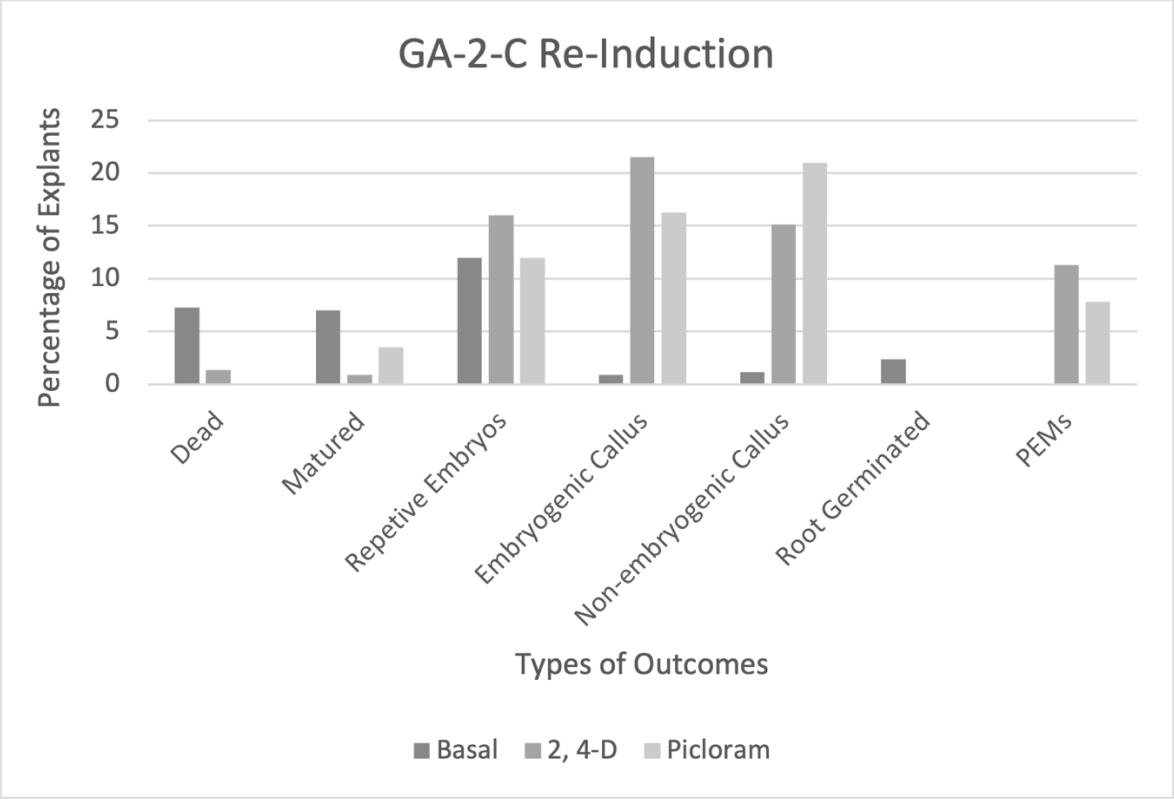

Figure 3.9: Outcomes of somatic embryos of the GA-2-C culture line re-induced on IMM supplemented with no PGRs, 2 mg/L 2, 4-D, or 0.2 mg/L picloram. This chart shows data for all 432 explants from the three replicates.

