SOMATIC EMBRYOGENESIS OF *MAGNOLIA* SPP. AND CULTIVARS

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Kathryn Marie Plotke

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Program:
Horticulture

March 2018

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Somatic Embryogenesis of *Magnolia* spp. and Cultivars

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Todd P. West
Chair

Dr. Wenhao Dai

Dr. R. Jay Goos

Dr. Chiwon W. Lee

Approved:

4/10/18
Date

Richard D. Horsley
Department Chair

ABSTRACT

This study focused on induction of somatic embryogenesis of *Magnolia* spp. and cultivars utilizing leaf and seed (immature and mature) tissues with attempted micropropagation experiments. In a preliminary experiment, direct embryo regeneration was successful in a single leaf tissue of *M*. 'Yellow Bird'. After various micropropagation experiments, microshoot proliferation rates decreased. As a result of minimal leaf material, mature seeds were utilized but had contamination issues. Subsequent experiments utilized immature seeds. *M*. 'Leonard Messel' and *M*. *stellata* had significantly greater embryo regeneration rates and *M*. 'Rosea', *M*. *stellata*, and *M*. *kobus* had greater callus induction rates. Woody Plant Medium had significantly greater rates of embryo regeneration as compared to Yellow Poplar medium. Further experimental measures including various collection times of immature seeds are necessary for an efficient regeneration protocol to support potential research utilizing floral-inducing genes to induce rapid breeding cycles for selection of magnolias with diverse floral characteristics.

ACKNOWLEDGEMENTS

I would like to thank Dr. Todd West for the opportunity he provided me as well as his guidance and support through this educational experience as my major advisor. Without Dr. West, this experience would not be possible. His mentorship provided me with scientific principles of horticultural research as well as biotechnological experience. Dr. West taught me the essentials of tissue culture as well as my former lab mate and friend Nate Maren who challenged me and guided me through the ins and outs of everyday lab work. Nate provided extensive knowledge on somatic embryogenic applications and spent long hours helping me prepare for experiments in the lab.

My gratitude also extends to the NDSU Woody Plant Improvement Program research specialist Greg Morgenson. Greg is a tree man. He taught me endless knowledge through daily tree quizzes and adventures. His passion and love for horticulture is truly inspiring. Greg inspires me to follow my dreams and continue to learn about the complexities of woody plants.

Most importantly I would like to acknowledge my family, especially my parents Mary and Jerry. They provided me with continued love and support as I began my journey to North Dakota for graduate school. Without my mother's guidance as my female role model growing up, I would not have the independence and willingness to embark on lone adventures. My father taught me to be thorough and diligent in any given task presented in life. I will forever be appreciative for them as they taught me the value of education.

DEDICATION

To my grandfather, Rufus R. Gonzales

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LIST OF ABBREVIATIONS

2,4-D .....2,4-Dichlorophenoxoxyacetic acid.

2iP .....6-(γ,γ-Dimethylallylamino) purine.

AC .....Activated Charcoal.

BA .....6-Benzylaminopurine.

GA_3 .....Gibberellic acid.

MS.....Basal salt and vitamin medium composition by Murashige and Skoog (1962).

*m*T .....*m*eta-topolin.

NAA.....6-(γ,γ-Dimethylallylamino) purine.

NDSU WPIP .....North Dakota State University Woody Plant Improvement Program.

NGP.....Northern Great Plains.

PTC$^{3™}$.....Plant Tissue Culture Contamination Control (PhytoTechnology Laboratories®, P.O. Box 12205, Shawnee Mission, KS 66282, USA).

S .....Basal salt and vitamin medium composition by Standardi and Catalana (1958).

SWPM.....A modified Woody Plant Medium (described by Lloyd and McCown 1980) formulation for *Syringa* spp. micropropagation described by Maren (2016).

WPM.....Woody Plant Medium described by Lloyd and McCown in 1980.

LIST OF DEFINITIONS

Hyperhydricity .....a stress induced change in physiological state as a result of excessive hydration, low lignification, impaired stomatal function, and weakened inherent strength causing microshoot malformation of tissue cultured plants.

Phenolic Compounds .....secondary metabolites consisting of an aromatic ring bearing one or more hydroxyl groups derived from the phenylpropanoid pathway, and considered as an important role in growth and reproduction as well as providing protection against pathogens and predators.

Post-anthesis .....occurring after a flower is fully open and functional.

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INTRODUCTION

Magnolia is a genus in Magnoliaceae (Magnolia family) and comprises of numerous species that are deciduous and evergreen trees and shrubs found in temperate to tropical climates. There are three subgenera (*Magnolia, Yulania, Gynopodium*) with approximately 210 different species of magnolia. The subgenera *Yulania* are the most important to the Northern Great Plains (NGP) because of their potential hardiness for this region. Species within *Yulania* include but not limited to: *M. acuminata* L. (cucumbertree), *M. denudata* Desr. (Yulan magnolia), *M. kobus* DC. (Kobus magnolia), *M. liliiflora* Desr. (lily magnolia), *M. stellata* (Sieb. & Zucc.) Maxim. (star magnolia). There are eight species of magnolia native to the United States and none native to North Dakota. Of the subgenus *Yulania*, only one species is native to the United States, *M. acuminata*.

Magnolias are best known for their spring inflorescence (flowers). Callaway (1994) reported that no group of trees and shrubs is more favorably known or more highly appreciated in gardens than magnolias, and no group produces larger or more abundant blossoms. One of the many reasons magnolias make such a magnificent floral display is the emergence of the inflorescence in such abundance to completely fill the canopy before leaves emerge, with the exceptions of cucumbertree (Beauchamp, 2009). Inflorescence is a showy flower with indistinguishable petals and sepals with collectively are referred to tepals. Flower color within *Yulania* range from white, pink to purple and also yellow. The magnolia flower has full potential to fill the canopy with rich, satiny, vibrant color and fragrance, but the northern continental climate including North Dakota puts limitations on inclusion in the landscape of these ornamental features. The blossoms of *M. stellata* and *M. kobus* produce white and sometimes tinged with pink, thin, delicate tepals but can be damaged by spring frosts because of their earlier

bloom time. *Magnolia acuminata* blossoms avoid damage caused by spring frosts because of their later bloom time, but lack ornamental value and hardiness in northern continental climates.

The admiration and interest of magnolia in the horticultural world is expressed in commercial landscapes as well in its natural habitat. After spring, other ornamental characteristics of magnolia extends into fall with showy red or orange fruit and into winter providing interest with the smooth gray or scaly brown bark. Besides showing nobility in the garden among other plants, magnolias have additional uses, which add importance to this group of plants. Many species attract wildlife by the brightly colored seed coat, which serve as food for birds and mammals. Magnolias also offer cut flowers and cut greenery for use in floral decoration, while smaller species and cultivars make fitting houseplants. Over the centuries, magnolia species (*M. acuminata*) have been harvested for timber and medicinal purposes as well (Callaway, 1994).

