

MICROPROPAGATION AND SOMATIC EMBRYOGENESIS IN FRANKLINIA

ALATAMAHA BARTRAM EX MARSHALL

by

DAVID GLENN BELESKI

(Under the Direction of Scott Merkle)

ABSTRACT

Franklinia alatamaha (Theaceae) is a monotypic genus, believed to be extinct in the wild since 1803. It exists solely as *ex situ* cultivated specimens. A thorough literature review including primary sources, previously unrecognized publications, and current studies in the field, provides a number of possible answers towards the ongoing debate over the species' origins and causes of its extirpation. In addition, a complete micropropagation system for the production of axenic shoot cultures and fully rooted potted plantlets was developed. A variety of plant growth regulator (PGR) combinations, as well as explant sources, were tested for the initiation of both dormant and active shoot cultures. The influence of gibberellic acid (GA₃) and various chelating compounds were tested for their applicability toward *in vitro* propagation. Finally a variety of treatments were tested for their effect on rooting the previously cultured shoots *in vitro* prior to hardening off.

INDEX WORDS: *Franklinia alatamaha*, Micropropagation, Organogenesis, Gordoniae, Theaceae, *in vitro* propagation, micropropagation, Plant Growth Regulator (PGR), plant conservation, ex-situ preservation, paleobotany, auxin, cytokinin, chelation, EDDHA, EDTA

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Discovery and History

Franklinia alatamaha Bartram ex Marshall, is a monotypic genus from within the family Theaceae (Prince and Parks, 2001; Yang et al., 2004). It is commonly referred to as Franklinia, Franklin Tree, Lost Gordonia, and Lost Camellia (Griffin and Blazich, 2008). The species was originally collected from a site in coastal Georgia near the banks of the Altamaha (Alatamaha, was a variant spelling used by Bartram) River in the vicinity of Fort Barrington, Georgia. The site of the only known population was located at approximately North 31° 29' Latitude, West 81° 57' Longitude. The original discovery of this species was made by John Bartram and his son, William, on the 1st of October, 1765. This discovery took place during the duo's now famous explorations throughout the eastern coast of the current United States. The *Franklinia* species has only been identified from the original type locality, and since 1803, there have not been any confirmed identifications of *Franklinia* plants *in situ* (Del Tredici, 2005; Harper and Leeds, 1937; Plummer, 1977). William Bartram was the first to comment on the rarity of the species, noting that he had not seen it anywhere else between Pennsylvania in the north, Florida in the south, and from the eastern coast, west to the Mississippi (Harper and Leeds, 1937). The original population, which, the Bartrams had discovered in 1765 and to which William subsequently returned to collect material from in 1773, was described as being only two or three acres in size (Harper and Leeds, 1937; Plummer, 1977). Thirty plus years later, when the well known botanist and plant collector John Lyon arrived at what is believed the same location, he

found just six to eight trees covering less than half an acre of land (Plummer, 1977). Since that date, (May 30th 1803), there has not been a single recorded discovery of a non-cultivated *Franklinia* (Del Tredici, 2005; Harper and Leeds, 1937; Plummer, 1977). According to some sources however, the last reliable sighting of *Franklinia in situ* was at some point in 1790, during a collecting expedition made by Moses Marshall (Harper and Leeds, 1937; Ranney and Fantz, 2006; Ranney et al., 2003; Rowland, 2006).

In 1785, William Bartram's cousin, Humphry Marshall, published the current botanical description of the species in his work Arbustum Americanum (1785). Prior to the description of *Franklinia alatamaha* Bartram ex Marshall, there are some published accounts of the plant being referred to as *Gordonia pubescens* (Aiton, 1814; L'Héritier de Brutelle et al., 1784; Lamarck and Poiret, 1786). L'Heritier lists, as separate species, both *Gordonia pubescens* as well as *Gordonia franklini* in his botanical work, Stirpes Novae aut minus cognitae (1785) (L'Héritier de Brutelle et al., 1784). While the original date of this publication is generally given as 1784, the later chapters, including chapter six, in which the *Gordonia* species are described, were not published until sometime either during or after 1785. As cited by the Biblioteca Digital del Real Jardin Botánico de Madrid, the chapter containing descriptions of the species within the Theaceae (Fasculus 6) was not actually published until the year of 1791 (L'Héritier de Brutelle et al., 1784). Support for this being a later publication than Marshall's can be found in that L'Heritier included as a synonym *Franklinia alatamaha* Marsh. Arbust. This is obviously a reference to Marshall's publication, Arbustum Americanum (L'Héritier de Brutelle et al., 1784). The names published by L'Heritier do not appear to have been botanically valid, as there are no records of a published description as *Gordonia pubescens* prior to the description by Marshall in his Arbustum Americanum of 1785. While the name does appear to have been in use, as even

William Bartram refers to the *Franklinia* material he collected during his 1773-1779 expedition by this name, his records were not published until 1791 (Del Tredici, 2005; Plummer, 1977; Rowland, 2006). The history of the binomial, *Gordonia pubescens*, is somewhat convoluted. Prince (2001) attributes this name to Antonio Jose Cavanilles. The earliest publication of Cavanilles', however, does not appear until sometime after 1785 (Lumbreras, 2005; Prince and Parks, 2001). In the cited publication of Cavanilles, the binomial is attributed to Lamarck (Lamarck and Poiret, 1786). While the publication date for this work is often listed as 1786, the portion containing the binomial *Gordonia pubescens* is considered to have been published on the 14th of April 1788. It seems most likely that the name originated with Dr. Daniel Solander. Funding for the Bartram's collecting expeditions of 1773-1778 was provided at least in part by Dr. John Fothergill and in exchange for his support, Bartram sent him samples of both living and mounted specimens that were collected on the expedition (Harper and Leeds, 1937; Plummer, 1977). These samples were then to be examined and described by Daniel Solander, who was a pupil of Linnaeus, as well as also being under the patronage of Fothergill (Harper and Leeds, 1937). It appears Solander did examine the *Franklinia* specimens, as in the early 1780's he "advised Bartram that the plant being called *Gordonia pubescens* was a "unique species" (Plummer, 1977). Unfortunately, Solander died in 1782 before any of his work could be published. Dr. John Fothergill also died within a year or two of Solander, with the result that the Bartram's' discoveries remained unpublished (Harper and Leeds, 1937).

Botanical Characteristics and Diseases

Franklinia may be grown as a woody ornamental plant. In cultivation, it takes the habit of a small tree or large shrub. The species grows in USDA agricultural zones 5-9, may attain heights of approximately 9 meters or more, and bears attractive, bright white, five-petaled, bright

yellow-stamened, perfect flowers from July until frost, generally. In some situations, it will flower as early as April, depending on the region where it is grown (Griffin and Blazich, 2008; Harper and Leeds, 1937). One of the plant's most desirable and commented on features, especially in areas lacking fall color, are its leaves, which turn a brilliant red prior to fall abscission (Griffin and Blazich, 2008). Specimens near the southern end of *Franklinia*'s range tend to be smaller and shorter lived, while those further north may attain great size and have long life spans (Del Tredici, 2005; Plummer, 1977; Rowland, 2006). Some of Bartram's original seedlings lived over 50 years and attained heights of over 40 ft at his garden in Philadelphia. Specimens in the Arnold Arboretum at Harvard University in Massachusetts are over 120 years old (Del Tredici, 2005).

One reason given for *Franklinia*'s infrequent use in ornamental plantings, especially in its native range, is its short life span (Koslow and Peterson, 1980; Plummer, 1977). The reduced life span has been connected to the species' high susceptibility to crown galls and root rot. These disorders have been linked to the pathogen *Phytophthora cinnamomi* (Koslow and Peterson, 1980; Meyer et al., 2009a; Plummer, 1977). In plants that developed these symptoms, no other pathogens were detected, leading to the conclusion that *P. cinnamomi* caused the symptoms and the plants' demise (Koslow and Peterson, 1980). When *Franklinia* was tested for resistance to this pathogen, the results were quite dramatic. Within 70 days of a *P. cinnamomi*-infected rice grain being inserted into the growth media, 100% of the experimental *Franklinia* plants had died, while control plants, which consisted of five non-inoculated plants per species, displayed no symptoms. In comparison, the closest known relatives of *Franklinia*, representatives of the genus *Schima* (*S. wallichii*, *S. khasiana*), and *Gordonia lasianthus*, were either unaffected, or displayed only slight symptoms 70 days post-inoculation with *P. cinnamomi*. These results

suggest that the species is extremely susceptible to infection by this pathogen and has little ability to limit the procession of damage once infection has occurred (Meyer et al., 2009a). Inter-generic hybrids of *Franklinia* crossed with either *Schima* or *Gordonia* displayed a significant increase in *Phytophthora* resistance (Meyer et al., 2009a).

Species Origin and Phylogenetics

The events leading to the extirpation of *Franklinia* in the wild, as well as its origins, have become the subject of a number of hypotheses. Among these, is the hypothesis that *Franklinia* is a displaced relic from a period of continental glaciation. According to this hypothesis, *Franklinia* would have existed throughout a greater range in the past, until southward expanding glaciers scraped bare the existing flora and the altered climate rendered the former homeland uncondusive to the continued existence of the species (Braun, 1955). Based on this theory, the claim is that the population of *Franklinia* discovered by the Bartrams had been driven either south or to lower elevations. The migration resulted in the remaining population of *Franklinia* being left stranded in a geographical region and/or climate which was unfavorable to its biological requirements after the glaciers receded and the climate reverted to its pre-glacial state (Braun, 1955). Some support for this hypothesis can be construed from the knowledge that there are a number of other plant species in the southeastern U.S. which seem to exist in a similar set of circumstances. One such species is the Georgia Plume (*Elliotia racemosa*), which is known to exist only in a small number of locations, primarily in the Altamaha-Ogeechee-Savannah river watersheds (Faircloth, 1970). *Torreya taxifolia* is also cited as another species which was displaced by glacial events (Schwartz, 2005). *Neviusia alabamensis* is a third example of a southeastern U.S. plant which is believed to have been left isolated in small populations as a result of glaciations (Freiley, 1994).

Further support for the hypothesis that *Franklinia* is a displaced species can be drawn from the field of phylogeography. As described by Tiffney (2001) ‘Phylogeography posits that the sequence of speciation events within a clade should parallel the geographic migration and isolation of members of the clade through time’ (Tiffney and Manchester, 2001). This field of research can be applied to the questions regarding the origin of *Franklinia*. This would be done through the processes known as Leaf Margin Analysis (LMA), and Leaf Physiognomy, which are based on a correlation between leaf morphology and climate. Using these techniques, it may be possible to elucidate the climate under which the species differentiated from its ancestors (Royer et al., 2005; Tiffney and Manchester, 2001). While a thorough study of *Franklinia* and its relatives has yet to be performed, a few key characteristics do tend to differentiate *Franklinia* from its close relative, *Gordonia lasianthus*, despite the fact that they are/were known to exist in identical habitats, within close proximity of one another (Harper and Leeds, 1937; Harper, 1906). In LMA and Leaf Physiognomy, a number of morphological traits are considered indicative of the climate in which a plant developed. Traits indicative of a hotter climate are as follows; fewer teeth on the leaf margin, smaller tooth area, less leaf dissection, high mass per area (density), and long leaf life. Traits correlated with a colder climate are generally the opposite of the traits listed previously, such as more teeth on the leaf margin, greater tooth area, higher degree of leaf dissection, low mass per area (density), and short leaf life. A comparison of these traits in *Franklinia* and *G. lasianthus* clearly demonstrates that *Franklinia* has a greater number of teeth per leaf, a greater tooth area, shorter leaf life, and a much higher cold tolerance (Ranney and Fantz, 2006). These differences could be taken as an indication that, at least in comparison to *G. lasianthus*, *Franklinia* evolved in a cooler climate with fewer growth days (Royer et al., 2005; Tiffney and Manchester, 2001). Further support of this argument could be derived from the fact

that, within the Theaceae, deciduous leaves are considered to be an evolved trait, existing only in some *Stewartia* spp. and *Franklinia* (Prince, 2002).

Further support for this hypothesis is offered from the field of phytogeography. While Bartram's records regarding the exact location of *Franklinia*'s discovery may be debated, the habitat in which *Franklinia* was located is clearly within the geographical region of what is now known as the Altamaha Grit Series (Ewan and Ewan, 1963; Harper and Leeds, 1937; Harper, 1906; Plummer, 1977). The Altamaha Grit series is a clearly defined geological horizon which is believed to have been deposited during the Pleistocene epoch (Harper, 1906). R.M. Harper, in his description of the phytogeography of the Altamaha Grit Series states that 'the whole flora [of the Altamaha Grit Series] without exception is believed to have come into the region since the Pleistocene period' (Harper, 1906). It is further stated that while "many of the species came into existence (i.e. evolved) since that time [the Pleistocene Epoch], others which are older have found their way in from distant regions" (Harper, 1906).

A second hypothesis, put forth by Gayther L. Plummer (1977) regarding the existence of a small, single population of *Franklinia*, is that the species is not in fact a native of the southeastern U.S. The support which is offered for this hypothesis is based largely upon the physiology of the known *ex situ*-cultivated specimens of the species. Plummer's support for his claim includes *Franklinia*'s noted cold tolerance, the vernalisation requirement of *Franklinia* seed, the late flowering period common to the species, the observation that a very low percentage of collected seeds contained ovules (<5%), historical accounts lacking comments regarding *Franklinia* seedlings, chromosome number; which Plummer believed was closer to known Asian relatives within the Theaceae (*Eurya*) versus *Gordonia*, as well as what he claimed to be some inconsistencies between modern observations and historical accounts with regard to

inflorescence location and timing (Plummer, 1977). Plummer takes these observations as support for the theory originally mentioned briefly by Lyon that *Franklinia* is most probably a species which is exotic to the continent of North America (Ewan and Ewan, 1963; Plummer, 1977). Lyon, who was a naturalist and botanical collector, traveled the eastern coast of the United States between the years of 1799-1814. His observations were recorded in his journal, which had remained unpublished prior to the 1963 publication of Ewan and Ewan 1963 (Ewan and Ewan, 1963). The possibility that the species arrived from parts unknown via human intervention as a result of the colonial era tea trade, is offered as the most logical conclusion (Plummer, 1977). While the evidence put forth by Plummer would seem to support the hypothesis that *Franklinia* is not a native of the region where it was discovered by Bartram, it does not lead one inevitably to the conclusion that it must be exotic to the continental U.S. In fact, he has been proven correct in his linking of *Franklinia* with Asian members of the Theaceae. However, the genus on which this hypothesis was based (*Eurya*) is no longer accepted as a member of the Theaceae (Plummer, 1977; Prince and Parks, 2001; Prince, 2007; Wu et al., 2003; Yang et al., 2004). Recent genetic analyses indicate that the Asian genus *Schima* is one of the three currently recognized genera within the tribe Gordoniae, the other genera being *Franklinia* and *Gordonia*. Thus, the evidence cited by Plummer does not necessarily indicate *Franklinia* has a recent Asian origin. The link between the flora of the Eastern U.S and Eastern Asia is fairly well known and can be demonstrated by a number of plant genera that have close relatives in both locations. Examples of this link are the genera *Gymnocladus*, *Liriodendron*, *Rhododendron*, and an example from the Theaceae itself, *Stewartia* (Braun, 1955). As to the data concerning seed germination, while it has been found that seed of this species does have a vernalisation requirement, it is a form of secondary dormancy, and if the

seeds are sown immediately after maturation, they will germinate readily. Plummer himself cites the work of Farmer and Chase (1977), which includes the secondary dormancy information as well as demonstrating that the vernalisation requirement is overcome by light exposure (Plummer, 1977).

