APPLICATION OF SOMATIC EMBRYOGENESIS FOR PROPAGATION OF WHITE ASH AND WHITE OAK TREES

by

JESSICA ELAINE MITCHELL

(Under the Direction of Scott Merkle)

ABSTRACT

Somatic embryogenesis *in vitro* propagation systems were used to clonally propagate two significant species of hardwood trees, white ash (*Fraxinus americana*) and white oak (*Quercus alba*). North America's native ash resource is threatened by emerald ash borer (*Agrilus planipennis* Fairmaire; EAB). Via somatic embryogenesis, we clonally propagated white ash genotypes *in vitro* that have demonstrated resistance to EAB, by initiating embryogenic cultures from zygotic embryos collected from putatively EAB-resistant trees, potentially eliminating the need to hybridize white ash with EAB-resistant Asian species or to employ gene transfer techniques to produce transgenic trees carrying genes for resistance to EAB. We also demonstrated the ability to clonally propagate white oak *in vitro* via somatic embryogenesis, and successfully applied a cryostorage and recovery protocol to embryogenic white oak tissues, providing a base system to which a gene transfer techniques could be applied to potentially accelerate production of genetically improved white oak trees.

INDEX WORDS: somatic embryogenesis, tissue culture, white ash, emerald ash borer, white oak

# APPLICATION OF SOMATIC EMBRYOGENESIS FOR PROPAGATION OF WHITE ASH AND WHITE OAK TREES

by

JESSICA ELAINE MITCHELL

B.S., Brenau University, 2013

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2015

# APPLICATION OF SOMATIC EMBRYOGENESIS FOR PROPAGATION OF WHITE ASH AND WHITE OAK TREES

by

JESSICA ELAINE MITCHELL

Major Professor: Scott Merkle
Committee: Kamal Gandhi
Dayton Wilde

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2015

ACKNOWLEDGEMENTS

I would like to thank Heather Gladfelter for first introducing me to plant tissue culture and graciously sharing her years of experience with me, becoming a great mentor and friend. I would also like to thank Dr. Scott Merkle and his entire lab for making the pursuit of a Master of Science a positive and rewarding experience. Thank you for kindness and for always being willing and ready to share your skills, knowledge, brilliant ideas, and advice with me, without which, this research project would not have been possible.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

# Introduction and literature review

## Major North American ash species

Green ash (*Fraxinus pennsylvanica*) and white ash (*Fraxinus americana*) are two ecologically and economically important hardwoods belonging to the family Oleaceae. They are long-lived and similar in properties, with good form and strong, tough wood with high shock resistance. White ash wood has especially high shock resistance, making it important for the production of handle stock, oars, and baseball bats. The demand for good white ash required for the production of such items has increased, which may be caused by an emphasis on recreation in North America (Stewart and Krajicek 1973). While white ash is the most commonly used native ash, green ash is the most widely distributed native ash. Like most trees, both species grow well in fertile, moist, well-drained soils, although white ash has more demanding soil fertility and moisture requirements, whereas green ash is much more adaptable to different soil conditions. Because of its adaptability, as well as its good form and high tolerance to insects and diseases, green ash is a popular ornamental tree in residential areas. White ash is also used as an ornamental tree for its similar properties (Burns and Honkala 1990). Both species bear opposite, pinnately compound leaves and winged fruits called samaras, which provide food for a variety of birds and small mammals. The species are also very grazing-tolerant, providing an important source of browse for wildlife and livestock, such as white-tailed deer and cattle. In addition to providing a food source, white ash and green ash provide cover for wildlife and livestock

(Gucker 2005; Griffith 1991). White ash is the largest of all the ashes, commonly reaching 70 to 80 feet tall or taller, while green ash is a medium-sized tree, commonly reaching 60 to 70 feet tall (Stewart & Krajicek 1973).

Other North American ashes include black ash (*Fraxinus nigra*), pumpkin ash (*Fraxinus profunda*), and Oregon ash (*Fraxinus latifolia*), also belonging to the family Oleaceae. Black ash and pumpkin ash grow in swampy areas, with black ash occurring in many northeastern U.S. states and Canada's eastern provinces, whereas pumpkin ash grows primarily in the Atlantic Coastal Plains and discontinuously along swamp margins and river bottoms in the southeastern U.S. Oregon ash is the only native species of ash in the Pacific Northwest, ranging from northern Washington to southern California (Stewart and Krajicek 1973). These three species do not carry the same commercial significance as white or green ash, but still have some applications and are ecologically important, providing seeds as food for birds and small mammals like squirrels, as well as providing browse for larger mammals, such as deer and moose (Stewart and Krajicek 1973). Black ash is strongly ring-porous, allowing the wood to split and bend easily. The wood is also relatively soft and light weight, making it ideal for making baskets and woven furniture. Because of this, black ash is culturally important to several Native American tribes who use the wood to make baskets, food storage barrels, and lacrosse sticks (Anderson & Nesom 2003). Much like white ash, pumpkin ash and Oregon ash are often used a source for handle stock. Oregon ash is also used for fuel, because it splits easily and has a high heat value. Although the leaves, twigs, flowers, and fruit of pumpkin ash are larger than those of white and green ash, the three trees share particularly similar properties, likely because pumpkin ash is considered to be a

true-breeding polyploid derivative of a cross between a diploid green ash and tetraploid white ash (Stewart and Krajicek 1973).

**EAB in North America**

The emerald ash borer (EAB) is a phloem-feeding beetle (Coleoptera: Buprestidae) native to northeastern China, Korea, Japan, Mongolia, Taiwan, and eastern Russia (Jendek 1994, Haack et al. 2002). In its native range, EAB is not considered a major pest, partly because the Asian ash species Manchurian ash (*Fraxinus mandschurica*) and Chinese or Korean ash (*Fraxinus chinensis*) are more resistant to EAB than North American species (Rebek et al. 2008). EAB first became established in North America in the urban forests of Detroit, Michigan in 2002, and its life cycle in North America appears similar to what is described in China (Chinese Academy of Science 1986; Yu 1992). In the spring, adults chew their way out of the stem, leaving D-shaped exit holes, which are characteristic of Buprestids, and feed on ash foliage, mainly causing aesthetic damage to trees. After 5-7 days of feeding, adults mate, and females continue to feed for 5-7 more days before laying eggs. Eggs are laid in bark crevices, and each female can lay between 50-90 eggs during the remainder of her 3-6 week lifespan. Larvae hatch within two weeks and feed on the phloem and cambium from July to autumn, leaving serpentine-shaped galleries packed with frass. Most larvae complete feeding between October and November, and prepupae overwinter in the sapwood or outer bark. Pupation occurs mid-April to May, and adults emerge about three weeks later. Larval feeding causes the most extensive damage by disrupting translocation and girdling the host tree, which can ultimately lead to death within 1-3 years. Other symptoms of EAB infestation include bark splitting, canopy dieback, and epicormic shoots from large branches or the trunk (Poland and McCullough 2006). Adults can be

identified by their characteristic metallic coppery-green color. Their bodies are slender and elongate, measuring 7.5-15 mm long. Larvae have a small brown head that is retracted inside an enlarged prothorax, and the body is white, flat, and slender, with a pair of brown, pincer-like appendages on the last abdominal segment (Haack et al. 2002).

Since first becoming established in North America in 2002, EAB has caused mortality of tens of millions of trees in Michigan alone. As of 2015, EAB has spread and killed tens of million more trees in 21 other U.S. states and two Canadian provinces, Ontario and Quebec (Herms & McCullough 2014). All North American ash species are susceptible to EAB, with black, green, and white ash being the most vulnerable (Anulewicz et al. 2008, Klooster et al. 2014). EAB adults are particularly attracted to trees stressed by factors such as girdling, but healthy trees are still susceptible to colonization and damage (Herms et al. 2004, Cappaert et al. 2005, Poland and McCullough 2006). EAB spreads by stratified dispersal, in which natural dispersal through adult flight expands infestations over short distances, and long-distance dispersal occurs through transportation of infested wood materials by humans (Herms & McCullough 2014). This long-distance dispersal causes localized satellite populations, which eventually grow and merge with other satellite populations, as well as the main invasion front, increasing the rate of spread and resulting damage to ash trees. The economic damage caused by EAB is estimated to be between $10 billion and $26 billion by 2020, including costs for treatment, removal, and replacement of infested trees in urban and suburban landscapes (Syndor et al. 2007, Kovaks et al. 2010, Kovaks et al. 2011, Syndor et al. 2011). As EAB continues to spread, the ecological and economic impacts are expected to rival or exceed those of chestnut

blight and Dutch elm disease, two of the most devastating invasive pathogens in North America of the 20th century (Herms & McCullough 2014).

**Ash susceptibility and resistance to EAB**

In attempt to slow the spread of EAB, federal, state, and provincial quarantines have been established to prevent transportation of infested ash firewood, logs, and nursery trees. Research is also being conducted at universities to better understand the beetle's life cycle, dispersal, and host selection. Further research is being conducted to discover the best ways to detect and control infestation. Types of control research include investigating native parasitoids, predators, and pathogens of EAB, classical biological control including the release of Asian parasitoids, insecticidal control, and integrated management strategies. Unfortunately, no control or prevention methods have been determined to be most effective, as assessment takes many years. In addition, federal funding for EAB research programs has decreased, despite well-documented economic impacts of EAB invasion. Focus is being placed on protecting high-value landscape trees with systemic insecticides, because it is the most cost-effective option (Herms & McCullough 2014). Resources for protecting ash in forested areas, however, are limited and little ecological and genetic research has been conducted to increase efforts to conserve ash in forests.

Hybrid breeding may be one method to propagate ash trees that are more tolerant to EAB than native North American species, as Asian ash species are likely to be more resistant due to their evolutionary relationship with EAB. Rebek et al. (2008) found that the Asian species Manchurian ash (*Fraxinus mandschurica*) was far more tolerant to EAB than green and white ash; however, a black ash x Manchurian ash hybrid remained highly susceptible to EAB, indicating that the hybrid did not inherit resistance from its resistant parent.

Although all North American *Fraxinus* species are susceptible to EAB, this study concentrates on the two most commercially and ecologically important species, green ash and white ash. “Lingering” ash trees are of particular interest to some researchers. These are healthy trees which are intermingled with dead ash trees where EAB has caused almost complete mortality of mature ash trees in a particular area. These trees have healthy canopies and while some show signs of past EAB infestations, others have no symptoms. Researchers are interested in discovering whether lingering ash trees have a genetic basis for their apparent resistance to EAB (Knight et al. 2012).

**White oak in North America**

White oak (*Quercus alba*) is a relatively slow-growing, long-lived species of hardwood belonging to the family Fagaceae. It is native to North America, with a geographical range that extends over the eastern half of the United States, from the east coast to the Missouri River and north into Southern Ontario and Quebec (Goldblum 2010). White oak has the greatest range of any oak species in the United Sates, lending itself to great ecological and economical significance; however, it has declined due to landscape disturbances and the introduction of exotic pests and diseases (Abrams 2003). It is a medium to large deciduous tree, commonly reaching 60 to 80 feet, although individuals may grow more than 100 feet on the most favorable sites. White oak is monoecious, with staminate catkins emerging from the base of new growth and pistillate flowers growing in the axils of new growth. White oak produces short-stalked, smooth, ovoid acorns that typically develop in pairs (Tirmenstein 1991). It grows well on a range of soil types and sites, on all but the driest shallow soils, as well as under varying climatic conditions. Its acorns provide a food source for over 180 species of birds and mammals, and the

twigs and foliage are browsed by deer. Due to its broad crown, dense foliage, and attractive red to violet fall color, white oak is often planted as an ornamental tree (Burns & Honkala 1990). White oak's wood is heavy, strong, and durable, making it a good source of wood for a variety of products, such as furniture, flooring, timbers, ships, and caskets. White oak wood is in particularly high demand by the distilling industry, due to its impermeable nature, strength, and flexibility, which are all necessary criteria for fabricating the barrels in which whiskey and bourbon are aged (Mosedale 1995).

**Ash *in vitro* propagation**

Two *in vitro* propagation methods have been reported for ash species, organogenesis and somatic embryogenesis. Organogenesis involves the formation of adventitious roots or shoots from undifferentiated cell mass. Somatic embryogenesis is the process by which an embryo is derived from a somatic cell, in a developmental pathway that parallels that of zygotic embryos. Organogenesis by adventitious shoot production has been reported for both green ash (Du and Pijut 2008) and white ash (Bates et al. 1992; Palla and Pijut 2011). Du and Pijut also produced transgenic green ash plantlets by *Agrobacterium*-mediated transformation of hypocotyl explants, which then produced transgenic adventitious shoots (2009). Somatic embryogenesis has been reported for multiple ash species, including white ash (Preece et al. 1989; Bates et al. 1992), narrow-leafed ash (*F. angustifolia*; Tonon et al. 2001), common ash (*F. excelsior*; Capuana et al. 2007) and Manchurian ash (*F. mandshurica*; Kong et al. 2012). However, only Tonon et al. (2001) described a system suitable for potential scalable ash somatic embryo production using suspension culture.

Until recently, somatic embryogenesis had not been reported for green ash. Li et al. (2014) produced embryogenic cultures from zygotic embryos of green ash and successfully germinated somatic embryos grown from suspension culture to produce somatic seedlings. Li et al. (2014) also described the developmental stages of zygotic embryos in the green ash seeds used for their induction experiments. This knowledge is especially important because it has been shown that there is typically a narrow zygotic embryo developmental window for explants that are capable of making repetitive embryogenic cultures of hardwood trees. As such, developmental stage of zygotic embryo explants has been shown to be a contributing factor for successful induction in yellow-poplar (Sotak et al. 1991), American chestnut (Carraway and Merkle 1997), pecan (Wetzstein et al. 1989) and Manchurian ash (Kong et al. 2012), with early and late stages of zygotic embryo development resulting in lower or no embryogenesis induction. Of the plant growth regulator (PGR) treatments used in Li et al.'s (2014) study, only cultures initiated on media with 2 mg/l 2,4-D and 0.5 mg/l BA produced an embryogenic response. The authors suggested more experiments with more treatments, more source trees, and larger sample sizes were needed before conclusions could be made regarding optimal zygotic embryo developmental stages or PGR treatments for embryogenesis induction.

**Oak *in vitro* propagation**

*In vitro* propagation methods have been reported for several European oak species, including cork oak (*Quercus suber*), pedunculate oak (*Quercus robur*), and holm oak (*Quercus ilex*), but there have been very few studies on somatic embryogenesis systems for North American oaks, such as white oak. In the cork oak and pedunculate oak, embryogenic cultures were successfully initiated using leaf explants from epicormic shoots forced to sprout from

branch segments (Hernandez et al. 2002; Toribio et al. 2003). Embryogenic cultures have also been initiated from shoot apex explants derived from mature pedunculate oak trees (San-Jose et al. 2010). Somatic embryogenesis and plant regeneration has been reported in holm oak using fertilized ovule explants (Barra-Jiminez et al. 2013). There has only been one previous report on the induction of embryogenic cultures in white oak, the oak species tested in this study. Previously, somatic embryogenesis was achieved in white oak using shoot apices and leaf explants of cultures derived from 6 to 7 year old trees (Corredoira et al. 2012), but there was no evidence that the system described in this study had the potential for scalable white oak somatic embryo production using suspension culture.