Cold hardiness survival is one of the key limiting factors for growing and breeding magnolias in the NGP. There are three factors involved with cold hardiness, wood hardiness (vegetative structures), flower-bud hardiness (reproductive structures) and root hardiness (Preece and Read, 2005). Cold hardiness survival of ornamental species including magnolia has been ongoing in North Dakota by the North Dakota State University Woody Plant Improvement Program (NDSU WPIP) since 1954 with magnolia beginning in 1996 (West, 2014). The Magnolia cold hardiness survival evaluations from the NDSU WPIP found that there is a large amount of variability among species and cultivars from the subgenera *Yulania* for cold hardiness survival (West, personal communication).

In 2006, NDSU WPIP released Spring Welcome® Magnolia (*Magnolia* x*loebneri* 'Ruth'). Among the *M.* x*loebneri* hybrids, Spring Welcome® flowers emerge from frost-resistant

buds, avoiding spring frosts. The flower buds are hardy below -37°C, which outperformed other Loebner hybrids evaluated (NDSU, 2006). Therefore, utilizing Spring Welcome® as a breeding parent is a valuable asset in this breeding program in efforts to increase cold hardiness in North Dakota.

Magnolia Breeding

Significant amount of breeding work has been conducted within *Yulania* producing selected species, cultivars and several interspecific crosses including *M. soulangeana* Soul.–Bod. (*M. denudata* x *M. liliiflora*, saucer magnolia) and *M.* x*loebneri* Kache (*M. kobus* x *M. stellata*, Loebneri magnolia) (Callaway, 2000).

Evaluations at the NDSU Horticulture Research Farm and Dale E. Herman Research Arboretum (Absaraka, ND, USA; Lat: 46.9859, Long: -97.3549) have included several different species and cultivars with mixed results. Many of the evaluations did not survive including *M. acuminata, M. kobus, M. tripetala, M. virginiana* ('Ned's Northern Belle' and 'Jim Wilson'), 'Ann', 'Waterlily', 'Rosea', and 'White Stardust' as a result of wood or root cold hardiness issues or extended flooding of planting area. Several cultivars have survived but have limited performance with regard to either wood or flower-bud cold hardiness are 'Ballerina', 'Merrill', 'Leonard Messel, 'Royal Star' and 'Yellow Bird' (NDSU, data not published).

As a result of cold winters in North Dakota, flower-bud hardiness is a major limiting factor when developing magnolias in a USDA cold hardiness zone 4 environment. One surviving cultivar evaluated, 'Yellow Bird' (*M. acuminata* x *M.* x*brooklynensis* G. Kalmbacher 'Eva Maria') only flowered below the snow line, limiting the ornamental features of this tree. *Magnolia* x*brooklynensis* (*M. acuminata* x *M. liliiflora*) hybrids, such as 'Yellow Bird', crossed with a pink or purple flower plant, such as *M. liliiflora* could produce a late-blooming pink or

purple form- potentially introducing a cold hardy pink or purple flowered magnolia into North Dakota (Callaway, 2000).

To our knowledge, *M. acuminata* shows wood hardiness, but uncertain about flower-bud hardiness as a result of a flood killing the juvenile plant at the NDSU Horticulture Research Farm. With its yellow flower color and potential cold hardiness, *M. acuminata* is an essential parent when trying to create late blooming hybrids. *Magnolia acuminata* is one of the tallest and cold hardiest magnolias native to the United States. The yellow-green flowers are inconspicuous and often hidden by foliage, but its geographical diversity makes it a valuable asset in a breeding program (Beauchamp, 2009). *Magnolia acuminata* var. *subcordata*, a smaller variety of *M. acuminata*, has a more pronounced yellow flower color and can be used to create distinct yellow flowered magnolia hybrids (Gardiner, 2000).

Magnolia stellata, another potential breeding parent, would be a benefit to this breeding program because of its wood and flower-bud cold hardiness. Even though *M. stellata* is a cold-hardy magnolia, some tepal damage occurs with late frosts. To eliminate this problem NDSU selections such as, Spring Welcome® and *M. stellata* NDSU Accession TS13074 (discovered by Lynn Morgenson in Bismarck, ND) are used as potential breeding parents. *Magnolia* x*loebneri* hybrids are among the toughest and most cold-hardy magnolias. Their abundant, star-like blossom adds a rich scent to the garden and their clean, white flowers show no tepal damage. These blossom characteristics show ornamental value in the landscape (Dirr, 2009). When crossed with *M. acuminata* hybrids, breeding objectives could include: larger flower, flower durability, and desirable flower color or shape. Breeding efforts aim to improve magnolia species for northern climates to increase wood-hardiness, flower-bud hardiness, flower color diversity, and tepal quality. The diversity of magnolia species in the NGP shrinks as winter

temperatures decrease; therefore, the overall goal of this breeding program is to develop a diverse magnolia collection, hardy in a USDA cold hardiness zone 4 environment that bloom late to avoid late frost.

Traditional Breeding

Traditional breeding relies primarily on integration of genetic material from the same genus or across sexually compatible species and depends on selection of plants according to their phenotype (Hansen, 1999). Once the selection of parent plants is made, traditional breeding methods are utilized such as hand pollination crosses. Considerations for traditional breeding methods include: available space, germplasm sources for pollen collection, and crossing into non-hardy source material, such as some *M*. *acuminata* hybrids. One major impediment of traditional breeding with subsequent selections is the long-lasting juvenile stage, which can take up to five to 10 years or more to develop floral organs (Flachowsky et al., 2009). Frequently, desired traits are visible but backcrossing is often essential to homogenize and fix desired traits. If desired traits are unexpressed, the process repeats. Thus, the goal of tree breeding is to increase the extent to which specific desired traits are expressed in each subsequent generation (Hadley et al., 2001).

Hybridization is a valuable asset in breeding because desirable characteristics in two or more species can be combined in one individual. With intermediate traits absent in parental species, utilizing intermediate hybrids stacks phenotypic traits such as flower color, form and habit, and bloom time. Many new hybrids are being developed that have potential to combine and enhance flower color, cold hardiness, and fragrance. Hybrid evaluations are an essential part to any breeding program. To ensure quality releases in the trade, hybrids are often grown from seed to maturity for complete phenotypic evaluation. Maturation time may be shortened by chip-

budding or side-grafting the hybrid on a mature tree, but time is still a major limitation (Callaway, 2000). Phenotypic evaluations for hybrid development occurs after a minimum of 10 years, which is the earliest elites can be advanced to the next hybridization cycle or as new releases. Breeders also test for true hybridity and determine plant fertility. In magnolia, interploid hybrids (hybridization between two different individuals of different ploidy level) are often produced and fertility of the progeny may be reduced, which limits generation advancements as well (Ranney and Gillooly, 2014). However, biotechnological approaches such as genetic engineering have been developed to shorten the juvenility period to accelerate the breeding process and improve genotypes in other species.