What Plummer claims to be a discrepancy between Bartram's records and the known time and form of inflorescence in cultivated *Franklinia*, is a subject taken up by the earlier work of Harper and Leeds (1937). These authors clearly indicate that, under certain conditions, the inflorescence timing and form does match those described by Bartram. While few would argue against the hypothesis that *Franklinia* possesses a number of characteristics which suggest an origin different from its site of discovery, they do not necessarily suggest it to be a recently introduced exotic to the North American continent. The evidence cited by Plummer (1977) supports the hypothesis that *Franklinia* evolved in a cooler climate, but it does not support any one region over another.

A third possibility which has been raised by some is that *Franklinia* is actually a sport or mutant form of *Gordonia lasianthus*. Mellinger (1969) attributes this theory to a Walter Harmer. The original source for this claim was not cited, however, and it remains currently unverifiable (Mellinger, 1969). While this hypothesis might have been a viable proposal at the time it was made, recent genetic analyses offer little support for this theory (Prince and Parks, 2001; Yang et al., 2004).

The combination of a number of factors leaves little doubt that *Franklinia alatamaha* is in fact a North American native woody plant. Most notably, the work of Yang and Yang et al. (2004) as well as, Prince and Parks (2001) offer support for this conclusion. These works examine phylogenetic relationships in the family Theaceae. Based on chloroplast encoded *rbcL*

and *matK* DNA, Prince and Parks (2007) concluded that *Franklinia* is a member of what they designate the tribe *Gordoniae sensu stricto*. This tribe is said to consist of the North and South American *Gordonia* species (*G. lasianthus* and *G. brandegeei*, and possibly other members currently in Laplacea), as well as all the species of the genus *Schima* which were examined. Excluded from this tribe are the Asian species of *Gordonia*, which are reassigned to the genus *Polyspora* (Prince and Parks, 2001). In reference to *Franklinia*, the authors state “Molecular data clearly distinguishes *Franklinia* from *Gordonia*, and places it as a closer relative of the Asian genus *Schima*” (Prince and Parks, 2001). A similar conclusion was reached by Yang and Yang et al. (2007). Their work examined the relationship between the genera of *Gordonia sensu strictu*, *Gordonia sensu lato* (the Asian members of *Gordonia*), *Schima*, and *Franklinia*. The relationships were tested using nuclear ITS, plastid *trnL-F*, and mitochondrial *matR* DNA. The authors reached the conclusion that the genera *Franklinia*, *Gordonia sensu stricto*, and *Schima* should be recognized as the current known members of the tribe *Gordoniae*. The authors, however, come to a slightly different conclusion concerning the relationship between *Franklinia* and the other genera of the tribe. While not discussed explicitly, the authors’ cladistic diagrams clearly indicate *Gordonia* and *Schima* diverged from a single common ancestor more recently than *Franklinia* (Yang et al., 2004). In a 2007 analysis of the phylogeny and evolutionary divergence times of *Apterosperma* and *Euryodendron* by Su et al. (Su et al., 2011), five genera of the Theaceae and 6 genera of Ternstroemiaceae, for a total of 37 species, were subjected to a Bayesian Evolutionary Analysis Sampling Tree (BEAST) analysis (Su et al., 2011). BEAST analysis allows for the simultaneous determination of “phylogeny, node ages, and substitution rates, and is used to date the divergence of plant and animal species” (Su et al., 2011). Estimated divergence times at the level of species, genus, tribe and family, are given for a number of

Camellia species, as well as representatives of *Stewartia* and *Schima*. *Apterosperma* is estimated to have diverged from *Camellia* 61.98 million years ago (MYA) and the tribe Theeae is estimated to have diverged approximately 110 MYA from the lineage leading to both *Stewartia* and *Gordonia*. *Stewartia* and *Gordonia* are estimated to have diverged from a common lineage approximately 80 MYA (Su et al., 2011). If this model proves to be an accurate portrayal of divergence in the Theaceae, it supports the hypothesis that the tribe Gordoniae is an endemic North American tribe. By extension, when taken with the paleobotanical data, a North American origin for the basal lineage of the Theaceae might find some support. The model put forth by this analysis clearly places the divergence of the Theeae from the shared lineage of the Stewartiae and Gordoniae into the earlier half of the Cretaceous period (Su et al., 2011). A divergence of this age would place the Last Common Ancestor (LCA) of the tribes of the Theaceae into a time period shortly after and/or during what would by necessity have been an extremely rapid period of radiation by the core Eudicots as they are now known to exist (Schönenberger et al., 2005; Su et al., 2011). This result would suggest that while the authors of these papers agree on *Franklinia* being a separate monotypic genus, the exact relation to the other members of the tribe Gordoniae is not as yet definitive.

While the aforementioned genetic analyses support the classification of *Franklinia* as a monotypic genus, it does not in itself lead inevitably to the conclusion that the species is endemic to the North American continent. The hypothesis put forth by Plummer (1977) anticipated the close relationship of *Franklinia* with an, at the time, unidentified Asian relative. These predicted relatives, which share the same number of chromosomes ($n=18$, $2n=36$) as *Franklinia*, are now known to exist as the genus *Schima* (Plummer, 1977; Yang et al., 2004). Another point made by Yang, and Yang et al. (2004) is that the exact chromosome number of *G. lasianthus* seems to be

in dispute. Of the two works cited by Yang and Yang (2004), one lists the chromosome number of *G. lasianthus* as $n=15$, while the more recent work cited found the haploid chromosome number to be $n=18$. If the more recent finding is correct, this would provide further support of a closely related genus being a native of North America (Yang et al., 2004).

The seeming ambiguity of the genetic evidence in regard to *Franklinia*'s affiliations within the tribe Gordoniae leaves open the possibility that despite *Gordonia lasianthus*' acknowledged status as a native, *Franklinia* could be a transplanted Asiatic species (Plummer, 1977). This hypothesis can be refuted quite effectively through a number of paleobotanical discoveries (Grote and Dilcher, 1989; Grote and Dilcher, 1992). In these works, a number of extinct Eocene Epoch (~56MYA) species are identified and described as members of the Theaceae (Grote and Dilcher, 1989; Grote and Dilcher, 1992). While all of the described species are placed within the tribe Gordoniae, of particular interest is *Andrewsiocarpon henryense* sp. nov. This species is described as being most similar to (though most likely not directly ancestral to) *Franklinia alatamaha* (Grote and Dilcher, 1989). A second species, *Gordonia lamkinensis* sp. nov. , is described as sharing features with *Gordonia lasianthus* and *Gordonia brandegeei*. As both of these species are considered members of *Gordonia sensu stricto*, this should be considered as strong support for the placement of *G. lamkinensis* within the tribe Gordoniae (Grote and Dilcher, 1992). These species were all identified from Middle Eocene Claiborne formations in Western Kentucky and Tennessee (Grote and Dilcher, 1989; Grote and Dilcher, 1992). Given the age of these deposits, as well as the number of species both identified and attributed to the tribe Gordoniae, it would seem reasonable to conclude that the North American continent has, at least since the Eocene Epoch, been a center of diversity in the tribe Gordoniae.

While the aforementioned publications, (Grote and Dilcher, 1989; Grote and Dilcher, 1992) provide strong support for the consideration of North America as a center of diversity in the *Gordoniae*, there is also a long history of Theaceous plant remains in the North American fossil record. Theaceous plant remains have been found throughout North America and range in age from the Cretaceous period continuously throughout the entire tertiary period (Grote and Dilcher, 1992). The fossil genus *Ternstroemites*, which is considered to include leaves representing a number of different Theaceous genera, including *Gordonia*, has been recorded in Cretaceous deposits in the southeastern United States (Grote and Dilcher, 1989). A large number of the Theaceous discoveries have been attributed to the genus *Gordonia* or are considered to be closely related (Grote and Dilcher, 1989; Grote and Dilcher, 1992). The ancient history of the *Gordoniae* on the North American continent suggests that there is no reason to doubt *Franklinia*'s recognition as a North American endemic species. The North American fossil record, in addition to further genetic evidence suggesting the divergence of the basal tribes of the Theaceae occurred in the mid to late Cretaceous Period, allows more than enough time for the further divergence of the *Gordoniae* in North America (Su et al., 2011). In his examination of the origins of North American Tertiary flora (Manchester, 1999) concludes that the genus *Gordonia* was present and well-represented in North America during the Eocene. Furthermore the conclusion is reached that the genus migrated from North America, crossing the proto-North Atlantic ocean to Europe and then on to Asia (Manchester, 1999). A citation of Mai (1981) provided in a publication by Grote and Dilcher (1989) claims *Franklinia* remains have been identified from Pliocene deposits in both Europe and Western Asia. The publication of Mai (1981) cites an Italian publication from the University of Florence (Principi, 1938). A review of the original publication (Principi, 1938), failed to provide support for this citation.

The comparatively ancient and well-represented fossil record of the *Gordoniae*, as well as published specimens from the two additional Theaceous tribes, on the North American and European continents raise the further question of the actual origins of the Theaceae. Grote and Dilcher (1989) comment on the disparity between the existing Theaceous distribution and the fossil record, which has only yielded two remains in this same area, in contrast to numerous finds in North America and Europe (Grote and Dilcher, 1989). As described by (Graham, 1964) the North American continent has had habitats suitable for continuous occupation since the Cretaceous, though the exact ranges may have varied. Specifically the Appalachian Mountains have been dated as being a relatively stable environment for the previous 230 million years (Graham, 1964). As a result of ongoing geologic processes such as uplift, weathering, deposition, erosion etc., fossils become increasingly rare with increasing age. Due to these geologic processes, it may prove impossible to determine the geographic origins of the LCA of the tribe *Gordoniae* as well as the Theaceae overall. As discussed previously, there exists a large and diverse number of floral species with a disjunct distribution between Eastern North America and Eastern Asia (Graham, 1964; Hsu, 1983; Tiffney and Manchester, 2001; Xiang et al., 2000). The concordance of work suggests that in the distant past, a circumboreal mesophytic forest existed in which the now disjunct species existed throughout a continuous range (Graham, 1964; Hsu, 1983; Manchester, 1999; Tiffney and Manchester, 2001; Xiang et al., 2000). While in some cases the disjunct distribution may have resulted from “migration events” (Hsu, 1983), the majority of disjunct eastern North America/Asia species are believed to have existed *in situ* since the time of the hypothesized panboreal mesophytic forest (Graham, 1964; Hsu, 1983; Manchester, 1999). The now disjunct distributions are believed to be the result of vicariance events in which genera with a formerly widespread distribution were extirpated from regions

which had previously been inhabited (Xiang et al., 2000). While the current center of diversity of the Theaceae is located in Asia, the fossil record for North America is much more extensive in both quantity and age (Grote and Dilcher, 1989).

In addition to genera with a disjunct distribution in these areas, there are species which exist as relic populations, though they most likely existed throughout a greater range in the past. As an example of this situation, (Graham, 1964) cites the case of *Franklinia*. A second example is the present distribution of the sole remaining species of *Ginkgo*, *Ginkgo biloba*. While *Ginkgo* is commonly thought of as an Asian endemic, the fossil record indicates in the past it has existed throughout the northern hemisphere (Manchester, 1999). As described by (Tiffney and Manchester, 2001) the current range of a relic species is derived from “differential extinction events as much as the species’ place of origin”. In addition to the previous example of *Ginkgo*, *Metasequoia* is another relic genus which, while currently found in Asia, was widespread throughout the northern hemisphere in the past (Tiffney and Manchester, 2001).

As both the southeastern United States and eastern Asia are considered to have been refuges from climatic change, the current distribution of a family, genus, or species might not adequately represent its former habitat or place of origin (Grote and Dilcher, 1989; Hsu, 1983; Tiffney and Manchester, 2001; Xiang et al., 2000). The greater amount of diversity within the Theaceae of Asia compared to North America in modern times is believed to be derived from a lower rate of extinction due to Pleistocene climatic events; this may also have been accompanied by an increased rate of speciation (Prince and Parks, 2001; Prince, 2002).

As there is a distinct lack of paleobotanical evidence of the *Theaceae* in Asia, it would appear premature to conclude that the family has its origins in Asia based solely on the current distribution of existent species (Grote and Dilcher, 1989).

A third field of study can also be seen as supporting the hypothesis that *Franklinia* is a native North American plant species. Plummer (1977) was the first to bring up the issue of *Franklinia*'s susceptibility to what he described as "cotton root rot organism". While what he refers to may or may not have been *Phytophthora cinnamomi*, it is now a well-known pathogen of *Franklinia* (Koslow and Peterson, 1980; Meyer et al., 2009a; Ranney and Fantz, 2006; Ranney et al., 2003). As demonstrated by Koslow and Peterson (1980) as well as Meyer and Ranney et al. (2009), *Franklinia* infected by *P. cinnamomi* will generally suffer a 100% mortality rate (Koslow and Peterson, 1980; Meyer et al., 2009a). Thus, one could reasonably describe *Franklinia* as having little or no resistance and/or tolerance to *Phytophthora cinnamomi*. As noted by Newcombe and Dugan (2010), the most likely explanation for an organism's lack of resistance to a pathogenic fungus is a lack of exposure and hence little or no evolved resistance (Newcombe and Dugan, 2010). This point is made by Zentmyer on a number of occasions as well (Zentmyer, 1988; Zentmyer, 1985). Meyer and Ranney et al. (2009) as well as Marshall and Cole (1986) demonstrated that resistance to *P. cinnamomi* varies significantly among members of the Theaceae. Among a test group consisting of *Abies fraseri*, *Franklinia*, *Gordonia lasianthus*, *Schima khasiana*, *S. wallichii*, and the inter-generic hybrids X *Gordonia grandifolia*, and X *Schimlinia floribunda*, *Franklinia* displayed the lowest level of resistance and/or tolerance to *P. cinnamomi* (Meyer et al., 2009a). In contrast to *Franklinia*; the Asian *Schima* species displayed the highest level of tolerance to *P. cinnamomi*. While *Gordonia* did tolerate the pathogen significantly better than *Franklinia*, it also displayed a more severe range of symptoms than the *Schima* species (Meyer et al., 2009a). Marshall (1986) conducted a similar experiment determining the susceptibility of a number *Camellia* species to *P. cinnamomi*. Ninety days post inoculation with *P. cinnamomi*, root samples were tested for the presence of the pathogen.

