ENHANCING CHESTNUT EMBRYOGENIC CULTURE PRODUCTIVITY AND SOMATIC EMBRYO QUALITY BY TARGETING STEPS IN THE EMBRYOGENESIS PROTOCOL

by

CHRISTINE THERESE HOLTZ

(Under the Direction of Scott Arthur Merkle)

ABSTRACT

In vitro mass propagation systems have the potential to help re-establish the American chestnut (*Castanea dentata*) tree, which was devastated by the chestnut blight fungus in the 20th century. Somatic embryogenesis (SE) has been successfully applied for clonal propagation of American chestnuts. We tested the applicability of our published protocol for American chestnut SE induction from seeds of hybrid backcross trees produced by The American Chestnut Foundation (TACF), as well as pure Chinese chestnut. The protocol was successful in producing somatic embryos from the advanced generation hybrid backcross TACF material. In an attempt to improve proliferation of embryogenic tissue in suspension culture, air-lift bioreactors were compared with the standard method, continuously shaken flasks. Air-lift bioreactors showed great potential to enhance embryogenic tissue proliferation. Additionally, RITA® temporary immersion bioreactors were tested to assess their potential for improving somatic embryo germination frequency, but results were inconclusive. Finally, to improve embryo quality, different treatments in which the embryo development medium was supplemented with DLbuthionine–[S,R]-sulfoximine (BSO) were tested. A histological analysis was performed on

embryos with different types of morphology. No significant treatment effects were found in the BSO study.

INDEX WORDS: Somatic embryogenesis, Air-lift bioreactor, Temporary immersion bioreactor, DLbuthionine–[S,R]-sulfoximine (BSO), Histology

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CHRISTINE THERESE HOLTZ

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CHRISTINE THERESE HOLTZ

Major Professor:	Scott Arthur Merkle
Committee:	Hazel Wetzstein Robert Cooper

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
August 2014

DEDICATION

This thesis is dedicated to Roger and Kathy Holtz (AKA mom and dad). For their relentless support from 800 miles away, their understanding of my financial woes, and their constant effort to help me even though they have little themselves.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

American chestnut and chestnut blight

The American chestnut, (*Castanea dentata*) was one of the most economically important angiosperm forest tree species in the eastern United States up until the early 1900s (Carraway and Merkle 1997). The range of the species extended from northern Georgia and Alabama through southern Maine and parts of Canada (Burnham 1988). Comprising up to 25 percent of the trees in the Appalachian forest and with a natural life expectancy of 500-1000 years, the American chestnut could reach up to 150 feet tall and 4 feet in diameter. The wood was straight-grained, highly rot-resistant, and did not warp or shrink, making it a major timber tree used for poles, pilings, posts, shingles, railroad ties, and furniture (Andrade and Merkle 2005; Vieitez 1995). Most hardwoods that produce high quality wood are not fast-growing; however, the American chestnut could grow up to 2.5 cm in diameter per year (Anagnostakis 1987). Because of its prolific stump and root sprouting abilities, when stands of American chestnut were harvested, the tree would naturally reestablish and replanting was not necessary (Burnham 1987). This ability also allowed the tree to outcompete other species, resulting in pure stands. The nuts that the tree produced were an important cash crop, and were high in nutritional value. They were very high in starch compared to other nuts and seeds, had a protein content of 6%, and were very low in fatty oils (Abrams and Nowacki 2008). At maturity, a single tree could produce over 100 kg of nuts annually (Vieitez 1995).

Until the early 1900s, the major disease problem for the American chestnut was root rot caused by the oomycete *Phytophthora cinnamomi* (Vieitez 1995). Sometime in the late 1800s, however, the chestnut blight fungus (*Cryphonectria parasitica*) was introduced to the United States on Asian chestnut nursery stock. *C. parasitica* is a necrotrophic ascomycete that produces spores spread by insects, birds, wind, and water. The fungus enters through cracks or wounds in the bark, proliferates rapidly, and produces sunken cankers that girdle the tree. After being discovered on American chestnut trees in the New York Zoological Park in New York City, it spread approximately 200 miles every 10 years. Within 50 years, 9 million acres of American chestnut trees were either dead or dying (Anagnostakis 1987; Burnham 1988; Vieitez 1995). Today, the tree can still be found in the understory due to its stump sprouting ability, but rarely does it survive beyond a few years. Natural reestablishment of the American chestnut is highly unlikely, as the blight remains an obstacle. Both *P. cinnamomi* and *C. parasitica* are the cause of major declines in chestnut trees, although clearly the blight had a more widespread effect. Both Asian chestnut species, Chinese chestnut (*Castanea mollissima*) and Japanese chestnut (*Castanea crenata*), are resistant to root rot, and have a high resistance to chestnut blight (Vieitez 1995).

Breeding efforts

As a part of the considerable effort to re-introduce the American chestnut to its native range, a significant amount of research has been put forth to produce and propagate American chestnut trees with blight resistance. One such approach is hybridization with Asian chestnut trees, which carry resistance to the fungus. It was suggested that crossing an American chestnut with a Chinese chestnut, backcrossing three times to an American chestnut parent—with selection against Chinese morphological type—then intercrossing these individuals twice, would

result in an individual with both American chestnut characteristics and the genes from Chinese chestnut responsible for blight resistance (Burnham 1988). The breeding program designed by Burnham and implemented by the American Chestnut Foundation (TACF) is described below. The first cross between an American and Chinese chestnut will result in an individual (F_1) with a genome proportion that is half Chinese and half American. The progeny of the first backcross to an American parent will have a genetic inheritance that is half American and half F_1; here the selection against Chinese morphological type will take place as the inheritance from the F_1 hybrid will vary from 100% American to 100% Chinese. On average, however, the F_1 donation will be half American and half Chinese, thus, with each generation of backcrossing, the Chinese chestnut genome proportion will decrease by an average of 1/2 each time, resulting in a third backcross generation that is 15/16 American chestnut and 1/16 Chinese chestnut (Burnham 1988; Hebard 2005). The first intercross between two B_3 trees results in a B_3F_2, and the second intercross, between two B_3F_2s, yields the final and best material that TACF currently produces, called the B_3F_3. Currently, TACF has harvested increasing numbers of B_3F_3 seeds which they are calling "Restoration Chestnuts 1.0". The 1.0 signifies that they are first in a series of potentially blight-resistant trees. In 2009, the first of these chestnuts were planted in real forest environments (Smith 2012).

In vitro propagation efforts

Although TACF has been successful in producing some hybrid material that is shown to be highly blight resistant (Hebard 2005), it is extremely desirable to have a system in place to clonally propagate this material. There are some significant advantages to the use of clonal propagation for the best material that TACF produces. Because the production of B_3F_3 trees is for the purpose of reestablishing the species in the wild, the assumption is that the individuals

with the highest amount of blight resistance will have the lowest mortality rate, and therefore be in the highest demand. Therefore, it would be wise to have a system in place to mass propagate these specific varietals, since open pollination results in various genotypes and mass controlled pollination has not yet been developed with chestnut.

One focus of current American chestnut research pertains to the issue of mass propagating blight-resistant genotypes once they are established. The use of an *in vitro* propagation system known as somatic embryogenesis (SE) has shown great possibility for this purpose. SE is a tissue culture process that produces clonal populations of structures resembling seed embryos which can be germinated to produce seedling-like plants (Merkle et al. 2008). This system has shown the potential to contribute to restoration of the American chestnut, both by producing trees engineered with blight-resistance genes, and by providing a method for mass propagation of blight resistant genotypes from the conventional breeding programs (Andrade and Merkle 2005). Along with substantial multiplying power (SE has the ability to produce virtually unlimited number of clonal embryos), the ability of SE material to be preserved in cryostorage allows for long term maintenance of the highest quality material (Giri et al. 2004; Merkle et al. 2013). SE has been identified by many researchers as the optimal *in vitro* regeneration system for economical mass clonal propagation of forest trees; again, the efficacy of SE is derived from its potential for high-frequency, large-volume clonal propagation (Merkle and Trigiano 1992 Carraway and Merkle 1997). One significant disadvantage of SE is that, for the majority of forest species, the process relies on immature tissues to produce embryos. Therefore, the material being propagated has a genetic make-up that is unproven until the resulting trees can be tested in the field.

The first successful SE in plants was reported by both Steward et al. (1958) and Reinert (1958), who were simultaneously experimenting with carrot root cultures. Somatic embryogenesis in woody plants was described not long after, starting with Citrus (*Citrus spp*.) (Ranga 1958; Maheshwari and Ranga 1958), Chinese cedar (*Biota orientalis*) (Konar and Oberoi 1965), and sandalwood (*Santalum album*) (Rao 1965). One of the earliest North American hardwood trees to be successfully cultured was sweetgum (*Liquidambar styraciflua*), in which SE was induced from seedling hypocotyls (Sommer and Brown 1980). The first successful SE for a coniferous species was not reported until a few years later with Norway spruce (*Picea abies*) (Hakman and Von Arnold 1985). Beginning in the 1980s, SE became widely reported in woody angiosperms and gymnosperms (Dunstan et al. 1995).

Induction of SE has been attempted many times with various chestnut species and chestnut hybrids. Cultures of a *C. mollissima* X *C. dentata* hybrid resulted in embryo-like protuberances (McPheeters et al. 1980; Skirvin 1981). Culture of a *C. sativa* X *C. crenata* hybrid resulted in mature somatic embryos, but germination of the embryos was incomplete (Vieitez et al. 1990). Vieitez et al. (1992) later reported production of plantlets from somatic embryos of the hybrid. About 100 of these chestnuts planted in soil survived and displayed normal growth patterns (Vieitez and Merkle 2005). The first case of SE in American chestnut was reported 20 years ago, but no plantlets were produced in that study (Merkle et al. 1991). Subsequently, the addition of specific amino acids to the embryo development media resulted in the first regeneration of American chestnut somatic seedlings, although only a few plantlets survived hardening off (Robichaud et al. 2004). Xing et al. (1999) reported successful production of 20 American chestnut plantlets, with four still surviving at the age of two when the study was published, but it is unclear if these plantlets were true somatic seedlings, since at least

some of the plantlets were derived from shoots that had to be rooted in a separate step. Andrade and Merkle (2005) reported plantlet production, hardening-off, and successful transition to the greenhouse for over 100 *C. dentata* somatic seedlings using a suspension culture-based system.

Since a system has already been developed to clonally propagate pure American chestnut trees via SE (Carraway and Merkle, 1997; Andrade and Merkle 2005), we believe it also has great potential to be used to multiply blight-resistant trees produced by TACF's hybrid backcross breeding program. However, since it is well-known that genotype exerts a strong influence on embryogenesis induction (e.g. Trolinder and Xhixian 1989; Brown et al. 1995; Park et al. 2011), it is possible that Chinese chestnut (CC) genes in the hybrid backcross material could affect the success of SE using the protocol established for American chestnut (AC). To date, there have been no published reports of somatic embryogenesis in either CC or hybrid backcross material.

Application of suspension cultures

The initial protocol for American chestnut SE called for the cultures to be maintained continuously on semi-solid media from initial tissue explant culture through somatic embryo development, maturation, and germination (Merkle et al. 1991). Research conducted altering the protocol of maintaining embryogenic cultures on semi-solid media to suspending them in liquid media in shaken flasks helped to advance the chestnut SE process. Changing the procedure yielded much higher germination and somatic seedling production rates than had been previously reported (Andrade and Merkle 2005). The use of suspension cultures has greatly increased output and increased the efficiency of mass propagation in many other species as well (Etienne and Berthouly 2002; Ducos et al. 2007; Tautorus at al. 1992; Stuart et al. 1987).

Although it has been established that the use of suspension cultures has facilitated somatic seedling production, this portion of the process still constitutes the greatest challenge,

because the proportion of germinating embryos is still insufficient for mass propagation (Johnson et al. 2008). Different methods of suspension culture, such as the use of bioreactors, are showing potential in scaling-up production efficiency beyond what has been accomplished using shaken flasks.

Use of bioreactors for plant cell cultures

In general, a bioreactor (also known as a fermenter) is any manufactured or engineered device such as a large chamber, or vat, which supports a biologically active environment. For *in vitro* plant cultures, bioreactors can be described as self-contained sterile environments that take advantage of liquid media and an inflow/outflow system to have significant control over the cultures and their microenvironment (Leathers et al. 1995). Although fermentation has been used since long before the underlying scientific principles were understood—there is significant evidence that beer, bread, yogurt, cheese, and wine were abundant in ancient Egypt—it wasn't until the mid-20^{th} century that modern fermentation technology got its start (El-Mansi et al. 2012). The discovery of penicillin's antibacterial properties prompted the innovation of a sterile fermentation device, since the organism is highly susceptible to contamination; thus the development of the stirred-tank bioreactor. Bioreactor technology was originally adapted and advanced for microbial and mammalian cell cultures, and later plant cell cultures, where the end product was a secondary metabolite (Sharma et al. 1992).

The first reported attempt at putting a suspension of plant cells into a fermenter was with carrot cells in the mid-1950s (Dalton 1978). Noguchi et al. (1977) reported the culture of tobacco cells in a 20 m^3 tank mixed by aeration. The large-scale propagation of tobacco cells for smoking material was also reported a few years later. The production was ended, however, when the experience of smoking tobacco cells was found to be most un-pleasurable (Hashimoto and

Azecki 1988). It has been argued that bioreactors are not adaptable for culture of plant cells since there are many differences between plant cells and microbes. Plant cells have a lower respiration rate, higher shear sensitivity, and they often grow as aggregates or clumps (Payne et al. 1987). Plant cells have weak cell walls relative to their size. Although cellulose creates a rigid cell wall in plants, the large size of plant cells increases their vulnerability, allegedly making them highly susceptible to even moderate amounts of shear damage (Merchuk 1990). When testing the effect of shear stress on the growth of pro-embryogenic masses (PEMs) and suspensor cells in Norway spruce, it was shown that PEM growth was negatively affected and that there was a critical shear stress point, beyond which no suspensor cells would develop (Sun et al. 2009). Other potential issues caused by bioreactors include mechanical damage and foam formation from constant aeration (Etienne and Berthouly 2002). When subjected to such stress, cells of certain species tend to rupture or become malformed, causing cell death (Denchev et al. 1992). However, when assessing the tolerance of *Catharanthus roseus* cells to shear force, Scragg et al. (1988) used high-shear impellers at agitation speeds up to 1000 rpm and observed no loss of viability. Additionally, many other plant cell cultures, such as shikonin (*Lithospermum erythrorhizon*), were found to be shear-tolerant and able to be grown, with no issues, in stirred tank bioreactors (Verpoorte et al. 1993; Westphal 1990).