Similar to the ash embryogenesis studies previously mentioned, these previous oak embryogenesis studies demonstrate the need to optimize oak somatic embryogenesis systems in order to make them potentially useful for mass clonal propagation or gene transfer applications. A reliable gene transfer system for white oak has the potential to greatly accelerate production of genetically improved white oak trees by facilitating the application of rapid cycle breeding, a strategy used to shorten breeding cycles in plants with long generation cycle that employs transgenics with early-flowering genes (van Nocker and Gardiner 2014).

**Study goals and objectives**

The overall goal of this research project was to demonstrate the potential to mass propagate EAB-resistant ash planting stock and white oak planting stock through application of a somatic embryogenesis system, to aid ash and white oak restoration efforts and production plantings. By cloning lingering ash trees through somatic embryogenesis, it may be possible to clonally propagate ash trees that are naturally resistant to EAB, eliminating the need to use gene

transfer techniques to produce transgenic trees that carry resistance to EAB. Furthermore, by focusing on explant type, developmental stage, PGR treatments, and other media treatments, the project aimed to develop a propagation system for lingering white ash and white oak with improved embryogenesis induction rates and improved germination and conversion rates of somatic seedlings, with the long-term goal of creating a scalable tissue culture system for this application.

**References**

Abrams MD (2003) Where has all the white oak gone?. BioScience 53(10):927-939

Anderson MK, Nesom G (2003) Black ash, *Fraxinus nigra* Marsh. USDA Natural Resources Conservation Service, National Plant Data Center. http://plants.usda.gov/plantguide/pdf/cs_frni.pdf. Accessed 6 October 2015.

Anulewicz AC, Mccullough DG, Cappaert DL, Poland TM (2008) Host range of the emerald ash borer (*Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae) in North America: results of multiple-choice field experiments. Environ Entomol 37(1):230-241

Barra-Jiménez A, Blasco M, Ruiz-Galea M, Celestino C, Alegre J, Arrillaga I, and Toribio M (2014) Cloning mature holm oak trees by somatic embryogenesis. Trees-Struct Funct 28(3): 657-667

Bates S, Preece JE, Navarrete NE, Vansambeek JW, Gaffney GR (1992) Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white ash (*Fraxinus americana* L.). Plant Cell Tiss Org Cult 31:21–29

Burns RM, Honkala BH (1990) Silvics of North America, vol. 2. United States Department of Agriculture

Cappaert D, McCullough DG, Poland TM, Siegert NW (2005) Emerald ash borer in North America: a research and regulatory challenge. Am Entomol 51(3):152-165

Capuana M, Petrini G, Di Marco A, Giannini R (2007) Plant regeneration of common ash (*Fraxinus excelsior* L.) by somatic embryogenesis. In Vitro Cell Dev Biol Plant 43(2):101-110

Carraway DT, Merkle SA (1997) Plantlet regeneration from somatic embryos of American

chestnut. Can J For Res 27(11):1805-1812

Chinese Academy of Science Institute of Zoology (1986) *Agrilus marcopoli* Obenberger. In: Agriculture insects of China (part 1). China Agriculture Press, Beijing, China, p 445

Corredoira E, San-José MC, and Vieitez AM (2012) Induction of somatic embryogenesis from different explants of shoot cultures derived from young *Quercus alba* trees. Trees-Struct Funct 26(3):881-891

Du, N. and Pijut, P.M. 2008. Regeneration of plants from Fraxinus pennsylvanica hypocotyls and cotyledons. *Scientia Horticulturae*, *118*(1), 74-79.

Du N, Pijut PM (2009) Agrobacterium-mediated transformation of *Fraxinus pennsylvanica* hypocotyls and plant regeneration. Plant Cell Rep 28(6):915-923

Goldblum D (2010) The geography of white oak's (*Quercus alba* L.) response to climatic variables in North America and speculation on its sensitivity to climate change across its range. Dendrochronologia 28(2):73-83

Griffith RS (1991) *Fraxinus americana*. In: Fire Effects Information System. US Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (Producer). http://www.fs.fed.us/database/feis/ Accessed 6 October 2015

Gucker CL (2005) *Fraxinus pennsylvanica*. In: Fire Effects Information System. US Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (Producer). http://www.fs.fed.us/database/feis/ Accessed 6 October 2015

Haack RA, Jendak E, Houping L, Marchant KR, Petrice TR, Poland TM, Ye H (2002) The emerald ash borer: a new exotic pest in North America. Newsletter of the Michigan Entomological Society. 47(3-4):1-5

Herms DA, Stone AK, Chatfield JA (2004) Emerald ash borer: the beginning of the end of ash in North America?. Special Circular-Ohio Agricultural Research and Development Center. pp 62-71

Herms DA, McCullough DG (2014) Emerald ash borer invasion of North America: history, biology, ecology, impacts, and management. Annu Rev Entomol 59:13-30

Hernández I, Celestino C, Toribio M (2003) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis. Plant Cell Rep 21(8):759-764

Klooster WS, Herms DA, Knight KS, Herms CP, McCullough DG, et al (2014) Ash (*Fraxinus* spp.) mortality, regeneration, and seed bank dynamics in mixed hardwood forests following invasion by emerald ash borer (*Agrilus planipennis*). Biol Invasions 16(4):859-873

Knight KS, Herms DA, Plumb R, Sawyer E, Spalink D, et al (2012) Dynamics of surviving ash (*Fraxinus* spp.) populations in areas long infested by emerald ash borer (*Agrilus planipennis*). Proceedings of the 4th international workshop on genetics of host-parasite interactions in forestry. pp 143-152

Kong DM, Preece JE, Shen HL (2012) Somatic embryogenesis in immature cotyledons of Manchurian ash (*Fraxinus mandshurica* Rupr.). Plant Cell Tiss Org 108(3):485-492

Kovacs KF, Haight RG, McCullough DG, Mercader RJ, Siegert NW, Liebhold AM (2010) Cost of potential emerald ash borer damage in US communities, 2009-2019. Ecol Econ 69(3):569-578

Kovacs KF, Mercader RJ, Haight RG, Siegert NW, McCullough DG, Liebhold AM (2011) The influence of satellite populations of emerald ash borer on projected economic costs in

US communities, 2010–2020. J Environ Manage 92(9): 2170-2181

Li D, Zhang J, Merkle SA (2014) Induction of green ash embryogenic cultures with potential for scalable somatic embryo production using suspension culture. Trees-Struct Funct 28(1):253-262

Mosedale JR (1995) Effects of oak wood on the maturation of alcoholic beverages with particular reference to whisky. Forestry 68(3):203-230

Palla KJ, Pijut PM (2011) Regeneration of plants from *Fraxinus americana* hypocotyls and cotyledons. In Vitro Cell Dev Biol Plant 47:250–256

Poland TM, McCullough DG (2006) Emerald ash borer: Invasion of the urban forest and the threat to North America's ash resource. J Forest 104(3):118-124.

Preece JE, Ji-Liang ZHAO, Kung FH (1989) Callus production and somatic embryogenesis from white ash. HortScience 24(2):377-380

Rebek EJ, Herms DA, Smitley DR (2008) Interspecific variation in resistance to emerald ash borer (Coleoptera: Buprestidae) among North American and Asian ash (*Fraxinus* spp.). Environ Entomol 37(1):242-246

San-José MC, Corredoira E, Martínez MT, Vidal N, Valladares S, Mallón R, Vieitez, AM (2010) Shoot apex explants for induction of somatic embryogenesis in mature *Quercus robur* L. trees. Plant Cell Rep 29(6):661-671

Sotak RJ, Sommer HE, Merkle SA (1991) Relation of the developmental stage of zygotic embryos of yellow-poplar to their somatic embryogenic potential. Plant Cell Rep 10(4):175-178

Stewart HA, Krajicek JE (1973) Ash… an American wood. USDA Forest Service.

Sydnor TD, Bumgardner M, Todd A (2007) The potential economic impacts of emerald ash borer (*Agrilus planipennis*) on Ohio, US, communities. Arboric Urban For 33(1):48

Sydnor TD, Bumgardner M, Subburayalu S (2011) Community ash densities and economic impact potential of emerald ash borer (*Agrilus planipennis*) in four Midwestern states. Arboric Urban For 37:84-89

Tirmenstein DA (1991) *Quercus alba*. In: Fire Effects Information System. US Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (Producer). http://www.fs.fed.us/database/feis/ Accessed 6 October 2015

Tonon G, Berardi G, Rossi C, Bagnaresi U (2001) Synchronized somatic embryo development in embryogenic suspensions of *Fraxinus angustifolia*. In Vitro Cell Dev Biol Plant 37(4):462-465.

Toribio M, Fernández C, Celestino C, Martínez MT, San-José, MC, Vieitez AM (2004) Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell Tiss Org 76(3):283-287

van Nocker S, Gardiner SE (2014) Breeding better cultivars, faster: applications of new technologies for the rapid deployment of superior horticultural tree crops. Horticulture Research 1

Wetzstein HY, Ault JR, Merkle SA (1989) Further characterization of somatic embryogenesis and plantlet regeneration in pecan (*Carya illinoensis*). Plant Sci 64(2):193-201

Yu C (1992) *Agrilus marcopoli* Obenberger (Coleoptera: Buprestidae). In: Xao G (ed) Forest I insects of China, 3rd edn. China Forestry Publishing House, Beijing, China, pp 400-401

CHAPTER 2

# Application of somatic embryogenesis for propagation of emerald ash borer-resistant white ash trees

J.E. Mitchell and S.A. Merkle. To be submitted to *Trees – Structure and Function*.

**Abstract**

White ash (*Fraxinus americana*) is an economically and ecologically significant genus of hardwood forest tree in eastern North America. Unfortunately, North America's native ash resource is currently threatened by the rapid spread of emerald ash borer (*Agrilus planipennis* Fairmaire; EAB), an exotic wood-boring beetle. Although management efforts are in place in attempt to control EAB, the pest is still spreading, and little research is being conducted on methods to mass propagate white ash to aid restoration efforts. One possible approach is to clonally propagate white ash genotypes that have demonstrated resistance to EAB *in vitro* via somatic embryogenesis. In this study, embryogenic white ash cultures were successfully initiated from zygotic embryos collected from putatively EAB-resistant "lingering ash" trees in Michigan, USA and cultured on a modified Woody Plant Medium with 2,4-dichlorophenoxyacetic acid and benzyladenine. Embryogenesis induction percentage varied with seed collection date, with immature embryos collected in August showing the highest average induction percentage (27%). Embryogenesis induction percentage did not vary significantly among the tested source trees. Supplementing germination medium with gibberellic acid (GA3), with or without activated charcoal (AC), improved somatic embryo germination and conversion. The conversion rate was 42% for embryos on medium supplemented with GA3 and 97% for embryos on medium supplemented with both GA3 and AC. Somatic seedlings transferred to potting mix and hardened off grew rapidly following transfer to the greenhouse, indicating that the trees may be used for clonal screening for EAB resistance within a few years.

**Key words**

*Fraxinus americana*, white ash, emerald ash borer, somatic embryogenesis, tissue culture

**Key message**

Clones derived from seeds of "lingering" white ash trees were produced through somatic embryogenesis, producing plantlets which can be screened to test if they are naturally resistant to emerald ash borer (EAB).

## Introduction

White ash (*Fraxinus americana*) is an ecologically and economically important hardwood forest tree native to North America. It is characterized by good form and strong, tough wood with especially high shock resistance, making it the most commonly used native ash for such products as baseball bats and tool handles. The species provides food for a variety of birds and small mammals in the form of its samaras, and it is an important source of cover and browse for wildlife and livestock, such as white-tailed deer and cattle (Burns and Honkala 1990; Gucker 2005; Griffith 1991). Unfortunately, white ash is currently under threat by the emerald ash borer (*Agrilus planipennis* Fairmaire; EAB), a phloem-feeding beetle (Coleoptera: Buprestidae) native to northeastern China, Korea, Japan, Mongolia, Taiwan, and eastern Russia (Jendek 1994; Haack et al. 2002). In its native range, EAB is not considered a major pest, partly because the Asian ash species Manchurian ash (*Fraxinus mandschurica*) and Chinese or Korean ash (*Fraxinus chinensis*) are more resistant to EAB than North American species (Rebek et al. 2008). EAB killed tens of millions of trees in Michigan after first becoming established in 2002, and has

since spread and killed tens of million more trees in 21 other U.S. states and two Canadian provinces, Ontario and Quebec (Herms & McCullough 2014). The economic damage caused by EAB is projected to be between $10 billion and $26 billion by 2020, including costs for treatment, removal, and replacement of infested trees in urban and suburban landscapes (Syndor et al. 2007; Kovaks et al. 2010; Kovaks et al. 2011; Syndor et al. 2011).

One potentially useful approach to managing EAB infestation is to identify and clonally propagate ash genotypes that are naturally resistant to EAB. Such EAB-resistant ash genotypes may have already been identified in the form of "lingering ash" trees, healthy ash trees which are intermingled with dead trees where EAB has caused almost complete mortality of mature ash trees in a particular area (Knight et al. 2012). Two *in vitro* propagation methods have been reported for ash species, organogenesis and somatic embryogenesis. Organogenesis by adventitious shoot production has been reported for both green ash (Du and Pijut 2008) and white ash (Bates et al. 1992; Palla and Pijut 2011). Du and Pijut (2009) also produced transgenic green ash plantlets by *Agrobacterium*-mediated transformation of hypocotyl explants, which then produced transgenic adventitious shoots. Somatic embryogenesis has been reported for multiple ash species, including white ash (Preece et al. 1989; Bates et al. 1992), narrow-leafed ash (*F. angustifolia*; Tonon et al. 2001), common ash (*F. excelsior*; Capuana et al. 2007) and Manchurian ash (*F. mandshurica*; Kong et al. 2012). However, only Tonon et al. (2001) described a system suitable for potential scalable ash somatic embryo production using suspension culture.

Until recently, somatic embryogenesis had not been reported for green ash, which is similar in its properties to white ash (Burns and Honkala 1990). Li et al. (2014) produced

embryogenic cultures from zygotic embryos of green ash and successfully germinated somatic embryos grown from suspension culture to produce somatic seedlings. Of the plant growth regulator (PGR) treatments used in Li et al.'s (2014) study, only cultures initiated on media with 2 mg/l 2,4-D and 0.5 mg/l BA produced an embryogenic response.

The overall goal of this study was to demonstrate the potential to clonally mass propagate EAB-resistant white ash through application of a somatic embryogenesis system, to aid ash restoration efforts. By clonally propagating lingering ash trees through somatic embryogenesis, it may be possible to produce ash trees that are naturally resistant to EAB, eliminating the need to hybridize white ash with EAB-resistant Asian species or to employ gene transfer techniques to produce transgenic trees carrying genes for resistance to EAB.