Camellia sasanqua was found to be entirely free from physical symptoms as well as statistically uninfected by the pathogen ($\alpha < .05$) when compared to the *C. sasanqua* control group. While the three remaining species tested (*C. japonica*, *C. reticulata*, *C. sinensis*) did display some physical symptoms, which the authors described as slow growth, only *C. sinensis* and *C. japonica* showed any significant level of infection. To illustrate the level of physical effects exhibited by the test subjects as a result of *P. cinnamomi* infection, the author makes the following statement. “ *C. sinensis*, in particular, showed very poor root growth, and whilst it still managed to produce a flush of growth in the spring, the lack of root growth would make it unsuitable for use in commercial grafting’ (Marshall and Cole, 1986). This is a dramatic difference from Meyer’s (2009) finding for *Franklinia*, in which every individual died within a 84 days post inoculation with *P. cinnamomi* (Meyer et al., 2009a). While the exact center of origin for *P. cinnamomi* has yet to be definitively determined, it is most likely that the pathogen originated in Asia. Some have claimed the center of origin to be somewhere in North or South America, Australia, Melanesia, or South Africa, but the most reliable evidence points towards an Asian origin (Newcombe and Dugan, 2010; Zentmyer, 1985). This conclusion is based on evidence of the presence of both the A1 and A2 mating types in an approximately equal ratio, as well as a two additional factors (Zentmyer, 1988). In other locations, such as the state of Georgia in the U.S., as well as a number of other Southeastern U.S. states, the A1 mating form is the predominant form present. In other locations, the A2 mating form predominates (Zentmyer, 1988) An Asian center of origin would explain data suggesting that *P. cinnamomi* has been present in Melanesia and Australia for a significant period of time. Some suggest that the pathogen could have been introduced to these regions during the Pleistocene Epoch or perhaps by prehistoric human migrations (Newcombe and Dugan, 2010; Zentmyer, 1977). While it has long been a source of

heated debate, the hypothesis supporting pre-Columbian agricultural exchange has benefited from recent technological advances such as radio-isotope analysis and DNA analysis. A pre-Columbian exchange of host material would provide a possible reason explanation as to why it might appear that *P. cinnamomi* was endemic in areas in which it was not actually native. Two of the most cited examples of pre-Colombian intercontinental exchange are the sweet potato (*Ipomoea batatas*) and the domestic chicken (*Gallus sp.*). While the sweet potato is widely acknowledged to have originated on the South American continent, it has been recorded throughout the Pacific since the first recorded European contact. A number of recent publications have supported pre-Colombian dates for the arrival of *I. batatas* on a number of different islands throughout Polynesia (Ladefoged et al., 2005). The Asian genus of *Gallus* has also recently been recorded in pre-Colombian middens from the coast of Chile and is supported through a number of radioisotope dates (Storey et al., 2007). While further research should be undertaken in order to either offer further support or refutation of these finds, the long history of human migration and trade still offers a reasonable explanation for the varying claims as to the center of origin of *P. cinnamomi*. Taiwan has been proposed as being located within the center of origin for *P. cinnamomi*, based on it fulfilling three criteria: (1) its populations contain an approximately equal ratio of mating types, (2) the pathogen is found in what are considered to be undisturbed sites, and (3) the native flora in the region appears to be fairly tolerant of the pathogen in comparison to exotic species (Zentmyer, 1988). If one accepts this line of reasoning, it can also be said that *P. cinnamomi* most likely should not be considered as native to the Southeastern U.S. The pathogen is known to have a large number of hosts among the southeastern flora, the majority of which are affected in an extremely negative manner when exposed to *P. cinnamomi* (Zentmyer, 1977; Zentmyer, 1988). Based on *Franklinia*'s noted lack of

resistance to *P. cinnamomi*, as well as the work which shows *P. cinnamomi* is not indigenous to the U.S., and the finding that Asian species of the Theaceae exhibit a much greater level of resistance and/or tolerance to *P. cinnamomi*, one can reasonably conclude that *Franklinia* and *P. cinnamomi* have had little or no interaction on an evolutionary timescale. This goes to support the conclusion that *Franklinia* is a native North American species.

In summary, when the totality of the evidence is considered, there can be only one reasonable conclusion concerning the origins of *Franklinia alatomaha*. The evidence to consider is the phylogenetic relationships within the Theaceae, specifically, the definitive status of *Franklinia* as a monotypic genus. In addition there is the paleobotanical data to consider, which clearly demonstrates a Middle Eocene Epoch presence in North America, for a number of species from within the tribe Gordoniae. When this evidence is taken in concert, with the data demonstrating *Franklinia*'s lack of resistance and/or tolerance to the Asian pathogen *Phytophthora cinnamomi*, there can be little doubt that *Franklinia alatomaha* is in fact a native of the North American continent.

Possible reasons for extinction of *Franklinia* in nature

As to the reasons for the extirpation of all known *Franklinia* in the wild; the exact cause and method of its demise may remain permanently unanswered. Some of the previously discussed findings may illuminate the possible reason for this occurrence.

As discussed by Plummer (1977), the species seems to fare better in regions of higher altitude and or latitude. *Franklinia* is reported to be hardy down to -38°F (Ranney and Fantz, 2006). *Franklinia* is a deciduous plant, in contrast to its close relative, *Gordonia*, which is evergreen, despite inhabiting the same area which *Franklinia* was discovered. This information, taken in combination with the vernalisation requirement of stored seed, points to an origin at

higher latitudes or altitudes than where it was originally discovered by the Bartrams (Griffin and Blazich, 2008; Plummer, 1977; Ranney and Fantz, 2006; Rowland, 2006). It is quite possible that a number of species in the southeastern United States are relics which were displaced by glaciations and/or climate change (Braun, 1955; Freiley, 1994; Schwartz, 2005). If *Franklinia* is in fact a relic which was stranded in an area unfavorable to its growth, this history could certainly be implicated in the eradication of this species in the wild.

Regardless of the cause, it is known from William Bartram's 1791 report that at that time, the population of *Franklinia* consisted of two to three acres of trees (Plummer, 1977). It is also known that Moses Marshall, the nephew of Humphry Marshall, had orders for what are variously described as, dozens or over 100 specimens of *Franklinia* during the period of 1787-1790 (Harper and Leeds, 1937; Plummer, 1977). While some dismiss the account of Lyon (1803), his journals, as published by Ewan and Ewan (1963), seem to give little reason to doubt the veracity of his claims (Ewan and Ewan, 1963; Rowland, 2006). Support for Lyon's claim of locating *Franklinia* can be found in a letter from David Landreth II. He directly attributes the collection of the rare plants, including "*Gordonias*", in his father's nursery, to John Lyon (Ewan and Ewan, 1963). While one cannot be sure from this statement that the *Gordonias* referred to were in fact, *Franklinia*, in the context of discussing rare native plants, it would seem probable, especially given the common usage of the synonyms *G. franklini* and *G. pubescens* at that time in history. If Lyon's reports are accurate, then an important piece of information regarding the *Franklinia* population of 1803 is available in addition to the other accounts. Lyon states that he found six to eight adult specimens of *Franklinia*, which did not extend over an area greater than one half acre (Ewan and Ewan, 1963; Plummer, 1977). If William Bartram reported the population to encompass two or three acres in 1791, and by 1803 the physical area covered by

the population had been reduced to half an acre, this shows a serious decline in the population (Ewan and Ewan, 1963; Harper and Leeds, 1937; Plummer, 1977). As John Lyon was a well-known collector of rare plants, it may be that he removed the last of the *Franklinia* at the time of his 1803 report. This explanation, however, seems somewhat improbable given his limited time at the site as well as the half acre size he specifies (Ewan and Ewan, 1963). If not, the reduction of the known population to less than ten adult specimens still posed a serious threat to the species' continued existence in the wild.

The study of Minimum Population Viability takes into consideration a wide range of factors in order to give a prediction concerning the minimum number of individuals a population must sustain in order to maintain a viable population over a certain period of time (Matthies et al., 2004; Menges, 1990; Menges, 2000). While the exact factors contributing to MPV are considered to vary by the analyses, the main point is that it is believed that once a population has been reduced to a certain number of individuals, it stands little chance of either continuing to prosper or to withstand the many threats which occur in the wild (Matthies et al., 2004; Menges, 1990; Menges, 2000). Due to the vagaries surrounding the population dynamics of *Franklinia* in the wild, it would be extremely difficult to construct an analysis for this species. Studies of other endangered plant species, however, can shed some light on what one might expect the minimum viable population of *Franklinia* to be in the wild. Mathies et al. (2004) found in a study of eight short-lived endangered plant species, that the minimum population required varied from 71-1276 individuals for a prediction of 90% confidence for survival of a population over a period of ten years (Matthies et al., 2004). For predictions over longer periods of time, larger populations are required to prevent the possibility of extinction. It is also believed that once a species passes below its lower MPV limit, it may enter an unrecoverable downward spiral

into extinction (Matthies et al., 2004). This belief has very important implications in the eradication of *Franklinia* in the wild. If, as stated by Lyon (1803), the population were reduced to eight individuals, the probability of the species going extinct in the wild might have been virtually assured (Ewan and Ewan, 1963; Plummer, 1977). A variable which may play an extremely important part in the extirpation of *Franklinia* is the recruitment rate of new individuals to the population. To date, there has been no genetic analysis regarding the level of genetic variation in the existent cultivated specimens. As pointed out by Del Tredici (2005), *Franklinia* has a habit of effectively propagating itself asexually via layering (Del Tredici, 2005). This behavior, in combination with low seedling recruitment rates, as found in the native *Gordonia*, may suggest that the plants found at the original site were clones of one or a few individuals, rather than product of a sexually reproducing population (Del Tredici, 2005; Gresham and Lipscomb, 1985). If this was the case at the site of discovery, it could mean that the population had possibly reached its MPV lower limit even prior to being discovered.

Franklinia is far from the only species known to exist, or to have existed, in a drastically reduced population. The Wollemi Pine (*Wollemia nobilis*) was discovered as a grove of approximately 100 individuals in an isolated part of Australia in 1994 (Peakall et al., 2003). The Wollemi Pine is now considered one of the most endangered tree species known to man. It is thought to be the sole remnant of what was previously believed to be an extinct genus from within the family Araucariaceae (Peakall et al., 2003). *Wollemia nobilis* also shares with *Franklinia* an extremely low tolerance and/or resistance to *P. cinnamomi*. While precautions have been enacted to protect the population, an inadvertent introduction of *Phytophthora* would pose a serious threat to the health and existence of the species in the wild (Bullock et al., 2000). In addition to *Wollemia*, there is the better known example of the *Ginkgo*, or Maidenhair Tree

(*Ginkgo biloba*). *Ginkgo* was believed, until recently, to be extinct in the wild, existing only through cultivated specimens. Recent discoveries suggest, however, that a relic population of *Ginkgo* trees survived Pleistocene glacial events that eliminated the majority of the species (Shen et al., 2004). The species is believed to have survived as a relic population due to a confluence of geographic factors which led to a climatically protected region in the Southwest of China (Shen et al., 2004). Other possible relic species include the previously mentioned *Elliotia racemosa* and *Neviusia alabamensis* (Braun, 1955; Faircloth, 1970; Freiley, 1994). Despite the fact that these species are not closely related, they all share a number of features which may have resulted from their relict existence or perhaps allowed them to persist in relic populations while other species became extinct. Perhaps the most notable feature shared by the majority of these plants is the propensity towards asexual/vegetative reproduction. Among *Franklinia*, this occurs through its habit of “self layering” (Del Tredici, 2005). In *Elliotia* asexual reproduction is hypothesized to occur through root sprouting and/or coppicing (Faircloth, 1970; Godt and Hamrick, 1999; Woo and Wetzstein, 2008). In *Ginkgo* and *Wollemia*, asexual reproduction takes place primarily through coppicing (Gong et al., 2008; Peakall et al., 2003). While this may prove to be a valuable trait, allowing severely reduced populations to remain in existence, it also could result in a further loss of genetic diversity. The loss of genetic diversity in an already reduced population may limit the species’ ability to survive if faced with threats of a biological and/or environmental nature (Matthies et al., 2004). The loss of diversity in an isolated population may lead to reduced fitness of offspring due to inbreeding, which promotes the accumulation of negative recessive traits (genetic load). Small populations also face the possibility of incompatible mating types and the loss of mutualistic species which may be required for survival (Matthies et al., 2004). A distinct lack of diversity has been noted in both

Neviusia and *Wollemia* (Freiley, 1994; Peakall et al., 2003). *Elliotia racemosa*, was found to have a relatively low amount of diversity at both the species and population levels (Godt and Hamrick, 1999). However, in the same study, the majority of the genetic variation which was detected existed within populations, not among populations (Godt and Hamrick, 1999). To date, there are no published data concerning the exact level of genetic diversity or lack thereof within *Franklinia*. Despite the lack of data, a number of publications state that *Franklinia* contains either “little or minimal levels” of intraspecific genetic diversity (Meyer et al., 2009a; Ranney and Fantz, 2006; Ranney et al., 2003). While *Franklinia* is known to spread vegetatively, it is also known to produce viable seed. Thus, in comparison to other relics which reproduce primarily asexually, *Franklinia* might have a much wider genetic base. Also, the claim has been made that one of the reasons for suspecting there to be limited diversity in *Franklinia* is that all of the plants in cultivation descend directly from Wm. Bartram’s original 5 seed-grown specimens; of which only two survived to fruiting (Del Tredici, 2005; Rowland, 2006). This information may not be accurate, as the historical record demonstrates. A number of sources all seem to agree that one of the reasons for Moses’ Marshall’s expedition of 1790 was to collect *Franklinia*. Moses and his father, Humphry, had orders for over 100 *Franklinia* in February, 1787, and “as many as possible” in August of the same year, followed by orders for a number of both *Franklinia* and what is listed as *Gordonia pubescens* [syn. *Franklinia alatamaha*] in 1789 (Ewan and Ewan, 1963; Harper and Leeds, 1937). As with Marshall, Lyon was on a commercial plant collecting expedition when he relocated the Bartram’s original *Franklinia* site in May 1803. During this period, records indicate he stored his collected material with Stephan Elliot, who states he used the material to describe a number of species. This evidence supports the claim that Lyon was in fact collecting during this expedition. Unfortunately, Lyon’s journal

entries during this period are sporadic and brief; he does mention locating *Franklinia*, but does not offer any information concerning collections. Given Lyon's commercial and academic interest in botany, it is probable that *Franklinia* would have been among his collection (Ewan and Ewan, 1963; Plummer, 1977; Rowland, 2006). There is also some uncertainty concerning the possibility of others having collected *Franklinia* from the original location for the nursery trade as well (Plummer, 1977). In his 1990 publication, Thomson cites records of *Franklinia* specimens 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, as stated by Bartram, this would leave little time for its propagation and subsequent transport to Europe, in order to be cataloged in the year of 1774 (Thomson, 1990). Marshall (1785) states 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 cultivated stock and the intraspecific diversity of *Franklinia* might prove to be greater than many have assumed.

While the actual extinction event may remain unknown, Dirr (Dirr, 2006) is a proponent of a hypothesis from Frank Galle, which suggests that farming and an unknown pathogen led to the extinction event (Rowland, 2006). Based on the previously discussed information concerning *Franklinia* and its susceptibility to the pathogen *Phytophthora cinnamomi*, it would seem likely this organism would be the culprit. Meyer et al (2009) suggest that the susceptibility to *P. cinnamomi* is a recessive heritable trait. If so, the already dwindling genetic base of *Franklinia* may have left it with no means to overcome this last threat to its existence (Meyer et al., 2009a).