Although shaken flasks are essentially bioreactors, there are a few significant differences between them and automated bioreactors when used for somatic embryo production in suspension culture. Advantages of using bioreactors over flasks include the potential to apply increased working volume, maintenance of a homogenous culture by mechanical or air-lift stirring, and control of the cultural and physical environment for the cultures—such as pH, dissolved oxygen and carbon dioxide (Ammirato and Styer 1985; Tautorus et al. 1992).

Producing an environment in which gaseous composition can be properly controlled has the potential to result in optimum growth and yield (Huang et al. 2006). For example: In all suspension cultures, tissue is immersed in liquid medium, an anaerobic environment. Shaken flasks introduce oxygen into the vessel with one method, constant gyratory shaking. The use of bioreactors increases the amount of control over the introduction of oxygen into the vessel. The three main methods of aeration in a bioreactor system include: air-lift, propeller, and magnetic bar stirring (Nishimura et al. 1993).

Somatic embryo development in bioreactors

Stirred tank bioreactor designs have proved suitable for some embryogenic cultures. Somatic embryos of black spruce (*Picea mariana*) were grown in a mechanically-stirred bioreactor with no observable signs of stress, and successfully matured to plantlets (Tautorus et al. 1992); the potential for large-scale production of poinsettia (*Euphorbia plucherimma*) and alfalfa (*Medicago sativa*) somatic embryos with vibration stir-bar bioreactors has also been reported (Preil et al. 1988; Chen et al. 1987). Because air-lift bioreactors do not contain any type of stirring blade, they are the most common type of bioreactor used for the growth of shear-sensitive cells (Allman 2012). The main advantage that results from the absence of a propeller or stir bar is that the aeration is distributed with approximate homogeneity throughout the bioreactor, as opposed to the uneven dissipation of energy that results from distribution via a mechanical device (Merchuk 1990). The principle behind air-lift bioreactors was first described by Le Francois in 1955 (Birch et al. 1987). Because of the lack of a mechanical system for aeration, they are relatively simple to construct (Merchuk 1990). Bubble column bioreactors work similarly to air-lift bioreactors by introducing oxygen to the system with the use of an air pump. In a study of carrot (*Daucus carota* L.) somatic embryo development, Teng et al. (1994)

compared three different types of suspension culture: spinner flask, air-lift bioreactor, and a screen column bioreactor that was aerated with a two-blade propeller. The air-lift bioreactor showed the highest biomass production. When the productivity of Siberian ginseng (*Eleutherococcus senticosus*) SEs was compared in bubble column bioreactors and shaken flasks, the bioreactors yielded higher productivity and secondary SE induction was more rapid, resulting in a higher number of germinated embryos from bioreactor cultures than shaken flasks (Yang et al. 2012). The productivity of Japanese yew (*Taxus cuspidata*) somatic embryos in bubble column bioreactors also yielded higher biomass than shaken flasks (Zhong et al. 2009). Stuart et al. (1987) reported successful alfalfa somatic embryo production using air-lift bioreactors, again comparing this method to shaken flasks.

Temporary immersion bioreactors

Improving the efficiency of embryogenic tissue proliferation using bioreactor technology is one way to improve propagation via somatic embryogenesis. Increasing the germination frequency of the embryos produced by the cultures, however, is a separate issue. Temporary immersion bioreactors (TIBs) have been shown to promote somatic embryo production, as well as germination, by incorporating considerable exposure to the gas phase, as well as short durations of liquid immersion. There are a few different TIB designs, including a rocker box system, which provides liquid immersion and gas phase by gently rocking the boxes containing the tissue back and forth, and a balloon-type bubble bioreactor which incorporates an ebb and flow immersion system (Weathers et al. 2012). Another type of TIB is the RITA® bioreactor (CIRAD, France). This system is a 1-l TIB unit with an upper compartment in which the plant material is placed and a lower compartment that is filled with the culture medium. Air pressure pushes the liquid medium through the holes in the upper compartment, immersing the tissue

(Etienne-Barry et al.1999). The medium, oxygenated by air flow, then returns to the bottom of the vessel and the process is repeated over again at intervals set by the operator.

There are many examples of improved propagation using temporary immersion systems. To evaluate mass propagation in pineapple plants, three different micropropagation techniques were compared and it was found that temporary immersion bioreactors increased the multiplication rate by 300% over liquid medium in flasks and 400% over conventional solid medium (Escalona et al. 1999). Temporary immersion bioreactors also increased multiplication rates in the meristem propagation of bananas (Alvard et al. 1993). For *in vitro* axillary shoot multiplication of Eucalyptus, the use of RITAs yielded benefits not yet obtained with the standard semi-solid protocols in place. Additionally, cold-tolerant Eucalyptus clones which have been extremely difficult to multiply, have responded favorably to RITA culture (McAlister 2005).

Temporary immersion systems have also shown success in improving the potential of large-scale somatic embryo production, and subsequently germination, of different species. *Coffea canephora* clones produced a total of 4.4 million pre-germinated embryos over a three year study, with a conversion rate of 95% (Ducos et al. 2007). With an immersion frequency of 1 minute every 12 or 4 hours, 85% of *Coffea arabica* embryos achieved the torpedo-shape (Albarran 2005). Along with a high proportion of torpedo-shaped embryos, tissues that underwent these immersion frequencies showed highly organized cell aggregates undergoing active cell division when evaluated in a histological study. Compared to conventional methods, there was improved consistency and synchronized multiplication, as well as a high level of plant recovery from clonal tea (*Camellia sinensis*) somatic embryos using a temporary immersion system (Akula et al. 2000). For rubber tree (*Hevea brasiliensis*), temporary immersion resulted

in substantially more consistent and synchronized somatic embryo development, reduced the number of abnormal embryos by half and stimulated a higher germination rate compared to gelled medium (Etienne et al. 1997). Etienne-Barry et al. (1999) reported successful germination of mass-cultured *Coffea arabica* somatic embryos using RITAs. Embryos regenerated using RITA® also showed morphological quality improvements in some species. The germination of *Theobroma cacao* embryos was not only successful in TIB, but certain combinations of immersion frequency and immersion duration increased the development of a pronounced hypocotyl (Niemenak et al. 2008). Research concerning the use of temporary immersion bioreactors (TIBs) in American chestnut has been minimal to date and no such research has been published.

Glutathione and somatic embryo development

The advancement of chestnut SE via the implementation of suspension cultures has increased the efficiency of American chestnut somatic embryo production from the initial protocol (Andrade and Merkle 2005). However, issues remain with the current system including low germination frequency, which has limited the success of somatic seedling production for the species. Another issue is poor somatic seedling quality. The embryos that are able to germinate often grow into somatic seedlings with abnormal morphology. Characteristics such as crooked stems, the presence of a small, fibrous root system instead of a vigorous taproot, and short internodes are often present. Some preliminary histological work has indicated that root and shoot meristems may be incomplete or lacking in a large proportion of American chestnut somatic embryos (Kormanick and Merkle, unpublished data). Thus cultural treatments that improve meristem formation may improve American chestnut somatic embryo germination and early somatic seedling growth.

The two redox forms of endogenous and exogenous glutathione have shown potential in influencing the success and quality of somatic embryogenesis for different plant species. Glutathione is a tripeptide and antioxidant that is found in most prokaryotic and eukaryotic cells (Noctor et al. 1998). The two redox forms of the compound that exist interchangeably are: reduced, the thiol form (GSH), and oxidized, glutathione disulfide (GSSG). Until the late 1990s, the study of glutathione was limited to physiological functions such as sulfur metabolism, inter-organ sulfur allocation, and detoxification of xenobiotic compounds (Potters et al. 2002; Noctor et al. 1998; May et al. 1998). One of the most widely acknowledged functions of the compound is its involvement in defense reactions against oxidative stress, as illustrated by the fact that GSH biosynthesis is stimulated when stressful conditions are present (Foyer et al. 2001; KunMing et al. 2004; Rausch et al. 2007; Gill et al. 2013). More recent studies of both glutathione redox states have been focused on the role of the compound in the cell cycle. Treating *Arabidopsis* roots with exogenous GSH increased mitotic activity, whereas the application of a GSH biosynthesis inhibitor—DL- buthionine-[S,R]-sulfoximine (BSO)—decreased it (Potters et al. 2002). Similarly, high endogenous GSH levels were correlated with rapid cell division for white spruce (*Picea glauca*) embryogenic tissue. When exogenous GSH was added to maintenance media of white spruce, cell proliferation increased; at low doses, it was also found to have a positive effect on somatic embryo conversion frequency (Belmonte et al. 2003; Stasolla et al. 2004). Conversely, the oxidized form of glutathione, GSSG, is low during the initial stages of cell differentiation and mitotic activity, and increases during embryo maturation (Belmonte et al. 2003). Further research has revealed that the production of the hormone ethylene in white spruce is under the control of the glutathione redox state. The accumulation of ethylene is directly associated with the formation of intercellular spaces, which were blamed for meristem abortion

at germination. A high GSH:GSSG ratio increases ethylene biosynthesis, but a low GSH:GSSG ratio (an oxidized cellular environment) reduces it, thereby decreasing the formation of intercellular spaces, and improving the quality of embryo formation (Belmonte et al. 2005). Consequently, the inclusion of GSH during the first few days of somatic embryo development, followed by a treatment of GSSG, enhanced embryo quality by improving the soundness of the shoot apical meristem. Thus, there is evidence that both redox forms of glutathione are equally important in producing quality embryos, since GSH promotes early cell proliferation and GSSH then promotes organized development (Yeung et al. 2005).

Since the main issues with American chestnut SE are somatic embryo quality and subsequent germination, rather than with embryonic cell proliferation, it would seem that the application of GSH would not be necessary. Conversely, the application of exogenous GSSG could potentially both increase the oxidative environment and increase embryo yield and quality. However, both GSH and GSSG are expensive chemicals, and supplementing embryogenic cultures with GSSG would not be economically feasible given the high levels of SE production needed (Belmonte and Stasolla 2007). An alternative strategy to using oxidized glutathione is the use of a reduced glutathione biosynthesis inhibitor such as BSO. Research has shown that using BSO in replacement of both GSH and GSSG lowers the cellular reduced glutathione state, improves embryo quality, and improves conversion frequency (Belmonte et al. 2006). When added to the maintenance media of white spruce (*Picea glauca*) embryogenic tissue, 0.01 mM BSO increased the total yield of fully developed embryos (Belmonte and Stasolla 2007). The application of BSO also greatly increased the percentage of morphologically normal white spruce embryos (i.e. embryos with four or more cotyledons). Anatomical studies showed that, when compared to the control embryos, which were often characterized by elongated cells and

intercellular spaces, the treated embryos showed no signs of intercellular spaces and the sub-apical domains were composed of tightly packed cells. Applications of BSO also significantly increased conversion frequency of rapeseed (*Brassica napus*) somatic embryos compared to controls. Further investigation of the mechanisms of BSO-mediated improvement with *B. napus* showed that the compound prevented cellular deterioration in the apical poles of the embryos (Stasolla et al. 2008). Additionally, several genes involved in functions such as antioxidant response and developmental processes were differentially expressed in the BSO treated embryos.

Current research on the effects of BSO on somatic embryogenesis is limited, and no studies have been published regarding its influence on SE in any hardwood forest tree, including American chestnut. However, the benefits of BSO on somatic embryo quality and embryo conversion in other species are encouraging.

There is a need for further research in order to rectify the major issues impeding the advancement of American chestnut somatic embryogenesis for chestnut clonal propagation. Both embryo quality and percent germination and conversion must be improved if mass propagation via SE is to be a useful for the reestablishment of the American chestnut. If these problems are adequately addressed, the SE system may not only aid in the reintroduction of the American chestnut, but can also be implemented for other hardwoods that are being threatened by pathogens and insect pests.

Research Objectives

As detailed in this thesis, over three years of culture initiations, we tested the effects of CC genome proportion on the success of SE induction using our standard protocol for culturing AC and, subsequently, protocols based on a published protocol for SE in European chestnut. Additionally, to test the efficacy of bioreactors, we compared the tissue growth process of

embryogenic suspension cultures from initial suspension to maximum growth potential using air-lift bioreactors and shaken flasks and investigated germination efficiency of American chestnut somatic embryos using RITA® TIBs. Finally, in order to analyze the effects that BSO has on American chestnut embryo yield, quality, and conversion, different levels of the compound were added to maturation media and we compared the resulting embryos to those produced using the standard protocol for American chestnut SE.

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CHAPTER 2

INFLUENCE OF SPECIES AND HYBRID STATUS ON INDUCTION OF SOMATIC EMBRYOGENESIS IN *CASTANEA*

C.T. Holtz, A.R. Tull and S.A. Merkle. To be submitted to *Canadian Journal of Forest Research*

Abstract

As part of the substantial effort to re-introduce the American chestnut (*Castanea dentata*) tree to its native range in the Eastern forests of North America, significant effort has been put forth to propagate trees with blight resistance using the process of somatic embryogenesis (SE). SE is applicable for clonally propagating blight-resistant trees produced by The American Chestnut Foundation's hybrid backcross breeding program, in which AC trees are hybridized with blight-resistant Chinese chestnut (CC) trees, followed by backcrossing to AC trees. It is possible that the proportion of (CC) in the hybrid backcross material can affect the success of SE using the protocol established for AC. Over three years of culture initiations, we tested the effects of relative AC/CC genome proportion and pollination type (control- versus open-pollination) on the success of SE induction using our standard protocol for culturing AC and, subsequently, a protocol for SE in European chestnut. With our standard AC protocol, open-pollinated AC and open and control-pollinated hybrid backcross B3F3 trees (approximately 15/16 American and 1/16 Chinese), successfully produced embryogenic tissue from the SE induction. There was a significant difference in the success of both AC and B3F3 compared to CC using this protocol ($p = 0.0037$). Using the European chestnut SE protocol, open-pollinated Chinese chestnut produced embryogenic tissue and subsequently somatic embryos. The success of SE for Chinese chestnut will allow a developmental comparison of American chestnut somatic embryos with a species carrying natural resistance to *C. parasitica*. The first B3F3 somatic embryos have successfully produced plantlets and more are in production. SE shows the potential for mass clonal propagation of elite advanced generation hybrid backcross trees for restoration, as well as for timber and nut production.