**Materials and methods**

Plant material

Fruit (samaras) from four "lingering" female white ash trees (designated 101, 102, 114, and 116) were harvested by Ohio State University collaborators in Michigan on July 7$^{th}$, August 8$^{th}$, and September 16$^{th}$ 2013. All four source trees were located in Indian Springs Metro Park Farmland Trail, Oakland County, MI. On the final collection date, samaras were harvested from only two trees, 102 and 116, and no cultures were initiated from the harvested fruit due to weevil-infested seeds, aborted seeds, or zygotic embryos that appeared to be fully mature. In 2013, the four mother trees were given a canopy rating of average to healthy and none of them showed signs of damage by EAB.

Culture initiation

Samaras were surface disinfested by washing them in 70% ethanol for 20 seconds, 10% Roccal-D Plus (9.2 % didecyl dimethyl ammonium chloride, 13.8 % alkyl dimethyl benzyl ammonium chloride, 1 % bis-n-tributyltin oxide; Pfizer) for 3 minutes, 70% ethanol for 20 additional seconds, 10% Roccal for 3 additional minutes, 50% Clorox (8.25 % sodium hypochlorite) for 5 minutes, sterile deionized water for 3 minutes, and sterile 0.01 N hydrochloric acid (HCl) for 3 minutes, followed by three additional rinses in sterile deionized water for 3 minutes each. Following surface disinfestation, samaras were dissected, and the zygotic embryos were cultured in 60 mm plastic Petri plates containing semisolid induction-maintenance medium (IMM; Andrade and Merkle 2005), which was a modified woody plant medium (WPM; Lloyd and McCown 1980), with 30 g/l sucrose, 1 g/l filter-sterilized L-glutamine, and either 2 (low auxin treatment) or 4 (high auxin treatment) mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), and gelled with 3 mg/L Phytagel (Sigma). Zygotic embryo explants were cultured either with or without surrounding seed tissues.

Four explants were cultured per plate, with explants on each plate grouped according to length. Explant were classified into three length categories: small (1.5 to 4.5 mm), medium (5 to 8 mm), and large (8.5 mm or larger). For each collection date, for each source tree, three repetitions (Petri plates) were used per each auxin treatment/explant type (naked zygotic embryo versus embryo with seed coat) combination. Cultures were incubated in the dark at 25°C. After 1 month, they were transferred to fresh medium with the same concentration of 2,4-D. Three months following initiation, each explant was scored for whether or not it produced embryogenic callus or somatic embryos, and percentages were calculated for each collection date, treatment

and genotype as well as the different combinations of these variables. Following arcsine transformation of the percentage data, one-way analysis of variance (ANOVA) was performed to test for the effects of initiation date, auxin treatment, explant treatment and their interactions on embryogenesis induction. Means comparisons were conducted using Tukey's HSD test. Data were analyzed using R statistical software (version 3.12; R Foundation for Statistical Computing).

Germination and conversion

Somatic embryos were harvested from two culture lines (designated FA-144-17B and FA-114-20B) that produced direct somatic embryos on induction medium containing 2,4-D, for germination tests. Embryos were moved to plates of semisolid embryo development medium (EDM; Andrade and Merkle 2005), which was a modified WPM basal medium containing no PGRs, and given a pre-germination cold treatment at 8º C for 15 weeks in the dark in a refrigerator. Following cold treatment, all embryos were transferred to 125 ml Erlenmeyer flasks containing 30 ml liquid EDM and shaken overnight on a rotary shaker at 90 rpm, in attempt to remove any residual 2,4-D. Before transfer to germination treatments the next day, embryos received a final wash using a Büchner funnel by pipetting approximately 200 ml of liquid EDM over the embryos under mild vacuum. Embryos were then transferred to 100 mm plastic Petri plates containing one of four treatments: semisolid EDM, semisolid EDM supplemented with 0.01 g/L gibberellic acid (GA3), semisolid EDM with 0.5 g/L activated charcoal (AC), or semisolid EDM with both AC and GA3. Ten somatic embryos were placed on each plate and 3 repetitions (plates) were used per treatment for each culture line. Plates with embryos were incubated in a lighted incubator under cool white fluorescent lights (100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) with 16

h day lengths at 25° C to encourage germination. Data were collected weekly to calculate the percentages of green embryos, germinated embryos (those exhibiting radicle elongation), and converted embryos (those producing roots and shoots). Following arcsine transformation, these data were analyzed by ANOVA to test for the effect of the four germination medium treatments on greening, germination and conversion. Means comparisons were conducted using Tukey's HSD test. Data were analyzed using R statistical software (version 3.12; R Foundation for Statistical Computing). Somatic seedlings were removed from in vitro conditions and potted in moistened peat:perlite:vermiculite (1:1:1) mix in plastic pots, which were placed on water-saturated perlite in a clear plastic dome-covered tray under cool white fluorescent lights (80 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) and 16 h day lengths.

**Results**

Culture initiation

Embryogenic callus and proembryogenic masses (PEMs) developed from some explants in as little as one month. PEMs were typically white-yellow to yellow in color, fine in texture, light, and malleable. Surprisingly, somatic embryos began to emerge from callus while it was still on media containing 2,4-D (Fig. 2.1), which usually inhibits the development of somatic embryos from embryogenic callus or PEMs of other hardwood species we have worked with until they are transferred to PGR-free medium (e.g. Merkle et al. 1990, Dai et al. 2004, Andrade and Merkle 2005). Overall, collection date had a significant effect on embryogenesis induction ($P < 0.001$), with explants collected on August 8$^{th}$ producing higher mean embryogenesis induction rates (27%) than July 10$^{th}$ explants (0%), according to Tukey's HSD test (Fig. 2.2). This suggests that early to mid-August is the optimal time for harvesting lingering white ash fruit

from the Michigan trees for induction of embryogenic cultures, as zygotic embryos are likely to be too immature in July to produce embryogenic cultures. Although no cultures were initiated from the September collection, the large, mature zygotic embryos found in that collection further suggested that August was the optimal time for harvesting, because mature zygotic embryo explants of green ash failed to produce any embryogenic cultures (Li et al. 2014). When each tree was analyzed individually, collection date did not always have a significant effect on embryogenesis. For Trees 101 and 114, collection date had a significant effect on embryogenesis induction ($P = 0.0406$; $P = 0.00701$). For Trees 102 and 116, collection date did not have a significant effect on embryogenesis induction ($P = 0.0819$; $P = 0.0511$). This suggests that genotypic differences may exist among the source trees; however, mother tree genotype did not have a significant effect on overall embryogenesis induction ($P = 0.467$), and no significant interaction was found between collection date and mother tree genotype ($P = 0.431$).

Although not tested statistically, zygotic embryo size may have impacted induction rate, with the highest mean percentage of embryogenic cultures produced from medium-sized (5-8 mm) zygotic embryos. (Fig. 2.3), meaning medium-sized embryos may be associated with a mid-August collection time, since collection date did have a significant effect on embryogenesis induction. This was not tested statistically, however, because there were not enough embryos of each size class for a fair comparison. Also, embryos of the same size class were cultured on the same plate, so embryo size was confounded with plate.

Although the mean percentage of embryogenic explants appeared higher for the high auxin treatment (4 mg/L) than the low auxin treatment (2 mg/L) (Fig. 2.4), this difference was not statistically significant ($P = 0.263$). There was also no significant interaction between source

tree and auxin treatment for embryogenesis induction (P = 0.495). Culturing zygotic embryos with or without their surrounding seed tissues had a significant effect on embryogenesis, with cultures initiated from naked zygotic embryos producing a higher percentage of embryogenic cultures (42%) than culturing embryos with their associated seed tissues (5%) (P <0.001) (Fig. 2.5).

Germination and conversion

Analysis of variance results indicated that the four germination treatments differed significantly (P <0.001) with regard to greening of somatic embryos, germination (radicle elongation) and conversion of embryos to somatic seedlings. With regard to greening, the two treatments with 10 mg/L GA3, with or without AC, resulted in higher percentages of embryos greening up than the two treatments lacking GA3, although they were not significantly different from each other (Fig. 2.6a). Neither GA3 nor AC alone improved germination frequency over the control, but the combination of GA3 and AC resulted in higher germination frequency (97%) than any of the other treatments (Fig. 2.6b). Both GA3 alone and in combination with AC improved conversion over the control and AC alone treatments, with the GA3 plus AC treatment giving a significantly higher conversion percentage (97%) than GA3 alone (42%; Fig 2.6c). Thus it appears that while GA3 alone promotes greening of white ash somatic embryos, it may be more beneficial in promoting germination and conversion in combination with AC.

Converted white ash somatic embryos transferred to potting mix under high humidity grew into vigorous somatic seedlings, which continued rapid growth following hardening off and transfer to the greenhouse (Fig. 2.7).

## Discussion

In previous studies, cotyledons of seedlings from mature white ash seeds induced direct and indirect somatic embryogenesis (Preece et al. 1989; Bates et al. 1992). To our knowledge, this is the first study to induce somatic embryogenesis from immature zygotic white ash embryos, although this has also been achieved in narrow-leafed ash (Tonon et al. 2001), common ash (Capuana et al. 2007), and Manchurian ash (Kong et al. 2012). Preece et al. (1989) obtained the highest percentage of somatic embryogenesis induction from white ash when culturing mature cut seeds on Driver and Kuniyuki (1984) walnut (DKW) medium supplemented with 2,4-D, whereas we successfully initiated embryogenic cultures from zygotic embryos derived from immature seeds on WPM supplemented with 2,4-D. Li et al. (2014) described the developmental stages of zygotic embryos in the green ash seeds used for their induction experiments. This knowledge is especially important because it has been shown that there is typically a narrow zygotic embryo developmental window for explants that are capable of making repetitive embryogenic cultures of hardwood trees. As such, developmental stage of zygotic embryo explants has been shown to be a contributing factor for successful induction in yellow-poplar (Sotak et al. 1991), American chestnut (Carraway and Merkle 1997), pecan (Wetzstein et al. 1989) and Manchurian ash (Kong et al. 2012), with early and late stages of zygotic embryo development resulting in lower or no embryogenesis induction. This is consistent with our results, as seeds collected in July did not produce embryogenic cultures and thus were likely too immature, whereas seeds collected in August did produce embryogenic cultures and were likely near the optimal developmental stage. Based on previous studies, surviving zygotic embryos

from our September collection were likely too mature to produce embryogenic cultures, which is why we did not culture any.

Similar to our results, Preece et al. (1989) obtained some somatic embryos on medium containing 2,4-D. Unlike our results, however, the greatest amount of embryogenesis occurred when Preece et al. (1989) transferred cultures to a secondary medium, which was Murashige and Skoog (1962) medium (MS) supplemented with the cytokinin 6-Benzylaminopurine (BA) or the auxin 1-Naphthaleneacetic acid (NAA), or DKW with no plant growth regulators. In our cultures, however, many somatic embryos developed on WPM supplemented with 2,4-D. When cultures where transferred to basal medium, we saw no further somatic embryo production.

Preece et al. (1989) reported only a 20% germination rate of white ash seeds on medium supplemented with GA3, which contrasts with our results. Our germination rate was 42% for embryos on media supplemented with GA3 and 97% for embryos on media supplemented with both GA3 and AC. In their GA3 experiment, Preece et al. (1989) used unstratified, whole, uncut seeds as explants on DKW medium, whereas we used somatic embryos which received a 15 – week cold treatment before being transferred to our germination media treatments, with WPM as the basal medium for germination. These factors, along with the addition of AC to our medium, may have improved the germination rate of our somatic embryos over that of the zygotic embryos reported by Preece et al. (1989).

Our results indicate that it is possible to clonally propagate white ash from seeds produced by lingering ash source trees. Since, in many cases, the trees from which seeds were collected were the only surviving ash trees in stands devastated by EAB, it is highly likely that both parents were lingering ash individuals. Thus, if there is a genetic basis for their resistance

to EAB, the genes involved in resistance are likely to have been passed to the seeds from which our embryogenic cultures were derived. We plan for the somatic seedlings produced from the cultures initiated in this study to be used in the future for clonal testing of resistance to EAB. By successfully inducing embryogenic cultures from explants gathered from lingering ash trees, our results are a promising step toward clonal propagation for potentially EAB-resistant trees. With further optimization of the culture system, it could eventually be scaled-up to mass produce clonal EAB-resistant planting stock to aid forest restoration in areas affected by EAB.

**Acknowledgements**

This research was supported by the USDA Forest Service, Southern Region National Forest System Genetics Program. We wish to thank Diane Hartzler and Dan Herms (Ohio State University) for assistance in collecting ash material.

**References**

Andrade G, Merkle SA (2005) Enhancement of American chestnut somatic seedling production. Plant Cell Rep 24(6):326-334

Bates S, Preece JE, Navarrete NE, Vansambeek JW, Gaffney GR (1992) Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white ash (*Fraxinus americana* L.). Plant Cell Tiss Org Cult 31:21–29

Burns RM, Honkala BH (1990) Silvics of North America, vol. 2. United States Department of Agriculture

Capuana M, Petrini G, Di Marco A, Giannini R (2007) Plant regeneration of common ash (*Fraxinus excelsior* L.) by somatic embryogenesis. In Vitro Cell Dev Biol Plant 43(2):101-110

Carraway DT, Merkle SA (1997) Plantlet regeneration from somatic embryos of American chestnut. Can J For Res 27(11):1805-1812

Dai J, Vendrame WA, Merkle SA (2004) Enhancing the productivity of hybrid yellow-poplar and hybrid sweetgum embryogenic cultures. In Vitro Cell Dev Biol Plant 40(4):376-383

Driver JA, Kuniyuki AH (1984). In vitro propagation of paradox walnut rootstocks. HortScience 18:506-509

Du Du N, Pijut PM (2008) Regeneration of plants from *Fraxinus pennsylvanica* hypocotyls and cotyledons. Sci Hort 118(1):74-79

Du N, Pijut PM (2009) Agrobacterium-mediated transformation of *Fraxinus pennsylvanica* hypocotyls and plant regeneration. Plant Cell Rep 28(6):915-923

Herms DA, McCullough DG (2014) Emerald ash borer invasion of North America: history, biology, ecology, impacts, and management. Annu Rev Entomol 59:13-30

Kong DM, Preece JE, Shen HL (2012) Somatic embryogenesis in immature cotyledons of Manchurian ash (*Fraxinus mandshurica* Rupr.). Plant Cell Tiss Org 108(3):485-492

Kovacs KF, Haight RG, McCullough DG, Mercader RJ, Siegert NW, Liebhold AM (2010) Cost of potential emerald ash borer damage in US communities, 2009-2019. Ecol Econ 69(3):569-578

Kovacs KF, Mercader RJ, Haight RG, Siegert NW, McCullough DG, Liebhold AM (2011) The influence of satellite populations of emerald ash borer on projected economic costs in US communities, 2010–2020. J Environ Manage 92(9): 2170-2181

Li D, Zhang J, Merkle SA (2014) Induction of green ash embryogenic cultures with potential for scalable somatic embryo production using suspension culture. Trees-Struct Funct 28(1):253-262

Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Combined Proceedings of International Plant Propagators' Society 30:421-427

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15(3):473-497

Palla KJ, Pijut PM (2011) Regeneration of plants from *Fraxinus americana* hypocotyls and cotyledons. In Vitro Cell Dev Biol Plant 47:250–256

Preece JE, Bates S (1995). Somatic embryogenesis in white ash (*Fraxinus americana* L.). In: Somatic Embryogenesis in Woody Plants. Springer Netherlands. pp 311-325

Preece JE, Ji-Liang ZHAO, Kung FH (1989) Callus production and somatic embryogenesis from white ash. HortScience 24(2):377-380

Sotak RJ, Sommer HE, Merkle SA (1991) Relation of the developmental stage of zygotic embryos of yellow-poplar to their somatic embryogenic potential. Plant Cell Rep 10(4):175-178

Sydnor TD, Bumgardner M, Todd A (2007) The potential economic impacts of emerald ash borer (*Agrilus planipennis*) on Ohio, US, communities. Arboric Urban For 33(1):48

Sydnor TD, Bumgardner M, Subburayalu S (2011) Community ash densities and economic impact potential of emerald ash borer (*Agrilus planipennis*) in four Midwestern states. Arboric Urban For 37:84-89

Tonon G, Berardi G, Rossi C, Bagnaresi U (2001) Synchronized somatic embryo development in embryogenic suspensions of *Fraxinus angustifolia*. In Vitro Cell Dev Biol Plant 37(4):462-465.