Genetic Engineering

Advancements in breeding time, productivity, and sustainability can be achieved through genetic engineering trees with altered dormancy, flowering, and architectural characteristics (Srinivasan et al., 2012). As a result of the long juvenility period in woody species, trees are unable to develop floral organs and fruits promptly. The reproductive stage of a tree is reached once the plant goes through a juvenility stage, which can last five to 40 or more years. Since no further genetic development is possible during juvenility, production of new hybrids is unattainable and only selections based on these juvenile traits can be made. These limitations make it nearly impossible to achieve timely breeding improvement goals. Additionally, commercial breeders experience difficulty when making crosses due to substantial plant height. With hundreds or thousands of crosses being made, proper equipment, infrastructure, and plenty of land is required (Flachowsky et al., 2009). Therefore, utilizing floral-inducing techniques is a potential tool for making floral selections in juvenile plants.

Conventional breeding techniques for early flowering are plausible with adequate genetic diversity. Because few, natural, early flowering genotypes in woody species are known, transgenic breeding methods are required. A variety of transgenic approaches have been tested, including a FasTrack breeding system, involving a tree flowering gene that produces generation cycles of one year or less (Scorza et al., 2012). In order to achieve early flowering, the transfer of genes must be directly involved in the flowering pathway. The MADS-box is a conserved, amino acid sequence and is found in the genes that contain the MADS-box gene family. These genes are found in many eukaryotic organisms and are responsible for encoding protein that binds specific DNA sequences in various biological functions (Flachowsky et al., 2009). Various biological functions include: the transition from vegetative to reproductive growth, determination of floral-organ identity, development of vegetative tissues, senescence, and winter dormancy (Saedler et al., 2001). Evidence indicates the importance of these genes in flowering plants, such as the formation of flower, including the development of reproductive structure and the control of flowering time (Ng and Yanofsky, 2001).

Plants evolved mechanisms to sense a favorable time to produce reproductive structures to promote propagation. In order for plants to transition from vegetative to reproductive development, the expression of MADS-box genes is required. There are three genetic pathways involved in flowering time. Ng and Yanofsky (2001) reported that the first is the autonomous pathway, which promotes the transition from the vegetative to the reproductive phase. Central to this pathway is the MADS-box gene *FLOWERING LOCUS C (FLC)*, which negatively regulates this transition. Next, the photoperiodic pathway (also called the long-day pathway) promotes flowering under conditions of long-day lengths and is controlled by genes such as CONSTANS (CO), which encodes a zinc-finger (structural motif) protein. Last, the day-length-independent

pathway (also called the gibberellin pathway) depends on the signals that are relayed by the plant hormone gibberellin (regulates growth and influences developmental processes), through the activation of the *LEAFY (LFY)* meristem identity gene. MADS- box genes such as *APETALA 1 (AP1)*, *CAULIFLOWER (CAL)*, *FRUITFULL (FUL)*, and *SHORT VEGETATIVEPHASE (SVP)* also control flowering time. The overexpression of these genes has been found to induce early flowering in many species.

The FasTrack breeding system, utilizing MAD-box genes, was created to induce first year flowering in *Betula* (birch), *Malus* (apple), and *Prunus* (plum) species (Flachowsky et al., 2007). Scorza et al. (2012) describes FasTrack breeding as a breeding system that uses a genetically engineered (GE) tree flowering gene that produces generation cycles of one year or less for rapid improvement of tree fruit and other specialty crops. The process begins with the insertion of an Early Continual Flowering Gene (ECF) into a parent plum through genetic engineering. The *BpMADS4* (ECF) gene of *Betula pendula* Roth (silver birch)-homolog with *FUL* and the *PtFT1* and *PtFT2* (ECF) genes of *Populus trichocarpa* Torr. & Gray (black cottonwood) -homologs of *FT*, are applied to produce a plum parent that can flower and fruit within a year. The GE parent plum enters the breeding program to combine traits of interest. The breeder will wait three to seven years for each generation, but with ECF, a generation can be made every year. In each generation, the highest quality plums are selected as parents. Once progeny is produced, the GE type with desirable characteristics is backcrossed with a non-GE type with desirable characteristics. The breeder then selects for non-GE type progeny with desirable traits. With conventional breeding, a single generation may take four years and three generations could take more than 12 years. However, FasTrack breeding would produce three generations in five years, which allows for rapid improvement and prospective cultivar release.

Plum trees transformed with *FT1* genes from *P. trichocarpa* along with *BpMADS4* promoter produced flowers within one to 10 months after transfer (Srinivasan et al., 2012). The FasTrack approach would be a beneficial tool for magnolia breeding because first year floral production helps screen out undesirable traits, while parents expressing traits of interest enter the breeding cycle rapidly.

The FasTrack approach involves the use of *Agrobacterium*-mediated transformations. Agrobacterium is a soil bacterium that causes tumorous growths or roots to develop at wound sites of infected dicotyledonous and monocotyledonous plants. During induction, T-DNA transfer is initiated when agrobacterium detects phenolic molecules released from the cells at the wound site. The phenolic compounds induce the expression of multiple virulence (*vir*) genes. The *vir* genes encode products responsible for processing and transferring the T-DNA across the bacterial membrane into the plant cell, which integrates into the plant genome at a random location. Ultimately, the infection site genes in the T-DNA region are removed and placed with genes of interest (Lee et al., 2009).

Overall, breeding with transgenic early flowering trees, such as the FasTrack breeding program is a potential tool to improve magnolia flowers. The time scale of a traditional breeding program suggests that in the first year, selection of parent crosses is made. Following first year parental selections, production of the first initial generation occurs. From the first initial generation, flower production may take up to five to 10 years or more. Desired traits may not be present after floral production in which further crosses are made and the process starts over. With utilizing an early-flowering gene, the plant is screened in the first year and one breeding cycle is reduced. This system allows for the rapid approach of intermediate selection as a result of floral production in the first year.

Developing a biotechnological method to induce early flowering in trees is important, however, a regeneration protocol for the host plant is necessary. Regeneration through direct somatic embryogenesis has been shown to make useful target material for gene transfer via *Agrobacterium* (Merkle et al., 1997). Kim et al. (2007) reported that micropropagation of *Magnolia* spp. is not sufficiently successful, however, several results have suggested *Magnolia* spp. can be propagated by somatic embryogenesis (Merkle and Watson-Pauley, 1993, 1994; Merkle and Wiecko, 1990; Merkle, 1999).

Somatic Embryogenesis

Giri et al. (2003) reported that in addition to regeneration through organogenesis, somatic embryogenesis offers the advantage of single cell regeneration and currently appears to be the most promising approach to introduce new genes into woody tree species, while Merkle (1997) reported that direct somatic embryogenesis has a lower probability of genetic variation than other propagation methods. Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos. Somatic cells within a plant contain all the genetic information necessary to create a complete and functional plant. The induction of somatic embryos is a result of the termination of a current gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression program (George et al., 2008). Embryo development is divided into two main phases: beginning with the zygote and finishing at the cotyledonary stage and maturation of the embryo followed by germination (Dodeman et al., 1997).