Introduction

The American chestnut was one of the most economically important angiosperm forest trees in North American, and comprised 25% of the Appalachian forest up until the early 1900s. When the chestnut blight fungus (*Cryphonectria parasitica*) was introduced in the late 1800s on Asian chestnut stock, it took approximately 50 years for 3.5 million American chestnut trees to be killed off. Natural reestablishment of the tree is highly unlikely, as the blight remains an obstacle (Anagnostakis 1987; Johnson et al. 2008; Vieitez 1995).

As a part of the considerable effort to re-introduce the American chestnut to its native range, a significant amount of research has been put forth to produce and propagate American chestnut trees with blight resistance. One approach is hybridization with Asian chestnut trees which carry resistance to the fungus. Burnham (1988) suggested that crossing an American chestnut with a Chinese chestnut and then backcrossing three times to an American chestnut parent—with selection against Chinese morphological type—would result in an individual with both American chestnut characteristics and the genes from Chinese chestnut responsible for blight resistance. The American Chestnut Foundation (TACF) was established to accomplish the plan that Burnham proposed.

Although TACF has been successful in producing hybrid material with improved blight resistance (Hebard 2005), the restoration program could be significantly enhanced by the availability of a system to clonally propagate this material. One focus of current American chestnut research pertains to the issue of mass propagating blight-resistant genotypes once they are established. An *in vitro* propagation method known as somatic embryogenesis (SE) has shown the potential to contribute to restoration of the tree, both by producing trees engineered with blight-resistance genes, and by providing a method for mass propagation of blight resistant

genotypes from the conventional breeding programs (Andrade and Merkle 2005). The efficacy of SE is derived from its potential for high-frequency, large-volume clonal propagation (Carraway and Merkle 1997). Induction of SE has been attempted many times with various chestnut species and chestnut hybrids. Culture of a *C. mollissima* X *C. dentata* hybrid resulted in embryo-like protuberances, though no true embryos were reported (McPheeters et al. 1980; Skirvin 1981). Mature and immature zygotic embryos of the hybrid *C. sativa* X *C. crenata* were cultured *in vitro* and produced somatic embryos, but germination of embryos was incomplete (Vieitez et al. 1990). Vieitez et al. (1992) later reported production of plantlets from somatic embryos of the hybrid. About 100 of these chestnuts planted in soil survived and displayed normal growth patterns (Vieitez and Merkle 2005). The first case of successful SE in American chestnut was reported over 20 years ago, but no plantlets were produced in that study (Merkle et al. 1991). Subsequently, the addition of specific amino acids to the embryo development media resulted in the first regeneration of American chestnut somatic seedlings, although only a few plantlets survived hardening off (Robichaud et al. 2004). Xing et al. (1999) reported successful production of 20 American chestnut plantlets, with four still surviving at the age of two when the study was published, but it is unclear if these plantlets were true somatic seedlings. Andrade and Merkle (2005) reported plantlet production, hardening off, and successful transition to the greenhouse of approximately 100 *C. dentata* somatic seedlings.

Since a system has already been developed to clonally propagate pure American chestnut trees via SE (Carraway and Merkle, 1997; Andrade and Merkle 2005), it also has potential to be used to multiply blight-resistant trees produced by TACF's hybrid backcross breeding program. However, it is well-known that genotype exerts a strong influence in embryogenesis induction (Trolinder and Xhixian 1989; Brown et al. 1995; Park et al. 2011); therefore, it is possible that

Chinese chestnut genes in the hybrid backcross material could affect the success of SE using the protocol established for American chestnut. To date, there have been no published reports of somatic embryogenesis in either Chinese chestnut or hybrid backcross material. Over three years of culture initiations, we tested the effects of Chinese chestnut genome proportion on the success of SE induction using a protocol for culturing American chestnut and, subsequently a protocol for SE in European chestnut (Vieitez 1995).

Materials and methods

Plant material

A total of 145 different chestnut genotypes were used to assess the impact of species or hybrid genotype on the success of embryogenesis induction. As shown in Table 1, along with American chestnut (AC) and Chinese chestnut (CC), tested genotypes included the following CC x AC hybrid and backcross types: F1 (1/2 Chinese and1/2 American), B1 (3/4 American and 1/4 Chinese), B2 (7/8 American and 1/8 Chinese), and B3F3 (15/16 American and 1/16 Chinese). The American chestnut, F1, B1, B2, and B3F3 material was all collected from TACF's research farm in Meadowview, VA. When TACF was first established, there were two first backcross (BC1) individuals used as mother trees: 'Clapper' (Little and Diller 1964) and the undescribed 'Graves' (Hebard 1994). These were the most advanced crosses available and they showed blight resistance similar to that of CC by AC hybrids (Hebard 2005); all of the B3F3 material we cultured was derived from one of these two lines. The CC parent of the B2 lines was cultivar 'Nanking' and that of the B1 material was genotype Mollissima 12. In 2010 and 2011, the F1 lines were all control pollinated with either 'Nanking' (Headland and Griffen 1976) or genotype 'Vanuxem' as the CC mother tree. The three F1 lines cultured in 2012 came from three different CC sources: Orrin, Meiling, and 72-211.

The TACF protocol for pollination technique varies among the different hybrids (Hebard 2005). First hybrids and straight backcrosses are produced using control pollination techniques. Intercross generations are produced by open pollination; however, the orchards are isolated with a distance of approximately 1 km between them to prevent unwanted fertilization. Other methods utilized to prevent pollination from undesired trees include emasculation, pruning, and removal of individual trees. The F1, B1, and B2 trees from which burs were collected in 2012 were pollinated from unknown sources. The orchards for the different hybrids are separated, but potential pollen sources would include pure AC, pure CC, and hybrids and backcrosses with different proportions of AC and CC genomes. The ranges of possible Chinese/American proportions for the different hybrid seeds we cultured were as follows: F1, 3/4 CC:1/4 AC to 1/4 CC:3/4 AC; B1, 3/8 CC:5/8 AC to 1/8 CC:7/8 AC; and B2, 7/16 CC: 9/16 AC to 1/16 CC:15/16 AC. On the other hand, the B3F3 nuts that we cultured in 2012 were from control pollinations between B3F2 parents, so the range of Chinese versus American chestnut genome is more accurately predicted to be approximately 15/16 AC: 1/16 CC. The open-pollinated CC burs were collected from trees located in a planting in Athens, Georgia and were cultured in early July each year. Seeds from these trees were almost certainly pure CC, since there were no other chestnut trees located within miles of their location. Shipments of immature AC and hybrid type burs were received in the beginning of August each year; not all hybrids types were initiated in all three years of the study (Table 1).

Culture initiation

In 2010, all of the species and hybrids were cultured using the standard protocol. Briefly, burs were dissected to remove the immature nuts, which were surface-sterilized by agitation in the following solutions: 70% ethanol for 20 sec; 10% Roccal-D Plus (9.2% Didecyl dimethyl

ammonium chloride, 13.8% Alkyl dimethyl benzyl ammonium chloride, 1.0% bis-n-tributyltin oxide. Exton, PA, Div. of Pfizer Inc., New York, NY) for 1 min (these first two steps are repeated twice); 50% Clorox (8.25% sodium hypochlorite) solution for 5 min; sterile water rinse for 3 min; 0.01N HCL for 3 min, and three subsequent rinses in sterile water for 3 min each. The nuts were then dissected to remove the immature seeds for culture, following methods described previously (Carraway and Merkle 1997; Merkle et al. 1991). Immature seeds were cultured in 60 X 15-mm plastic Petri dishes on semi-solid induction-maintenance medium (IMM; Andrade and Merkle 2005), which is a modified woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with vitamins of Schenk and Hildebrandt (1972), 4.0 mg l^{-1} 2,4-D, 0.5 g l^{-1} L-glutamine (filter sterilized and added to autoclaved medium following cooling) and 3% sucrose, at pH 5.65, gelled with 3.0 g l^{-1} Phytagel gellan gum (Sigma). All cultures were transferred to fresh IMM of the same type after one month, and at the end of the second month, they were transferred to secondary medium. Standard protocol secondary medium consists of IMM with only 2.0 mg l^{-1} 2,4-D. Cultures were scored for signs of proliferation of embryogenic material after 10 weeks. The result for each seed explant was recorded, and the percentage of seeds that produced embryogenic material was calculated for each source.

Cultures in 2011 and 2012 were initiated using the same protocol as 2010 cultures, with the addition of different media treatments. In 2011, different media treatments were introduced for the CC culture initiations only (Table 2). Briefly, nuts of each CC genotype were divided into two groups, and immature seeds were cultured on either standard IMM as described above, or on IMM supplemented with 1 mg l^{-1} 2,4-D and 1 mg^{-1} of 6-benzylaminopurine (BA). After the second month, explants initially cultured on IMM were transferred to either standard secondary IMM, or IMM without 2,4-D, but including 0.1 mg l^{-1} BA and 0.05 mg l^{-1} NAA.

Explants initially cultured on IMM with 1 mg l^{-1} 2,4-D and 1 mg l^{-1} BA were transferred to either the same media, or IMM without 2,4-D, but including 0.1 mg l^{-1} BA and 0.05 mg l^{-1} NAA.

Based on the results of the 2011 initiations, only two treatments were used for initiation of all species and hybrid types in 2012 (Table 3). For all initiations, cultures were scored for signs of proliferation type after 10 weeks. Plates with seeds producing embryogenic tissue were maintained and transferred to fresh media every 3 to 4 weeks; these cultures were subsequently put through the rest of the SE system (see below) for somatic embryo and somatic seedling production. The result for each seed explant was recorded and the percentage of seeds that produced embryogenic material was calculated for each source.

Somatic embryo production

Somatic embryos were generated from proembryogenic masses (PEMs) grown in suspension cultures using the technique described by Andrade and Merkle (2005). Briefly, suspension cultures were initiated by inoculating 0.50 g of tissue into 125- ml Erlenmeyer flasks. Each vessel was filled with 30- ml of liquid IMM. Suspensions in the flasks were maintained in the dark at 25°C and aerated by shaking on a gyratory shaker at 100 rpm. Suspensions were maintained in the shaken flasks for 45 days and were fed at two week intervals by aspirating out the old media and adding 30 ml of fresh liquid media. At day 45, the tissue from each flask was fractionated using a top sieve size of 860 μm and a bottom sieve size of 140 μm. The fractionated tissue remaining in the bottom sieve was inoculated back into the flasks for 4 more days, and the flasks were filled with liquid embryo development media (EDM), which is the same as IMM, but lacking 2,4-D. At day 4, the fractionated tissue was collected onto a nylon mesh and transferred to Petri plates holding semi-solid EDM; approximately 1/10 ml of material was transferred on to each plate. After 3 to 4 weeks, the plates were checked for embryo growth.

Embryos that were 2 to 4-mm long were selected and transferred onto a new plate of the same media type. These embryos were kept in the dark and allowed to grow for 2 more weeks before given a cold pre-germination treatment, which consisted of being wrapped securely in aluminum foil, and then placed into a dark refrigerator at 8°C, for twelve weeks. Following cold treatment, the embryos were placed into GA-7 vessels (Magenta Corp.) filled with semi-solid germination medium (GM) which consists of the same ingredients as EDM but excluding the L-glutamine, and including 1 g l^{-1} activated charcoal. The GA-7 vessels were placed into an incubator with cool white fluorescent light (100 μmol m^{-2} s^{-1}) with a 16h photoperiod at 25°C. Final germination and conversion data were collected after three months.

Statistical analysis for this data was performed using SAS (SAS Institute Inc. 2011). For all cultures initiated with the standard protocol from 2010 through 2012, an analysis of variance combined for all three years was conducted. Additionally, all hybrid types as well as the AC and CC cultures were compared from 2011 and 2012, using induction data from both the standard protocol and experimental treatment 4. All proportion data was arcsine transformed. The type of test run for this analysis was a two-way factorial design with fixed effects.

Results and Discussion

In 2010, no embryogenesis was induced from either the CC or the F1 seeds. When the Chinese chestnut cultures were scored at 10 weeks, some of the tissue proliferation resembled American chestnut embryogenic tissue. However, after transferring it a third time, the tissue was found to be non-morphogenic (Fig. 2.1a-c). Successful embryogenesis in these experiments is defined as the production of repetitively embryogenic tissue. Both B3F3 and American chestnut seed explants produced embryogenic tissue and subsequently somatic embryos (Fig. 2.1 d-e).

Induction frequencies for the B3F3 and American chestnut initiations were 0.99% and 2.12% respectively (Fig. 2.2a).

Research conducted on the phylogeny of *Castanea* indicates that the genus originated in Asia, migrated west to Europe, and then to North America (Lang et al. 2007). Based on this information, we theorized that CC is probably more closely related to European chestnut (*Castanea sativa*) than AC and that embryogenesis induction protocols based on a report for European chestnut by Vieitez (1995) might be successful for CC embryogenesis induction. The tissue culture protocol for European chestnut initiations differs from the AC protocol in that a different auxin, naphthaleneacetic acid (NAA), is used instead of 2,4-D, and the addition of a cytokinin, N_6-benzyladenine (BA). In 2011, three different experimental media treatments that incorporated BA and NAA were used, along with the American chestnut standard protocol, for the Chinese chestnut culture initiations (Table 2.1). Using the AC standard protocol, CC again had zero percent induction, but both American chestnut and B3F3 explants again produced embryogenic cultures, with induction frequencies of 1.53% and 1.65%, respectively (Fig. 2.2b). Using the experimental media treatments, CC cultures initiated with treatment 4 (primary medium, IMM supplemented with 1 mg l^{-1} 2,4-D and 1 mg^{-1} of BA and secondary medium, 0.1 mg l^{-1} BA and 0.05 mg l^{-1} NAA) produced embryogenic tissue with an induction frequency of 1.25% (Fig. 2.2). These cultures subsequently produced somatic embryos (Fig. 2.3a-d).