Wetzstein HY, Ault JR, Merkle SA (1989) Further characterization of somatic embryogenesis and plantlet regeneration in pecan (*Carya illinoensis*). Plant Sci 64(2):193-201

## Figures

**Fig. 2.1** Embryogenic callus and somatic embryo production from immature white ash zygotic embryo explants. **a.** Original zygotic embryo explant with newly developing callus. **b.** Typical white ash embryogenic callus. **c** and **d.** Embryogenic callus with somatic embryos beginning to emerge. **e** and **f.** Clusters of somatic embryos that arose from embryogenic callus. Scale bar represents 1 mm.

**Fig. 2.2** Mean embryogenesis induction frequencies for each source tree (101, 202, 114, 116) for each collection date in 2013. The means represent 4 zygotic embryos per plate, with 3 plates used for each collection date for each source tree per each auxin treatment/explant type (naked zygotic embryo versus embryo with seed coat) combination. Bars represent standard error.

**Fig. 2.3** Total (**a**) and individual tree (**b**) mean embryogenesis induction frequencies for small (1.5 to 4.5 mm), medium (5 to 8 mm), and large (8.5 mm or larger) explants. The means represent 4 zygotic embryos per plate, with 3 plates used for each collection date for each source tree per each auxin treatment/explant type (naked zygotic embryo versus embryo with seed coat) combination. Bars represent standard error.

**Fig. 2.4** Total (**a**) and individual tree (**b**) mean embryogenesis induction frequencies for each induction medium treatment. The means represent 4 zygotic embryos per plate, with 3 plates used for each collection date for each source tree per each auxin treatment/explant type (naked zygotic embryo versus embryo with seed coat) combination. Bars represent standard error.

**Fig. 2.5** Total (**a**) and individual tree (**b**) mean embryogenesis induction frequencies for each explant type zygotic embryos with our without seedcoat). The means represent 4 zygotic embryos per plate, with 3 plates used for each collection date for each source tree per each auxin

treatment/explant type (naked zygotic embryo versus embryo with seed coat) combination. Bars represent standard error.

**Fig. 2.6** Effects of germination medium on ash plantlet production for each of the four germination media treatments: semisolid EDM (Control), semisolid EDM containing 0.01 g/L gibberellic acid (GA3), semisolid EDM containing 0.5 g/L activated charcoal (AC), and semisolid EDM containing both 0.5 g/L activated charcoal and 0.01 g/L gibberellic acid (GA3 and AC). Treatments sharing the same letter are not significantly different according to Tukey's HSD test. **a**. Mean percentages of greening somatic embryos. **b.** Mean percentages of total germination (radicle elongation) **c.** Mean percentages of converted germinants. Bars represent standard error.

**Fig. 2.7** White ash somatic seedlings (**a**) approximately one month following transfer to potting mix, and (**b**) during the second season of growth in the greenhouse.

**Fig. 2.1**

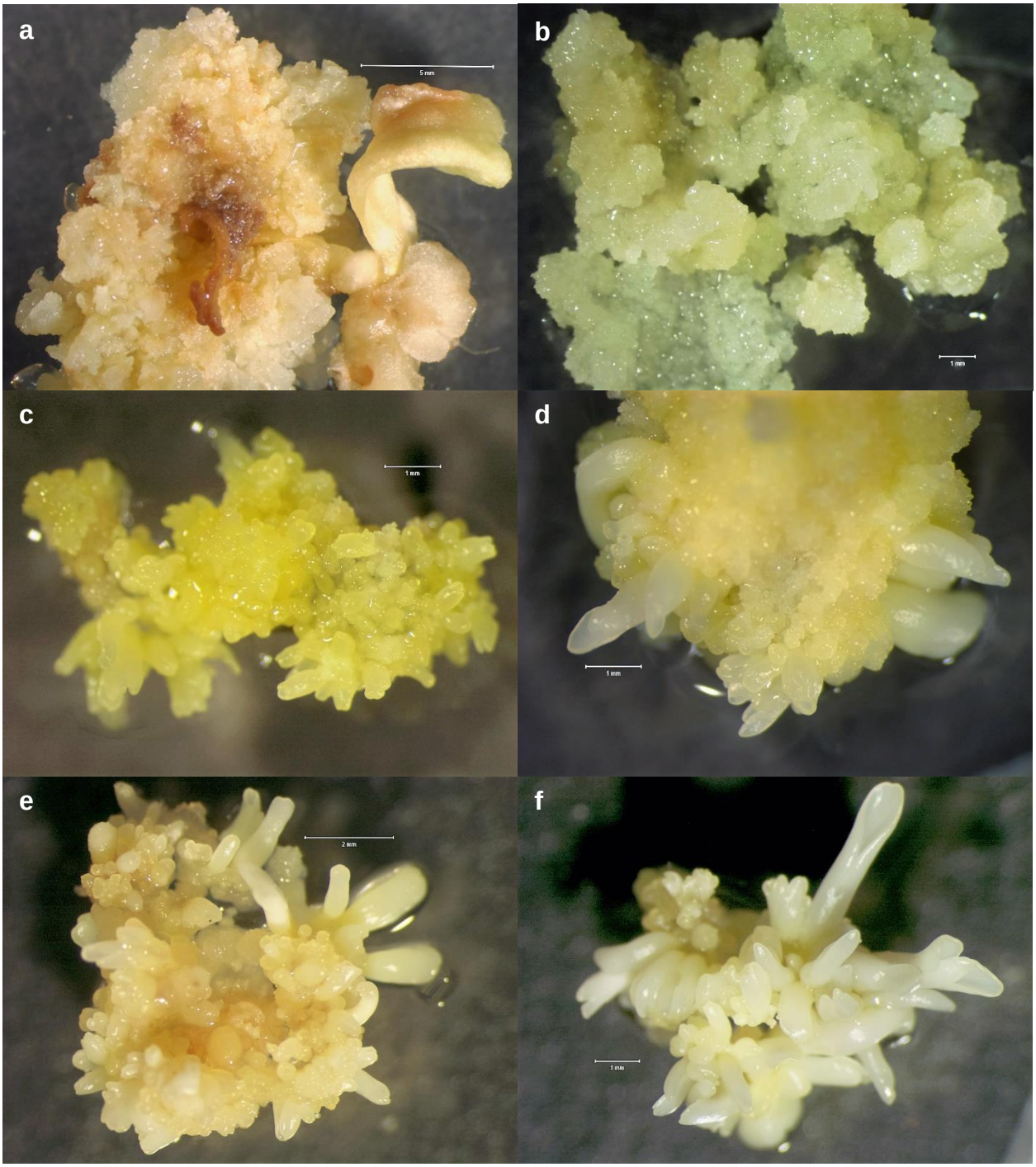


**Fig. 2.2**

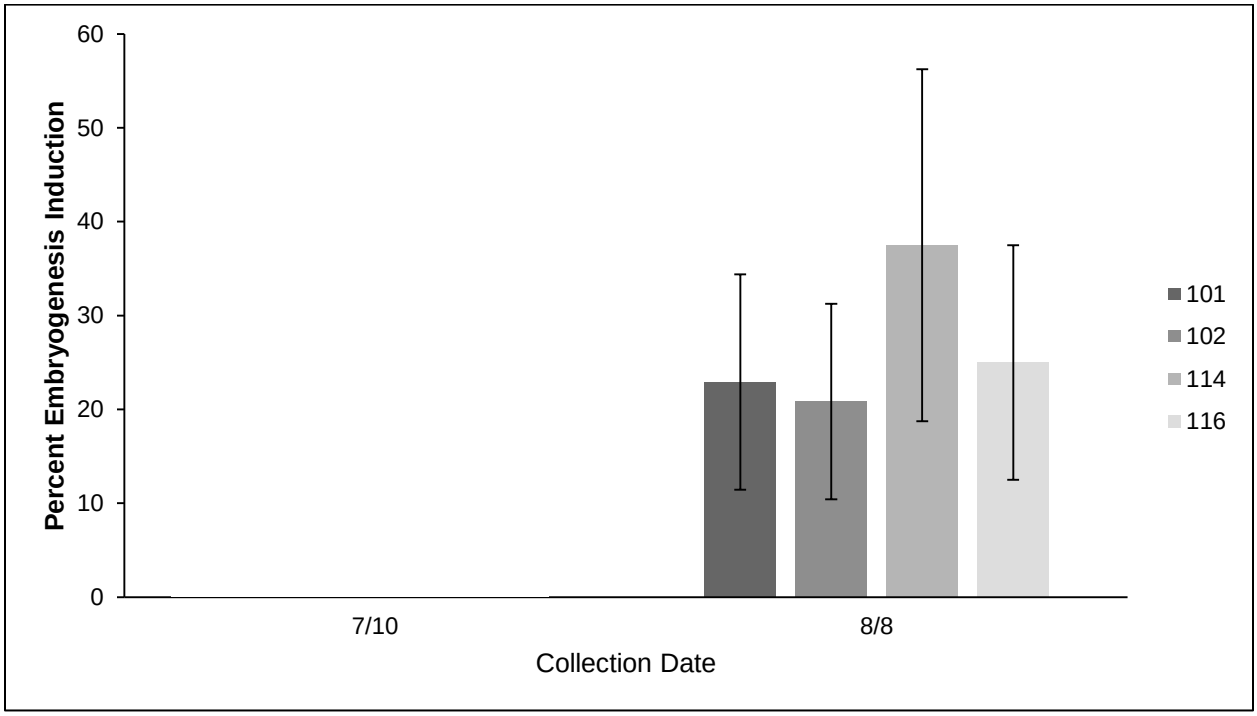
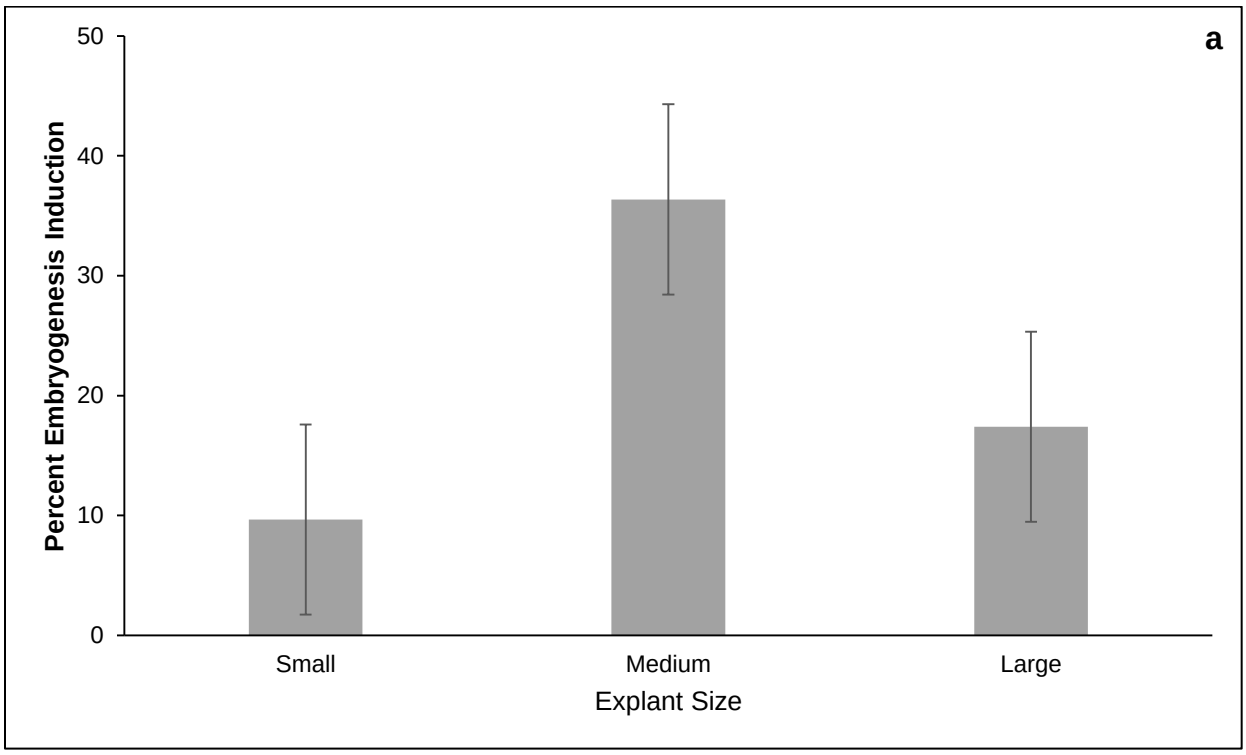

60
50
40
30
20
10
0
Percent Embryogenesis Induction
7/10
8/8
Collection Date
101
102
114
116

**Fig. 2.3**


a
Percent Embryogenesis Induction
50
40
30
20
10
0
Small
Medium
Large
Explant Size