A further condition may have influenced the demise of *Franklinia* in its type locality. Prior to European settlement, as well as during the early colonial period, the area of southern coastal Georgia was inhabited by a native American culture known as the Guale (Saunders, 2000). The Altamaha River is claimed to derive its name from a leader of this group, who lived in the region during the 1500's. During this period, it is hypothesized that there was a great deal of anthropomorphic environmental changes (Mann, 2011; Saunders, 2000; Stewart, 2002). The Guale in addition to many other Native American groups of the southeast, are believed to have employed fire as a method of resource management (Mann, 2011; Stewart, 2002). Early colonial reports note an open "parklike" character to the land as well as chronicling an extensive lack of wood for fires and construction (Saunders, 2000). As proposed by Stewart (2002) most of the land in this region consisted of an ecology which was characteristic of an altered environment (Stewart, 2002). In support of this hypothesis, Stewart (2002) quotes an earlier work by Greene (1931) in which Greene bases his hypothesis on the published accounts of Bartram, which describe the use of Indian burning throughout this region (Stewart, 2002). If the area was extensively modified throughout history, as these authors suggest, this could have played a significant role in the elimination of *Franklinia* (Saunders, 2000; Stewart, 2002). The effect of fire on a population of *Franklinia* is unknown. It has been noted that fire could play an important role in recruitment of *Franklinia's* closest North American relative, *Gordonia lasianthus* (Gresham and Lipscomb, 1985). Successful recruitment in *G. lasianthus* is said to be dependent on "severe site disturbance"(Gresham and Lipscomb, 1985). From what has been noted concerning the reproduction of *Franklinia* in cultivation it may be possible to make some inferences between the two species. Plummer (1977), reached the conclusion that *Franklinia* was probably not actively reproducing by the time it was seen by Lyon in 1803 (Plummer, 1977).

In their studies of germination in *Franklinia*, Farmer and Chase (1977) noted that it seems that the seed has an obligate light requirement for germination. They state that germination without exposure to light is virtually nil (Griffin and Blazich, 2008). This finding could suggest an important correlation between recruitment in *G. lasianthus* and *Franklinia*. A disturbed site such as that required for *G. lasianthus* would also allow for a *Franklinia* seed's light requirement to be met. It could be that successful recruitment in *Franklinia* was linked to indigenous land management practices. This could possibly explain the timing of *Franklinia*'s final demise. If *Franklinia*'s tenuous grasp on continued existence was being aided through Native American land management practices, the elimination of the Native American population and land management practices could explain the lack of recruitment in historical records, the lack of recruitment would then lead to the eventual elimination of the population.

The arrival of Europeans may have also affected the recruitment rate of *Franklinia* through the introduction and spread of feral swine. Feral pig bones were noted in burial mounds predating Catholic missions in the immediate area, and had presumably spread from early introductions by Spanish explorers (Saunders, 2000). The arrival of feral hogs to a specific location generally preceded actual Native American-European contact due to the hogs rapid reproduction rates and lack of natural predators (Mann, 2011). The arrival of feral hogs to an area often resulted in a rapid and drastic change in the local environment. This is generally a result of the hogs' feeding habits, which involve rooting up seedlings and consuming seeds both from underground and above the surface (Mann, 2011; Stewart, 2002). This leads to a serious decrease in the rate of recruitment especially in species which had previously depended on seed reserves within the soil (Stewart, 2002). If *Franklinia* did traditionally maintain its populations

in a similar fashion to *G. lasianthus*; the impact of feral hogs consuming dormant seeds could be devastating (Gresham and Lipscomb, 1985; Griffin and Blazich, 2008).

Micropropagation

A micropropagation system for the production of *Franklinia* would have a number of potential applications, the first being a method to rapidly produce large numbers of axenic plants. Production of such plants would be of use to the nursery trade and to groups attempting to reintroduce the species to the wild. It could also be a way to promote diversity in the species. If it can be determined whether plants currently in cultivation are of varying genotypes, they could then be preserved *in vitro* for use in breeding programs. A micropropagation system could also aid in the development of morphogenic regeneration systems, such as adventitious shoot (AS) production, or somatic embryogenesis (SE) propagation systems, as well as transgenics, through the ability to provide axenic material for explants to initiate these processes (Mondal, 2004A). The availability of embryogenic or adventitious shoot-based regeneration systems, in combination with gene transfer technology, has the potential to reduce, or possibly eliminate, the specific pathogen issues affecting *Franklinia* by engineering the tree with anti-microbial genes. For example, the orchid, *Gastrodia elata*, has been found to produce a protein, Gastrodia Anti-Fungal Protein, that confers resistance to a number of plant pathogens, including *Phytophthora spp.* The gene has been introduced into a diverse selection of plant species, where it confers fungal resistance (Cox et al., 2006). It stands to reason that this gene or others conferring *Phytophthora* resistance could be introduced in to *Franklinia* (Cox et al., 2006).

Other potential biotech applications exist that would be able to utilize an embryogenic or adventitious shoot regeneration system as well. These could be employed for induced mutagenesis (IM) and ploidy manipulation (PM) as methods to increase the genetic diversity of

the species (Mondal, 2004A; Touchell et al., 2008). Induced mutagenesis has been used to increase disease resistance in many commercial crop varieties. The reason SE is preferable for use in conjunction with induced mutagenesis is that as the amount of plant material being mutated increases, the chances of a chimera generation also increase, resulting in plants composed of mutated and non-mutated cells (Ahloowalia and Maluszynski, 2001; Touchell et al., 2008). . Since somatic embryos and the plantlets regenerated from them are derived from single cells, there is less likelihood of chimeras. The rationale for the use of a SE system in ploidy manipulation is the same, primarily to avoid producing chimeric plants. Ploidy manipulation, which can affect a number of the horticulturally desirable traits related to chromosome number, is most often employed for its effects of increased overall flower size, seedless fruit production, and in breeding sterile triploid hybrids (Jones et al., 2008).

In Vitro Propagation of Theaceae Members

There has been no previously published work concerning the *in vitro* propagation of *Franklinia alatamaha*. Previous *in vitro* propagation work concerning related species has been primarily limited to the genus *Camellia*. The body of work on the genus *Camellia* is relatively extensive considering the lack of work on other *Theaceae* genera. Aside from *Camellia*, the sole publication dealing with the micropropagation of the Theaceae is the work of (McGuigan et al., 1997), which details the limited micropropagation of *Stewartia pseudocamellia* (McGuigan et al., 1997). The application of methods developed for use in *Camellia* towards *Franklinia* is in some ways a less than ideal. The time span encompassed since the divergence of the tribe Theaeae from the lineage of *Franklinia* may be such that methods developed for *Camellia* spp. may not be optimal, or perhaps even ineffectual, when applied to the micropropagation of *Franklinia*.

***Camellia* Micropropagation**

The micropropagation of *Camellia* has been undertaken primarily for use in the tea trade. Tea, in this context, may consist of *Camellia sinensis*, *C. assamica*, *C. assamica* sub spp. *lasiocalyx*, as well as a large number of inter-specific hybrids between these and other *Camellia* species (Mondal, 2004B). Tea is considered to be one of, if not the, most popular non-water beverages throughout the world and represents a significant portion of the economic output of a number of countries (Akula and Dodd, 1998; Mondal, 2004A). “Due to limits on the amount of arable land that can be devoted to the crop, as well as significant disease and pest concerns, there is a significant demand for improved yields and hardier cultivars” (Akula and Dodd, 1998; Frisch and Camper, 1987; Mondal, 2004A). There are estimated to be at least 600 varieties of tea in cultivation, which may consist of hybrids of the previously mentioned species as well as an unknown number of wild *Camellia* species (Mondal, 2004B). This great variety is hypothesized to be a result of the high amount of outbreeding and genetic instability in the *Camellia* genus (Mondal, 2004B). As a result of the heterozygous, outbreeding nature of sexually produced plants, it can be extremely difficult to maintain desired traits in plants propagated from seed. Thus, the ability to vegetatively propagate *Camellia* species is highly desirable. Asexual propagation in the form of cuttings is reported to suffer from low success rates and long propagation periods (Mondal, 2004A; Mondal, 2004B). Thus, micropropagation, due to its potential to provide virtually unlimited quantities of rapidly cloned plants, has been investigated for its application in the mass propagation of cloned elite cultivars of *Camellia* (Akula and Dodd, 1998; Mondal, 2004A; Mondal, 2004B). In the comprehensive review of tea biotechnology by Mondal (Mondal, 2004B), published methods of tea micropropagation are cited and detailed

(Mondal, 2004B). The micropropagation methods reviewed employed a wide range of tea varieties and cultivars, plant growth regulators (PGRs), and growth media.

The media formulation of Murashige and Skoog (Murashige and Skoog, 1962) is the primary nutrient formula for the micropropagation of the tea plant (Mondal, 2004B). As cited by Mondal (Mondal, 2004B) some previous research has also made use of media based on Woody Plant Medium, Hellers medium, and the medium of Nitsch and Nitsch (Mondal, 2004B). The MS medium employed in some work has been modified in a number of ways, through the addition and/or substitution of various components (Akula and Dodd, 1998; Mondal, 2004B; Pedroso and Pais, 1993).

As noted by Mondal (Mondal, 2004B) the most common plant growth regulators employed in the micropropagation of tea have consisted of a combination of benzyl amino purine (BAP) and indole butyric acid (IBA). This combination has been found to be effective across a wide range of concentrations. In the case of BAP, the effective concentration for both culture initiation and shoot multiplication ranges from 1- 6 mg/L. Indole butyric acid has been found to be effective at concentrations of 0.01 – 2 mg/L (Mondal, 2004B). In addition to this combination of PGRs the auxins indole acetic acid (IAA) and naphthalene acetic acid (NAA) have also been employed successfully in the micropropagation of tea. BAP is the primary cytokinin utilized for this purpose, although dimethylallyl amino purine (isopentyladenine, 2-*Ip*), and kinetin (N⁶ furfuryl adenine) have also been found to have some limited success (Mondal, 2004B).

In addition to auxins and cytokinins, a number of micropropagation procedures also include the use of a gibberellic acid (GA₃). As with the previously mentioned PGR's, GA₃ has been used at a variety of different concentrations (0.1 – 10 mg/L media) (Mondal, 2004B).

Exogenous sources of gibberellins, unlike auxins and/or cytokinins, are usually not considered a necessity for successful micropropagation of most species. In some plant species however, it has been found that shoot cultures fail to elongate normally and that the addition of exogenous gibberellins often remedies this problem, producing what is considered to be a more desirable pattern of growth (George et al., 2008). In a number of publications dealing with micropropagation in *Camellia spp.* it has been found that the inclusion of exogenous GA₃ is required for a normal pattern and duration of shoot multiplication (Mondal, 2004B). While there is currently a lack of research concerning the physiological reasons for an exogenous GA₃ requirement in the micropropagation of some *Camellia spp.*, one possible explanation is that the plants have become acclimated to a source of exogenous gibberellins. In a survey of endophytic fungi isolated from samples of *Camellia sinensis* collected in Indonesia, a number of different fungal species were identified (Agusta et al., 2006). Based on RAPD analysis, one of the primary fungi identified as an endophyte of *Camellia sinensis* was a species of *Fusarium*. This fungus was determined to be a close relative of the species most commonly known as *Gibberella fujikuroi* (now recognized as *F. moniliforme*) (Agusta et al., 2006). While the final determination of the species isolated in this study remains undetermined, it is clearly a close relation of *F. moniliforme* (Agusta et al., 2006). *F. moniliforme* is the source from which gibberellic acid was originally isolated. In addition to *F. moniliforme*, a number of other *Fusarium* species are known to produce gibberellic acid in quantifiable amounts (Mitter et al., 2002). While it has not at this time been determined whether the species isolated from *Camellia sinensis* is a producer of gibberellins, it is a distinct possibility. The identification of a *Fusarium* species closely related to *F. moniliforme* as an endophyte of *Camellia sinensis* suggests that, at

least in some *Camellia spp.*, a relationship may exist in which an endophytic gibberellin producing *Fusarium* species contributes a physiologically active source of gibberellins.

The relevance of this work to the micropropagation of *Camellia spp.* lies in the proposed relationship with a gibberellin producing species of *Fusarium*. It has been conclusively demonstrated that endofungi contribute secondary metabolites to the host plant in certain host/endophyte relationships. This form of relationship has been noted within a great variety of plant species including, but not limited to, the Poaceae, and Convolvulaceae (Kucht et al., 2004). It has also been noted that certain treatments will result in the elimination of the endophytic fungi, resulting in the elimination of specific secondary metabolites (Kucht et al., 2004). A number of *Fusarium* species, including *F. moniliforme*, have been found to exist in endophytic non-pathogenic relationships in Gymnosperm and Angiosperm species (Kuldau and Yates, 2000). If the isolated *Fusarium* species, or a closely related species, maintains a habitual endophytic relationship with *Camellia*, resulting in the presence of additional gibberellins, it may be that the host plant has evolved a mechanism to either make beneficial use of the excess gibberellin or the host has minimized the potential detrimental effects of excess gibberellin through lowered endogenous gibberellin production. This relationship could explain the apparent requirement for gibberellin supplementation in micropropagated camellia. If, through the process of disinfestation (which is typically carried out when initiating cultures), the gibberellin contributing endophyte was eliminated, the resulting axenic cultures would be left with a physiology unaccustomed to having to supply the plant's required amount of gibberellins. This deficit of fungal gibberellins would then result in the axenic culture's failure to thrive without the application of exogenous gibberellins.

Micropropagation of *Camellia* species outside the *Camellia* “tea” complex has also met with some success (Mondal, 2004B; Pedroso, 1995). Published reports of micropropagation in non-tea *Camellia* species include *C. japonica*, *C. oleifera*, *C. reticulata*, *C. sasquana* and the hybrid cultivar *C. X williamsii* cv. Debbie (Mondal, 2004B; Pedroso-Ubach, 1991; Pedroso, 1995; Pedroso and Pais, 1993; Tosca, 1996; Tosca et al., 1991). As with tea *Camellias*, the primary PGRs employed for the micropropagation of other *Camellia* species are also BAP and IBA, both of which have been used in a similar range of concentrations as those listed for *C. sinensis* (Mondal, 2004B). Higher levels of auxins (up to 10 mg/l) were claimed to be required for the micropropagation of some cultivars (Mondal, 2004B).

The great variety of micropropagation conditions utilized for the propagation of tea may have a relationship to the previously mentioned genetic diversity of the tea plant. It has been found that different cultivars of tea may show a significantly different response to identical treatments (Kato, 1996; Mondal, 2004B).