To further investigate the theory that the amount of CC genome proportion has an impact on the success of somatic embryogenesis using the standard AC protocol, in 2012 we cultured all species and hybrids that were made available to us from the TACF breeding program using the successful treatment (treatment 4) from the 2011 CC initiations and the standard AC protocol. Because initiation of large numbers of AC and B3F3 cultures was the objective of

another project in 2012, the seed explant sample sizes for these two groups were much larger than for the other species and hybrids. The sample sizes for each species and hybrid were as follows: AC, 3911; B3F3, 4934; B2, 1022; B1, 643; F1, 530; CC, 410. With the standard AC protocol, induction frequencies for AC and B3F3 were 2.4% and 0.54%, respectively (Fig. 2.2c). The B2 hybrid also produced embryogenic tissue at 1.53% using the standard AC culture regime. Although this percentage suggests that B2 had a similar induction frequency to AC and B3F3, the sample size for B2 was much smaller, as stated above. Along with B2 hybrid, the F1 hybrid also produced embryogenic tissue using the standard AC protocol, but the tissue was not repetitively embryogenic. The B2 cultures were repetitively embryogenic and these cultures were capable of producing mature somatic embryos (Fig. 2.4). Neither the B1 nor CC cultures produced embryogenic tissue using the standard protocol, but embryogenic cultures were again induced from CC explants using the experimental protocol. With the experimental treatment, one of the B2 cultures produced one flush of embryos directly from the cultured explant, but the embryos were not repetitive and therefore the culture could not be maintained. Cultures initiated with the experimental treatment had the following induction percentages: AC, 0; B3F3, 0; B2, 0; B1, 0; F1, 0; CC, 2.50 (Fig. 2.2).

The results of the statistical analyses for the combined data from all three years using the standard protocol indicated that there were significant differences in embryogenesis induction frequencies among the species and hybrids ($P=0.0037$; $F=3.73$; $DF=5$). Tukey's test revealed that embryogenesis induction frequencies for AC and B3F3 were not significantly different from each other, but both were significantly higher than CC. When all hybrid types as well as the AC and CC cultures were compared, using induction data from both the standard protocol and experimental treatment 4, analysis of variance results indicated significant

differences ($P<0.001$; $F=6.81$; $DF=4$) among species and hybrids, and that the interaction between species/hybrid and media treatment was also significant ($P=0.0221$; $F=2.90$; $DF=4$). The results of these analyses suggest that the proportion of Chinese chestnut genome does have an effect on the induction of somatic embryogenesis using the standard protocol for American chestnut. The significant interaction between species/hybrid and treatment is due to the fact that the standard SE protocol works for pure American chestnut, and B3F3 and B2 hybrids, but not Chinese chestnut, or the B1 or F1 hybrids, while the European chestnut SE protocol (our experimental treatment 4) was successful for Chinese chestnut, but not for other Chinese x American hybrids. It is unclear, without further research, what is causing the proportion of Chinese chestnut genome to make a difference, but it is possible that Chinese chestnuts have either different alleles or, more likely, differences in gene expression, giving higher endogenous levels of auxin and/or lower levels of cytokinin, thereby creating the need for a medium with both lower auxin content and the addition of a cytokinin for embryogenesis induction.

One important conclusion provided by the culture initiation experiment was that there is substantial evidence that the standard protocol for initiating embryogenic cultures from American chestnut seeds can be applied for initiating embryogenic cultures of B3F3 hybrids, thereby providing the potential to clonally propagate the most desirable blight-resistant hybrid backcross material from conventional chestnut breeding programs. However, the standard protocol for inducing somatic embryogenesis in American chestnut was not successful for Chinese chestnut, or for F1, B1, or B2 hybrids. Thus, it appears that there may be some threshold of Chinese chestnut genome proportion, beyond which the standard AC SE induction protocol is not effective. Subsequent research testing alternative SE protocols by introducing a medium with both a lower auxin potency (NAA instead of 2,4-D), and supplemented with

cytokinin (BA) led to the successful production of somatic embryos from Chinese chestnut and non-repetitive embryogenic cultures from B2 and F1 cultures.

We believe that clonal propagation of the best hybrid backcross chestnut material will play an important role in the program for chestnut restoration. Thus, one important goal of the research presented here was to address possible issues with the propagation of material from TACF's backcross breeding program using the successful protocol already established for the production of American chestnut plantlets. While we demonstrated that the protocol developed for American chestnut is applicable to B3F3 material, as well as other hybrid chestnuts, it is essential that continued progress be made in the improvement of the chestnut somatic embryogenesis system if it is to be applied on an operational level for TACF's restoration program.

Acknowledgements

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Table 2.1. Species and hybrid types cultured each year of the experiment

2010	2011	2012
American chestnut	American chestnut	American chestnut*
B3F3	B3F3	B3F3*
F1	Chinese chestnut*	B2*
Chinese chestnut		B1*
		F1*
		Chinese chestnut*

* Species and hybrids cultured using both the standard protocol and experimental treatment

Table 2.2. Treatments used for Chinese chestnut culture initiations in 2011. Treatment 1 is the standard protocol for American chestnut culture initiations.

Primary medium	Secondary medium
1. WPM with 4 mg/l 2,4-D	WPM with 2 MG/L 2,4-D
2. WPM with 4 mg/l 2,4-D	WPM with 0.1 mg/l BAP and 0.05 mg/l NAA
3. WPM with 1 mg/l 2,4-D and 1mg/l BAP	WPM with 1 mg/l 2,4-D and 1 mg/l BAP
4.* WPM with 1 mg/l 2,4-D and 1mg/l BAP	**WPM with 0.1 mg/l BAP and 0.05 mg/l NAA**

*Treatment number 4 was the successful protocol for Chinese chestnut

Figures 2.1- 2.4

Fig. 2.1. Tissue proliferation and somatic embryo production from 2010 culture initiations. **a**. Chinese chestnut tissue looking comparatively embryogenic. Scale bar = 1mm. **b**. Embryogenic American chestnut tissue. Scale bar = 1mm. **c**. Non-morphogenic Chinese chestnut tissue. Scale bar = 1mm. **d**. Embryogenic B3F3 tissue. Scale bar = 1mm. **e**. B3F3 somatic embryos. Scale bar = 1mm.

Fig. 2.2a-c: Induction frequency for all species and hybrids cultured with the standard and experimental protocol in **a**. 2010 **b**. 2011 and **c**. 2012 (Chinese chestnut was the only species/hybrid to be cultured with the experimental protocol in 2011).

Fig. 2.3: 2011 Chinese chestnut culture initiations. **a-b**. embryogenic proliferation from CC cultures. **c-d**. Chinese chestnut somatic embryos.

Fig. 2.4: Repetitive B2 somatic embryos.

Fig. 2.1

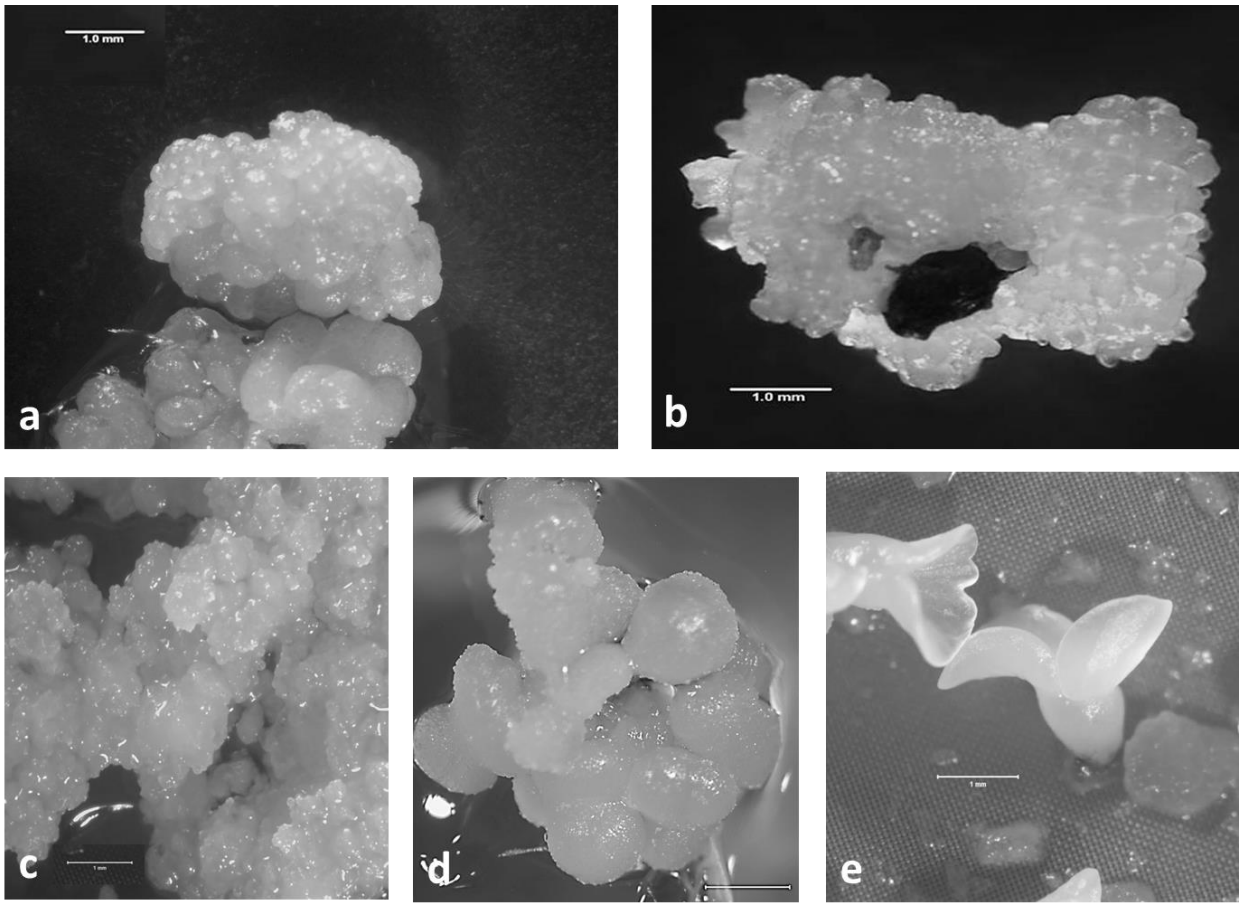
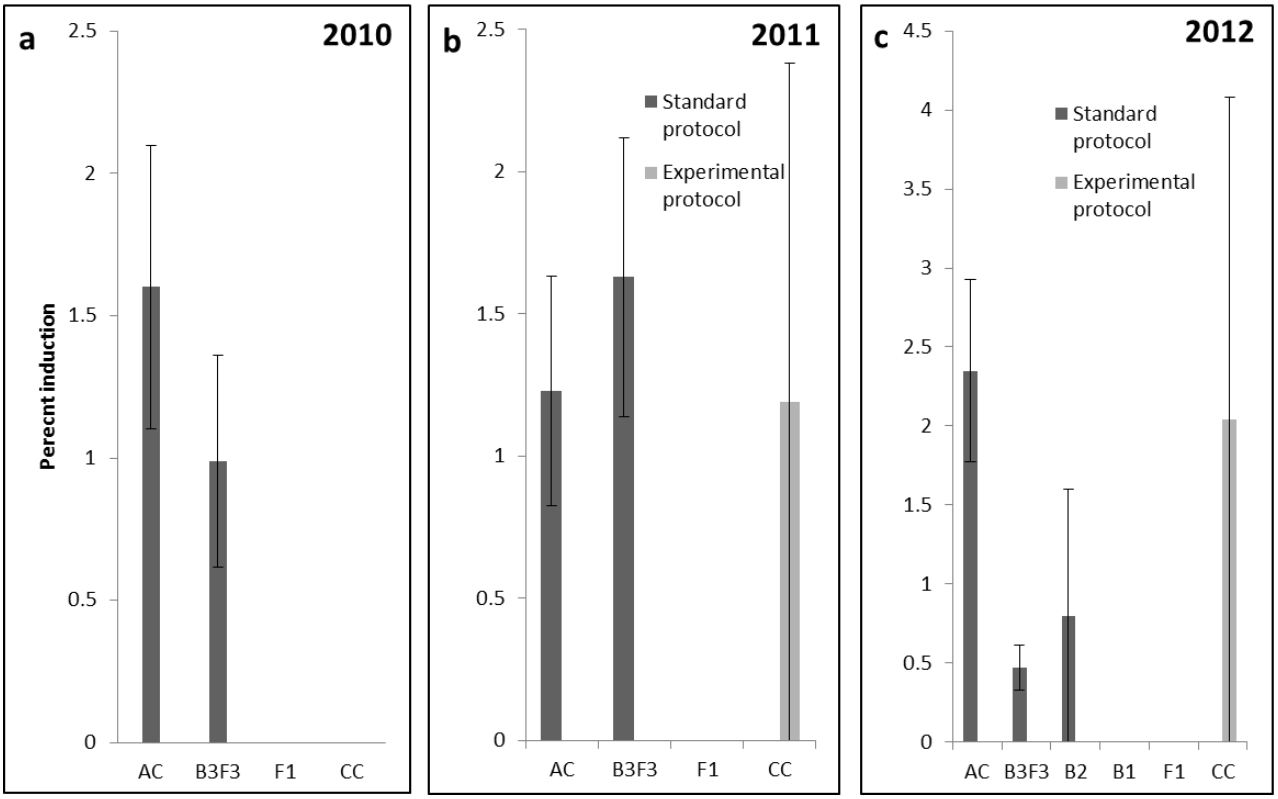


Fig. 2.2a-c


a
2010
Perecnt induction
2.5
2
1.5
1
0.5
0
AC
B3F3
F1
CC
b
2011
Standard protocol
Experimental protocol
2.5
2
1.5
1
0.5
0
AC
B3F3
F1
CC
c
2012
Standard protocol
Experimental protocol
4.5
4
3.5
3
2.5
2
1.5
1
0.5
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AC
B3F3
B2
B1
F1
CC