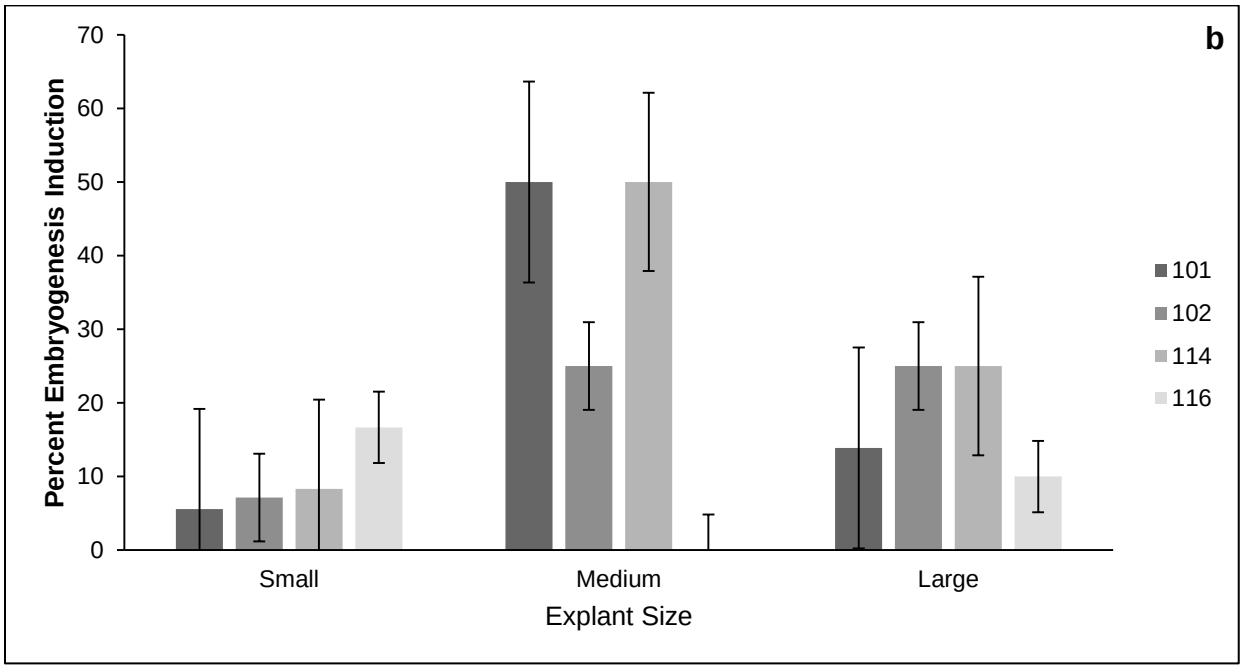

b
Percent Embryogenesis Induction
70
60
50
40
30
20
10
0
Small
Medium
Large
Explant Size
101
102
114
116

**Fig. 2.4**

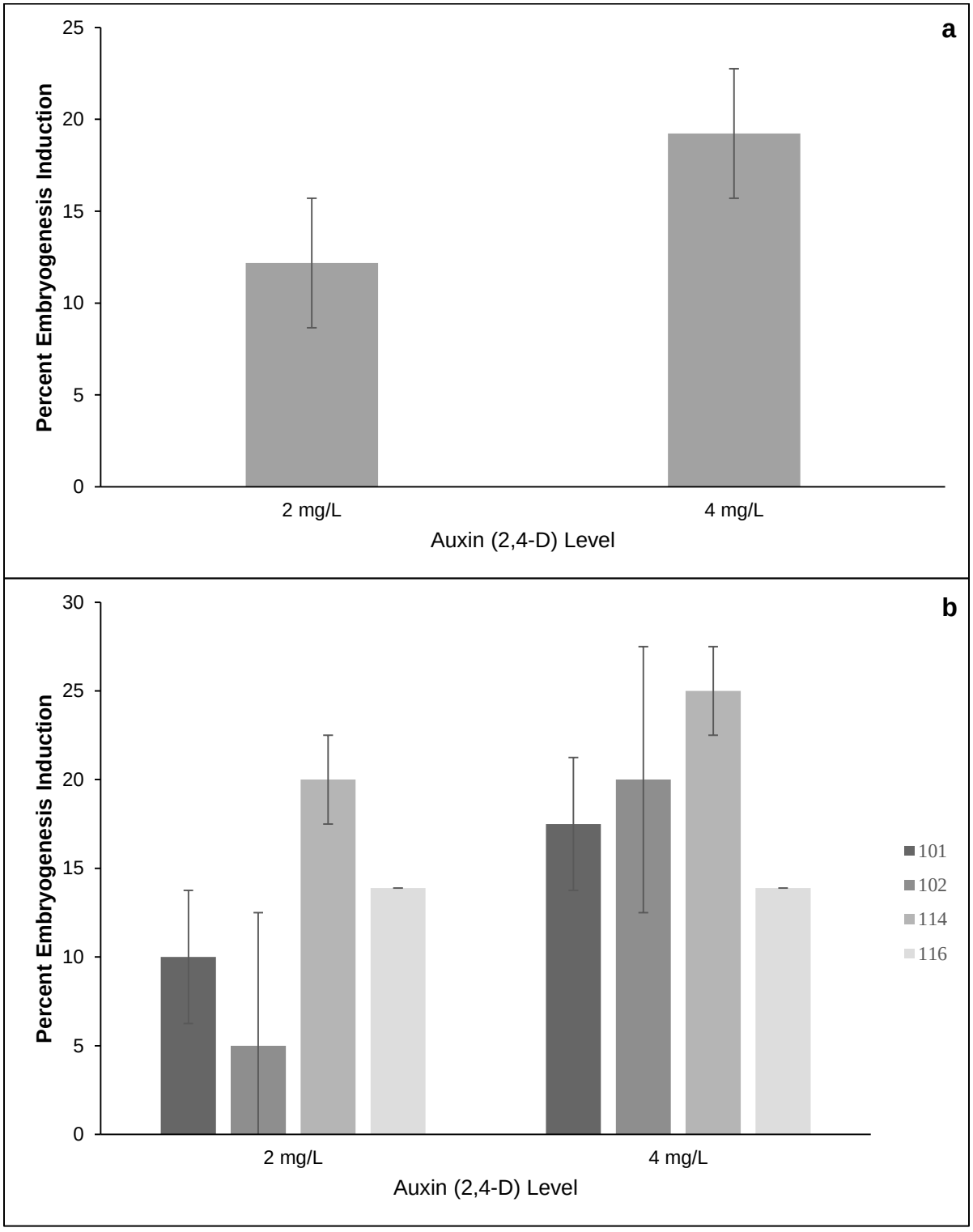
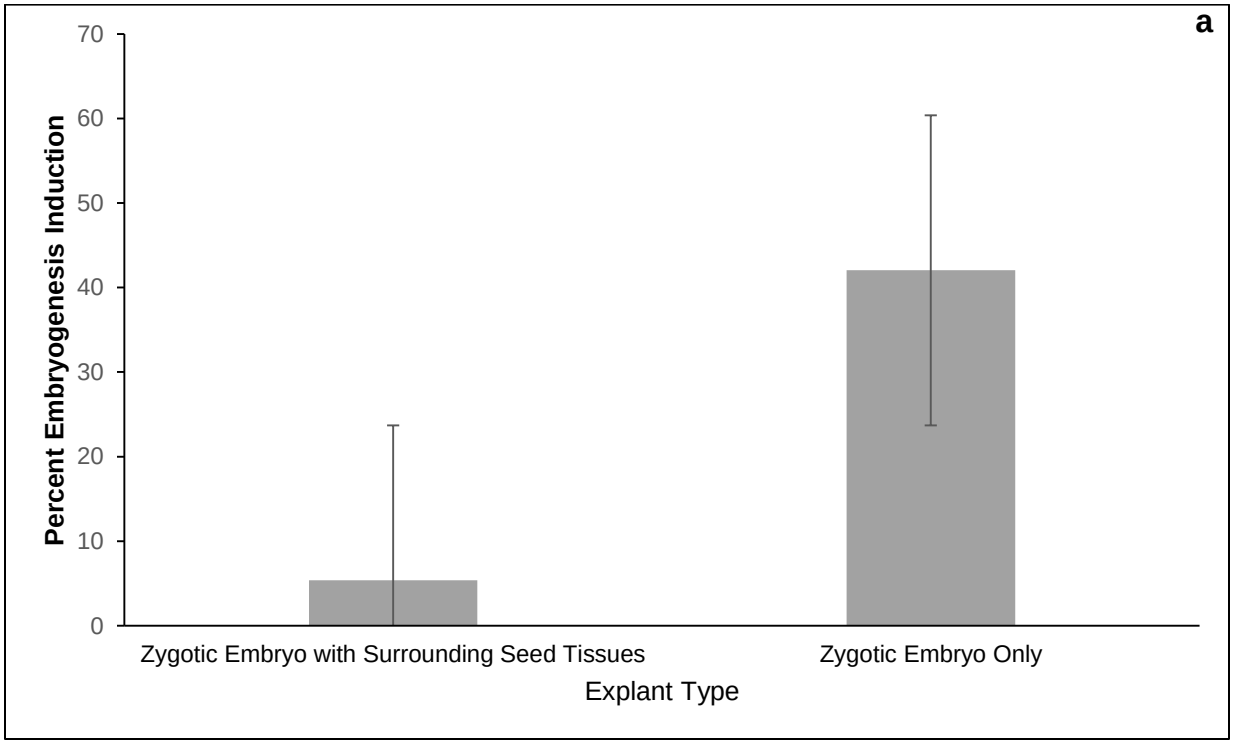

a
Percent Embryogenesis Induction
25
20
15
10
5
0
2 mg/L
4 mg/L
Auxin (2,4-D) Level
b
Percent Embryogenesis Induction
30
25
20
15
10
5
0
2 mg/L
4 mg/L
Auxin (2,4-D) Level
101
102
114
116

**Fig. 2.5**


a
Percent Embryogenesis Induction
70
60
50
40
30
20
10
0
Zygotic Embryo with Surrounding Seed Tissues
Zygotic Embryo Only
Explant Type

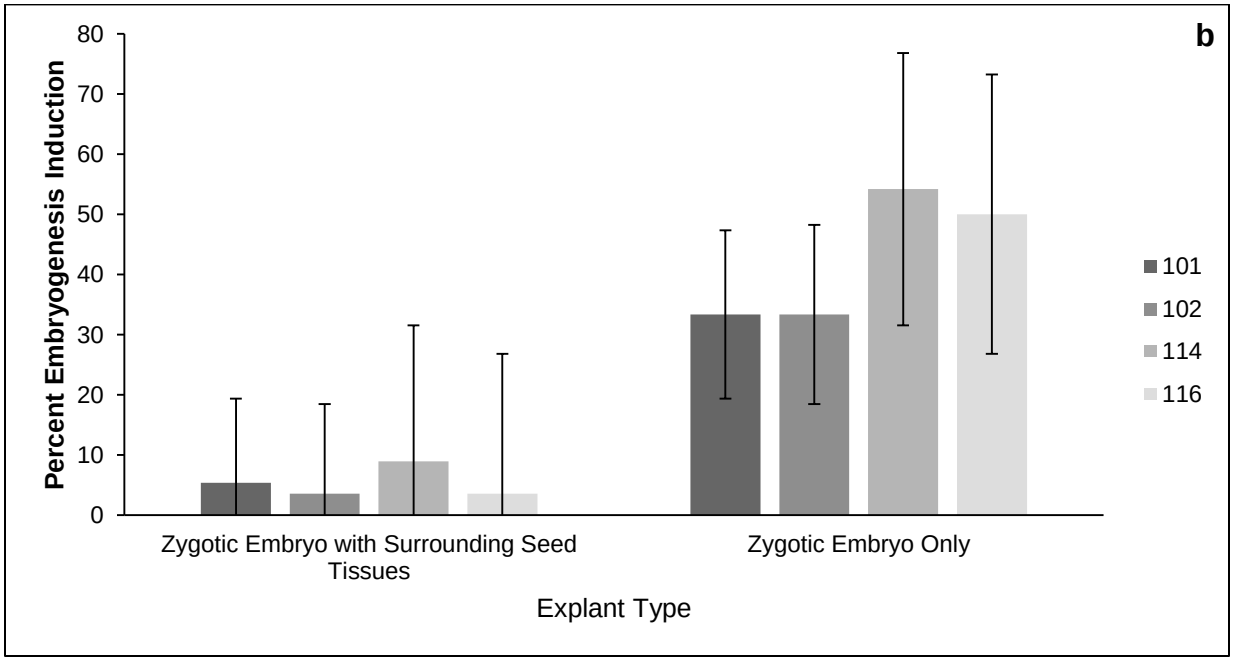

b
Percent Embryogenesis Induction
80
70
60
50
40
30
20
10
0
101
102
114
116
Zygotic Embryo with Surrounding Seed Tissues
Zygotic Embryo Only
Explant Type

**Fig. 2.6**

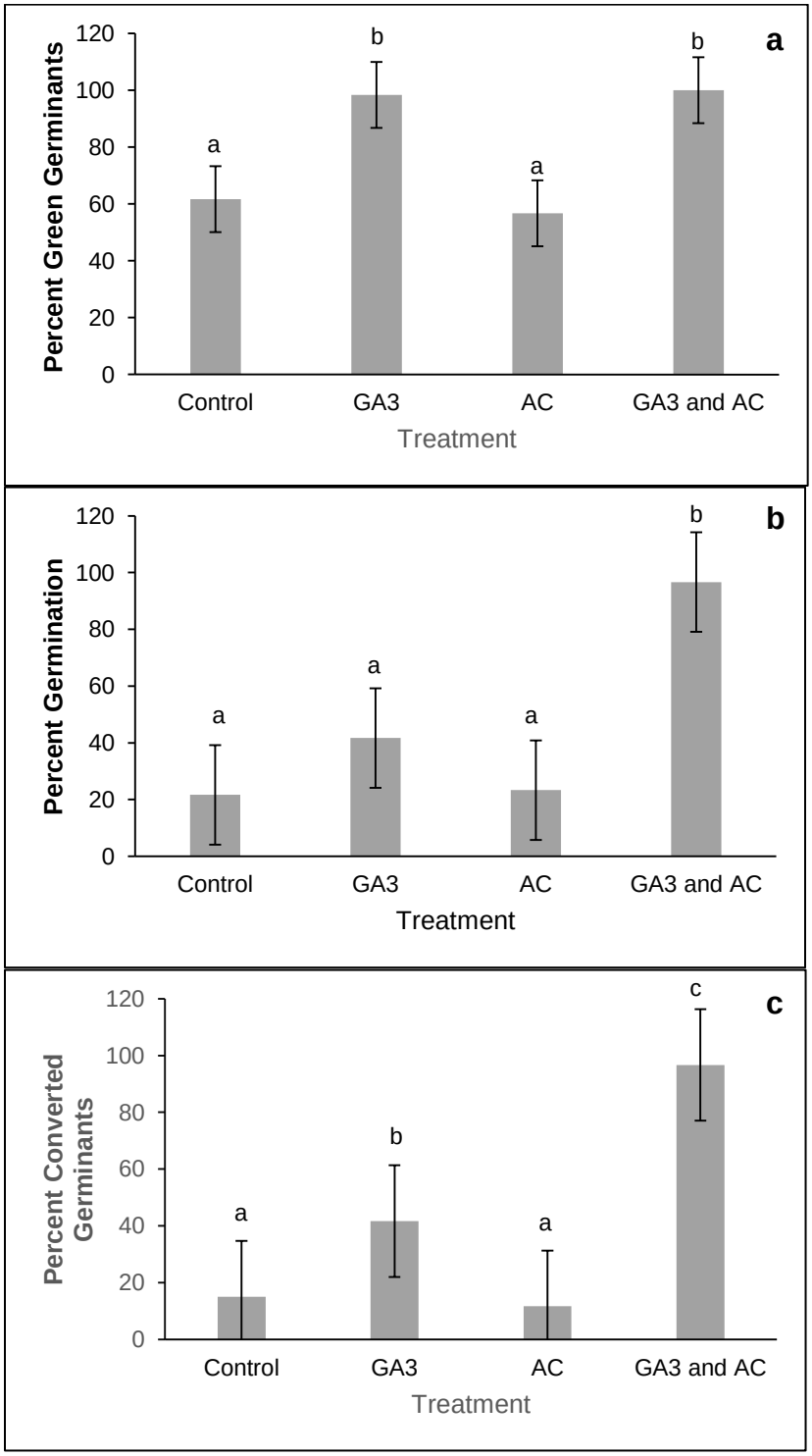

a
Percent Green Germinants
120
100
80
60
40
20
0
b
b
a
a
Control
GA3
AC
GA3 and AC
Treatment
b
Percent Germination
120
100
80
60
40
20
0
a
a
a
b
Control
GA3
AC
GA3 and AC
Treatment
c
Percent Converted Germinants
120
100
80
60
40
20
0
a
b
a
c
Control
GA3
AC
GA3 and AC
Treatment

**Fig. 2.7**


CHAPTER 3

# Application of somatic embryogenesis for propagation of white oak trees

J.E. Mitchell, P. Montello, R. Tull, S.A. Merkle. To be submitted to *Trees – Structure and Function*.