Organogenesis in *Camellia* species

Organogenesis in *Camellia* species has been achieved using a variety of methods. In the case of adventitious buds were produced through the use of single node explants on MS media supplemented with 0.5 mg/L BAP + 0.1 mg/L IBA (Kato, 1996). In the work of (Pedroso and Pais, 1993), organogenic callus was derived from micropropagated leaf explants cultured on a modified MS medium after immersion in a 1 g/L IBA- supplemented MS liquid medium. (Pedroso and Pais, 1993). In *Camellia X williamsii* Cv. Debbie, organogenic callus was obtained from micropropagated internodal explants as well as leaf explants with a variety of PGR supplements. Organogenic callus was produced with the addition of IBA in combination with BAP or thidiazuron (TDZ), as well as tri-iodobenzoic acid (TIBA) at a variety of

concentrations (Tosca, 1996; Tosca et al., 1991). The conditions reported by Kato and Pedroso (Kato, 1996; Pedroso, 1995; Pedroso and Pais, 1993) were also reported to lead to both direct and indirect embryogenesis simultaneously with the organogenic growth (Akula and Dodd, 1998; Pedroso and Pais, 1993).

Somatic Embryogenesis in *Camellia* Species

A successful system for the induction of somatic embryogenesis in *Camellia* would aid a number of applications. Somatic embryogenesis in tea would allow for rapid propagation of genetically identical plants which could be utilized for the production of superior cultivars. It also could aid in the breeding of new cultivars through haploid plant generation, which could then be utilized in breeding programs (Mondal, 2004B). This could be extremely beneficial when applied to *Camellia*, as a great variety of ploidy levels are found in the genus and haploid plants could then be crossed with varieties containing uneven chromosome sets, restoring fertility and allowing for previously incompatible crosses.

Somatic embryogenesis in *Camellia* has been induced starting from a variety of organs and structures. Cotyledons, immature embryos, and immature leaves and petioles have been the most common explant material for the induction of somatic embryogenesis (Mondal, 2004B). Somatic embryogenesis in *Camellia* has also been initiated directly from nodal explants. The rate of initiation was reported to be 60 percent of cultures producing embryos, using the combination of 0.1 mg/L IBA, 0.5 mg/L BAP, and 3mg/L GA-3, followed by a period of growth with no PGR supplementation (Akula and Dodd, 1998). In a 2006 publication by Kato, immature leaves derived from long term *in vitro* propagated shoots of *Camellia sinensis* var. *assimica* cv. Tingamira Normal were the source of direct somatic embryos. All treatments were equally successful in the variety *Camellia sinensis* var. *assimica* cv. Tingamira Normal. In

contrast, *C. sinensis* var. *assimica* SRL73 failed to produce any embryos in the same experiment, as did the hybrid tea clones Akane and Benikaori. The *C. sinensis* var. *sinensis* cultivar Yabukita successfully produced embryos in a maximum of 2.8 % of cultures and cv. Sayamamidori also failed to produce embryos in the treatments described (Kato, 1996). As this study demonstrated, the induction of somatic embryogenesis in *Camellia* can be highly variable in relation to genotype (Kato, 1996; Mondal, 2004B).

C. japonica and its hybrids have also been reported as having successfully producing embryos in vitro. *C. japonica* embryos were produced via immersion of leaves in MS media supplemented with BAP and either IBA or 2,4-D. With a 20 minute immersion in 1g/L IBA solution followed by incubation in darkness for 11 days, a maximum of 100% of leaf explants were found to undergo embryogenesis (Pedroso and Pais, 1993). Leaf explants of *C. Japonica* were found to produce somatic embryos when cultured initially in liquid media supplemented with a combination of 2,4-D and IBA, followed by a period with BAP and IBA supplementation (Pedroso, 1995) When shoot explants were utilized in the same procedure, induction of embryogenesis was significantly reduced, with basal sections resulting in embryogenesis in only 2-3% of explants, and stem portions resulting in embryogenesis in 15.1% of explants (Pedroso, 1995).

Role of Iron and Chelating Agent in Micropropagation

In previous work by Pedroso (Pedroso-Ubach, 1991) the MS media was modified through the substitution of 5 mg/l of Fe^{3+} citrate for the normally employed 27.8 mg/l of Fe^{3+} EDTA . In the final media composition, the mass of iron per liter of MS medium (Murashige and Skoog, 1962) was 5.56 mg/L of elemental iron, while in the medium formulated by Pedroso (Pedroso-Ubach, 1991; Pedroso, 1995; Pedroso and Pais, 1993) utilizing Fe^{3+} citrate the mass of

elemental iron per liter of final medium was 1.15 mg/L of media. The authors indicated that this modification was necessary in order to obtain embryogenic cultures. A number of publications have analyzed the effect of Fe-EDTA as a medium supplement and inclusion of Fe-EDTA has been reported to result in a number of chemical reactions with both other media components and endogenous phytohormones (Ben-Yehoshua and Biggs, 1970; Hangarter and Stasinopoulos, 1991; Kamnev et al., 2001; Kovács et al., 2006). Fe-EDTA for inclusion into media is generally prepared from Na₂EDTA, which is reacted with FeSO₄ * 7H₂O in the presence of atmospheric oxygen. The desired result of this reaction is the compound Na₂FeEDTA (Murashige and Skoog, 1962; Steiner and van Winden, 1970). The formation of Na₂FeEDTA ideally results in Fe³⁺, however this seems to be a relatively incomplete reaction based on standard colorimetric analysis of standard stock solutions. Analysis of stock solutions seems to indicate that prepared stock solutions contain a combination of Fe³⁺ and Fe²⁺ oxidation states (Steiner and van Winden, 1970). Assuming a pure grade of Na₂FeEDTA a number of issues remain. The initial reaction within prepared MS media is believed to be a light promoted degradation of the EDTA molecule by the Fe³⁺ cation. This is considered to be a form of Fenton reaction or is sometimes termed a Fenton-like reaction (Ghiselli et al., 2004). This reaction is believed to result in both the destruction of the EDTA chelating molecule as well as the reduction of the Fe³⁺ cation to a Fe²⁺ cation. This same type of reaction is commonly employed for the removal of EDTA as well as Total Organic Compounds (TOC) in industrial waste water treatment. One application is in the treatment of pulpwood processing effluent (Ghiselli et al., 2004) This has the overall effect of removing soluble iron from the growth medium (Ghiselli et al., 2004; Hangarter and Stasinopoulos, 1991). In addition, the breakdown products of this same reaction are believed to also act as growth inhibitors, the effect of which are considered a separate factor from the effect

of the insoluble iron (Hangarter and Stasinopoulos, 1991). The incorporation of Fe-EDTA has also been linked to the degradation of IAA in growth media (Ben-Yehoshua and Biggs, 1970; Hangarter and Stasinopoulos, 1991). In a 1988 publication by (Dunlap and Robacker, 1988), up to 80% of exogenous IAA was destroyed within seven days when cultures on MS medium were exposed to light (Dunlap and Robacker, 1988). The Fe^{3+} cation is in this case believed to react with both the pyrrole and carboxylic acid portions of indole carboxylic acids (Kamnev et al., 2001; Kovács et al., 2006). While IAA has been the source of most investigations into this reaction, it has been shown that the reaction occurs with IAA, IBA, and IPA (indole propionic acid) (Kovács et al., 2006). In addition to degradation of the EDTA molecule it is highly probable this reaction would also affect the cytokinin content *in vitro*. It has been noted that in media supplemented with Fe^{3+} EDDHA, there is a significant increase in cytokinin-like effects in comparison to media supplemented with Fe^{3+} EDTA (Sopory and Maheshwari, 1973). In studies relating to the degradation of purines, the Fenton reaction has been utilized as a control method due to its recognized ability to degrade purines. Adenine is specifically cited as a degradation substrate. The presence of an amine group is cited as enhancing the degradation of the parent molecule (Scholes and Weiss, 1953). Further published material hypothesized that the chemical degradation of naturally occurring plant cytokinins has a direct influence on plant senescence (Frimer et al., 1983). The overall effect of these reactions on the growth of *in vitro*-propagated plant tissue is at this time unknown. It has, however, been conclusively demonstrated that there is a definite influence on plant growth and media composition as a result of these chemical reactions.

Interactions between growth medium components result in a complex and incompletely defined mixture of secondary and tertiary reaction products. Growth media for *in vitro*

propagation which contains undefined chemical components has long been a concern in the field of *in vitro* plant propagation (Blakeslee and Satina, 1944; Murashige and Skoog, 1962). Undefined media components can result in a serious source of unaccounted variability especially when they are either derived from natural products or are the result of uncontrolled, unknown, or unaccounted for reactions. Given that these reactions most likely contribute to analysis errors, negatively impact the reproducibility of studies, and lead to less than optimum growth patterns and or rates it would be highly advantageous to eliminate or minimize these reactions. A number of factors which contribute to these reactions have been identified. Consequently, by eliminating or minimizing these influences one might improve the accuracy of analyses, the reproducibility of studies, and optimize growth *in vitro*. The three primary substrates which have been investigated for their involvement in these reactions are iron, EDTA, and IAA (Ben-Yehoshua and Biggs, 1970; Dunlap and Robacker, 1988; Hangarter and Stasinopoulos, 1991; Kamnev et al., 2001; Kovács et al., 2006; Schönenberger et al., 2005). The iron content of the medium is a primary component involved in the light-catalyzed degradation of IAA ((Dunlap and Robacker, 1988). This reaction was reduced or eliminated through a number of treatments. It was found that by eliminating light, nitrate salts, or Fe³⁺ EDTA, the degradation of IAA was reduced. If both nitrates and Fe³⁺ EDTA were eliminated, the IAA levels remained equal to those of the controls. It was also found that there was a positive relationship between an increase of pH from 4 – 7 and the amount of IAA remaining in media. While all of these factors offer a potential method of controlling the undesired reaction, some of the alternatives are likely to be less desirable than the reaction they would be designed to remedy. The authors suggest the possibility of employing liquid media which would be supplemented with a different form of iron, a periodic replenishment of IAA or the inclusion of a more stable derivative, and/or a

periodic adjustment of pH (Dunlap and Robacker, 1988). Subsequent work by (Hangarter and Stasinopoulos, 1991) determined a number of specific parameters affecting this reaction. A primary finding was that the Fe³⁺ EDTA compound itself is subject to a photo-catalyzed reaction. The catalysis of the reaction was promoted primarily by light from the ultra-violet and blue wavelengths. The use of filtered wavelengths of light significantly reduced light-catalyzed reactions involving Fe³⁺ EDTA, although this was determined via reaction products, as opposed to Fe³⁺ EDTA concentrations, so a direct quantification of the remaining Fe³⁺ EDTA could not be made based on this work (Hangarter and Stasinopoulos, 1991). As the destruction of EDTA is believed to be driven by the same type of reaction as the destruction of IAA, the authors proposed that filtered light would prevent the destruction of this media component as well (Hangarter and Stasinopoulos, 1991). In a study of the mechanisms involved in the reduction of substrates by Fe-EDTA, it was determined that without an external energy supply (in this case electricity), the reduction of substrates by Fe³⁺ EDTA was highly influenced by the ratio of metal to chelate (i.e. iron: EDTA ratio). A high ratio of chelating ligand (EDTA) to iron reduced the ability of the iron to reduce experimental substrates (Engelmann et al., 2003). This study, however, was not conducted in the context of nutritional media and as such may not be directly applicable to *in vitro* propagation media. While the elimination of iron and/or nitrate from the media might solve the problem of molecular degradation of indoles it would most likely result in media which was inappropriate for most uses due to deficiency of iron and/or nitrate. While the complete elimination of light *in vitro* would aid in the preservation of indoles by reducing the catalyzing agent, light is considered to be one of the requirements necessary for most stages of plant life. The elimination of light would however be applicable in situations such as organogenesis or embryogenesis, where light is often excluded intentionally. The finding by

(Hangarter and Stasinopoulos, 1991) which concluded that the elimination of blue and UV wavelengths was sufficient to minimize or reduce this type of reaction between Fe^{3+} and EDTA may be applicable in a wider range of circumstances. This solution presents some potential issues as well however. While it was found that the elimination of the stated wavelengths minimized the reaction of Fe^{3+} with EDTA, this could possibly shift the reaction mechanism towards other substrates in the medium. It was found that indole carboxylic acids, which possess different length carboxylic acid moieties, react to different spectra (Kovács et al., 2006). If the reaction between Fe^{3+} and another substrate (e.g. an indole carboxylic acid) was catalyzed at an un-excluded wavelength, the reaction might occur to the exclusion of EDTA but to the detriment of another substrate. Also as many plants are known to utilize wavelengths in the red and blue spectrums preferentially, the exclusion of blue light could potentially lead to physiologic consequences. Inclusion of EDTA in growth medium could result in adverse effects, even if degradation reactions were controlled. In the normal utilization of Fe^{3+} -EDTA within media, it is believed that chelated iron is reduced to Fe^{2+} , which is then utilized by the plant, while the chelating agent is believed in most cases to be left in the growth substrate (Orera et al., 2009). If this occurs in growth medium, the chelating agent would most likely recombine with other metals in the medium, resulting in an increased uptake of these elements, as the iron content of the medium decreased Fe^{3+} -EDTA (Orera et al., 2009). In some cases, the chelation of other metals, especially copper, when present in addition to Fe^{3+} , Fe^{2+} resulted in a higher level of degradation than that induced solely by the presence of Fe^{3+} , Fe^{2+} (Ghiselli et al., 2004)

The most efficient method of resolving these interactions would most likely be the inclusion of a chelate which is less prone to such reactions. A chelating agent which has been increasingly utilized, in part to minimize these concerns, is Fe^{3+} ethylenediamine-N,N-di (ortho-

hydroxyphenyl) acetic acid (Fe^{3+} EDDHA) (Orera et al., 2009). It is reported that this chelate is un-reactive when pH is between 4 and 8 (Orera et al., 2009). The substitution of Fe^{3+} EDDHA for the traditionally utilized Fe-EDTA would appear to be an efficient one-step process for the elimination of undesired reactions between media components. This substitution would ideally eliminate or significantly reduce the undesirable reactions between Fe^{3+} , EDTA, and auxins. The elimination of these reactions could result in a more consistent medium composition which, as described previously, would be beneficial in a number of ways.

CHAPTER 2
MICROPROPAGATION AND IN FRANKLINIA ALATAMAHA BARTRAM EX.
MARSHALL

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Abstract

Franklinia alatamaha is a monotypic genus, believed to be extinct in the wild since 1803. It exists solely as ex situ cultivated specimens. There have been no previous publications regarding micropropagation or in vitro propagation of the species. A system for the micropropagation of this species would serve as a precursor for future development of a process for the production of transgenic lines resistant to *Phytophthora cinnamomi*.

A number of plant growth regulator combinations were tested for the induction of shoot cultures utilizing explants of both dormant and actively growing shoots. The optimization of shoot culture growth was then tested via the use of gibberellic acid (GA₃) as well as comparing the effect of Fe-EDTA against that of Fe-EDDHA. Shoots were then tested for rooting ability via a number of treatments.

It was found that among plant growth regulator combinations tested for the induction of shoots, MS media supplemented with 2mg/L BAP and .2mg/L IBA was most effective. It was subsequently found that the addition of 10mg/L gibberellic acid (GA₃) enhanced shoot elongation as did the replacement of Fe-EDTA with Fe-EDDHA. Rooting was most effective at ½ strength MS or MS with 1mg/L IBA.

Key Words: *Franklinia alatamaha*, micropropagation, in vitro propagation, plant growth regulator (PGR), indole-3-butyric acid (IBA), Fe-EDTA, Fe-EDDHA, gibberellic acid (GA₃), Murishige and Skoog (MS).