Fig. 2.3

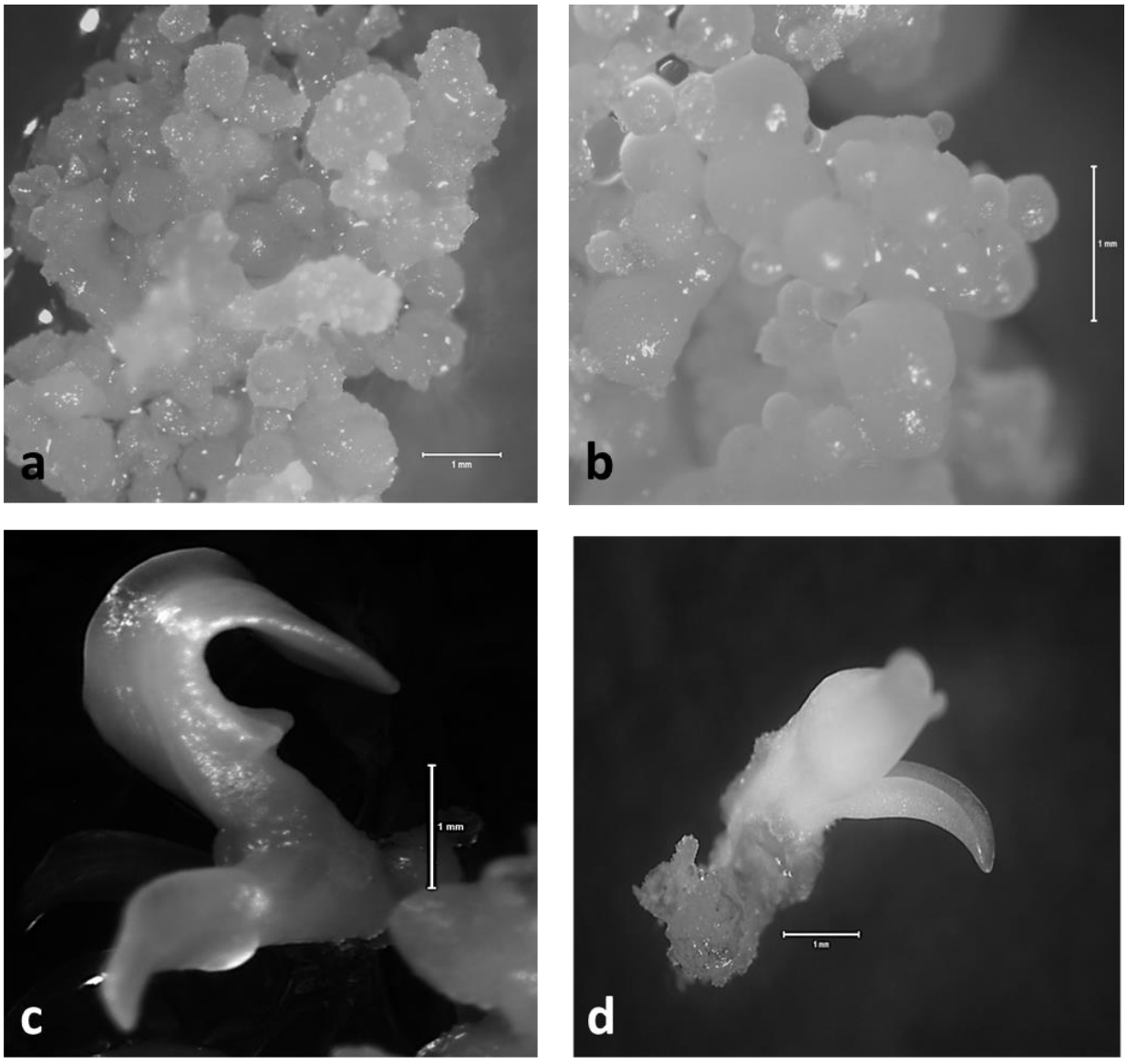
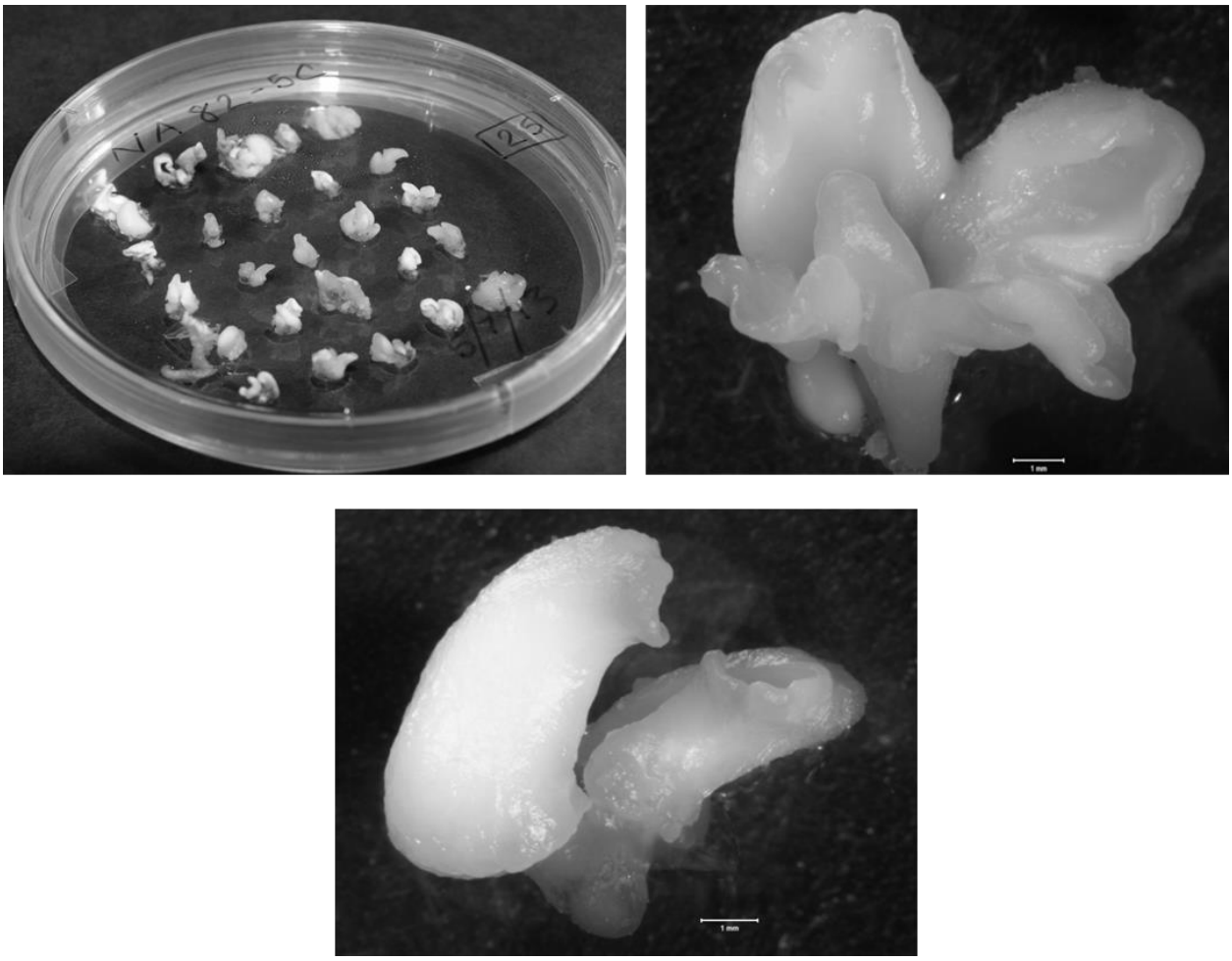


Fig. 2.4



CHAPTER 3

THE USE OF TEMPORARY IMMERSION AND AIR-LIFT BIOREACTORS TO ENHANCE THE AMERICAN CHESTNUT SOMATIC EMBRYOGENESIS SYSTEM

Holtz, C.T., Kong, L., Merkle, S.A. To be submitted to *Plant Tissue and Organ Culture*

Abstract

Somatic embryogenesis (SE) has been successfully applied for clonal propagation of American chestnut trees, including transgenic trees engineered with genes potentially conferring resistance to chestnut blight. Growing embryogenic chestnut cultures in suspension culture has improved the efficiency of embryogenic tissue proliferation over semi-sold medium, but issues remain with inefficient proliferation and low somatic embryo germination rates. The standard method to maintain suspension cultures is shaken flasks, however, air-lift and temporary immersion bioreactors (TIB) have been used to enhance the productivity of embryogenic cultures of multiple species. With the goal of improving the productivity of the American chestnut SE system, we tested two different types of bioreactors on two different steps in the SE process. First, we tested the effects of air-lift bioreactors on embryogenic tissue proliferation, comparing this method to shaken flasks. Second we conducted an experiment evaluating the efficiency of RITA® TIBs in increasing the proportion of somatic embryos that germinate, comparing this to the standard method of germination on semisolid medium in GA7s. Proliferation rates in air-lift bioreactors showed significant differences from shaken flasks and percent growth was higher in bioreactors by as much as 60%. RITA TIBs show potential to increase germination and conversion rates, although no statistical analysis was run due to insufficient sample size.

Introduction

The American chestnut was one of the most economically important angiosperm forest trees in North American, and comprised up to 25 percent of the Appalachian forest up until the early 1900s. When the chestnut blight fungus (*Cryphonectria parasitica*) was introduced in the late 1800s on Asian chestnut stock, it took approximately 50 years for 3.5 million American chestnut trees to be killed off. Natural reestablishment of the tree is highly unlikely, as the blight remains an obstacle (Anagnostakis 1987; Johnson et al. 2008; Vieitez 1995).

As a part of the considerable effort to re-introduce the American chestnut to its native range, a significant amount of research has been put forth to produce and propagate American chestnut trees with blight resistance using back-cross breeding, genetic transformation, and somatic embryogenesis (SE) (Andrade and Merkle 2005; Hebard 2005). The initial published protocol for American chestnut SE called for the cultures to be maintained continuously on semi-solid media from initial tissue explant culture through somatic embryo development, maturation, and germination (Merkle et al. 1991). Changing the SE procedure to suspending the cultures in liquid medium in shaken flasks for proliferation yielded much higher somatic embryo and somatic seedling production rates than had been previously reported (Andrade and Merkle 2005).

Although it has been established that the use of suspension cultures has facilitated chestnut somatic seedling production, the process still fails to produce sufficient germinable embryos for mass propagation (Johnson et al. 2008). Different methods of suspension culture, such as the use of bioreactors, are showing potential in scaling-up production efficiency beyond what has been accomplished using shaken flasks.

Although shaken flasks are essentially bioreactors, there are a few significant differences between them and automated bioreactors when used for somatic embryo production in

suspension culture. Advantages of using bioreactors over flasks include the potential to apply increased working volume, maintenance of a homogenous culture by mechanical or air-lift stirring, and control of the cultural and physical environment for the cultures—such as pH, dissolved oxygen and carbon dioxide (Ammirato and Styer 1985; Tautorus et al. 1992).

The three main methods of aeration in a bioreactor system include: air-lift, propeller, and magnetic bar stirring (Nishimura et al. 1993). Because air-lift (or bubble-column) bioreactors do not contain any type of stirring blade, they are the most common type of bioreactor used for the growth of shear-sensitive cells, such as plant cells (Allman 2012). The main advantage that results from the absence of a propeller or stir bar is that the aeration is distributed with approximate homogeneity throughout the bioreactor, as opposed to the uneven dissipation of energy that results from distribution via a mechanical device (Merchuk 1990). In a study of carrot (*Daucus carota* L.) somatic embryo development, Teng et al. (1994) compared three different types of suspension culture: spinner flask, air-lift bioreactor, and a screen column bioreactor that was aerated with a two-blade propeller. The air-lift bioreactor showed the highest biomass production. When the productivity of Siberian ginseng (*Eleutherococcus senticosus*) SEs was compared in bubble column bioreactors and shaken flasks, the bioreactors yielded higher productivity and secondary SE induction was more rapid, resulting in a higher number of germinated embryos from bioreactor cultures than shaken flasks (Yang et al. 2012). Growing Japanese yew (*Taxus cuspidata*) somatic embryos in bubble column bioreactors also yielded higher biomass than shaken flasks (Zhong et al. 2009). Stuart et al. (1987) reported successful alfalfa somatic embryo production using air-lift bioreactors, again comparing this method to shaken flasks.

Temporary immersion bioreactors

Improving the efficiency of embryogenic tissue proliferation using bioreactor technology is one way to improve propagation via somatic embryogenesis. Increasing the germination frequency of the embryos produced by the cultures, however, is a separate issue. Temporary immersion bioreactors (TIBs) have been shown to promote somatic embryo production, as well as germination, by incorporating increased exposure to the gas phase as well as short durations of liquid immersion. Different TIB designs include a rocker box system, which provides liquid immersion and gas phase by gently rocking the boxes containing the tissue back and forth, and a balloon-type bubble bioreactor which incorporates an ebb and flow immersion system (Weathers et al. 2012). Another type of TIB is the RITA® bioreactor (CIRAD, France). This system is a 1-l TIB unit with an upper compartment in which the plant material is placed and a lower compartment that is filled with the culture medium. Air pressure pushes the liquid medium through the holes in the upper compartment, immersing the tissue (Etienne-Barry et al.1999). After a timed period, the medium, oxygenated by air flow, is allowed to return to the bottom of the vessel and the process is repeated at intervals set by the operator.

Temporary immersion systems have shown success in improving the potential of large-scale somatic embryo production, and subsequently germination, of different species. *Coffea canephora* clones produced a total of 4.4 million pre-germinated embryos over a three year study, with a conversion rate of 95% (Ducos et al. 2007). With an immersion frequency of 1 minute every 12 or 4 hours, 85% of *Coffea arabica* embryos achieved the torpedo-shape (Albarran 2005). Along with a high proportion of torpedo-shaped embryos, tissues that underwent these immersion frequencies showed highly organized cell aggregates undergoing active cell division when evaluated in a histological study. Compared to conventional methods,

TIBs improved consistency and synchronized multiplication, and gave higher levels of plant recovery for tea (*Camellia sinensis*) somatic embryos (Akula et al. 2000). For rubber tree (*Hevea brasiliensis*), temporary immersion resulted in substantially more consistent and synchronized somatic embryo development, reduced the number of abnormal embryos by half, and stimulated a higher germination rate compared to gelled medium (Etienne et al. 1997). Etienne-Barry et al. (1999) reported successful germination of mass-cultured *Coffea arabica* somatic embryos using RITAs. Embryos regenerated using RITA TIBs also showed morphological quality improvements in some species. The germination of *Theobroma cacao* embryos was not only successful in TIBs, but certain combinations of immersion frequency and immersion duration increased the development of a pronounced hypocotyl (Niemenak et al. 2008).

To test the efficacy of bioreactors for the American chestnut SE process, we compared the growth process of embryogenic suspension cultures using air-lift bioreactors and shaken flasks. We also investigated the effect RITA TIBs on germination efficiency of American chestnut somatic embryos.