After fertilization, the zygote divides asymmetrically, resulting in a smaller apical cell and a larger basal cell. The embryo develops from the apical cell and the suspensor develops from the basal cell. The suspensor cells attach the embryo to the endosperm, which serves as a nutrient outlet for the developing embryo. Further cell division leads to the globular stage. As

the cotyledons begin to form, the globular shape is lost and the formation of two cotyledons gives the embryo a heart-shaped appearance. The embryo continues to develop into a torpedo shape and the shoot and root apical meristem are established. Following the previous period of cell division, the embryo maturation phase occurs (George et al., 2008). Goldberg et al. (1994) reported that the maturation program is responsible for synthesizing large amounts of storage products, inducing water loss, preventing premature germination, and establishing a state of dormancy.

Plant regeneration via somatic embryogenesis is a result of the differentiation of somatic embryos directly from the explant without any intervening callus phase or indirectly after callus induction (Williams and Maheswaran, 1986). Direct regeneration occurs when embryos originate directly from explant tissues, while indirect regeneration requires extra callus phase. There are some reports of direct embryo regeneration using immature seeds of a few species of the Magnoliaceae family including: *M. virginiana* L., *M. fraseri* Walt., *M. acuminata* var. *subcordata* Michx., *M. macrophylla* Michx., and *M. obovata* Thunberg (Merkle and Wiecko, 1990; Merkle and Watson-Pauley, 1993; Kim et al., 2007). Merkle and Wiecko (1990) reported somatic embryo regeneration from only one immature seed of M. *acuminata* var. *subcordata*. However, to our knowledge, no direct embryo regeneration from leaf tissue of any magnolia has been reported. Embryogenic cultures are an attractive target for gene transformation because the regeneration of plants from single cells is a requirement for agrobacterium mediated gene transfers to achieve homogeneously transformed plants (Giri et al., 2003).

Research Objectives

The objective of this research was to develop an efficient somatic embryogenic regeneration protocol for magnolia. Many biotechnological experiments were performed in

anticipation of vigorous nodal and embryogenetic cultures to support continued research conducted by the NDSU WPIP. In efforts to improve magnolia in the NGP, an approach to develop a genetic transformation protocol utilizing early flowering genes would provide rapid breeding cycles for the prospective selection of magnolias with diverse floral characteristics.

MATERIALS AND METHODS

Source of Microplant Stock Material

Magnolia 'Yellow Bird' microplants maintained from Stage II multiplication cultures previously established on Murashige and Skoog (1962) (MS) medium, 2µM 6-benzylaminopurine (BA), 30 $g \cdot L^{-1}$ sucrose, and solidified with 0.8% (w/v) agar at the Woody Plant Improvement Lab at NDSU. Cultures were maintained at 23 ± 1°C with a 16h photoperiod (36-40 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) supplied by cool white florescent lamps. All microplants were kept under standard culture conditions unless otherwise noted.

Stage II Micropropagation

Axillary shoots were sub-cultured on a four-week growing cycle on MS medium. Medium was formulated with 1 $mL \cdot L^{-1}$ Plant Tissue Culture Contamination Control (PTC$^{3™}$, PhytoTechnology Laboratories®, P.O. Box 12205, Shawnee Mission, KS 66282, USA), 2µM BA, and 30 $g \cdot L^{-1}$ sucrose. Prior to autoclaving, media was adjusted to a 6.0 pH with 1 N HCL or 1 N KOH, melted with 0.8% (w/v) agar, and dispensed into Magenta™ GA-7-3 vessels (V8380, Sigma-Aldrich® Co., 3050 Spruce Street, St. Louis, MO 53103, USA) containing 50 mL of liquid in each vessel. Vessels were autoclaved at 121°C at 15 PSI for 20 min and subsequently cooled for agar solidification. At the end of each growing cycle, microplants were sub-cultured under a sterile laminar flow hood and transferred to new vessels.

Shoot Proliferation Experiment

A factorial of basal medium compositions, carbohydrate sources, plant growth regulator (PGR) compositions, and PGR concentrations were used to determine shoot proliferation rates. Nodal explants were placed in 25 X 150 mm test tubes containing 15 $mL \cdot L^{-1}$ MS or Standardi and Catalana (1958) (S) medium (Appendix Table A-1), 30 $g \cdot L^{-1}$ sucrose or fructose, with 0, 2, 4,

or 8 μM BA, in combination with 0 or 0.5 μM 1-naphthaleneacetic acid (NAA). All treatments included 0.8% (w/v) agar with the pH adjusted to 6.0 prior to autoclaving.

After 4 weeks, data was taken on microshoot number. This experiment was arranged as a completely random design (CRD) and conducted once as a result of limited plant material. In each treatment (32), there were 5 replications. One explant was placed in each test tube. Data was analyzed using the General Linear Model (GLM) of SAS 9.3 (SAS Institute, Inc., 2018). Only shoots >5mm long were counted and used for data analysis. Means were analyzed based on a mean separation analysis using Fisher's Least Significant Difference (LSD) test for paired comparisons.

Cytokinin Composition and Concentration Experiment

Microshoot growth response was evaluated by the presence of three different cytokinins; BA, *meta*-topolin (*m*T), and 6-(γ,γ-dimethylallylamino) purine (2iP) at 0, 2, 4, or 8 μM. The basal medium consisted of MS basal salts and vitamins, 30 $g \cdot L^{-1}$ sucrose, and solidified with 0.8% (w/v) agar. The pH was adjusted to 6.0 with 1 N HCL or 1 N KOH prior to autoclaving. Microshoots were placed in test tubes (25 X 150 mm) containing 15 $mL \cdot L^{-1}$ of treatment medium.

Activated Charcoal Experiment

Explant health was evaluated by the addition of activated charcoal (AC). The basal treatment medium consisted of 0, 0.5, 1, 2, or 4 $g \cdot L^{-1}$ AC, MS basal salts and vitamins, 2 μM BA, 30 $g \cdot L^{-1}$ sucrose, and solidified with 0.8% (w/v) agar. The pH was adjusted to 6.0 with 1 N HCL or 1 N KOH prior to autoclaving. Microshoots were placed in test tubes (25 X 150 mm) containing 15 $mL \cdot L^{-1}$ of treatment medium.

Activated Charcoal and Cytokinin Composition Experiment

Microshoot health and growth vigor was evaluated by the addition of AC and two different cytokinin combinations. Treatment medium consisted of MS basal salts and vitamins, 30 $g \cdot L^{-1}$ sucrose, 0.8% (w/v) agar, and a factorial arrangement treatment of AC (0 or 2 $g \cdot L^{-1}$) and BA (0 or 2 μM) or 2iP (0 or 2 μM). The pH was adjusted to 6.0 with 1 N HCL or 1 N KOH prior to autoclaving. Nodal explants were placed in test tubes (25 X 150 mm) containing 15 $mL \cdot L^{-1}$ of treatment medium.