Introduction

Franklinia alatamaha Bartram ex Marshall, is a monotypic genus from within the family Theaceae (Prince and Parks, 2001; Yang et al., 2004). It is commonly referred to as Franklinia, Franklin Tree, Lost Gordonia, and Lost Camellia (Griffin and Blazich, 2008). The species was originally collected from a site in coastal Georgia near the banks of the Altamaha (Alatamaha, was a variant spelling used by Bartram) River in the vicinity of Fort Barrington, Georgia. The site of the only known population was located at approximately North 31° 29' Latitude, West 81° 57' Longitude. The original discovery of this species was made by John Bartram and his son, William, on the 1st of October, 1765. This discovery took place during the duo's now famous explorations throughout the eastern coast of the current United States. The *Franklinia* species has only been identified from the original type locality, and since 1803, there have not been any confirmed identifications of *Franklinia* plants *in situ* (Del Tredici, 2005; Harper and Leeds, 1937; Plummer, 1977).

Franklinia may be grown as a woody ornamental plant. In cultivation, it takes the habit of a small tree or large shrub. The species grows in USDA agricultural zones 5-9, may attain heights of approximately 9 meters or more, and bears attractive, bright white, five-petaled, bright yellow-stamened, perfect flowers from July until frost, generally. In some situations, it will flower as early as April, depending on the region where it is grown (Griffin and Blazich, 2008; Harper and Leeds, 1937). One of the plant's most desirable and commented on features, especially in areas lacking fall color, are its leaves, which turn a brilliant red prior to fall abscission (Griffin and Blazich, 2008). Specimens near the southern end of *Franklinia*'s range

tend to be smaller and shorter lived, while those further north may attain great heights greater than 50 ft and have long life spans (Del Tredici, 2005; Plummer, 1977; Rowland, 2006). One reason given for *Franklinia*'s infrequent use in ornamental plantings, especially in its native range, is its short life span (Koslow and Peterson, 1980; Plummer, 1977). The reduced life span has been connected to the species' high susceptibility to crown galls and root rot. These disorders have been linked to the pathogen *Phytophthora cinnamomi* (Koslow and Peterson, 1980; Meyer et al., 2009a; Plummer, 1977).

A micropropagation system for the production of *Franklinia* would have a number of potential applications, the first being a method to rapidly produce large numbers of axenic plants. Production of such plants would be of use to the nursery trade and to groups attempting to reintroduce the species to the wild. It could also be a way to promote diversity in the species. If it can be determined whether plants currently in cultivation are of varying genotypes, they could then be preserved in vitro for use in breeding programs. A micropropagation system could also aid in the development of morphogenic regeneration systems, such as adventitious shoot production, or somatic embryogenesis propagation systems, as well as transgenics, through the ability to provide axenic material for explants to initiate these processes (Mondal, 2004A). The availability of embryogenic or adventitious shoot-based regeneration systems, in combination with gene transfer technology, has the potential to reduce, or possibly eliminate, the specific pathogen issues affecting *Franklinia* by engineering the tree with anti-microbial genes. For example, the orchid *Gastrodia elata* has been found to produce a protein, *Gastrodia Anti-Fungal Protein*, that confers resistance to a number of plant pathogens, including *Phytophthora sp.* The gene has been introduced into a diverse selection of plant species, where it has conferred fungal resistance (Cox et al., 2006). It stands to reason that this gene or others conferring *Phytophthora*

resistance could be introduced into *Franklinia* (Cox et al., 2006). There has been no previously published work concerning the *in vitro* propagation of *Franklinia*. Previous *in vitro* propagation work concerning related species has been primarily limited to the genus *Camellia*. The body of work on the genus *Camellia* is relatively extensive considering the lack of work on other *Theaceae* genera. Aside from *Camellia*, the sole publication dealing with the micropropagation of the *Theaceae* is the work of (McGuigan et al., 1997), which details the limited micropropagation of *Stewartia pseudocamellia* (McGuigan et al., 1997).

In order to determine an effective method of micropropagation for *Franklinia*, a number of experiments were undertaken. The first of these experiments examined methods by which axenic shoot cultures could be successfully and consistently initiated. A number of further experiments were then conducted for the optimization of *Franklinia* shoot growth *in vitro*. The final stage of research undertaken in the development of a complete micropropagation system for *Franklinia* was testing the ability of micropropagated shoots to root *in vitro* in relation to a number of factors such as, nutrient concentration, plant growth regulator (PGR) type and concentration, and previous growth conditions of the elongating shoot cultures.

Materials and Methods

Culture initiation from dormant plant material

Plant material utilized for the initiation of *in vitro* shoot cultures was obtained from seed grown *Franklinia* plants maintained under greenhouse conditions. The plants which were used as a source of explants had been under cultivation for a period of at least three years at the time experiments began. Dormant shoots of the previous year's growth were collected from 10 different seed grown mother plants on January 20, 2010 and separated into single-node segments. The length of the explants was approximately 1-2 cm, with the axillary bud located

approximately midway between the apical and basal cut surfaces. The explants were surface disinfested with the following procedure: Explants were briefly washed in an aqueous 2% solution of Micro 90 detergent (Fischer Scientific) and room temperature tap water. After rinsing with tap water, explants were wrapped in dampened paper towels and stored in plastic bags prior to surface disinfestation. Surface disinfestation and all following aseptic media preparation or explant transfer were carried out in a laminar air flow hood to exclude competitive or pathogenic organisms. The disinfestation procedure consisted of the following: Aliquots of the previously rinsed explants were immersed and magnetically stirred in a 70% ethanol (ETOH, ethyl hydrate) aqueous solution for one minute. Explants were then immersed with stirring in a 1.05% sodium hypochlorite (Clorox) solution, with the addition of 0.19% Tween 20 for 10 minutes. After 10 minutes, the explants were removed from the bleach solution and rinsed in three sequential baths of deionized water for 5 min each. Explants were then inserted horizontally into semisolid Murashige and Skoog (1962) basal medium in a 60mm X 15mm diameter Petri dish to a level just below the axillary node. All media was supplemented with Gamborg's B5 vitamins (Gamborg et al. 1968), 30g/L sucrose, 2mg /L BAP, 0.2 mg/L IBA and 7g/L Phytagar (Gibco Laboratories). Half of the media used was also supplemented with a combination of 50 mg/L gentamicin sulfate and 16mg/L chloramphenicol. All media were brought to a pH of 5.75 prior to being autoclaved for 20 minutes at 121° C @ 18-21PSI. All vessels and implements in contact with explants after disinfestation were also autoclaved for at least 15 minutes under the same conditions. Petri dishes containing explants on media were sealed with Parafilm, then incubated 30 days in a Percival Science growth chamber, at 22-25°C under cool white fluorescent light with 16 h day lengths at approximately $70\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

After 6 weeks, explants were scored on whether the dormant axillary bud had elongated. In addition, the number of explants successfully disinfested was determined based on the presence or absence of fungal or bacterial contaminants. The shoot initiation rate was also recorded in relation to the mother plant from which the individual explants had been derived.

Culture initiation from actively growing plant material

The explants utilized in this experiment consisted of a bulked collection of the current season's shoot growth from a random selection of the same seed grown plants described above, but were collected during May 2010. Explants consisted of nodal segments along with approximately 1cm of internodal growth on the distal side of the node. The explants were surface sterilized via the method described by Tosca et al. (1992) for hybrid *Camellia* material. This process consists of a 1 minute immersion in an aqueous solution of 70% ethanol, followed by a 15 minute immersion period in an aqueous solution of 2% NaOCl with stirring. The explants were then rinsed three times in sterile de-ionized water and stored in sealed sterile Petri dishes with hydrated filter paper prior to being cultured (Tosca et al., 1991). Explants were disinfested en-mass prior to treatment assignment in order to minimize any effects between treatments due to the disinfestation procedure. Explants were cultured individually in 150X25mm borosilicate glass tubes containing 25 ml of semi-solid medium, to prevent cross contamination. The explants were inserted into the growth medium with the distal end down. Growth medium consisted of Murashige and Skoog's (1962) basal salts supplemented with Gamborg's B5 (Gamborg et al. 1968) vitamins, 30g/L sucrose, and one of eight PGR treatments. Two levels of IBA were tested (0.1mg/L, 0.2mg/L), in combination with four levels of BAP (0.2mg/L, 0.5mg/L, 1.0mg/L, 2.0mg/L) in a factorial design (Table 1.1). All media was brought to a pH of 5.75 prior to being autoclaved for 20 minutes at 121° C @ 18-21 PSI. All vessels and implements in contact with

explants after disinfestation were also autoclaved for at least 15 minutes under the same conditions. Twelve randomly selected explants were assigned per treatment. Explants were cultured for six weeks at a temperature of 22-25°C, under Cool White fluorescent light at approximately $70\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with 16 h day lengths. After six weeks, cultures were evaluated for the number of shoots produced as well as whether the resulting shoots were determined to be of normal or abnormal quality.

GA₃ Influence

In vitro propagated *Franklinia* shoot cultures maintained on Medium#8 (Table 2.1.) were used as a source of experimental material. In order to minimize confounding variables, the shoots used for this experiment were paired between a control group and a treatment group. The media utilized for the experiment consisted of Medium #8 in the control group, and Medium #8 supplemented with 10mg/L of gibberellic acid-3 (GA₃), as a filter sterilized solution, as the treatment group. Each explant consisted of a non-apical, non-active axillary node and the complete adjoining distal internodal stem segment. In order to pair explants between the control group and GA₃ treatment group, two shoots from a single elongating shoot culture which were of approximately equal length and diameter were selected, the apical node removed, and the lower nodal segments cut to an equal length to provide a pair of explants as close as possible in both development and size. Explants were cultured individually in Magenta GA-7 polycarbonate culture vessels supplied with 100ml of growth medium. Shoot elongation was measured from the intersection of any new shoots and the original node. In the case of multiple axillary bud activation, both new shoots were measured and the measurements combined to give a total quantity in millimeters of shoot elongation per explant. Elongation was measured with a digital Vernier Caliper accurate to $\pm 0.02\text{mm}$. All measurements were rounded to the nearest millimeter.

Explants were cultured in matched pairs, the control and treatment pairs were initiated in an alternating fashion such that if the control culture was inoculated first in one pair of explants, the following pair would be inoculated in a reciprocal fashion e.g. the GA₃ treatment explant would be initiated first, followed as quickly as possible by the initiation of its control. Initiations were performed in this manner to minimize any variation in subsequent growth which might have resulted from the time the explant spent within the laminar air flow hood. Following culture inoculation, control/treatment pairs were maintained as a pair throughout the duration of the experiment (6 weeks in a Percival Science temperature controlled growth chamber at 22-25°C, under cool white fluorescent light at approximately 70 μmol · m⁻² · s⁻¹ with 16 h day lengths. In order to minimize any effect resulting from placement within the growth chamber, pairs were maintained in parallel columns running from the back to front of the growth chamber shelf. Control/treatment pairs were arranged in an alternating fashion in order to eliminate any effect which would be imparted from having all of one group (e.g. GA₃ treatment or control) in a single row parallel to its designated pair member. The data recorded at the conclusion of the experiment were analyzed using a T-test: Paired Two Sample for Means.

FeEDTA/FeEDDHA Comparison

In vitro propagated *Franklinia* shoot cultures maintained on Medium#8 (2mg/L BAP, 0.2mg/L IBA) (Table 2.1) were used as a source of experimental material. In order to minimize confounding variables, the shoots used for this experiment were paired between a control group and a treatment group. The media utilized for the experiment consisted of Medium #8 as the control, and Medium #8 supplemented with an equimolar quantity of iron EDDHA [ethylenediamine N, N, di (ortho hydroxyphenyl) acetic acid]. The concentration of iron employed in both media was 100 μM (~5mg elemental Fe/L). Explants were cultured

individually in Magenta GA-7 polycarbonate culture vessels supplied with 100ml of growth medium. Following culture inoculation, control/treatment pairs were maintained throughout the duration of the experimental period (6 weeks) in a Percival Science growth chamber at 22-25°C, under Cool White fluorescent light at approximately $70\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with 16 h day lengths. The experiment was conducted twice, the first time with 57 experimental pairs, and the second with 108 experimental pairs.

Rooting

All shoots used for this experiment were derived from previously described shoot production experiments. Of the shoots employed in this experiment, 66% were produced on Medium #8 (2mg/L BAP, 0.2mg/L IBA), and 33% were produced on Medium #8 supplemented with 10mg/L GA₃. Six treatments were tested for the rooting of *Franklinia* shoots in vitro (Table 2.2.). Shoots of the same age and approximately the same length all resulting from one culture period (6 weeks) were used as experimental material. Explants were cultured individually in Magenta GA-7 polycarbonate culture vessels supplied with 100ml of growth medium. PGR treatments for the induction of rooting consisted of either a 1 min immersion of the freshly excised shoots proximal end to a depth of one centimeter in an aqueous IBA solution, or the insertion of the proximal end to a depth of 1 cm in semi-solid medium supplemented with IBA. After treatment, shoots were incubated in a Percival Science growth chamber at 22-25°C, under Cool White fluorescent light at approximately $70\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with 16 h day lengths. After six weeks the cultures were evaluated for the number of rooted shoots as well as the quality of the roots produced.

Results and Discussion

Culture initiation from dormant plant material

Axenic shoot cultures were successfully initiated from nodal segments of dormant *Franklinia* plants across all tested treatments. The rate of successful shoot culture initiation ranged from 22% (mother plant #7 antibiotic medium) to 100% in four treatments (Mother Plant #4, #9 on MS basal medium; Mother Plant #2, #3 on antibiotic medium). Analysis of variance results indicated that the rate of successful culture initiation varied significantly among mother plants ($P < 0.001$), although most mother plants had initiation rates of over 70% (Figure 2.2). Across all mother plants, no significant variation was found between the antibiotic medium versus the non-antibiotic medium (Figure 2.1.). The most likely reason for this result was that the use of an antibiotic medium treatment was included based on extremely limited preliminary experiments. A bacterial contaminant had occurred post disinfestation in some cultures. The inclusion of a wide spectrum antibiotic combination was included to determine if the bacterial contamination had survived disinfestation or if it was introduced at a later point. The results of this experiment as well as years of subsequent culture suggest the contaminant was introduced at some point post disinfestation.

The influence of ortet on the rate of successful culture initiation may be due to a number of factors. A genotypic influence has been noted in a number of plant species in regard to plant tissue culture amenability, including other species of limited distribution such as *Elliottia racemosa* (Radcliffe et al., 2011). The exact extent of genetic influence on the tissue culture amenability of *Franklinia* is difficult to determine, due to the current lack knowledge concerning the amount of genetic variability within the species. A second potential factor influencing this result is physiological variation among the source plants utilized in the experiment. The explant

material was sourced from a random selection of greenhouse-propagated plants. While every effort was made to obtain explants from plants in good health which were maintained in an identical manner, it is possible for greenhouse and propagation methods contributed to physiological variability among the plants.