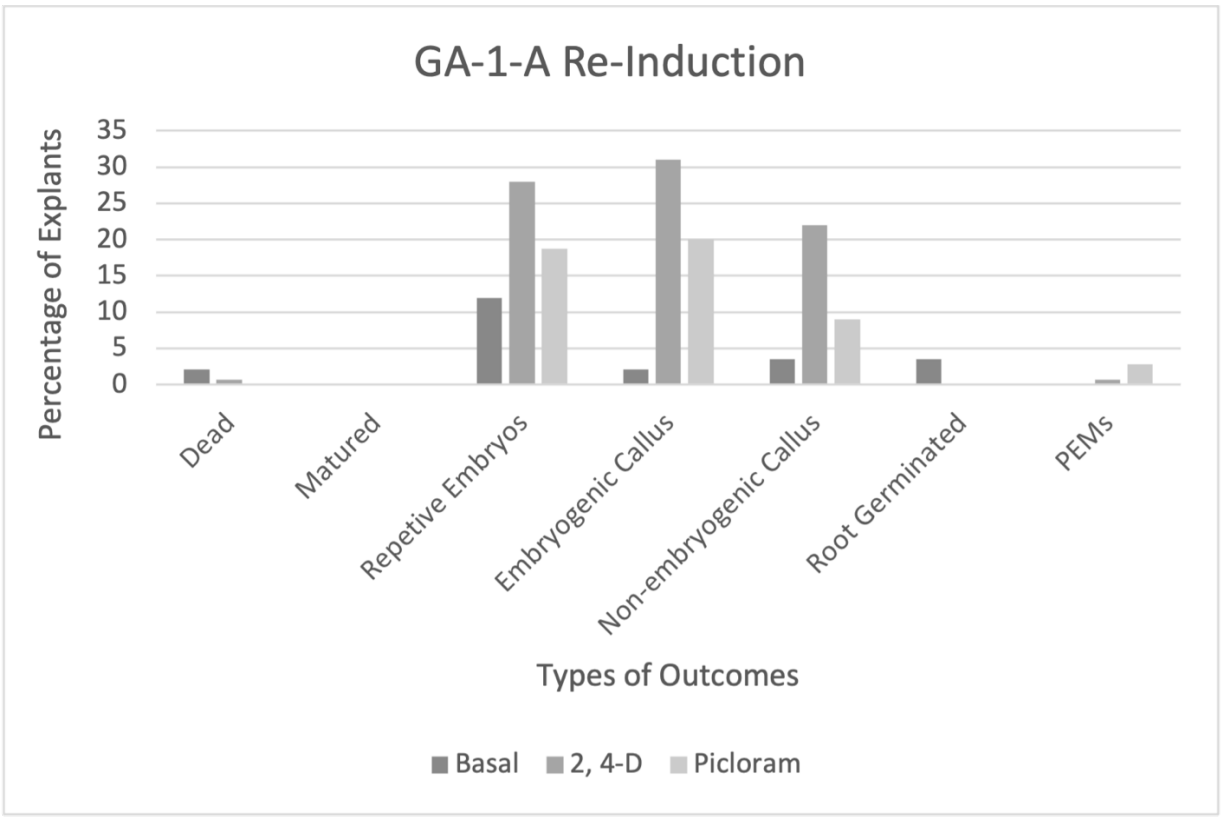

Figure 3.10: Outcomes of somatic embryos from the GA-1-A culture line re-induced on IMM supplemented with no PGRs, 2 mg/L 2, 4-D, or 0.2 mg/L picloram. This chart shows data for all 144 explants used in this single replicate.