Embryo Regeneration from Leaf Tissue Experiment

In a preliminary experiment, leaf tissues were harvested from established *M.* 'Yellow Bird' explant stock material at the end of a four-week growing cycle. Leaves were trimmed into segments and placed (midrib down) into sterilized 60 mm petri dishes containing Syringa Woody Plant Medium (SWPM) as described by Maren (2016). SWPM contained 10 $g \cdot L^{-1}$ sucrose, 10 $g \cdot L^{-1}$ maltose, 5 μM trans-zeatin (Z007; Caisson Laboratories, 836 South 100, East Smithfield, UT 84335, USA), 1.45 μM calcium D-gluconate monohydrate (G4625; Sigma-Aldrich® Co.), and 277 μM additional myo-inositol. The medium was solidified with 0.4% (w/v) agar and 0.14% (w/v) Gelrite® (CP Kelco U.S., Inc., Cumberland Center II, 3100 Cumberland Boulevard Suite 600, Atlanta, GA 30339, USA) and adjusted to a post autoclaved pH of 5.4. Under the laminar flow hood, 0, 5, 10, 15, or 20 μM picloram (systemic herbicide, 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) was filter sterilized into each petri dish. Treatments were cultured in an incubator (Model 818; Precision Scientific, 170 Marcel Drive, Winchester, VA 22602, USA) in the dark at 25 ± 1°C.

Subsequent to the preliminary experiment, leaf tissues were harvested from explant stock material at the end of another four-week growing cycle. Leaves were trimmed into segments and placed (midrib down) in sterilized petri dishes (60 mm) containing MS, 30 $g \cdot L^{-1}$ sucrose, 0.8% (w/v) agar, and a factorial arrangement treatment of trans-zeatin (0 or 5 µM) and picloram (0, 5, 10, 15, or 20 µM). The pH was adjusted to 6.0 prior to autoclaving. Treatments were cultured in an incubator (Model 818; Precision Scientific) in the dark at 25 ± 1°C.

Somatic Embryogenesis utilizing Mature Seed Experiment

M. acuminata var. *subcordata* mature seeds were obtained from previous collection at the University of Wisconsin-Madison Arboretum (1207 Seminole Highway, Madison, WI 53711, USA). Seeds were stored with moist peatlite in sealed plastic bags in a 2°C cold-storage facility at the NDSU Lord and Burnham greenhouse (Fargo, ND 58102, USA) for 180 days or more upon use. Hard mature seeds were rinsed in distilled water for 1 h and then placed in the laminar flow hood under sterile conditions. Seeds were placed in a sealable 500 mL Pyrex® (CLS13951L; Sigma-Aldrich® Co.) glass vessel, immersed in 70% ethanol (EtOH), and shaken for 2 min. The EtOH was decanted, replaced with distilled deionized water (ddH_2O) (18.3 MΩ), shaken for 5 min, and rinsed. Disinfestations solutions were prepared with 4, 5, 6, 7, 8, 9, or 10% (w/v) sodium dichloroisocyanurate dihydrate (35915; Sigma-Aldrich® Co.) and 500 µL TWEEN® 20 (Polysorbate 20, P1379; Sigma-Aldrich® Co.). Solutions were poured over seeds and vigorously shaken for 30 min. Solutions were decanted, replaced with ddH_2O, shaken for an additional 10 min, and rinsed. This disinfestation protocol was repeated 5 times. To decrease the risk of contamination, seeds were immersed in 0.01 M HCL, shaken for 3 min, and rinsed with ddH_2O.

The seeds were excised from their hard seed coat utilizing a crescent end cutting pliers, bisected longitudinally, and the halves were placed downward in sterilized petri dishes (60 mm) on MS medium. The medium was supplemented with 30 $g \cdot L^{-1}$ sucrose, 0.1 $g \cdot L^{-1}$ myo-inositol, 0.3% (w/v) gellan gum, 0, 1, 5, or 10 µM gibberellic acid (GA_3), and adjusted to a 5.7 pH prior to autoclaving. Treatments were cultured in an incubator (Model 818; Precision Scientific) in the dark at 25 ± 1°C. After one month, seeds were transferred to ½ MS with no supplemental PGRs.

After 6 weeks, data was taken on callus induction or embryo regeneration. This experiment was arranged as a completely random design (CRD). In each treatment, there were 5 replications and 3 samples per replication. Data was analyzed using the General Linear Model (GLM) of SAS 9.3 (SAS Institute, Inc., 2018). Means were analyzed based on a mean separation analysis using Fisher's Least Significant Difference (LSD) test for paired comparisons.

Somatic Embryogenesis and Callus Induction from Immature Seed Experiment

Immature (green) *M. acuminata*, *M. kobus*, *M. stellata*, *M.* 'Leonard Messel', *M.* 'Rosea' seed was collected on July, 1, 2017 from the Morton Arboretum (Lisle, IL 60532, USA) and *M. acuminata* seed was collected from the University of Wisconsin-Madison Arboretum to compare source tissue for regeneration. Immature fruit was rinsed in distilled water and washed with 500 µL Tween® 20. Seeds were extracted from the exocarp using a grafting knife, rinsed under distilled water, and placed in a sealable Pyrex® (CLS13951L; Sigma-Aldrich® Co.) glass vessel. Within the laminar flow hood, tissues were immersed in 70% EtOH and shaken for 20 sec. EtOH was decanted and 10% bleach (8.25% NaOCI) was poured over the seeds. Vessels were vigorously shaken for 5 min and decanted. Tissues were rinsed with sterile ddH_2O for 5 min for

three intervals. Observations indicated that seeds turned dark brown, therefore 5% bleach disinfestation solutions were used in Run 2 instead of 10% bleach in Run 1.

The seeds were bisected longitudinally and halves were placed downward in sterilized petri dishes (60 mm) on Yellow-Poplar conditioning medium as described by Merkle and Sommer (1986) or Lloyd and McCown (1980) (WPM) medium. There were four different treatment combinations evaluated. Treatment one consisted of Yellow-Poplar conditioning medium, supplemented with 1 $g \cdot L^{-1}$ casein hydrolysate, 0.1 $g \cdot L^{-1}$ Myo-Inositol, and no PGRs. Treatment two consisted of Yellow-Poplar conditioning medium, supplemented with 1 $g \cdot L^{-1}$ casein hydrolysate, 0.1 $g \cdot L^{-1}$ myo-inositol, 9 μM 2,4-dichlorophenoxoxyacetic acid (2,4-D), and 1.1 μM BA. Treatment three consisted of WPM, supplemented with no PGRs. Treatment four and five consisted of WPM, supplemented with 2.3 or 4.5 μM 2,4-D. All treatments contained 40 $g \cdot L^{-1}$ sucrose, 0.8% (w/v) agar and were adjusted to a pH of 5.8 prior to autoclaving. All treatments were cultured in an incubator (Model 818; Precision Scientific) in the dark at 25 ± 1 °C.

After 6 weeks, data was taken on callus induction or embryo regeneration. This experiment was arranged as a completely random design (CRD). In each treatment (5), there were 6 replications and 3 samples per replication. Data was analyzed using the General Linear Model (GLM) of SAS 9.3 (SAS Institute, Inc., 2018). Means were analyzed based on a mean separation analysis using Fisher's Least Significant Difference (LSD) test for paired comparisons.