**Abstract**

White oak (*Quercus alba*) is an economically and ecologically significant species of hardwood forest tree in eastern North America. Although it has the greatest range of all oak trees in North America, it has declined due to landscape disturbances and the introduction of exotic pests and diseases. In this study, embryogenic white oak cultures were initiated from seeds derived from immature acorns and cultured on a modified Woody Plant Medium with 2,4-dichlorophenoxyacetic acid and 6-benzyladenine. Embryogenesis induction percentage did not vary significantly between the two tested source trees. Once established, embryogenic white oak cultures grew rapidly in suspension cultures, which produced relatively synchronous populations of somatic embryos following size-fractionation and plating. Supplementing germination medium with gibberellic acid (GA3), with or without activated charcoal (AC), did not significantly improve somatic embryo germination (radicle elongation) or conversion. Overall, the mean frequency for germination was 37% and the mean frequency for conversion was 6.67%. Embryogenic white oak tissue tested in a cryostorage and recovery experiment showed 100% recovery and regrowth. Our somatic embryogenesis propagation system has the potential to be scaled-up and optimized for use in mass clonal propagation of white oak or gene transfer applications.

**Keywords**

*Quercus alba*, white oak, somatic embryogenesis, tissue culture, cryostorage

**Key message**

Clones derived from seeds of white oak trees were produced through somatic embryogenesis, demonstrating the potential to mass propagate white oak trees.

**Introduction**

White oak (*Quercus alba*) is a relatively slow-growing, long-lived species of hardwood forest tree belonging to the family Fagaceae. It is native to North America, with a geographical range that extends over the eastern half of the United States, from the east coast to the Missouri River and north into Southern Ontario and Quebec (Goldblum 2010). White oak has the greatest range of any oak species in the United Sates, lending itself to great ecological and economical significance; however, it has declined due to landscape disturbances and the introduction of exotic pests and diseases (Abrams 2003). It is a medium to large deciduous tree, commonly reaching 60 to 80 feet, although individuals may grow more than 100 feet on the most favorable sites. White oak is monoecious, with staminate catkins emerging from the base of new growth and pistillate flowers growing in the axils of new growth. The tree produces short-stalked, smooth, ovoid acorns that typically develop in pairs (Tirmenstein 1991). It grows well on a range of soil types and sites, on all but the driest shallow soils, as well as under varying climatic conditions. Its acorns provide a food source for over 180 species of birds and mammals, and the twigs and foliage are browsed by deer. Due to its broad crown, dense foliage, and attractive red to violet fall color, white oak is often planted as an ornamental tree (Burns & Honkala 1990). White oak's wood is heavy, strong, and durable, making it a highly desirable for a variety of products, such as furniture, flooring, timbers, ships, and caskets. White oak wood is in

particularly high demand by the distilling industry, due to its impermeable nature, strength, and flexibility, which are all necessary criteria for fabricating the barrels in which whiskey and bourbon are aged (Mosedale 1995).

*In vitro* propagation methods have been reported for several European oak species, including cork oak (*Quercus suber*), pedunculate oak (*Quercus robur*), and holm oak (*Quercus ilex*), but there have been very few studies on somatic embryogenesis systems for North American oaks. In the cork oak and pedunculate oak, embryogenic cultures were successfully initiated using leaf explants from epicormic shoots forced to sprout from branch segments (Hernandez et al. 2002; Toribio et al. 2003). Embryogenic cultures have also been initiated from shoot apex explants derived from mature pedunculate oak trees (San-Jose et al. 2010). Somatic embryogenesis and plant regeneration has been reported in holm oak using fertilized ovule explants (Barra-Jiminez et al. 2013). There has only been one previous report on the induction of embryogenic cultures in white oak, the oak species tested in this study. Previously, somatic embryogenesis was achieved in white oak using shoot apices and leaf explants of cultures derived from 6 to 7 year old trees (Corredoira et al. 2012). Plantlet production was reported, but there was no evidence that the repetitively embryogenic system described in this study had the potential for scalable white oak somatic embryo production using suspension culture.

These previous oak embryogenesis studies demonstrate the need to optimize oak somatic embryogenesis systems in order to make them potentially useful for mass clonal propagation or gene transfer applications. A reliable gene transfer system for white oak has the potential to greatly accelerate production of genetically improved white oak trees by facilitating the application of rapid cycle breeding, a strategy used to shorten breeding cycles in plants with long

generation cycle that employs transgenics with early-flowering genes (van Nocker and Gardiner 2014). The overall goal of this study was to demonstrate the potential to clonally mass propagate white oak planting stock through application of a somatic embryogenesis system, to aid white oak restoration efforts and production plantings.

**Materials and methods**

Plant material

Immature white oak acorns were collected from two trees (designated QA-F and QA-B) on the University of Georgia campus in 2015. Collections took place on July 9$^{th}$, July 12$^{th}$ (QA-B only), July 15$^{th}$, and July 24$^{th}$ (QA-F only).

Culture initiation

After collection, acorns were surface disinfested by washing them in 70% ethanol for 20 seconds, 10% Roccal-D Plus (9.2 % didecyl dimethyl ammonium chloride, 13.8 % alkyl dimethyl benzyl ammonium chloride, 1 % bis-n-tributyltin oxide; Pfizer) for 3 minutes, 70% ethanol for 20 additional seconds, 10% Roccal for 3 additional minutes, 50% Clorox (8.25 % sodium hypochlorite) for 5 minutes, sterile deionized water for 3 minutes, and sterile 0.01 N hydrochloric acid (HCl) for 3 minutes, followed by three additional rinses in sterile deionized water for 3 minutes each. Following surface disinfestation, acorns were dissected to remove developing embryos from the acorns. Even at the earliest collection date (July 9), only a single zygotic embryo was found in each acorn, the other five ovules in the nut (Stairs 1964) having previously aborted. The two cotyledons of the immature embryos were very loosely attached to the very small embryo axis and generally became detached during acorn and seed coat dissection (Fig. 3.1a). Cotyledons with any attached axis tissues were cultured, one embryo per plate, in 60

mm plastic Petri plates containing semisolid induction-maintenance medium (IMM; Andrade and Merkle 2005), which was a modified woody plant medium (WPM; Lloyd and McCown 1980), with 30 g/l sucrose, 1 g/l filter-sterilized L-glutamine, and either 2 (low auxin treatment) or 4 (high auxin treatment) mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), and gelled with 3 mg/L Phytagel (Sigma). At least five zygotic embryo explants were cultured for each collection date/source tree/auxin treatment combination. A total of 95 seeds were cultured, 56 from QA-F and 39 from QA-B.

Cultures were incubated in the dark at 25°C. After 1 month, they were transferred to fresh medium with the same concentration of 2,4-D. Three months following initiation, each explant was scored for whether or not it produced somatic embryos or proembryogenic masses (PEMs), and percentages were calculated for each collection date, treatment and genotype as well as the different combinations of these variables. Following arcsine transformation of the percentage data, one-way analysis of variance (ANOVA) was performed to test for the effects of initiation date, auxin treatment, source tree and their interactions on embryogenesis induction. Means comparisons were conducted using Tukey's HSD test. Data were analyzed using R statistical software (version 3.12; R Foundation for Statistical Computing). After the third month, all cultures were maintained on semisolid IMM with 2 mg/L 2,4-D, with transfer to fresh medium every three weeks.

Somatic embryo production

To produce somatic embryos, PEMs were selected from seven of the most prolific cultures and approximately 0.5 g of material was inoculated into30 ml of liquid IMM in 125 ml Erlenmeyer flasks to initiate suspension cultures. Suspension cultures were grown in the dark for

3 weeks on a gyratory shaker at 100 rpm, fed by pipetting out spent medium and pouring in 30 ml of fresh medium, and grown for an additional 3 weeks. After 6 weeks, PEMs were fractionated on Cellector® stainless steel sieves (Bellco Glass). The fraction between 38 µm and 140 µm was collected on filter paper using a Büchner funnel under mild vacuum and the filter with PEMs was cultured on semisolid embryo development medium (EDM; Andrade and Merkle 2005), which was a modified WPM basal medium containing no PGRs. Cultures were incubated in the dark at 25º C.

Germination and conversion

Somatic embryos that arose from plated suspension cultures were picked, moved to fresh plates of EDM, and allowed to enlarge for 2-3 weeks in the dark at 25º C. Two culture lines that produced the highest number of well-formed embryos, one from each source tree (designated QA-F-B and QA-B-K), were selected to test the effects of cold treatment and medium supplements on germination. After enlargement, embryos were given a pre-germination cold treatment of either 8 or 12 weeks by storing them in a refrigerator at 4º C. Following cold treatment, embryos were moved to GA-7 vessels (Magenta Corp.) containing one of four types of media: semisolid EDM, semisolid EDM supplemented with 0.01 g/L gibberellic acid (GA3), semisolid EDM with 0.5 g/L activated charcoal (AC), or semisolid EDM with both AC and GA3. For line QA-F-B, 5 embryos were placed in each GA-7 and 4 repetitions were used for each cold treatment and medium treatment combination. For line QA-B-K, 3 embryos were placed in each GA-7 and 2 repetitions were used for each cold treatment and medium treatment combination. Embryos were observed weekly and final data were collected after 6 weeks to calculate the percentages of green embryos, germinated embryos (those exhibiting radicle elongation), and

converted embryos (those producing roots and shoots). Following arcsine transformation, these data were analyzed by ANOVA to test for the effect of the four germination medium treatments on greening, germination and conversion. Means comparisons were conducted using Tukey's HSD test. Data were analyzed using R statistical software (version 3.12; R Foundation for Statistical Computing). When converted somatic embryos had at least one expanded leaf, they were removed from culture and planted in moistened peat:perlite:vermiculite (1:1:1) mix in plastic pots, which were placed on water-saturated perlite in a clear plastic dome-covered tray under cool white fluorescent lights (80 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) and 16 h day lengths.

Cryostorage and recovery

Four embryogenic culture lines were selected to test if our white oak cultures could recover from cryostorage (two QA-F lines and two QA-B lines). As a pretreatment, cultures were inoculated into 50 ml Erlenmeyer flasks containing 5 ml of liquid IMM supplemented with 0.4 M sorbitol and inclubated in the dark at 25º C on a gyratory shaker for 24 hours. Then cultures were inoculated into liquid IMM supplemented with 0.4 M sorbitol and 5% dimethylsulfide (DMSO) as a cryoprotectant, and 1.8 ml from each embryogenic suspension line were pipetted into pre-chilled (4 °C) 2 ml cryovials (Nalgene) and placed into a pre-chilled (4 °C) Mr. Frosty freezing container (Nalgene). Three repetitions were used for each line, for a total of 12 cryovials. The freezing container was placed in an ultralow freezer at -70° C overnight, which allowed the temperature of the material in the vials to slowly drop to -70º C . The next day, the cryovials were removed from the ultralow freezer and placed into a cryobox, which was placed in a Forma Cryomed II freezer and held in liquid nitrogen at -196° C for 5 days. Cryovials were then removed from liquid nitrogen and transferred from the cryobox to a floating tray in a 37 °C

water bath for 2 minutes to thaw. Next, vial contents were poured through a 30 μm pore size nylon mesh (Lamports Filter Media, Inc.), which was placed over two layers of filter paper and paper towels. Embryogenic tissue collected on the nylon mesh was then placed onto fresh semisolid IMM in 100 mm plastic Petri plates and incubated in the dark at 24 °C. The mesh and embryogenic tissue were transferred to fresh semisolid IMM hourly for two hours and then at 24 hours to dilute residual DMSO.

**Results**

Culture initiation

Twenty-nine embryogenic cultures were initiated from seed explants derived from immature acorns collected from the two source trees, 16 from QA-F and 13 from QA-B.. While most of the cultures appeared to start out producing repetitive somatic embryos (Fig. 3.1b, c, d), after a few sub-cultures, most eventually changed to proliferating as PEMs or mixtures of callus and globular-stage embryos (Fig. 3.1e, f).

Overall, collection date did not have a significant effect on embryogenesis induction ($P = 0.393$), and Tukey's HSD test confirmed that no significant difference existed between the collection dates (Fig. 3.2). In addition, there was no significant difference between the two source trees ($P = 0.871$) and there was no significant interaction between collection date and source tree genotype ($P = 0.851$). Since two of the collection dates were for one source tree only, the effect of date was also analyzed separately for each tree, and there still was not a significant effect ($P = 0.446$ for QA-F; $P = 0.75$ for QA-B). Mean embryogenesis induction frequencies were 39.25% for the July 9$^{th}$ collection date, 20% for the July 12$^{th}$, 40% for July 15$^{th}$, and 0% for July 24$^{th}$. Although collection date did not have a significant effect on embryogenesis induction,

the data suggests early to mid-July may be the optimal time for harvesting immature acorns for induction of embryogenic cultures, since embryogenic cultures were induced from acorn seeds collected on all dates except for July 24th. This would need to be confirmed by adding earlier and later collection dates in future initiation experiments and by collecting acorns from more source trees to determine if there is a genotypic difference in the zygotic embryo developmental window among different white oak trees.

There was also no significant difference between the high auxin treatment (4 mg/L 2,4-D) and the low auxin treatment (2 mg/L 2,4-D) on embryogenesis induction (P = 0.847. Mean embryogenic frequencies for each auxin treatment were similar at 29.6% for the high auxin treatment and 32.9% for the low auxin treatment.