Materials and methods

Culture initiation

American chestnut (*Castanea dentata*) embryogenic cultures were initiated from immature seeds as described in Merkle et al. (1991). Briefly, burs were dissected to remove the immature nuts, which were surface-sterilized using the following sequence: 70% ethanol for 20 sec; 10% Roccal-D Plus (9.2% Didecyl dimethyl ammonium chloride, 13.8% Alkyl dimethyl benzyl ammonium chloride, 1.0% bis-n-tributyltin oxide. Exton, PA, Div. of Pfizer Inc., New York, NY) for 1 min (these first two steps are repeated twice); 50% Clorox (8.25% sodium

hypochlorite) for 5 min; sterile water rinse for 3 min; 0.01N HCL for 3 min, and three subsequent rinses in sterile water for 3 min each. The nuts were then dissected to remove the immature seeds for culture, following methods described previously (Carraway and Merkle 1997; Merkle et al. 1991). Immature seeds were cultured in 60 X 15-mm plastic Petri dishes on semi-solid induction-maintenance medium (IMM; Andrade and Merkle 2005), which is a modified woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with vitamins of Schenk and Hildebrandt (1972), 4.0 mg l^{-1} 2,4-D, 0.5 g l^{-1} L-glutamine (filter sterilized and added to autoclaved medium following cooling) and 3% sucrose, at pH 5.65, gelled with 3.0 g l^{-1} Phytagel gellan gum (Sigma). All cultures were transferred to fresh IMM of the same type after one month, and at the end of the second month, they were transferred to the same medium, but with only 2.0 mg l^{-1} 2,4-D. Embryogenic cultures were maintained by transfer to fresh IMM with 2 mg/l 2,4-D every three weeks.

Testing culture proliferation in air-lift bioreactors

Three American chestnut embryogenic culture genotypes (WB484-3, AM54-1, and CD322-21) were utilized to compare growth rates in suspension culture between shaken flasks and air-lift bioreactors. The flask and bioreactor arrangement can be viewed in Fig. 3.1. Each genotype was cultured in three shaken 250 ml flasks and three bioreactors. Each vessel was filled with 100-ml of liquid IMM and then inoculated with 0.50 g of embryogenic tissue.

The air-lift bioreactors were constructed using 250 ml Pyrex bottles with 0.20 μm filter heads, and aerated using a Gast 2.35 amp (115 v) vacuum pump (Rankin Biomedical Corporation, Holly, MI). The bioreactors were kept at a 45º angle by laying them on an angled shelf with inserts to fit the individual bottles. The configuration of the air-lift bioreactor system is illustrated in Fig. 3.2. Air was first pumped through a bottle containing deionized water to

humidify it before entering the bioreactors, thus slowing evaporation of the liquid media in the bioreactors. The moist air was then pumped into the bioreactors to aerate them and to create sufficient agitation to break up the cell clumps. Suspensions in the bioreactors were aerated at 100-120 ml min^{-1}. Suspensions in the flasks were maintained by shaking on a gyratory shaker at 100 rpm.

All treatment units were maintained in the dark at 25° C and fed at two week intervals by pouring out the old media and adding 100 ml of fresh liquid media. Tissue volume measurements were performed each week for each treatment unit, under sterile conditions. Settled cell volume (SCV) for both flasks and bioreactors were measured in four steps. First, most of the liquid medium was extracted and temporarily stored in an extra, sterile flask. The tissue and remaining liquid was then poured into a graduated cylinder and allowed to settle for 3 minutes; the SCV was then recorded. The contents of the graduated cylinder were then poured back into the treatment unit. Finally, the liquid medium that was set aside was added back to the treatment unit. After every measurement, the values were recorded to track the growth progress. Measurements were taken once a week for seven weeks and the experiment was replicated twice. Data from both replications was combined and analyzed using the SAS statistical analysis software (SAS Institute Inc. 2011). Analysis of variance (PROC GLM of SAS) was used to test the effect of vessel type on proliferation efficiency.

Testing temporary immersion for somatic seedling production

Plant material

American chestnut somatic embryos were generated from proembryogenic masses (PEM) grown in suspension cultures using the technique described by Andrade and Merkle (2005). Briefly, suspension cultures were initiated by inoculating 0.50 g of tissue into 125-ml Erlenmeyer flasks

containing 30 ml of liquid IMM. Suspensions were maintained in the dark at 25°C on a gyratory shaker at 100 rpm. The tissue was maintained in the flasks for 45 days and fed at two week intervals by aspirating out the old medium and adding 30 ml of fresh liquid medium. At day 45, the tissue from each flask was size fractionated using stainless steel CELLECTOR sieves (Bellco Glass), as described previously by Andrade and Merkle (2005). Briefly, suspensions were poured through nested sieves with pore sizes of 860 μm and 140 μm, such that cell clumps with diameters between the two pore sizes were collected on the 140-μm sieve. PEMs collected on the 140-μm sieve were backwashed from the sieve with liquid embryo development medium (EDM; Andrade and Merkle 2005) using a pipette, and collected on Nitex nylon mesh (30 μm pore size; Sefar America, Depew, N.Y.) using a Büchner funnel under mild vacuum. The nylon mesh with the tissue was then transferred to semi-solid EDM in a 100 mm plastic Petri plate; approximately 10 ml of material (SCV) was cultured on each plate. Cultures were incubated in the dark at 24°C. Plates were checked for embryo development after 3-4 weeks. Embryos that were 2 to 4 mm long were transferred to a fresh plate of EDM and incubated in the dark for 2 weeks prior to being placed into pre-germination cold treatment. Plates were wrapped securely in aluminum foil, and placed into a dark refrigerator at 8° C, for twelve weeks.

Germination in temporary immersion bioreactors

Three transgenic American chestnut lines (RMxTH-22B, AxW3-46B, and WB484-3) were used to test germination potential using the standard method of semi-solid germination medium (GM; Andrade and Merkle 2005) in GA7 vessels versus liquid GM in the RITA TIBs. Briefly, plant material was placed in the upper compartment and the lower compartment was filled with the liquid media. At set immersion frequencies, controlled by a timer, the pump turned on and air pressure pushed the liquid through the holes in the upper compartment,

immersing the tissue. When the pump shut off, the medium, oxygenated by air flow, returned to the bottom of the vessel. Figs. 3.3a and 3.3b show an assembled RITA and plant material in the upper compartment. A Tetra Whisper 60 gallon aquarium air pump (Spectrum Brands Inc. Blacksburg VA) was used to aerate the system in this experiment.

Following removal from cold treatment, somatic embryos from each of the three lines were divided evenly among three RITA bioreactors and three GA7 vessels, for a total of nine bioreactors and nine GA7 vessels. The GA7 vessels were filled with 100 ml of semi-solid GM, and the TIBs were filled with 200 ml of liquid GM. Semi-solid GM included 0.5 g/L activated charcoal, while liquid GM lacked the activated charcoal. Each treatment unit contained 13 embryos. The immersion frequency for the TIBs was once every twelve hours and the immersion duration was 1 min. All treatment units were maintained at 25°C under cool white fluorescent light (100 μmol m^{-2} s^{-1}) with a 16h photoperiod. The TIBs were fed every other week by pouring the spent medium out of the reservoir and pouring fresh medium in. After 6 weeks embryos in the GA7 vessels and the RITAs were scored for germination (radicle elongation) and conversion (production of both roots and shoots).

Results and Discussion

Culture proliferation in air-lift bioreactors

Data from both replications of the experiment were combined for analysis. Following an initial lag, embryogenic chestnut tissue proliferation was significantly higher in air-lift bioreactors than in shaken flasks. Figure 3.4 shows the comparison of tissue volume for each week starting at week 0 and ending at week 7. All genotypes were combined in the average volume for each vessel. At the first, second, and third weeks of growth, tissue in the flasks yielded approximately 21%, 11%, and 2% more volume than bioreactors respectively. Analysis

of variance using repeated measures showed that the comparison of growth between weeks three and four indicated a significant event took place (p=0.029; F=6.02; DF=1). The growth in these weeks went from 2% higher growth in flasks to 22% higher growth in bioreactors.

The repeated measure ANOVA also showed that throughout the experiment there was a significant difference in vessel type over time (p= 0.0004, F=6.70, and DF= 6). ANOVA was also performed for each individual week of the experiment to assess the significance of vessel type at each point. At weeks four, five, six, and seven, the growth in the bioreactors was significantly higher than the growth in flasks with p-values of 0.07 (F=3.59; DF=1), 0.01 (F=7.57; DF= 1), 0.001 (F=14.97; DF=1), and 0.03 (F=5.80; DF=1), respectively. The difference in yield between the bioreactors and flasks continued to increase each week from the first week that bioreactor tissue volume initially surpassed that of the flasks. By weeks six and seven, bioreactor tissue had grown to the point where more media and more space were needed to sustain it, whereas the flasks still had a low enough density to continue growing (Fig. 3.5). If the experiment had continued beyond these weeks, the tissue would have been transferred to larger vessels and more media would have been added, to allow continued growth.

Germination in temporary immersion bioreactors

Comparison of somatic embryo germination in GA-7 vessels and RITAs provided some evidence that the RITA temporary immersion bioreactors could improve germination rates, although the sample size was too small to be analyzed statistically. For genotype WB484-3, six embryos in the RITAs produced shoots, although no roots were produced, so conversion did not take place. For genotype AxW3-46B, two embryos in the RITA and two embryos in the GA-7s produced shoots. Additionally, five embryos in the GA-7s and 3 embryos in the RITAs produced roots; however, only one embryo from a RITA converted. Genotype RxTH-22B

produced no roots or shoots in either the GA7s or RITAs. This particular genotype has an extremely low success rate for germination and conversion in the standard somatic embryogenesis protocol implemented in our lab, so these results were not surprising. Fig. 3.6 and Fig. 3.7 show the shoot growth in the RITAs and the one GA-7. A small preliminary germination experiment conducted with genotypes AM54-1, WB484-3, CD322-21 showed RITA conversion rates of 6%, 30%, and 5% higher than the rates in the GA7s, respectively. Both of these experiments suggest that there is future potential for RITA TIBs to positively influence the germination rate of American chestnut somatic embryos. Further studies should be conducted to assess the full capability of RITAs in the American chestnut somatic embryogenesis process.

The comparison of suspension culture techniques showed that the use of air-lift bioreactors had a significant, positive impact on the growth efficiency of embryogenic chestnut cultures compared to shaken flasks. Because of the effectiveness of bioreactors in increasing output of tissue volume in this study, this method of suspension culture should be applicable to help advance the use of SE for the mass propagation of both transgenic and hybrid backcross American chestnut with blight resistance genes. Additionally, RITA TIBs should be the subject of further American chestnut SE studies to assess their capability to enhance the SE germination process.

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Figures 3.1-3.7

Fig. 3.1a-b: Vessel types for the proliferation experiment. **a**. 250 ml Erlenmeyer flasks on a gyratory shaker. **b**. Air-lift bioreactors.

Fig. 3.2: Air-lift bioreactor configuration for the growth of embryogenic American chestnut tissue.

Fig. 3.3a-b: **a**. RITA temporary immersion bioreactor set-up. **b**. Plant material in the upper compartment of the TIB and medium in the lower compartment.

Fig. 3.4: Effect of vessel type (bioreactor vs. flask) on tissue growth rate for all genotypes combined starting at week 0 and ending at week 7. Values are the means of three vessels and bars represent standard error. Bars with different letters are significantly different.

Fig. 3.5a-b: Comparison of tissue density after several weeks of the proliferation experiment between **a**. Flasks **b**. Air-lift bioreactors.

Fig. 3.6: Somatic embryo shoot growth in RITA TIBs

Fig. 3.7: Somatic embryo shoot growth in GA7s.