RESULTS AND DISCUSSION

Because magnolias have been the center of horticultural interest for centuries, breeding and vegetative propagation methods have been well established. However, micropropagation has not been sufficiently effective (Kim et al., 2007). *In vitro* microcultured magnolias have poor performance compared to most other microcultured species because the absence of improved *in vitro* methodologies (Shi et al., 2002). In efforts to develop a somatic embryogenic regeneration protocol by improving *in vitro* methodologies, many experiments were preformed to increase the microplant stock population and overall plant health (shoot proliferation, cytokinin, and AC experiments). A sufficient microplant population with vigorously growing healthy plants is essential when designing and attempting various biotechnological experiments. Increasing population size and developing healthy experimental plant tissues was the initial step to supplement the somatic embryogenic regeneration protocol objective and experiments.

Shoot Proliferation Experiment

Medium composition, carbohydrate source, PGR compositions and concentration(s), and their interactions were analyzed to determine their effects on shoot multiplication rates. Data indicated that only medium composition had a significant effect on microshoot number (Appendix Table A-2). Shoot number was significantly higher (1.9) in MS media as compared to S media (Table 1). MS culture media has been widely used for *in vitro* magnolia production while S media has been successful for saucer magnolia. Biedermann (1987) reported higher proliferation rates and overall quality of *M.* 'Yellow Bird' microshoots grown on MS. However, it is important to note the statement made by McCown and Sellmer (1987) that the responses of *in vitro* grown plants to the nutrient medium are highly species-specific and can vary from little differences in growth to life or death reactions.

Table 1. *Magnolia* 'Yellow Bird' microshoot multiplication rates per nodal explant on different medium compositions *in vitro*.

	Mean[a]
Medium Composition	Microshoot Number
MS	1.9a
S	1.3b

[a] Means followed by the same letter are not significantly different ($\alpha<0.05$) based on mean separation analysis using a least significant difference (LSD) for paired comparisons.

Sucrose is the most widely used carbohydrate source in woody plant micropropagation, however sucrose is not always most efficient for shoot proliferation (Pua and Chong, 1985). Kamenicka (1998) reported fructose, mannose, and xylose were the most effective carbon source on shoot proliferation for saucer magnolia followed by sucrose. However, analyzed *M.* 'Yellow Bird' data indicated no significant difference between sucrose and fructose on microshoot number (Appendix Table A-2).

PGR compositions and various concentrations had no significant effect on shoot proliferation rates (Appendix Table A-2). However, Parris (2011) obtained higher proliferation rates on MS media supplemented with 2 µM BA. In previous *in vitro* studies on *M. grandiflora* L. (southern magnolia) and saucer magnolia 3.1 µM BA were used to induce microshoots (Ibrahim et al., 2011).

Since medium composition had a significant effect on shoot proliferation rates, the interaction between medium and carbohydrate, medium and BA, and medium and NAA were analyzed. There was no significant difference between interactions (Appendix Table A-2).

With magnolia axillary shoot proliferation, an average of 2 shoots per shoot is not sufficient enough for an increase in population size because microshoots varied in size and health. Lower proliferation rates may be caused by the relatively large *in vitro* shoots and leaves produced, subsequently limiting the number of shoots that can be grown in each culture vessel.

The high levels of phenolic compounds magnolias exude and physiological microshoot malformation were also observed during data collection. Phenolic compounds produced by magnolias reduces the efficiency of microculture which leads to lower shoot proliferation rates. Microshoots appeared translucent and leaves became necrotic or translucent with expanded midribs resulting in excessive hydration (hyperhydricity). This physiological distortion decreases overall microshoot health and vigor. The results of this experiment indicated that further experimentation (cytokinin and AC experiments) was necessary to improve *in vitro* proliferation rates and microculture conditions for magnolia.

Cytokinin Composition and Concentration Experiment

Several cytokinins have been used to induce shoot proliferation for magnolia. As mentioned by Marinescu (2008), BA has shown to produce higher shoot multiplication rates than 2iP in saucer magnolia, but has shown to induce hyperhydricity (Bairu et al., 2007; Werbrouck et al., 1996). Similar in structure to BA, the naturally occurring cytokinin (*m*T) has not been shown to induce hyperhydricity and is effective for several species *in vitro* (Meyer et al., 2009; Amoo et al., 2011). In our study, BA, *m*T, and 2iP at multiple concentrations (0, 2, 4, or 8 μM) were used to increase microshoot multiplication rates and culture conditions. Data was not shown because shoot proliferation was unsuccessful, however microshoots supplemented with 2 μM 2iP had the best overall visual appearance (dark green leaves and stems). Microshoots from stock populations continue to exhibit symptoms of hyperhydricity resulting in no microshoot multiplication.

Activated Charcoal and Cytokinin Composition Experiments

Visual observations indicated that magnolias exude high levels of phenolic compounds in tissue culture. During micropropagation, shoot proliferation has been reported to be difficult as a

result of the presence of phenolic acid (Biedermann, 1987; Sakr et al., 1999). The presence of phenolic compounds reduces the efficiency of magnolia microculture resulting in reduced plant health and growth vigor (Sarker and Maruyama, 2002). The addition of AC has been known to significantly decrease phenolic oxidation through absorption (Thomas, 2008). In the AC experiment, microshoots appeared healthy (dark green leaves and shoots), but shoots failed to multiply. Data was not presented because axillary shoot proliferation was unsuccessful. Visual observations indicated that AC absorbed unwanted substances (phenolic compounds) in addition to potentially absorbing essential hormones and added PGRs vital for growth vigor. These conclusions were made because in the shoot proliferation experiment, there was an increase in microshoot number per explant, but when AC was added to the medium (in the AC experiment) shoots failed to multiply. AC may be responsible for the absorption of phenolic compounds because microshoots appeared greener and healthier when the medium was supplemented with AC. AC has been linked to the absorption of various cytokinins including BA *in vitro* (Thomas, 2008) which may explain no microshoot multiplication. The previous statement may explain why microshoots were smaller and did not multiply when AC was added to the medium.

Subsequent to the AC experiment, the addition of AC with two different cytokinins (BA and 2iP) were evaluated. Observations indicated that leaves were greener and shoots were less hyperhydric, however data was not shown because shoot proliferation regeneration was unsuccessful.

Embryo Regeneration from Leaf Tissue Experiment

To date, no direct embryo regeneration from leaf tissue of any *Magnolia* spp. has been reported. In a preliminary experiment, direct embryo regeneration was successful in a single leaf

tissue of ‘Yellow Bird’ on SWPM supplemented with 5 µM trans-zeatin and 15 µM picloram. The embryo reached heart-stage (Fig. 1), but aborted during maturation phase.

Figure 1. Somatic embryo regeneration via leaf tissue in heart stage of embryo formation of *Magnolia* x*brooklynensis* ‘Yellow Bird’. Scale bar = 10mm.

After shoot proliferation, cytokinin, and AC experiments, microshoot proliferation rates decreased and plant health declined. Stage II micropropagated population rates of microplants were exceptionally low and plant material was limited as a result of micropropagation capacity of magnolia cultures. Low population rates resulted in minimal leaf material for experimental use. It became a challenge to acquire healthy leaf (green) material suitable for embryo regeneration. Observable growing conditions affected experimental methods, however the addition of picloram and trans-zeatin may be responsible for direct embryo regeneration via leaf tissue.