Somatic embryo production, germination and conversion

PEMs from all seven of the lines grown in suspension culture proliferated rapidly in liquid medium and produced somatic embryos following size-fractionation and plating on semisolid EDM, producing up to 400 well-formed embryos per 0.5 g of starting material, depending on culture line (Fig. 3.3). Analysis of variance results indicated that the four germination treatments applied following pre-germination cold treatment of the embryos did not differ significantly with regard to greening of somatic embryos (P = 0.402; Fig. 3.4a), germination (radicle elongation) (P = 0.242; Fig. 3.4b), and conversion of somatic embryos to somatic seedlings (P = 0.227; Fig. 3.4c). These results were confirmed with Tukey's HSD tests. Furthermore, the two cold treatments, 8 weeks and 12 weeks, did not differ significantly with regard to greening of somatic embryos (P = 0.323), germination (P = 0.959), or conversion (P = 0.716). Also, there was no significant interaction between cold treatment and medium treatment

on embryogenesis induction for greening of somatic embryos (P = 0.403), germination (P = 0.711), or conversion (P = 0.184). Overall, the mean frequency for greening of somatic embryos was 100%, the mean frequency for germination was 37%, and the mean frequency for conversion was 6.67%. Germination and conversion percentage differed significantly between the two tested culture lines. (P = < 0.001), with 54.4% germination for line QA-F-B and only 2.1% germination for line QA-B-K. Culture line also had a significant effect on conversion (P = 0.035), with 10% conversion for QA-F-B and 0% conversion for QA-B-K.

Somatic seedlings transferred to potting mix under high humidity grew slowly, often taking several weeks between the emergence of new leaf (Fig. 3.5a). Some somatic seedlings survived hardening off and continued to produce new leaves following removal from the humidifying chambers (Fig. 3.5b).

Cryostorage and recovery

A 100% recovery rate was obtained for all of the embryogenic white oak tissue samples we put into cryostorage. Tissue proliferated quickly and had the same appearance as a typical healthy embryogenic white oak culture prior to cryostorage (Fig. 3.6).

**Discussion**

Many hardwood forest trees are characterized by a relatively narrow developmental window during which zygotic embryo explants are capable of making repetitive embryogenic cultures. As such, developmental stage of zygotic embryo explants has been shown to be a contributing factor for successful induction in yellow-poplar (Sotak et al. 1991), American chestnut (Carraway and Merkle 1997), pecan (Wetzstein et al. 1989) and Manchurian ash (Kong et al. 2012), with early and late stages of zygotic embryo development resulting in lower or no

embryogenesis induction. Since we successfully initiated embryogenic cultures from both of our source trees, it appears that our three earliest collection dates fell into a relatively broad zygotic embryo developmental window for white oak. Our explants from the July 24$^{th}$ collection date were likely too mature to induce embryogenic cultures. If we had collected before July 9$^{th}$, explants may have been too immature, but it is also possible that the induction percentage from pre-cotyledonary embryos could be even higher than for the stages that we tested. This could be tested in future experiments by adding earlier and later collection dates to determine just how broad the developmental window for white oak embryogenesis induction really is. Although we did not find a significant interaction between source tree and collection date, this could also be further tested by adding more genotypes along with more collection dates.

To our knowledge there has only been one previous report on the induction of embryogenic cultures in white oak, but our study differs from that of Corredoira et al. (2012) in that embryogenesis was inducted from immature seed explants. Previously, somatic embryogenesis was achieved in white oak using shoot apices and leaf explants of cultures derived from 6 to 7 year old trees (Corredoira et al. 2012). Unlike our study, however, there was no evidence that the previously reported system had the potential for scalable somatic embryo production. Using suspension culture, our embryogenic cultures proliferated quickly and successfully produced hundreds of somatic embryos when plated. Our system has the potential to be scaled-up and optimized for use in mass clonal propagation or gene transfer applications, especially in combination with our cryostorage and recovery protocol. Applying a gene transfer system to our *in vitro* propagation system could potentially accelerate production of genetically improved white oak trees by employing transgenics with early-flowering genes to facilitate rapid cycle

breeding. Using this method, by clonally propagating white oak trees with the most desirable properties, more white oak trees would be available sooner to aid forest restoration and production of high-quality and high-demand products like wooden furniture and barrels used in the distilling industry. Cryopreservation would allow transgenic embryogenic tissue to be stored, recovered, and multiplied as needed.

**References**

Abrams MD (2003) Where has all the white oak gone? BioScience 53(10):927-939

Andrade G, Merkle SA (2005) Enhancement of American chestnut somatic seedling production. Plant Cell Rep 24(6):326-334

Barra-Jiménez A, Blasco M, Ruiz-Galea M, Celestino C, Alegre J, Arrillaga I, and Toribio M (2014) Cloning mature holm oak trees by somatic embryogenesis. Trees-Struct Funct 28(3): 657-667

Burns RM, Honkala BH (1990) Silvics of North America, vol. 2. United States Department of Agriculture

Carraway DT, Merkle SA (1997) Plantlet regeneration from somatic embryos of American chestnut. Can J For Res 27(11):1805-1812

Corredoira E, San-José MC, and Vieitez AM (2012) Induction of somatic embryogenesis from different explants of shoot cultures derived from young *Quercus alba* trees. Trees-Struct Funct 26(3):881-891

Goldblum D (2010) The geography of white oak's (*Quercus alba* L.) response to climatic variables in North America and speculation on its sensitivity to climate change across its range. Dendrochronologia 28(2):73-83

Hernández I, Celestino C, Toribio M (2003) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis. Plant Cell Rep 21(8):759-764

Kong DM, Preece JE, Shen HL (2012) Somatic embryogenesis in immature cotyledons of Manchurian ash (*Fraxinus mandshurica* Rupr.). Plant Cell Tiss Org 108(3):485-492

Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel,

*Kalmia latifolia*, by use of shoot-tip culture. Combined Proceedings of International Plant Propagators' Society 30:421-427

Mosedale JR (1995) Effects of oak wood on the maturation of alcoholic beverages with particular reference to whisky. Forestry 68(3):203-230

San-José MC, Corredoira E, Martínez MT, Vidal N, Valladares S, Mallón R, Vieitez, AM (2010) Shoot apex explants for induction of somatic embryogenesis in mature *Quercus robur* L. trees. Plant Cell Rep 29(6):661-671

Smith DR (1996) Growth Medium. US Patent No. 5,565,355

Sotak RJ, Sommer HE, Merkle SA (1991) Relation of the developmental stage of zygotic embryos of yellow-poplar to their somatic embryogenic potential. Plant Cell Rep 10(4):175-178

Stairs GR (1964) Microsporogenesis and embryogenesis in Quercus. Botan Gaz 125:115-121.

Tirmenstein DA (1991) *Quercus alba*. In: Fire Effects Information System. US Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (Producer). http://www.fs.fed.us/database/feis/ Accessed 6 October 2015

Toribio M, Fernández C, Celestino C, Martínez MT, San-José, MC, Vieitez AM (2004) Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell Tiss Org 76(3):283-287

van Nocker S, Gardiner SE (2014) Breeding better cultivars, faster: applications of new technologies for the rapid deployment of superior horticultural tree crops. Horticulture Research 1

Walter C, Grace LJ, Wagner A, White DWR, Walden AR, Donaldson SS, Hinton H, Gardner RC, Smith DR (1998) Stable transformation and regeneration of transgenic plants of

*Pinus radiate* D. Don. Plant Cell Rep 17:460-468

Wetzstein HY, Ault JR, Merkle SA (1989) Further characterization of somatic embryogenesis and plantlet regeneration in pecan (*Carya illinoensis*). Plant Sci 64(2):193-201

## Figures

**Fig. 3.1.** White oak embryogenic culture initiation. **a.** Dissected acorn collected on July 9, showing detached cotyledons of zygotic embryo. **b.** Embryogenic tissue arising from explanted cotyledon. **c.** Direct somatic embryos arising from cotyledon explants. **d.** Proembryogenic masses proliferating in established culture. **e.** Mixture of callus and somatic embryos in established culture. Bar in all photos = 1 mm.

**Fig. 3.2.** Mean embryogenesis induction frequencies for each source tree (QA-B and QA-F) for each collection date in 2015. Only embryos from tree QA-B were cultured on July 12$^{th}$ and only embryos from QA-F were cultured on July 24$^{th}$. Bars represent standard error.

**Fig. 3.3.** White oak somatic embryos developing after suspension cultures were plated onto semisolid EDM. Bar = 1 mm.

**Fig. 3.4.** Effects of cold treatments (8 and 12 weeks) and germination medium on white oak plantlet production for each of the four germination media treatments: semisolid EDM (Control), semisolid EDM containing 0.01 g/L gibberellic acid (GA3), semisolid EDM containing 0.5 g/L activated charcoal (AC), and semisolid EDM containing both 0.5 g/L activated charcoal and 0.01 g/L gibberellic acid (GA3 and AC). Treatments sharing the same letter are not significantly different according to Tukey's HSD test. **a**. Mean percentages of greening somatic embryos. **b.** Mean percentages of total germination (radicle elongation) **c.** Mean percentages of converted germinants. Bars represent standard error.

**Fig. 3.6.** White oak somatic seedling production. **a.** Somatic embryo conversion in GA-7 vessel. **b.** Somatic seedlings 2 months following transfer to potting mix.

**Fig. 3.5.** White oak embryogenic tissue after initial removal from cryostorage (**a** and **b**) and subsequent regrowth (**c** and **d**).

**Fig. 3.1**

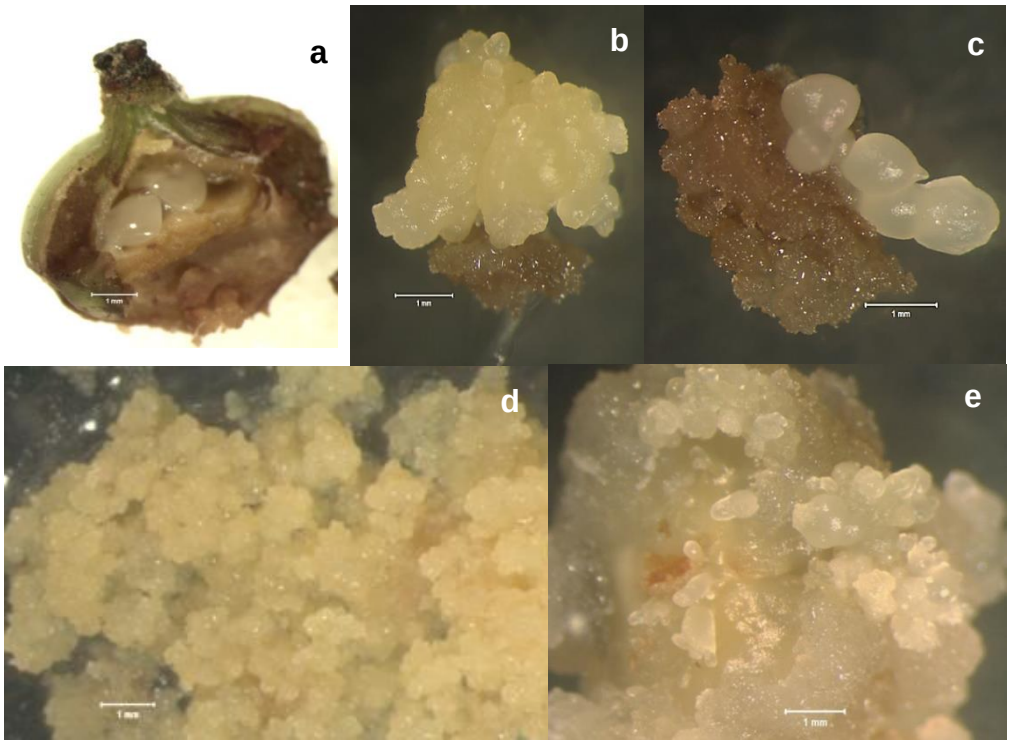


**Fig. 3.2**

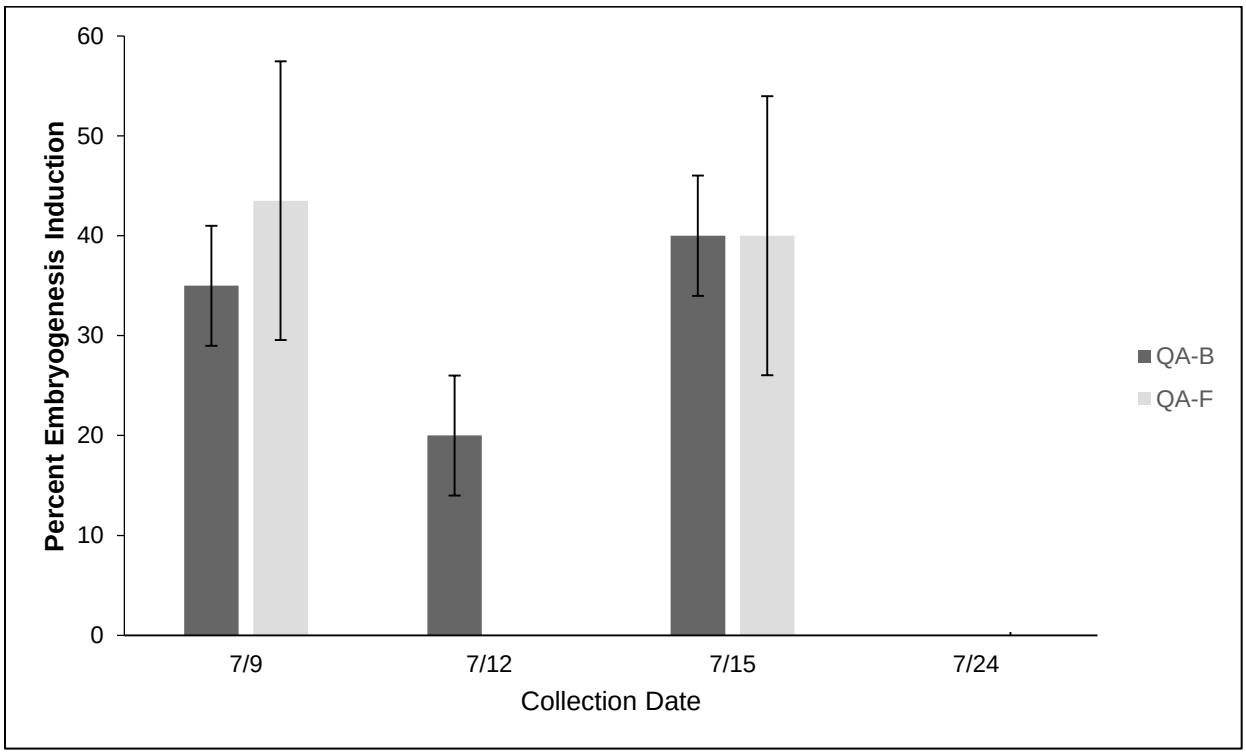

60
50
40
30
20
10
0
Percent Embryogenesis Induction
7/9
7/12
7/15
7/24
Collection Date
QA-B
QA-F