Fig. 3.1

Fig. 3.2

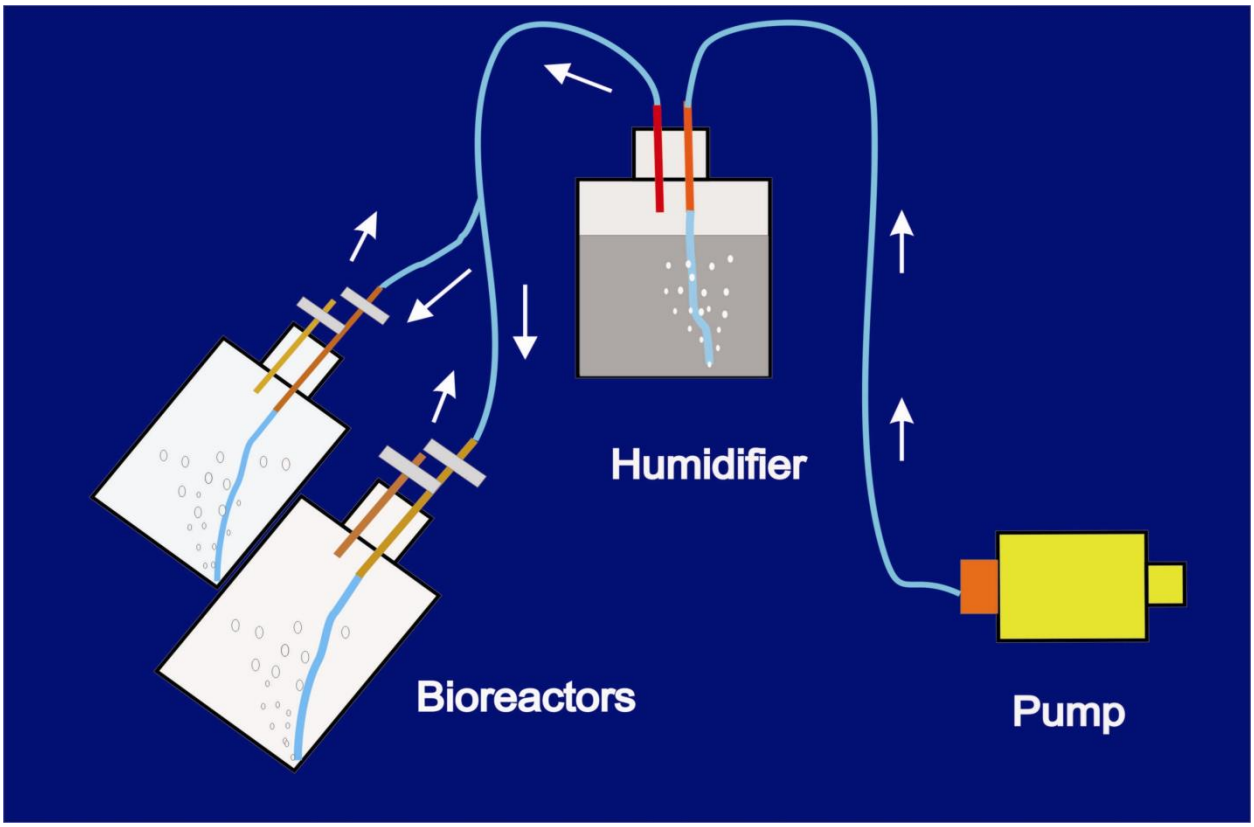


Fig. 3.3

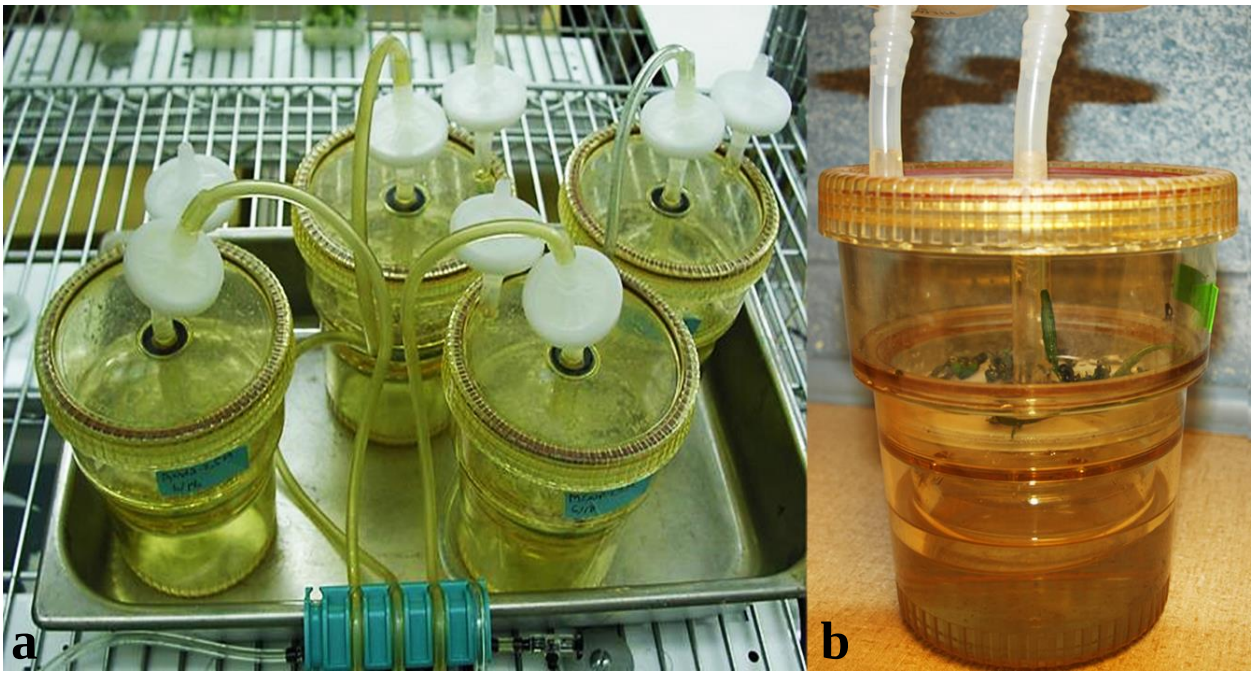
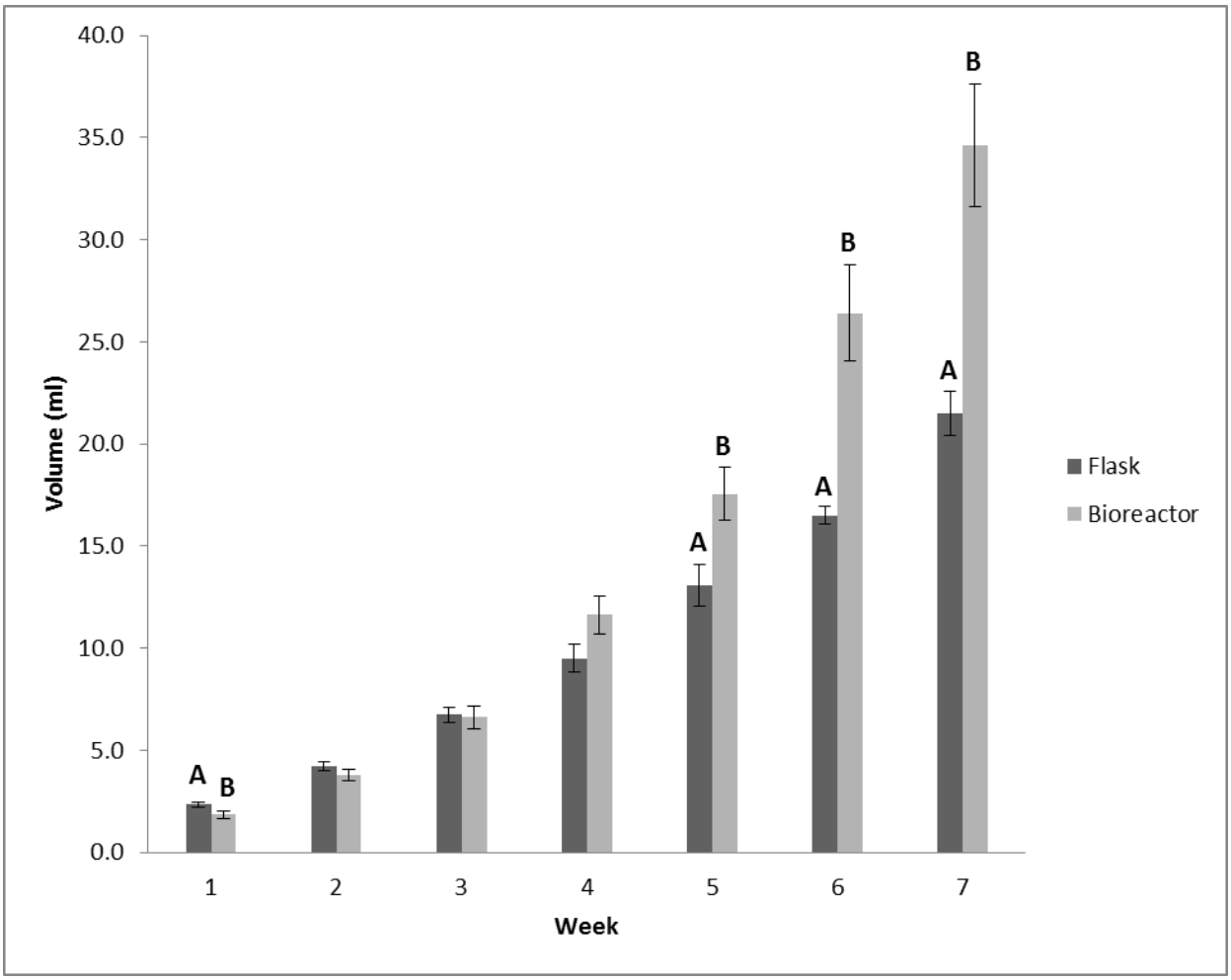


Fig. 3.4


40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Volume (ml)
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B
A
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B
1
2
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4
5
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Week
Flask
Bioreactor

Fig. 3.5

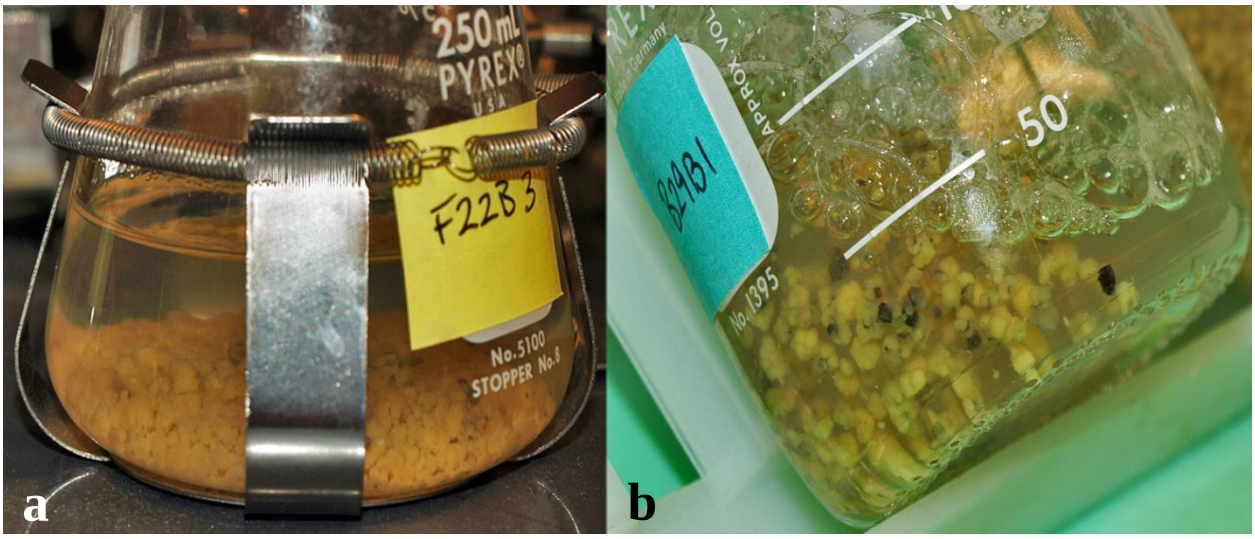
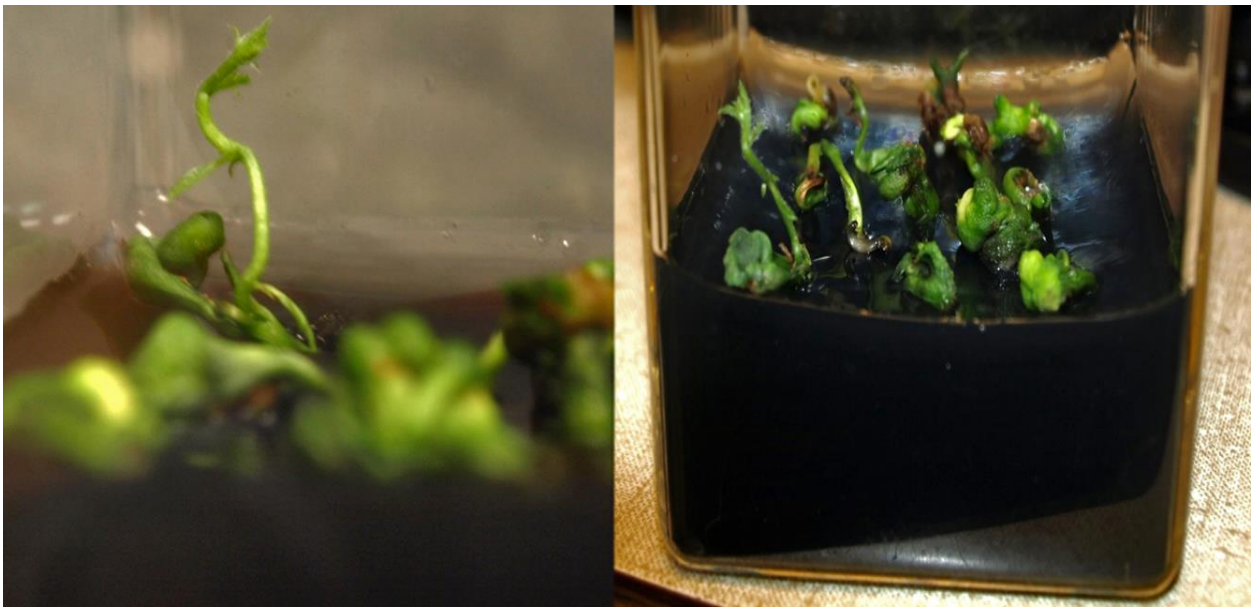


Fig. 3.6

Fig. 3.7



CHAPTER 4

EFFECT OF DL-BUTHIONINE–[S,R]-SULFOXIMINE (BSO) ON AMERICAN CHESTNUT SOMATIC EMBRYO QUALITY AND A HISTOLOGICAL ASSESSMENT OF SOMATIC EMBRYOGENESIS AND CONVERSION

Holtz, C.H., Wetzstein, H.Y., Merkle, S.A. To Be Submitted to *The Canadian Journal of Forest Research*

Abstract

As part of the effort to re-store the American chestnut tree to its native range in the Eastern forests of North America, significant effort has been put forth to propagate trees with blight resistance using the process of somatic embryogenesis (SE). Application of SE to American chestnut has been successful in producing clonal populations of many different genotypes and has the potential to aid in the reintroduction of this dominant forest tree to the wild. However, issues in the current SE system include low somatic embryo germination and conversion rates. Some preliminary histological work indicated that root and shoot meristems may be incomplete or lacking in a large proportion of American chestnut somatic embryos. Thus, cultural treatments that improve meristem formation may improve American chestnut somatic embryo germination and early somatic seedling growth. DL- buthionine-[S,R]-sulfoximine (BSO) is a reduced glutathione inhibitor that has shown potential in improving somatic embryo quality for both white spruce (*Picea glauca*) and rapeseed (*Brassica napus*). For experiments with American chestnut somatic embryos, nine different media treatments were compared to assess if BSO improved meristem integrity and subsequent germination and conversion. Analysis of variance results indicated that none of the tested BSO treatments had a significant effect on embryo yield or embryo quality; however, there were some significant differences in these variables among genotypes. Histological analyses of the somatic embryos also gave some insight into the structural quality and chemical make-up of American chestnut somatic embryos.

Introduction

The American chestnut was one of the most economically important angiosperm forest trees in North America, and comprised 25% of the Appalachian forest up until the early 1900s. When the chestnut blight fungus (*Cryphonectria parasitica*) was introduced to the United States in the late 1800s on Asian chestnut stock, it took approximately 50 years for 3.5 million American chestnut trees to be killed off. Natural reestablishment of the tree is highly unlikely, as the blight remains an obstacle (Anagnostakis 1987; Johnson et al. 2008; Vieitez 1995).

As a part of the considerable effort to re-introduce the American chestnut to its native range, an *in vitro* propagation method known as somatic embryogenesis (SE), has shown the potential to contribute to restoration of the tree, both by producing trees engineered with blight-resistance genes, and by providing a method for mass propagation of blight resistant genotypes from conventional breeding programs (Andrade and Merkle 2005). The advancement of American chestnut SE via the implementation of suspension cultures has increased the efficiency of somatic embryo production from the initial protocol (Andrade and Merkle 2005). However, issues remain with the current system, including low germination frequency, which has limited the success of somatic seedling production for the species. Another issue is poor somatic seedling quality. The embryos that are able to germinate often grow into somatic seedlings with abnormal morphology. Some preliminary histological work has indicated that root and shoot meristems may be incomplete or lacking in a large proportion of American chestnut somatic embryos (Merkle, unpublished data). Thus, cultural treatments that improve meristem formation may improve American chestnut somatic embryo germination and early somatic seedling growth.