The subsequent experiment evaluated trans-zeatin in factorial combination with picloram. Data was not presented because the limited number of leaf material and lack of tissue uniformity.

Experimental leaf tissues were hyperhydrated, hyperhydrated-green, or green. Minimal callus production and small embryos were observed on few green leaf tissue.

The primary objective was to develop a somatic embryogenesis system, initially utilizing leaf tissue. As a result of lacking enough quality leaf tissue, somatic embryogenesis utilizing seeds was evaluated.

Somatic Embryogenesis utilizing Mature Seed Experiment

Disinfestation protocols prepared with 4, 5, 6, 7, 8, 9, or 10% (w/v) sodium dichloroisocyanurate dihydrate for *M. acuminata* var. *subcordata* mature seeds were ineffective. Contamination was present in all seeds cultured. Initial contamination was detected in most seeds whereas others appeared clean. After the first month, contamination-free seeds were aseptically transferred to new treatment medium, but contamination appeared within days upon transfer. The presence of seedborne bacterial pathogens resulted in poor tissue culture establishment, therefore embryogenesis was ineffective.

Somatic Embryogenesis and Callus Induction from Immature Seed Experiment

Kim et al. (2007) reported that *Magnolia* spp. can be propagated by somatic embryogenesis using *M. macrophylla* Michx., *M. virginiana*, *M.* 'Yellow Bird', *M. fraseri* Walt. immature seeds as suggested in research conducted by Merkle's group (Merkle and Watson-Pauly, 1993; 1994; Merkle and Wiecko 1990; Merkle, 1999). Immature (green) *M. acuminata*, *M. kobus*, *M. stellata*, *M.* 'Leonard Messel', *M.* 'Rosea' seed were placed on Yellow-Poplar conditioning medium supplemented with 9 μM 2,4-D and 1.1 μM BA or WPM supplemented with 2,4-D (2.3 or 4.5 μM). Data was analyzed on a categorical yes or no with respect to callus induction or embryo regeneration from the source seed material.

Results indicated that specie/cultivar type has a significant effect on callus induction, however the interaction between treatment medium and specie/cultivar had no effect on callus induction (Appendix Table A-3). *M.* 'Rosea', *M. stellata*, and *M. kobus* were significantly different from M. 'Leonard Messel' and *M. acuminata* (IL and WI source) for callus induction. *M.* 'Rosea', *M. stellata*, and *M. kobus* had significantly greater callus percentage rates compared to *M.* 'Leonard Messel' and *M. acuminata* (IL and WI source) (Table 2). Visual observations indicated that *M.* 'Rosea' produced large, healthy callus masses via immature seed (Fig. 2).

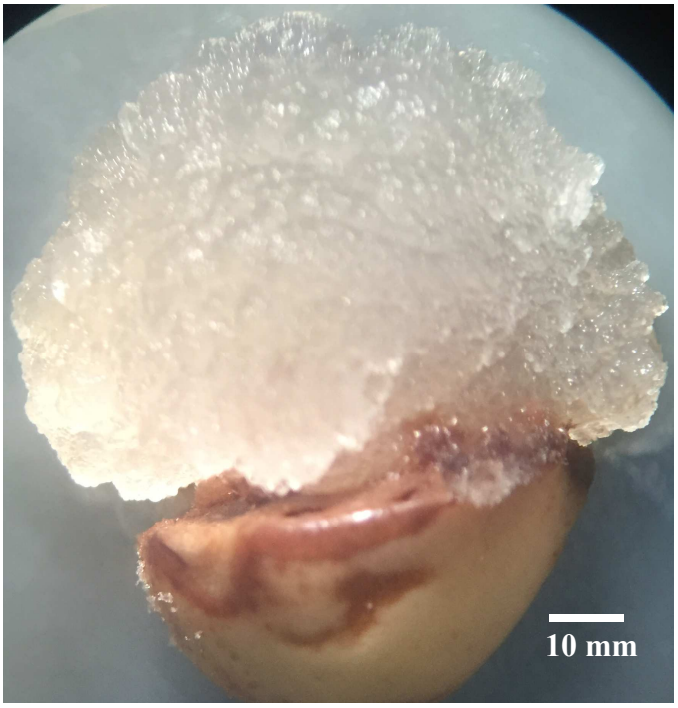

Figure 2. *Magnolia* 'Rosea' callus induction via immature seed. Scale bar = 10 mm.

Table 2. Callus induction percentage rates of *Magnolia* spp. and cultivars.

	Mean[a]
Magnolia Specie/Cultivar	Callus Induction
'Rosea'	0.68a
stellata	0.61a
kobus	0.61a
'Leonard Messel'	0.53b
acuminata (IL)	0.50b
acuminata (WI)	0.50b

[a] Means followed by the same letter are not significantly different ($\alpha<0.05$) based on mean separation analysis using a least significant difference (LSD) for paired comparisons.

Results indicated that treatment medium and specie/cultivar type has a significant effect on embryo regeneration, however the interaction between treatment medium and specie/cultivar had no effect on embryo regeneration (Appendix Table A-4). WPM had the greatest embryo regeneration percentage rates compared to Yellow Poplar conditioning medium (Table 3). *M*. 'Leonard Messel' and *M*. *stellata* (Fig. 3) had the greatest embryo regeneration percentage rates as compared to other evaluated species while *M*. 'Rosea' and *M*. *acuminata* (IL and WI) had the least (Table 4).

Data was not analyzed or presented for Run 2 because all immature seeds were contaminated. Dark brown seeds may have been from the release of phenolic compounds and seed oxidation from seed bisection instead of the concentration of bleach. Biederman (1987) indicated that explants should be taken in December to March after dormancy is broken because phenolic acid is lowest in magnolias. Observations concluded that phenolic acid was high when seeds were collected in July. Even though phenolic acid was high in July, it was important to collect at the time of developing fruits post-anthesis for experimental purposes.

Table 3. Embryo regeneration percentage rates on different medium compositions and Plant Growth Regulators.

Medium Composition	2,4-D (μM)	BA (μM)	Mean[a] Embryo Regeneration
WPM	0	0	0.55a
WPM	2.3	0	0.52ab
WPM	4.5	0	0.53ab
Yellow Poplar	0	0	0.5b
Yellow Poplar	9	1.1	0.5b

[a] Means followed by the same letter are not significantly different ($\alpha<0.05$) based on mean separation analysis using a least significant difference (LSD) for paired comparisons.

Table 4. Embryo regeneration percentage rates of *Magnolia* spp. and cultivars.

Magnolia Specie/Cultivar	Mean[a] Embryo Induction
'Leonard Messel'	0.56a
stellata	0.54ab
kobus	0.51bc
'Rosea'	0.50c
acuminata (IL)	0.50c
acuminata (WI)	0.50c

[a] Means followed by the same letter are not significantly different ($\alpha<0.05$) based on mean separation analysis using a least significant difference (LSD) for paired comparisons.