**Fig. 3.3**

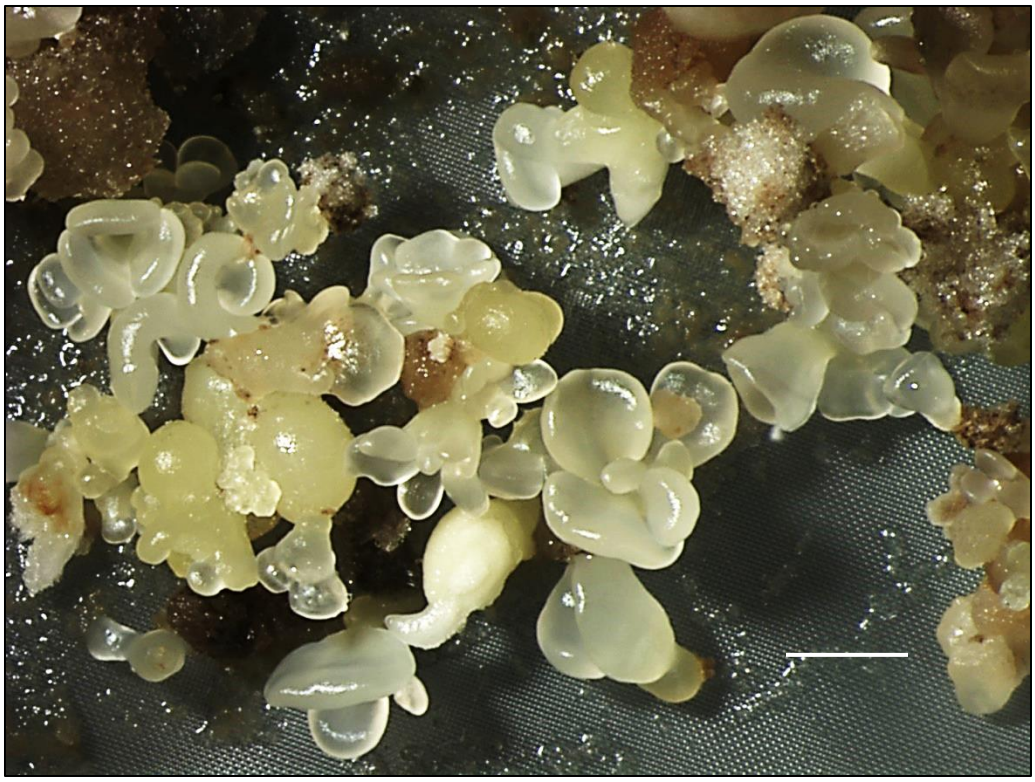


**Fig. 3.4**

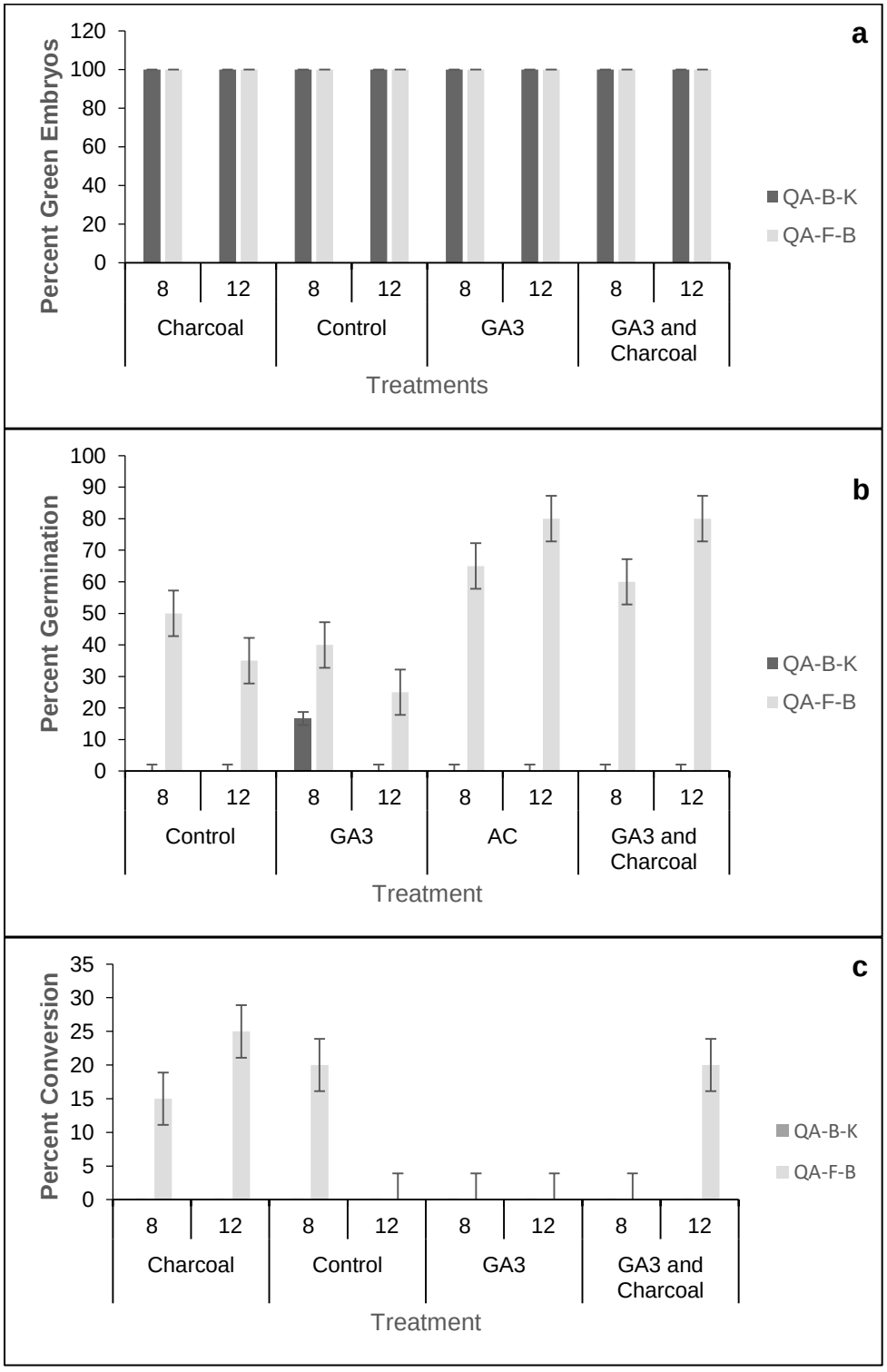

a
Percent Green Embryos
120
100
80
60
40
20
0
8
12
Charcoal
Control
GA3
GA3 and Charcoal
Treatments
QA-B-K
QA-F-B
b
Percent Germination
100
90
80
70
60
50
40
30
20
10
0
Control
GA3
AC
GA3 and Charcoal
Treatment
c
Percent Conversion
35
30
25
20
15
10
5
0
Charcoal
Control
GA3
GA3 and Charcoal
Treatment

**Fig. 3.5**

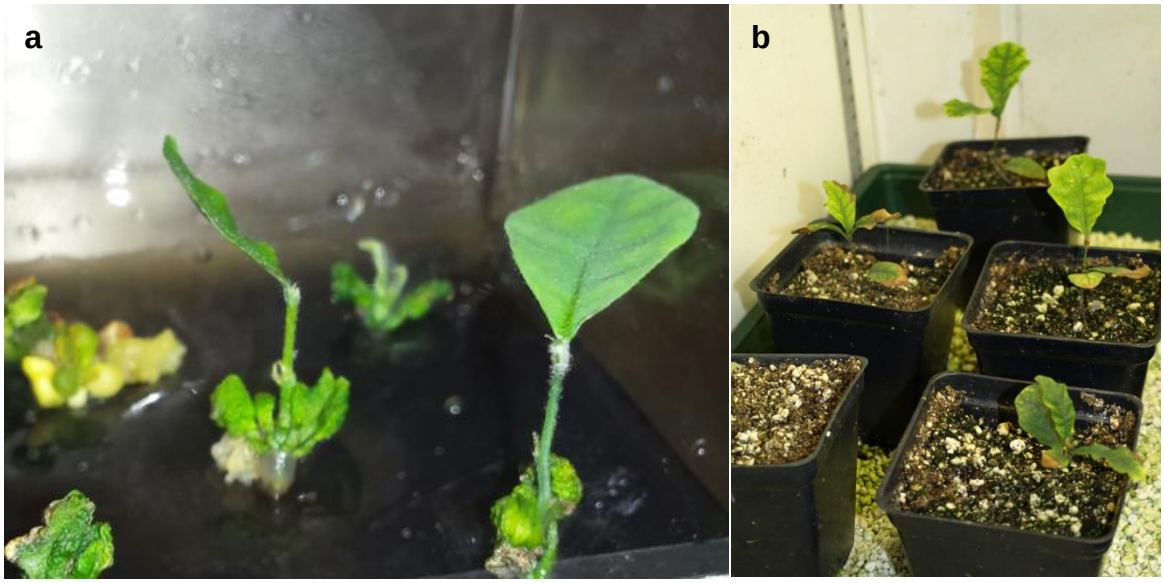
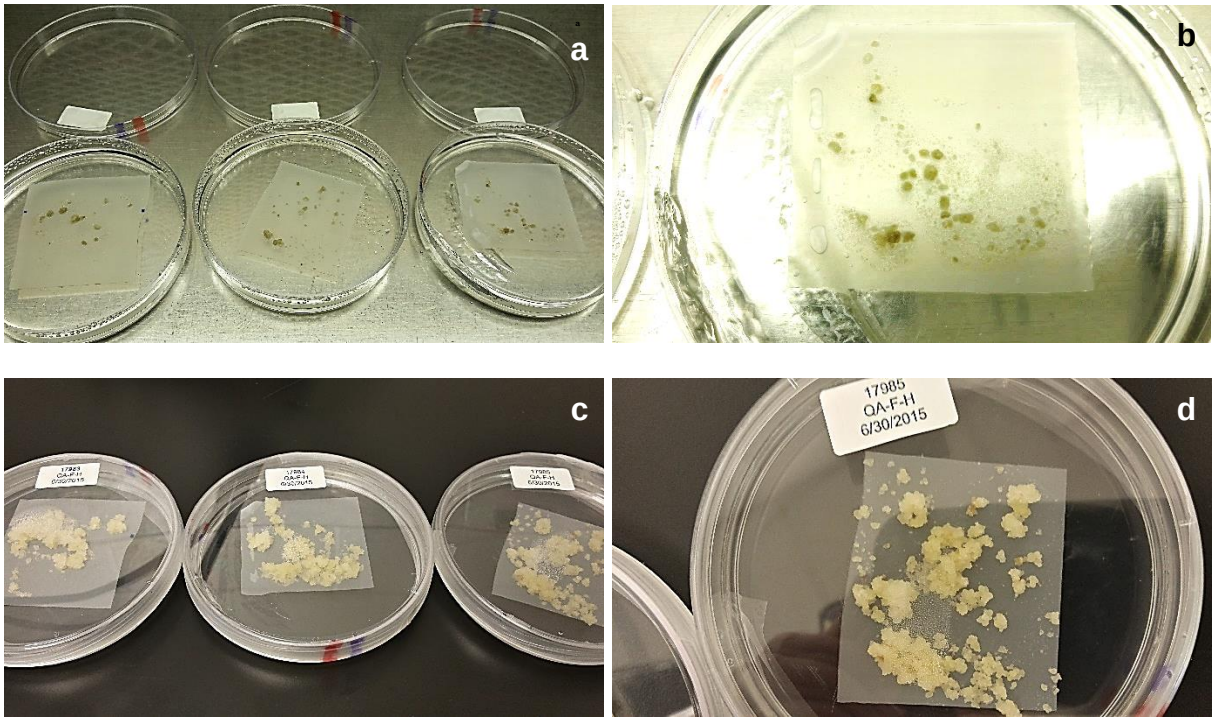


**Fig. 3.6**



CHAPTER 4

# Conclusion

This thesis detailed the initiations of embryogenic cultures from immature seed explants of both white ash white oak trees, demonstrating the potential to scale-up our somatic embryogenesis propagation system to clonally mass propagate hardwood trees with desirable qualities. After testing initiation treatments and after embryogenic cultures from each species proliferated and became established, we tested the effects of cold treatment and media treatments on germination and conversion. In each case, somatic seedlings were obtained. Some white oak somatic seedlings survived hardening off and continued to produce new leaves following removal from the humidifying chambers, while some white ash somatic seedlings grew much more vigorously and continued rapid growth following hardening off and transfer to the greenhouse.

Collection date had a significant effect on embryogenesis induction for white ash but not for white oak, although white oak appeared to have a relatively broad zygotic embryo developmental window during which embryogenesis could be induced. Although embryogenic cultures and somatic seedlings were obtained for both species, more collection dates and more source trees could be added in future initiation experiments to better define our collection window, as well as to determine if there is a genotypic difference between zygotic embryo development times. For both white ash and white oak, there was no statistical difference between our high and low auxin treatments on embryogenesis induction, with embryogenic cultures

arising from both treatments for both tree species. While this shows that 2,4-D is an effective auxin for induction of embryogenic cultures of both white ash and white oak, more auxin levels could be tested to pinpoint the most effective auxin concentration for embryogenesis induction.

The same four media treatments were tested for their effects on germination and conversion rates for each of the species: semisolid EDM (Control), semisolid EDM containing 0.01 g/L gibberellic acid (GA3), semisolid EDM containing 0.5 g/L activated charcoal (AC), and semisolid EDM containing both 0.5 g/L activated charcoal and 0.01 g/L gibberellic acid (GA3 and AC). For the white ash somatic embryos, the four treatments differed significantly with regard to greening of somatic embryos, germination (radicle elongation) and conversion of embryos to somatic seedlings, with the highest percentage of germination and conversion occurring on the medium with both GA3 and AC. For white oak, however, there was no significant difference among the four media treatments on greening, germination, or conversion. Somatic embryos from the line QA-F-B appear to have responded more strongly to the treatments, and it was the only line of the two to exhibit conversion, and only when AC was present in the medium. Some QA-B-K somatic embryos germinated on medium containing GA3, although they did not convert. Although the AC only and GA3 in combination with AC media treatments appeared to yield the highest percentage of germination for white oak, the effect of medium treatment on embryogenesis was not significant. Thus, while it appears that for both white ash and white oak, AC and GA3 medium supplements may improve germination or conversion, more concentrations of AC and GA3 and different combinations of those concentrations need to be tested, along with more repetitions and more genotypes, to further

optimize germination and conversion of white ash and white oak somatic embryos and to test for a significant effect on germination and conversion.

A 100% recovery rate was obtained for all of the embryogenic white oak tissue samples we put into cryostorage, showing that cryopreservation is a viable method to allow transgenic embryogenic tissue to be stored, recovered, and multiplied as needed for this species in order to quickly clonally mass propagate trees with desirable properties. In contrast, by clonally propagating putatively EAB-resistant “lingering” white ash trees via somatic embryogenesis, we demonstrated the use gene transfer techniques to produce trees resistant to EAB may not be necessary.