Culture initiation from actively growing plant material

At the conclusion of the experimental period, significant ($P=0.01$) variation was found among PGR treatments. The treatment with 0.2mg/L IBA and 0.5mg/L BAP (Medium #6) induced shoot production from the highest number of explants (75%; data not shown). When shoot quality, as well as the percentage of explants producing them, was taken into consideration, however, 0.2mg/L IBA and 2.0mg/L BAP (Medium #8) gave the highest number of explants producing well formed shoots (58%) (Figure 2.2.). The next highest initiation rate for well-formed shoot production (33%) was accomplished on both Medium #6, and on 0.2mg/L IBA and 1.0mg/L BAP (Medium #7). While Medium #6 resulted in the greatest overall number of shoots, more than half of the initiated shoots demonstrated hyper-hydrated and/or deformed patterns of growth. Deformations included leaves reduced to elongated filamentous structures without expanded blades, as well as excessive production of callus from the growth point, and adventitious roots emerging from both shoots and leaves.

GA₃ Influence

Shoots produced with the GA₃ treatment were markedly longer from those propagated on the control medium (Medium #8). The mean internode length of shoots supplemented with GA₃ was much greater based on a preliminary visual inspection. Paired t-test results indicated that the GA₃ treatment significantly increased shoot length in comparison to the control. The mean shoot

length increase of the GA₃ treatment (5.5cm was significantly greater than that of the control medium (Figure 2.3; Figure 2.4).

The GA₃ group shoots appeared healthy and, due to the elongated internodal regions, were much of a much higher quality for use in subsequent shoot multiplication cultures, as the greater distance between nodes allowed for individual nodes to be easily excised and propagated. The longer internodes greatly increased the efficiency of production, as previously produced shoots were more compact, which resulted in many explants with multiple nodes. With the ability to isolate single nodes for subsequent culture, a greater number of explants could be obtained from a given number of shoot cultures. Thus, the number of cultures that must be maintained to supply a desired quantity of shoots is lowered, reducing costs associated with the production of shoots through a reduction in utilized materials, labor associated with maintaining cultures, energy (e.g. for lighting), and space.

FeEDTA/FeEDDHA Comparison

A dramatic difference in both shoot length and leaf color was apparent when the two iron treatment groups were compared (Figure 2.5). Pair wise t-test results indicated that shoot lengths differed significantly ($p < 0.00001$) between the two treatments. In the first experiment, the mean increase in shoot length for the FeEDDHA-treated cultures was 22.26 mm over the mean shoot length for the FeEDTA-treated cultures (Figure 2.6). When the experiment was repeated, similar results were obtained. A 95% confidence interval for the first experiment was 16.297, 28.223, and the second experiment had a 95% confidence interval of 24.487, 32.503. Thus, using FeEDDHA as the iron source resulted in a substantial increase in the growth of *Franklinia* shoot cultures in vitro.

The reasons for this large increase in shoot length may involve a number of factors. A primary explanation for the growth increase would be that FeEDDHA provides iron in a form that is more subject to assimilation by the shoot cultures, in effect supplying a level of iron which may be lacking in media supplemented with FeEDTA. This effect may result from the influence of an increased ratio of Fe³⁺ iron to Fe²⁺ iron. In FeEDTA, especially when the solution is produced through the combination of Na₂EDTA and FeSO₄, iron may not exist entirely as the Fe³⁺ anion (Steiner and van Winden, 1970). It has been shown that iron must be present in the Fe³⁺ state in order for its incorporation by shoots (Ben-Yehoshua and Biggs, 1970; Dunlap and Robacker, 1988; Hangarter and Stasinopoulos, 1991). It has also been demonstrated that inappropriate pH levels within the Fe-EDTA solution, as well as insufficient conditions required for the oxidation reaction leading to the conversion of Fe²⁺ into Fe³⁺, can negatively influence the complete conversion of Fe²⁺ into Fe³⁺ (Steiner and van Winden, 1970). A second possible source of influence could be the interaction of Fe-EDTA with other molecules within the medium and/or plant tissue. FeEDTA has been found to promote the UV-catalyzed degradation of auxins, including those of synthetic origin, as well as naturally occurring forms (Ben-Yehoshua and Biggs, 1970; Dunlap and Robacker, 1988; Engelmann et al., 2003; Gordon and Weber, 1951; Hangarter and Stasinopoulos, 1991; Kamnev et al., 2001). It is hypothesized that the mechanism responsible for Fe-EDTA-promoted degradations is a form of Fenton reaction, in which free radicals are liberated (Ben-Yehoshua and Biggs, 1970; Dunlap and Robacker, 1988; Ghiselli et al., 2004; Kamnev et al., 2001). The photo-degradation of auxin compounds in media supplemented with FeEDTA would have the effect of altering the PGR levels between the experimental and control groups. A third possible factor influencing shoot length is the proposed toxicity of some degradation products of the EDTA (e.g. formaldehyde) (Hangarter and

Stasinopoulos, 1991). If the degradation of EDTA releases growth-inhibiting chemicals within the medium, as previously reported, an inhibition of shoot growth would be a likely result.

While the overall effect of any of these influences is measured as an increase in shoot elongation as a result of the replacement of FeEDTA with FeEDDHA, the mechanism by which this occurs may be due more to the failings of FeEDTA than any true growth promoting ability of FeEDDHA.

While it was not an effect tested for in this experiment, subsequent cultures employing Fe-EDDHA as a source of iron have been observed to produce callus which appears to be organogenic (D. Beleski, unpublished data). During the maintenance of elongating shoot cultures, nodal sections taken from below the apical meristem have been occasionally found to produce a reddish callus, which subsequently produced numerous leaf buds. Excising the callus and transferring to medium of the same composition resulted in the continued development and eventual elongation of the buds. The resulting shoots were of good quality and morphologically indistinguishable from those produced via axillary buds. Attempts at deliberately inducing this growth failed, yet it appears this growth sporadically reoccurs when FeEDDHA is employed. We have never observed growth of this callus in media employing EDTA, despite the same cultures having been continuously maintained for over 3 years.

Rooting

The success rate of rooting in vitro varied significantly with treatment (Figure 2.7.). Treatments that included a brief immersion (dip) in 1g/L IBA varied in rooting success from a low of 0% for shoots previously grown on medium supplemented with GA₃, to a high of 80% for shoots without prior exposure to GA₃, when rooted on ½-strength MS basal salts medium.

Overall, rooting percentage of shoots with previous exposure to GA₃ and those without did not significantly differ for shoots that received the IBA dip treatments.

Rooting success of treatments in which shoots were cultured on media supplemented with IBA varied with the quantity of IBA contained in the rooting medium. No shoots cultured on medium supplemented with 1g/L IBA were successfully rooted. In medium supplemented with 1mg/L IBA, 100% and 90% of shoots derived from cultures grown on medium with and without GA₃ successfully rooted, respectively (Figure 2.7.). As with the treatments subjected to an IBA dip, there was no significant difference between shoots previously cultured on medium with GA₃ and those without previous exposure to GA₃.

While the rooting of *Franklinia* shoots has been investigated previously, the reports indicated that *Franklinia*'s rooting ability is somewhat variable. In softwood cuttings, levels of IBA greater than 1g/L IBA inhibited the percentage of successfully rooted cuttings when applied as a dip treatment (Dirr, 2006; Sun and Bassuk, 1991). In hardwood cuttings, tested levels resulted in rooting inhibition when compared to a control in which no IBA was applied (Dirr, 2006; Meyer et al., 2009b). In the report by Meyer (2009), the lowest tested concentration of K-IBA was 2.5g/L. While the actual level of IBA in this treatment would be significantly lower than the equivalent concentration of pure IBA (m.w. K-IBA =241.3; m.w. IBA = 203.24), this level would still be equivalent to 2.105g/L of uncompounded IBA. According to Dirr's (Dirr, 2006) results, this would be an inhibitory concentration. Sun (1991) reported that IBA levels over 1g/L dramatically inhibited bud break in treated *Franklinia* cuttings. In softwood cuttings of *Franklinia*, Meyer (2009) reported no relationship between IBA treatment and rooting success. Our finding that the most successful rooting treatments involved culture on MS basal salts supplemented with 1mg/L IBA agrees with these previous findings on rooting in the greenhouse.

A direct comparison between the levels of IBA employed in the ex vitro work and the current experiment are not appropriate due to the use of different application and propagation methods. While the optimum level of IBA reported by Dirr (2006) for the rooting of softwood cuttings (1g/L dip) was tested, the use of semi-solid media for in vitro propagation most likely gave a considerably higher exposure of shoots to the IBA solution. Plants in nursery or greenhouse conditions would be subject to leaching, which would reduce the level of residual IBA on the shoots. Shoots rooted in vitro on semi-solid medium would remain in close proximity to any residual IBA. The variation in residual IBA levels is also a probable reason for the inhibitory effect of 1g/L IBA in this experiment, in contradiction to the findings of Dirr (2006).

Plantlets resulting from the rooting of the in vitro propagated shoots were removed from in vitro conditions for hardening off. This process entailed the manual removal of semi-solid media from the rooted shoots, followed by gentle rinsing with room temperature tap water. Plantlets were then planted out in autoclaved 3 inch square polypropylene containers in an autoclaved soilless mix composed of a 1:1:1 mixture of Peat moss: horticultural charcoal: horticultural pumice (Figure 2.8.). This mix was employed because previous attempts at rooting the micropropagated shoots ex vitro had been subject to damping off, and this mix is normally employed in the propagation of xeriphytic species, which are highly sensitive to excessive moisture (Anderson and Brown, 2001). Plantlets were successfully acclimatized to ambient temperature and humidity in a purpose-built acrylic hardening off chamber at 22-25°C, under Cool White fluorescent light at approximately $70\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with 16 h day lengths.

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Table 2.1. Plant growth regulator combinations tested for induction of proliferating shoot cultures of *Franklinia alatamaha*.

Media Designation	IBA Concentration	6-BAP Concentration
Medium #1	0.1 mg/L	0.2mg/L
Medium #2	0.1 mg/L	0.5mg/L
Medium #3	0.1 mg/L	1.0mg/L
Medium #4	0.1 mg/L	2.0mg/L
Medium #5	0.2mg/L	0.2mg/L
Medium #6	0.2mg/L	0.5mg/L
Medium #7	0.2mg/L	1.0mg/L
Medium #8	0.2mg/L	2.0mg/L

Table 2.2. Sources of shoots (plant growth regulator treatment in shoot proliferation culture) and rooting induction treatments for micropropagated *Franklinia alatamaha* shoots

Treatment #	Shoot production medium	Root induction treatment
# 1	Medium #8 ¹	1/2 strength MS + 1g/L IBA dip
# 2	Medium #8	MS + 1g/L IBA dip
# 3	Medium #8	MS supplemented with 1.0 g/L IBA
# 4	Medium #8	MS supplemented with 0.001 g/L IBA
# 5	Medium #8 + 10mg/L GA ₃	MS + 1.0g /L IBA dip
# 6	Medium #8 + 10mg/L GA ₃	MS supplemented with 0.001 g /L

¹Medium #8 plant growth regulators were 0.2 mg/l IBA and 2 mg/l BA

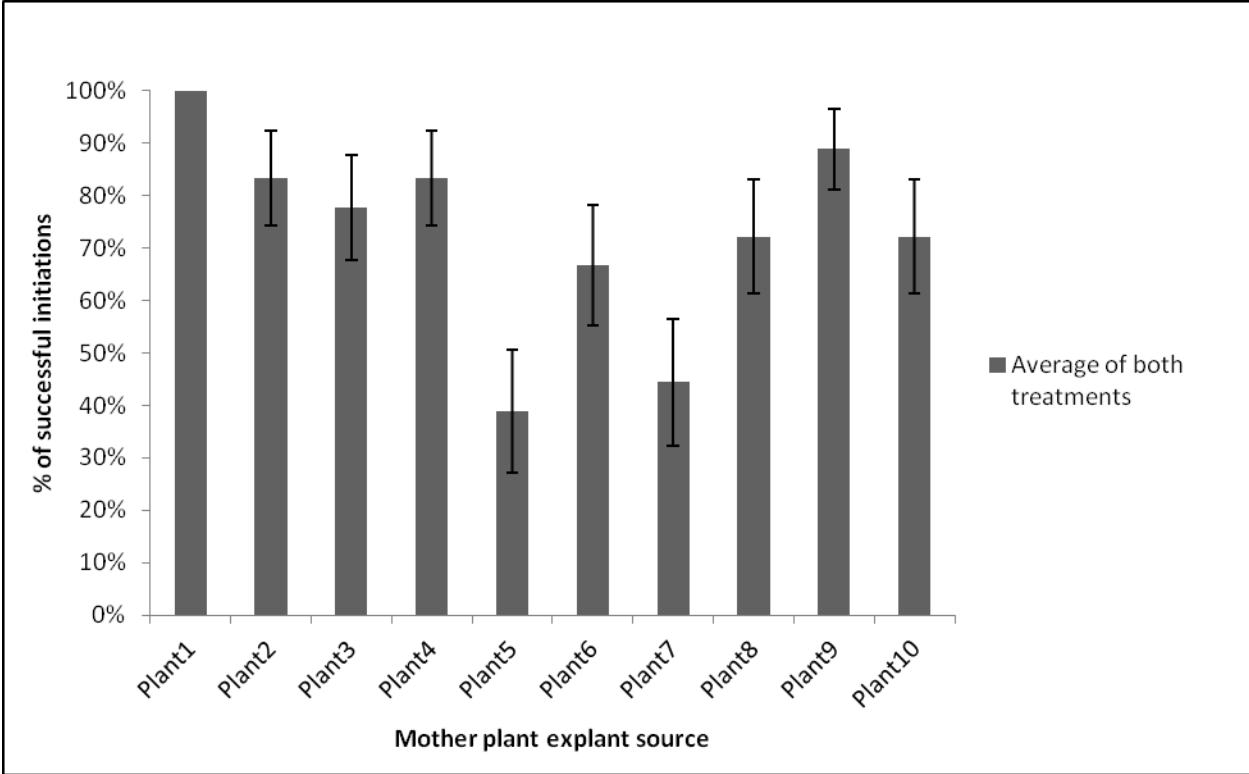


Figure 2.1. Percentages of successful shoot culture initiations from nodal segments of dormant shoots, by mother plant. Values represent the average of 18 nodal explants per treatment (n=180). Bars represent standard error.