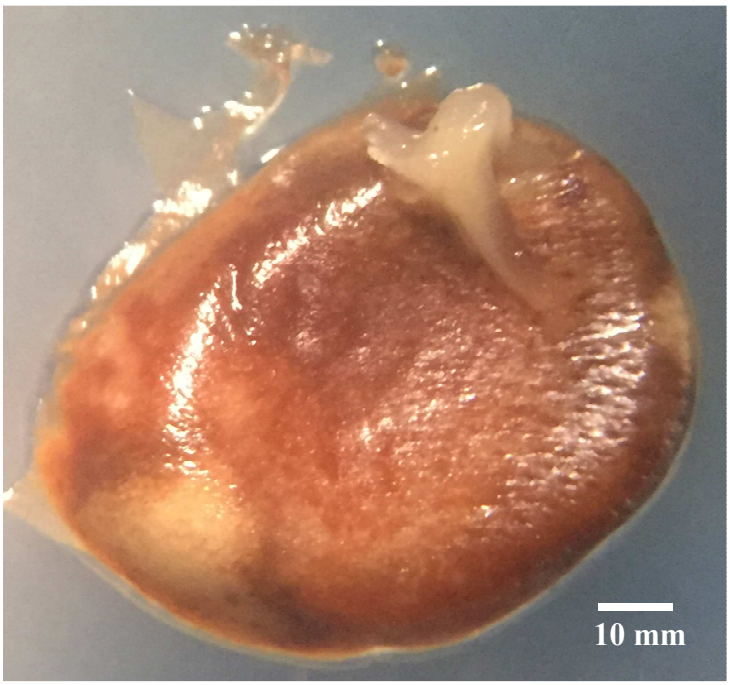

Figure 3. *Magnolia stellata* embryo regeneration via immature seed. Scale bar = 10mm.

CONCLUSION

Results indicated that MS medium significantly performed better than S medium with respect to microshoot number, but not sufficient for adequate microshoot population increase. Cytokinin (composition and concentration) and carbohydrate source had no effect on shoot multiplication rates or plant health. Observations indicated that addition of AC produced greener leaves and less hyperhydrated shoots but failed to induce microshoot proliferation. Over time, microshoot quality declined *in vitro* and increasing the microshoot population became unsuccessful. Microshoot culture may not be effective for increasing population size for experimentation, however, research indicates that somatic embryogenesis is feasible for *Magnolia* spp. (Kim et al., 2007; Merkle, 1999).

Further improvement of micropropagation protocols should involve: 1) various field collection times for magnolia tissue culture, 2) the use of various culture medium and PGRs, and 3) the use of various compounds or chemicals to decrease the effects of phenolic compounds on subsequent growth.

Initial somatic embryogenesis induction experiments utilized leaf tissues, however low population rates and unhealthy explants resulted in minimal leaf material for subsequent experimental use. As a result of inefficient leaf material, mature seeds were utilized for somatic embryogenesis induction experiments. Mature seeds were contaminated in culture after various disinfestation protocols. Subsequent somatic embryogenesis experiments utilized immature seeds.

Results indicated that *M*. 'Rosea', *M*. *stellata*, and *M*. *kobus* had significantly greater callus induction rates compared to *M*. 'Leonard Messel' and *M*. *acuminata* (IL and WI source) utilizing immature seed tissues. For somatic embryo regeneration, WPM medium had

significantly greater rates of regeneration as compared to Yellow Poplar conditioning medium utilizing immature seed tissues. *M*. 'Leonard Messel' and *M*. *stellata* had significantly higher regeneration rates as compared to *M*. 'Rosea' and *M*. *acuminata* (IL and WI).

Further improvement of somatic embryogenesis utilizing immature seed protocols should involve: 1) various field collection times for inducing callus or embryo regeneration from immature seed, 2) the use of various culture mediums and PGRs, and 3) the use of various compounds or chemicals to decrease the risk of phenolic compounds released. Embryo regeneration results observed in this study may lead to a regeneration protocol produced by the NDSU WPIP to develop an early flowering genetic transformation protocol to induce rapid breeding cycles for the prospective selection of magnolias with diverse floral characteristics.

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APPENDIX

Table A-1. Composition of Standardi and Catalana (1958) S micropropagation medium.

	mass ($mg \cdot L^{-1}$)	μM
Organics		
Myo-Inositol	100	555.0622
Thiamine HCL	4	11.8599
Phosphates and Oxides		
Potassium Phosphate Monobasic	270	1984.0395
Boric Acid	6.2	100.2701
Sodium Molybdate	0.25	1.0335
Potassium Iodide	0.8	4.8193
Cobalt Chloride	0.025	0.1051
Nitrates		
Ammonium Nitrate	400	4997.5012
Potassium Nitrate	1800	17804.1543
Calcium Nitrate	1200	5081.5160
Sulfates		
Magnesium Sulfate Heptahydrate	360	1460.5648
Manganese Sulfate Monohydrate	1	5.9165
Zinc Sulfate Heptahydrate	8.6	29.9130
Cupric Sulfate Pentahydrate	0.025	0.1001
Iron		
Disodium Ethylenediaminetetraacetic Acid	37.25	100.0698
Ferrous Sulfate Heptahydrate	27.85	100.1799

Table A-2. Analysis of variance (ANOVA) for the effects of medium composition, carbohydrate source, BA and NAA concentrations on microshoot multiplication rates of *Magnolia* x*brooklynensis* 'Yellow 'Bird' after 4 w incubation (23 ± 1°C in light).

Variable: Microshoot Number				
Source of Variation	df	MS	F	Pr > F
Rep	4	0.63	0.60	0.6667
Medium	1	11.56	10.84	0.0012
Carbohydrate	1	0.06	0.05	0.8186
BA	3	2.54	2.38	0.0719
NAA	1	2.26	2.12	0.1479
Medium*Carbohydrate	1	3.21	3.10	0.0803
Medium*BA	3	1.19	1.12	0.3447
Medium*NAA	1	1.80	1.69	0.1951
Error	144	1.07		
Total	159			

Table A-3. Analysis of variance (ANOVA) for the effects of medium treatment composition and various *Magnolia* spp. and cultivars on callus induction rates after 6 w incubation (25 ± 1°C in dark).

Variable: Callus Induction				
Source of Variation	df	MS	F	Pr > F
Rep	5	0.061	0.99	0.4236
Treatment	4	0.099	1.60	0.1720
Specie/Cultivar	5	0.47	7.61	<.0001
Treatment*Specie/Cultivar	20	0.097	1.57	0.0557
Error	505	0.062		
Total	539			

Table A-4. Analysis of variance (AVONA) for the effects of medium treatment composition and various *Magnolia* spp. and cultivars on embryo regeneration rates after 6 w incubation (25 ± 1°C in dark).

Variable: Embryo Regeneration				
Source of Variation	df	MS	F	Pr > F
Rep	5	0.0163	0.93	0.4582
Treatment	4	0.0417	2.39	0.0500
Specie/Cultivar	5	0.0563	3.23	0.0070
Treatment*Specie/Cultivar	20	0.0239	1.37	0.1311
Error	505	0.0174		
Total	539			