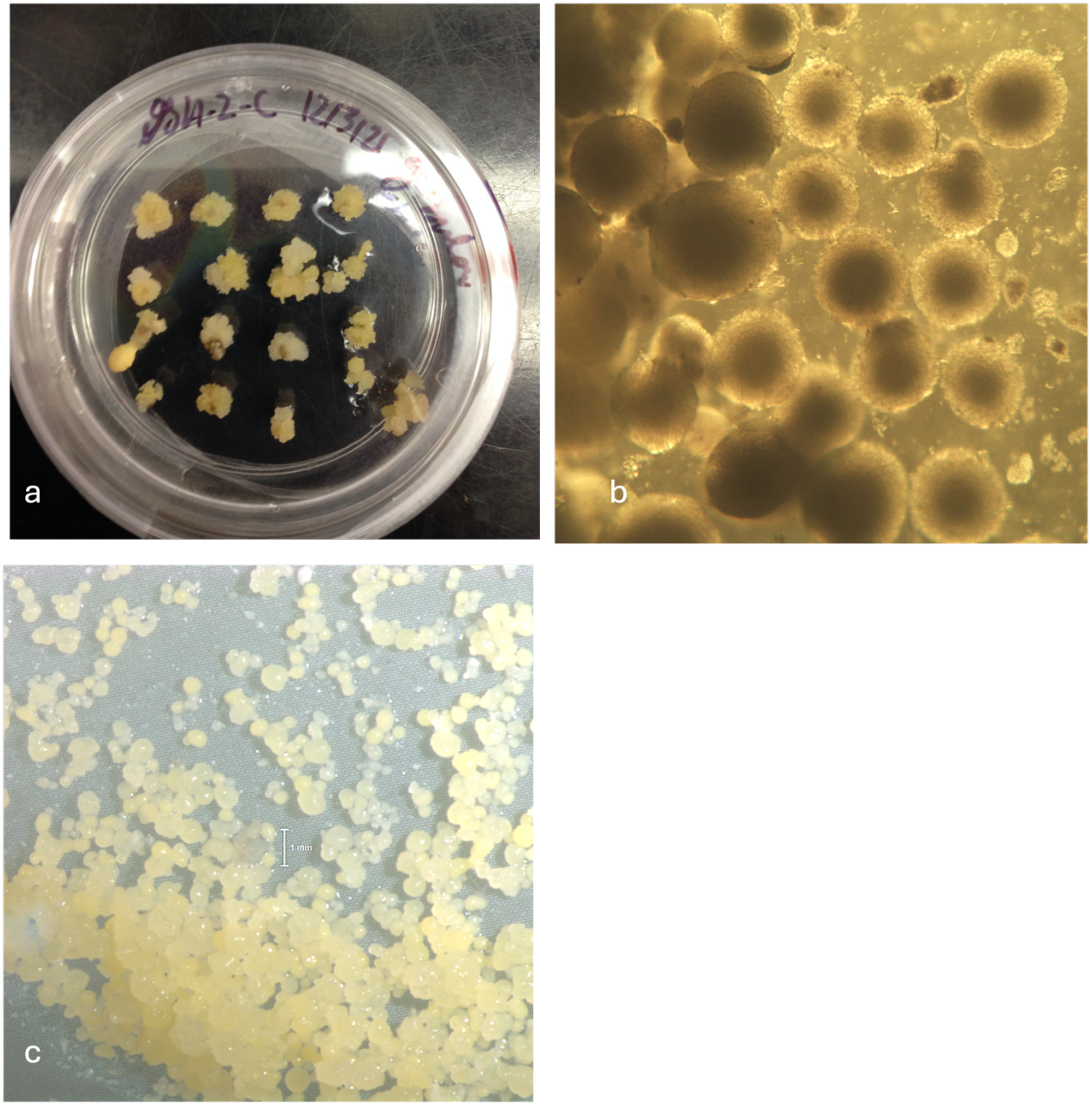

Figure 3.11: Re-induced embryogenic material growing on semi-solid media (a), re-induced Georgia oak embryogenic cultures from line GA-2-C growing in suspension culture in medium supplemented with 2 mg/L 2, 4-D (b), and immediately after being plated on nylon mesh following removal from suspension (c).

CHAPTER 4

CONCLUSION

Redbay, swampbay, and sassafras somatic embryogenesis

Somatic embryogenesis was successfully induced for the first time in redbay (*Persea borbonia*), swamp bay (*Persea palustris*), and sassafras (*Sassafras albidum*). Zygotic embryos used as explants for embryogenesis induction experiments were sourced from Dr. Jason Smith at the University of Florida, the University of Georgia main campus in Athens, and US Forest Service plots at the Warnell School of Forestry and Natural Resources' Whitehall Forest. Experiments involving redbay and swamp bay were performed in 2020 and 2021, and the experiment involving sassafras was conducted in 2022. Developing zygotic embryos were collected on two different dates for redbay and swamp bay, and only once for sassafras. Induction rates for all three species were very low, with only one explant from redbay and one explant from swamp bay producing embryogenic cultures. Four sassafras explants produced embryogenic cultures, which overall was slightly higher than either *Persea* species. For all of the successful somatic embryogenesis inductions, 0.2 mg/L picloram was used in the induction medium (Woody Plant Medium supplemented with glutamine). Due to the low induction rates, it was impossible to discern whether or not collection time, genotype, or plant growth regulator (PGR) treatment affected the production of embryogenic cultures. Although embryogenesis induction rates were very low and the cultures have yet to produce complete plantlets, the fact that somatic embryo production was achieved for all three Lauraceae species offers hope that eventually this approach can be further developed to use for clonal propagation and associated technologies such as cryopreservation for germplasm conservation.

Georgia oak and Oglethorpe oak somatic embryogenesis

Multiple embryogenic lines of two rare oak species, Georgia oak (*Quercus georgiana*) and Oglethorpe oak (*Quercus oglethorpensis*), were produced for the first time from zygotic embryo explants from immature acorns collected in the summer of 2021. Four Georgia oak embryogenic culture lines and two Oglethorpe oak embryogenic culture lines were produced during the study. All SE initiations occurred on WPM, and both the 0.2 mg/L picloram and the 2 mg/L 2, 4-D treatments produced embryogenic cultures. Availability of Oglethorpe oak acorns to utilize for the induction experiment was severely limited by poor acorn production from the accessible trees. A few months after the initial induction, embryogenic lines that were previously had been proliferating well began to decline. In an effort to preserve these lines, experiments were undertaken to determine the effectiveness of "re-inductions" using embryogenic material that had been grown in liquid suspension. These experiments produced embryogenic cultures that grew prolifically. Eventually, a small number of Georgia oak somatic seedlings lines were produced. They grew slowly, but otherwise seemed typical and healthy initially. Most did not successfully acclimatize following potting and died. One, however, has continued to grow and thrive. While somatic embryos were produced from the Oglethorpe oak cultures, they failed to produce plantlets. As with the Lauraceae research, while more work remains to optimize SE systems for these two rare oaks, the production of somatic embryos from them, and the regeneration of somatic seedlings of Georgia oak, are promising steps toward the eventual application of somatic embryogenesis for their propagation and germplasm conservation.