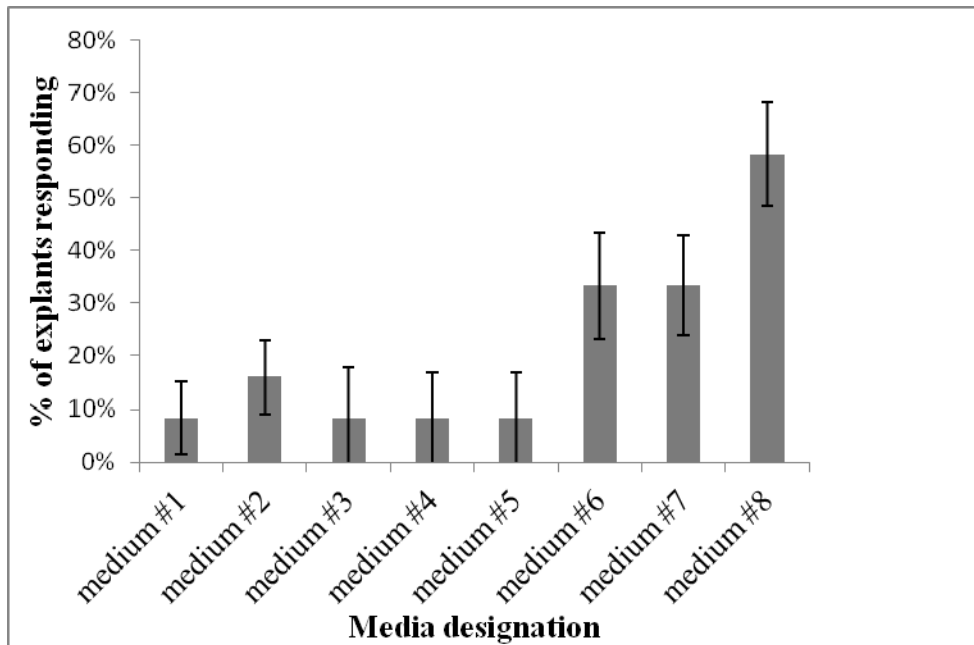


Figure 2.2. Percentages of nodal segments producing proliferating shoot cultures by Media 1-8 (see Table 2.1 for media descriptions). Data are the means of 24 nodal explants per treatment (total n=128 from actively growing shoots sourced from randomly selected seed-propagated greenhouse plants. Bars represent standard error.



Figure 2.3. Variation in growth between control medium (Medium 8) and medium supplemented with 10 mg/LGA₃ after one culture period (6 weeks). GA₃ supplemented medium is on the right.

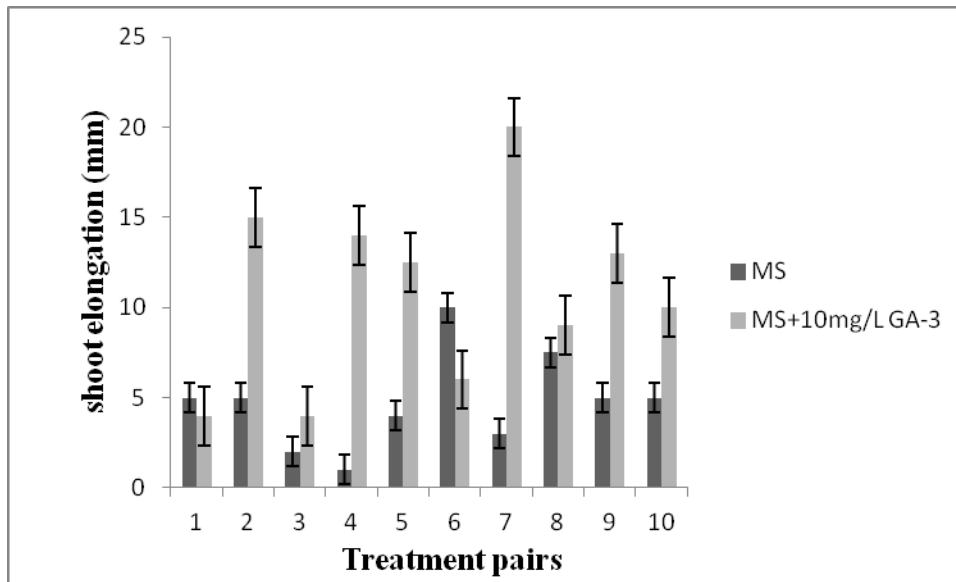


Figure 2.4. Effect of GA₃ on shoot length in paired treatments. Values are the means of overall shoot length increase as measured pre- and post-treatment of 10 replicates containing 4 shoots (pseudo-replicates) per Magenta GA-7 culture vessel. Bars represent standard error.



Figure 2.5. Paired comparison of FeEDDHA and FeEDTA effects on shoot elongation in *Franklinia alatamaha*. Fe-EDDHA treatment is on the left.

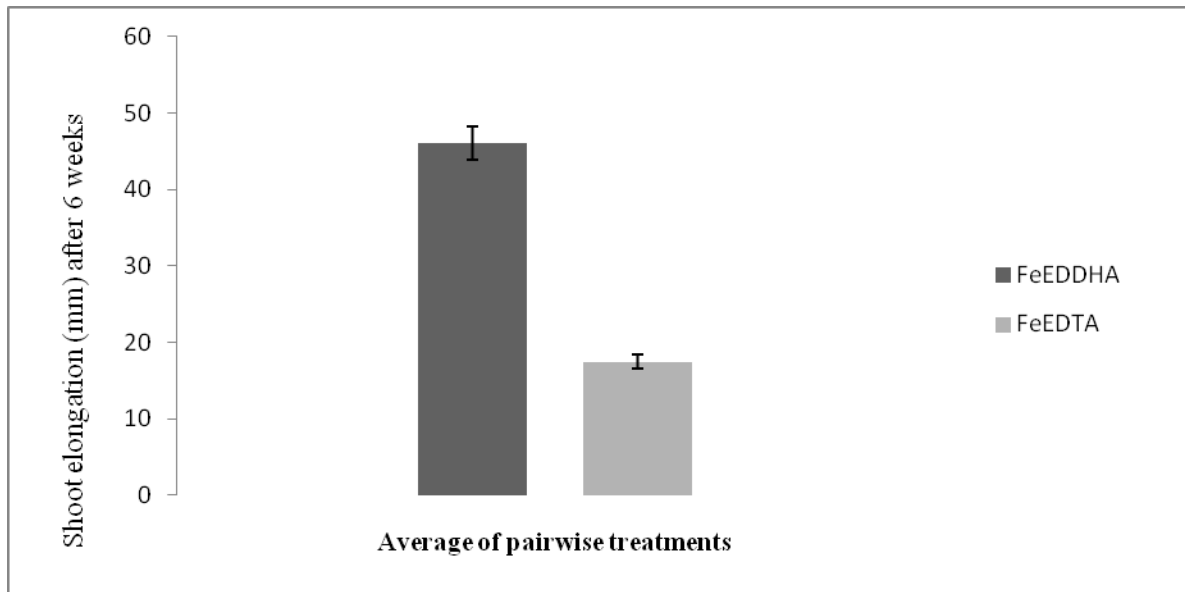


Figure 2.6. Comparison of *Franklinia* shoot elongation between FeEDDHA and FeEDTA treatments. Values are the combined average of increase in shoot length derived from a total of 108 paired experimental units. Four matched pairs of explants derived from a single shoot culture were divided between two Magenta GA-7 culture vessels. Twenty-seven paired replicates containing 4 pseudo-replicates (4 paired shoots per replicate) were measured both prior to and at the completion of the experimental period. The combined average increase in shoot length of all paired comparisons is presented. Bars represent standard error.

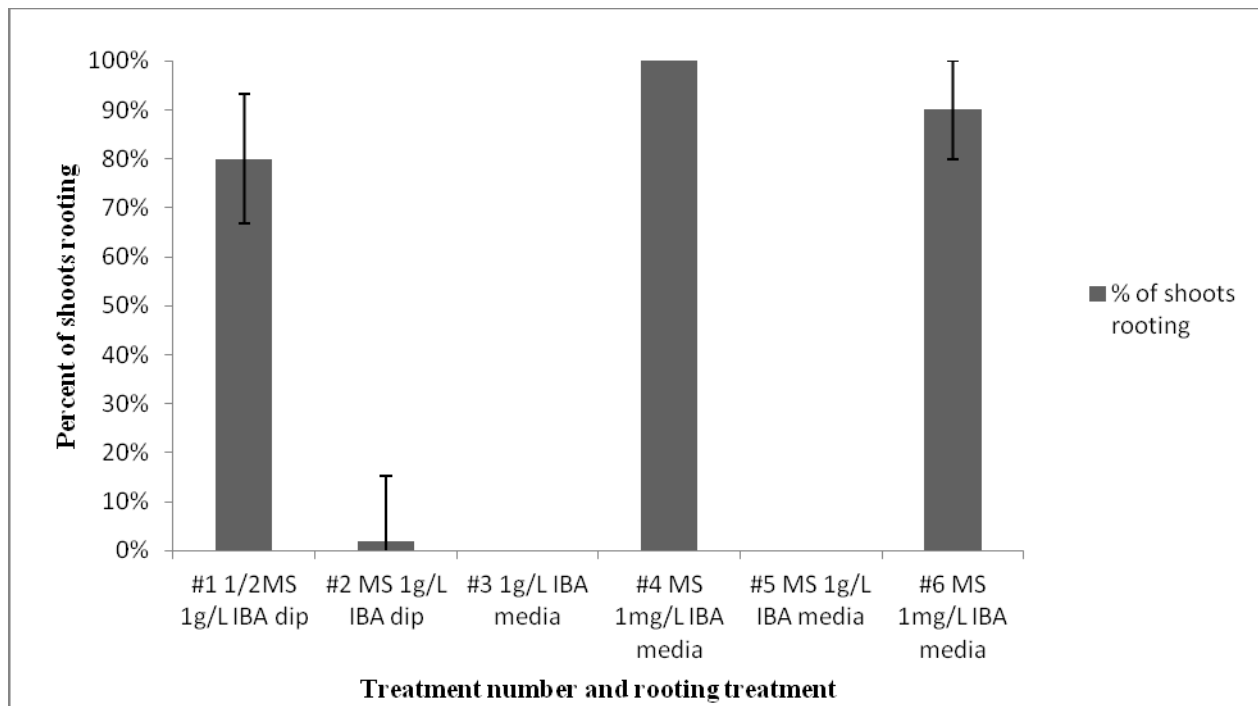


Figure 2.7. Effect of previous GA₃ exposure and root induction treatment on percentage of shoots successfully rooting. Shoots in Treatments 1-4 were derived from cultures maintained on MS medium supplemented with 2mg/L BAP and 0.2mg/L IBA. Shoots in Treatments 5-6 were derived from MS medium containing 2mg/L BAP, 0.2mg/L IBA and 10mg/L of GA₃. Values are the means of 10 shoots per treatment. Bars represent standard error



Figure 2.8. Franklinia plantlet following removal from in vitro conditions. A typical fully rooted in vitro propagated Franklinia plantlet after hardening off.

CHAPTER 3

CONCLUSION

A number of conclusions can be gleaned from the results of our work with in vitro propagation of *Franklinia*. These findings apply both to the literature research as well as the previously unreported development of a system for the production of axenic *Franklinia* shoots and fully hardened off plantlets.

A number of these findings relate to the origins of the *Franklinia* species. While the exact age and geographical origin of the family Theaceae is still far from certain, it has most likely been present on the North American continent for a substantial proportion of its history. If one accepts the age hypothesized from the cited paleobotanical evidence, the family has been represented in the form of all three basal lineages of the Theaceae, possibly since the Cretaceous period.

The Tribe Gordoniae, of which *Franklinia* is now believed to constitute one of the three existent genera, has been present on the North American continent possibly since the Cretaceous as well. As noted by Grote and Dilcher (1989,1992) there is “unequivocal evidence supporting the presence of the Theaceae... and most resembling the current tribe Gordoniae, especially the monotypic genus *Franklinia* by or before the middle Eocene” (Grote and Dilcher, 1989; Grote and Dilcher, 1992). Given the extensive range of the Gordoniae in North America throughout time and distance, as well as the great variety of species identified, it is highly probable that in addition to the tribe originating or diverging in North America, the Genus of *Franklinia* is likely an endemic species of the continent where it was originally identified. This conclusion is further

supported by the other acknowledged relict species which have been identified in the southeastern United States.

As to the reasons for the extirpation of *Franklinia* in the wild, it is highly probable that it was not due to a single factor, but resulted from multiple contributing factors. These factors would most likely consist of climate change and/or anthropic influences. The eastern United States is believed to have been experiencing a climatic period known as The Little Ice Age at precisely the time that the species was discovered. In addition, the end of the Wisconsin Glacial Episode coincided with the end of the Pleistocene epoch. The species may have spread south or existed as a relict population in a sheltered region during this period and was subsequently left stranded in an unsuitable habit, if its former range was reoccupied more rapidly by other species or its original range was altered in such a fashion that it was no longer suitable for the species. Anthropic influences would likely include changes in land use patterns, loss of habitat, and the introduction of exotic species such as *Phytophthora cinnamomi* and *Sus spp.*, and over-collection of the last remaining plants for the burgeoning intercontinental nursery trade by plant collectors (e.g. Marshall, Lyon).

The possibility that others, aside from Bartram, may have collected the species from the wild for the nursery trade could mean that there remains more genetic diversity within the species than previously hypothesized. If a number of genetically distinct plants entered into cultivation via plant collectors, as Lyon's records indicate, the offspring of these plants may still exist in ex situ collections. If there is remaining genetic variation beyond that of the progeny of the Bartrams' original collection, it would be highly valuable for the long term preservation of the species, as it could offer the possibility of resistance to current or future health threats to the

species. If a natural resistance to *Phytophthora* were found, it could allow the species to be reintroduced to the wild in or around its original collection site.

While there are no published reports of genetic diversity within *Franklinia*, the fact that there was variation in the success of shoot culture initiation among different seed grown plants may indicate some level of genetic variability, at least with in regard to in vitro propagation. If this genetic variation exists with regard to micropropagation ability, it most likely would indicate variability in other traits as well.

It has now been shown conclusively that *Franklinia* is amenable to in vitro propagation. This would allow for the extremely rapid propagation of genetically distinct axenic clones, which could then be preserved ex situ and used in organized breeding programs, as has been done with other species with extremely low genetic variability. The preservation of distinct genetic lines in vitro would offer some level of protection for the species against any future threats or until such a point that existent threats could be managed to allow for re-introduction.

The finding that *Franklinia* growth responds much better in response to treatment with FeEDDHA as opposed to FeEDTA suggests that this form of iron chelate could benefit *Franklinia* grown on the landscape as well. The species appears to be subject to inter-veinal leaf chlorosis, both in vitro and in potting mix, which is generally considered to be a symptom of iron deficiency. The ability to remedy this condition would improve the overall health of a specimen, perhaps aiding in the plant's ability to resist pathogens and extending its lifespan in areas known to harbor *P. cinnamomi*.

While shoot elongation as a result of GA₃ application is a well-known response in plants, the finding that *Franklinia* grown in vitro responds with normal healthy growth, as opposed to those without exogenous gibberellins, which are often characterized by stunted shoots may

indicate that the species is naturally a host to an endophyte which may contribute gibberellins of some form (Thomson, 1990). This possibility is supported by reports that *Camellia* is a host to known relatives of gibberellin-producing species of endofungi (Agusta et al., 2006). As in vitro *Camellia* cultures are known to often grow poorly in vitro, it may be that this is a common form of relationship in the Theaceae (Mondal, 2004B).

Our observation that high levels of exogenous auxins inhibit rooting in vitro- propagated *Franklinia* supports the previous work of Dirr (Dirr, 2006), who reported an inhibitory effect of IBA on the rooting of *Franklinia* at concentrations of 1 g/L or higher.

In conclusion it can be stated that *Franklinia alatamaha* is now known to be capable of being micropropagated successfully. The in vitro propagated shoots can serve as a source of axenic material for further studies, and in addition provide a method for the rapid production of fully developed hardened off axenic plants of this attractive ornamental woody species in vitro.

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