The two redox forms of endogenous and exogenous glutathione have shown potential in influencing the success and quality of somatic embryogenesis for different plant species. Glutathione is a tripeptide and antioxidant that is found in most prokaryotic and eukaryotic cells (Noctor et al. 1998). The two redox forms of the compound that exist interchangeably are: reduced, the thiol form (GSH), and oxidized, glutathione disulfide (GSSG). The exogenous application of GSH to early stages of embryogenic tissue has been shown to increase both cell proliferation and somatic embryo conversion frequency of white spruce (*Picea glauca*) and rapeseed (*Brassica napus*) (Belmonte et al. 2006; Stasolla et al. 2004). Conversely, when an application of a GSH biosynthesis inhibitor—DL- buthionine-[S,R]-sulfoximine (BSO)—was applied, the mitotic activity slowed down (Potters et al. 2002). During the first stages of embryogenic culture in white spruce, GSH levels declined steadily. On the other hand, GSSG was low during the initial stages of cell differentiation and mitotic activity, and increased during embryo maturation (Belmonte et al. 2003). The production of the hormone ethylene in white spruce is under the control of the glutathione redox state (Belmonte et al. 2005). The accumulation of ethylene is directly associated with the formation of intercellular spaces, which were blamed for meristem abortion at germination. A high GSH:GSSG ratio increased ethylene biosynthesis and a low GSH:GSSG ratio (an oxidized cellular environment) reduces it, thereby decreasing the formation of intercellular spaces, and improving the quality of embryo formation (Belmonte et al. 2005). Consequently, the inclusion of GSH during the first few days of somatic embryo development, followed by a treatment with GSSG, enhanced embryo quality by improving the soundness of the shoot apical meristem. Thus, there is evidence that both redox forms of glutathione are equally important in producing quality embryos, since GSH promotes early cell proliferation and GSSH then promotes organized development (Yeung et al. 2005).

Since the main issues with American chestnut SE are somatic embryo quality and subsequent germination, rather than with embryogenic cell proliferation, the application of exogenous GSSG could potentially both increase the oxidative environment and improve somatic embryo yield and quality. An alternative and more economically feasible strategy to using oxidized glutathione is the use of a reduced glutathione biosynthesis inhibitor such as BSO (Belmonte and Stasolla 2007). Replacing both GSH and GSSG with BSO lowered the cellular reduced glutathione state, and improved embryo quality and morphology of white spruce (*Picea glauca*) somatic embryos (Belmonte et al. 2006; Belmonte and Stasolla 2007). Application of BSO also significantly increased conversion frequency of rapeseed (*Brassica napus*) somatic embryos compared to controls. Further investigation of the mechanisms of BSO-mediated improvement with *B. napus* showed that the compound prevented cellular deterioration in the apical poles of the embryos (Stasolla et al. 2008). No studies regarding the influence of BSO on SE in any hardwood forest tree, including American chestnut, have been published. However, the benefits of BSO on somatic embryo quality and conversion in other species are encouraging.

There is need for further research in order to rectify the major issues impeding the advancement of American chestnut somatic embryogenesis for chestnut clonal propagation. Both embryo quality and percent germination and conversion must be improved if mass propagation via SE is to be a useful for the reestablishment of the American chestnut. If these problems are adequately addressed, the SE system can prove to not only aid in the reintroduction of the American chestnut, but can also be implemented for other hardwoods that are being threatened by pathogens and insect pests. Given the promising results of applying BSO with other species, the objectives of the current study were to test BSO for its potential to improve

meristem integrity of American chestnut somatic embryos, and their subsequent germination and conversion.

Materials and Methods

Culture initiation

American chestnut (*Castanea dentata*) embryogenic tissue was produced through somatic embryogenesis from immature seeds, as described by Merkle et al. (1991). Briefly, burs were dissected to remove the immature nuts, which were surface-sterilized by agitation in the following solutions: 70% ethanol for 20 sec; 10% Roccal-D Plus (9.2% Didecyl dimethyl ammonium chloride, 13.8% Alkyl dimethyl benzyl ammonium chloride, 1.0% bis-n-tributyltin oxide. Exton, PA, Div. of Pfizer Inc., New York, NY) for 1 min (these first two steps are repeated twice); 50% Clorox (8.25% sodium hypochlorite) solution for 5 min; sterile water rinse for 3 min; 0.01N HCL for 3 min, and three subsequent rinses in sterile water for 3 min each. The nuts were then dissected to remove the immature seeds for culture, following methods described previously (Carraway and Merkle 1997; Merkle et al. 1991). Immature seeds were cultured in 60 X 15-mm plastic Petri dishes on semi-solid induction-maintenance medium (IMM; Andrade and Merkle 2005), which is a modified woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with vitamins of Schenk and Hildebrandt (1972), 4.0 mg l^{-1} 2,4-D, 0.5 g l^{-1} L-glutamine (filter sterilized and added to autoclaved medium following cooling) and 3% sucrose, at pH 5.65, gelled with 3.0 g l^{-1} Phytagel gellan gum (Sigma). All cultures were transferred to fresh IMM of the same type after one month, and at the end of the second month, they were transferred to secondary medium. Standard protocol secondary medium consists of IMM with only 2.0 mg l^{-1} 2,4-D. Plates with cultures containing embryogenic material were maintained by transfer to fresh medium every 4 weeks.

Somatic embryo production and maturation treatments

Four American chestnut genotypes were used for the experiment: AM54-1, CD322-21, AM58-1, and THxRM-15B. Somatic embryos for the experiment were generated from proembryogenic masses (PEMs) grown in suspension cultures, following the technique described by Andrade and Merkle (2005). Briefly, suspension cultures were initiated by inoculating 0.50 g of tissue into 30 ml of liquid IMM in125-ml Erlenmeyer flasks. Each of the four clones was suspended into three separate flasks for a total of 12 flasks. Suspensions in the flasks were maintained in the dark at 25°C and aerated by shaking on a gyratory shaker at 100 rpm. The tissue was maintained in the shaken flasks for 45 days and was fed at two week intervals by aspirating out the old medium and adding 30 ml of fresh liquid medium. At day 45, the tissue from each flask was fractionated using nested sieves, with a top sieve pore size of 860 μm and a bottom sieve pore size of 140 μm. The fractionated tissue remaining on the bottom sieve was retained and inoculated into flasks for four more days; each of these flasks was then filled with 30 ml of one of three different treatments of liquid embryo development media. The three different media treatments were: (1) standard embryo development medium (EDM; Andrade and Merkle 2005) with no BSO, (2) EDM supplemented with 0.01 mM of BSO, or (3) EDM supplemented with 0.1 mM of BSO. EDM is the same composition as IMM, but lacking 2,4-D. The semi-solid medium treatments were the same as the liquid medium treatments, but included 3 g l^{-1} of Phytagel (Sigma). Each genotype was suspended into three flasks, each with one of the three liquid treatments, flasks were incubated in the dark at 25º C on a gyratory shaker at 100 rpm. On day 4, the fractionated tissue was collected onto a nylon mesh and transferred to Petri plates containing one of the three semi-solid medium treatments (Table 4.1). Each flask was separated onto 6 different plates so there were 2 plates of each treatment for each genotype.

Approximately 1/10 ml of material was transferred on to each plate. The plates were scored every week for embryo production and quality.

All embryos 2 to 4-mm long were evaluated, classified into one of four quality categories or "classes", and then transferred to a new plate containing semi-solid EDM with the same treatment as the plate they were picked from. The defining characteristics for each class were: 1, no well-defined radicle; 2, well-defined radicle with one cotyledon; 3, well-defined radicle with more than 2 cotyledons; 4, well-defined radicle with two cotyledons (this was considered to be the embryo morphology of the highest quality) (Fig. 4.1). Total embryo yield from each treatment, and proportion of each class for each treatment were calculated. Although class 1 embryos were counted, there were so few of these that this class was left out of the analysis. Following this evaluation, all plates were placed into a dark refrigerator and held at 8° C for twelve weeks for pre-germination cold treatment. Following cold treatment, the embryos were placed onto 100 ml of semi-solid germination medium (GM; Andrade and Merkle 2005) in GA-7 vessels (Magenta Corp.). GM is the same as EDM, but supplemented with 1 g l^{-1} activated charcoal and lacking L-glutamine. The GA-7 vessels were placed into an incubator under cool white fluorescent light (100 μmol m^{-2} s^{-1}) and a 16h photoperiod at 25°C. The embryos were monitored for shoot and root growth over three months. The proportions of somatic embryos that successfully germinated (exhibited root elongation) and those that converted into somatic seedlings were recorded. PROC GLM of SAS (SAS Institute Inc. 2011) was used to analyze line and treatment effects on total somatic embryo yield, embryo yields within the different quality categories, and somatic embryo germination and conversion, using analysis of variance. Two additional replications of the experiment were performed.

Histological assessment

Ten embryos were collected from each embryo class just before they were placed in pre-germination cold treatment, and prepared for light microscopy (LM). Embryos were fixed in Histochoice tissue fixative (Amresco, Solon, OH) and dehydrated through a graded ethanol series from 25% to 95%. Tissues were then infiltrated and embedded into glycol methacrylate (JB-4 Embedding Kit; Polysciences, Warrington, PA). Infiltration was with daily changes in an EtOH:JB-4 series [75:25, 50:50, 25:75, 5:95, and 0:100 (by volume) three times]. Tissues were finally embedded into JB-4 medium. Serial sections, 5 μm thick, were cut using a rotary microtome (Microm, Heidelberg, Germany), mounted on slides, and stained by immersion in 0.1% toluidine blue. Sections were observed and photographed with a BX51 Research Microscope (Olympus America, Center Valley, PA). Staining for starch, protein and lipids was also conducted. For lipid detection, sections were stained in 0.3% Sudan black B (w/v) in 70% ethanol, and de-stained in 70% ethanol. Starch was visualized by staining sections in a 50% IKI solution, and for protein detection, sections were stained in 0.025% Coomassie Brilliant Blue, 40% methanol, and 7% acetic acid.

Results and Discussion

Somatic embryo maturation treatments

Assessment of BSO treatment effectiveness was based on overall embryo yield and on the morphology of embryos produced. Embryo yields for each line and each treatment are shown in Fig. 4.2. For each genotype, the proportions of embryo production for each quality category for each treatment are shown in Fig. 4.3a-c. Genotype THxRM-15B is not included in the figures because after several weeks, no embryo production had occurred. Figs. 4.2 and 4.3 show no evidence that any of the BSO treatments were better than the control. The other two

replications of the experiment also indicated that there were no significant differences among BSO treatments.

Analysis of variance results testing the effects of genotype and treatment on total embryo yield and the proportion of embryo yield per class indicated that genotype was significant in all replications of the experiment. AM54-1 was significantly higher in overall embryo yield (p = <0.0001; F-score=18.26; DF, 2). Using Tukey's test to compare the means showed that AM54-1 also produced a significantly higher number of class 3 (p-value=0.0001; F-score=16.08; DF, 2) and class 2 embryos (p-value=<.0001; F-score=18.21; DF, 2). Tukey's test also indicated that AM58-1 yielded significantly higher number of class 4 embryos than the other lines (p-value=0.01; F-score=5.68; DF, 2).

ANOVA for the effects of genotype and treatment on germination and conversion proportion gave similar results to those described above for embryo yield and proportion of embryo yield per class. Genotype had a significant effect on both germination (p=0.04; F=4.06; DF=2) and conversion (p=0.05; F=3.77; DF=2). Treatment had no effect on germination or conversion. Although the ANOVA results indicated no difference among treatments, we did observe that some germinating embryos produced strong taproots in the GA-7s (Fig. 4.4). Strong taproot elongation is a crucial step in the development of somatic seedlings, but since there was no shoot growth, conversion did not take place. Further research should be conducted to improve this aspect.

Two additional replications of this experiment showed similar results. Therefore, there is no clear indication that BSO improved embryo quality. There was also no evidence of treatment by line interaction effects on this variable. Although the results of previous BSO research by others were encouraging, the previous studies were limited to a few authors, and performed on

just two species: white spruce, a conifer, and rapeseed, a herbaceous angiosperm in the cabbage family. As previously stated, there has been no published research on BSO in somatic embryogenesis of an angiosperm forest tree. It is possible that one of the reasons we did not see a treatment effect from the BSO is that the storage reserves for American chestnut are quite different from those of conifers and rapeseed. Conifer embryos have small cotyledons and therefore have a small amount of storage compared to the ex-albuminous chestnut embryos. *B. napus* embryos contain high oil reserves, whereas chestnuts are very low in fat and high in starch. For these reasons, BSO could have a different effect on the outcome of embryo development of chestnut.

Histological assessment

Despite the lack of evidence that BSO treatments can improve the quality of American chestnut somatic embryos, the histological examination of these embryos was enlightening. We conducted histological assessments to understand more about the cellular development of somatic embryos, as well as to ascertain if there are any developmental differences in the embryos of different classes, which would in turn allow us greater understanding of the quality of the different embryo morphological types. The ideal American chestnut somatic embryo has a well-defined radicle and two well-formed cotyledons (we call this category of embryos class 4). Based on our experience, this is the type of embryo that has the highest rates of germination and conversion; those embryos with more than two, or fused cotyledons are the second best (class 3), and those with one cotyledon perform the worst (class 2) of these classes (Merkle et al. unpublished).

Micrographs of the general structure of the embryos, in classes 4, 3, and 2 are shown in figures 4.5, 4.6, and 4.7, repectively. For the class 4 embryos, the morphology appears to be best

suited to normal elongation from the shoot apical meristem (SAM). Based on the qualifications for class 3 embryos—having three or more cotyledons—this is the embryo class with the most variable embryo morphology. Figs. 4.6a - 4.6b and 4.6c - 4.6d show two different embryos in class 3. The embryo in 4.6a and 4.6b has a fused cotyledon, and serial sections confirm the lack of SAM development. Conversely, the embryo shown in 4.6c and 4.6d appears to be developing somewhat normally in the central zone, so its abnormal morphology may not be disruptive to standard somatic seedling development. Fig. 4.7 shows a class 2 embryo with one cotyledon. The point of contact between the hypocotyl and cotyledon is clear from this image, but there is no obvious development of the SAM. There seems to be a fair amount of cell division going on at the right side of the hypotcotyl tip, where the cells appear very compact and organized. This cellular activity suggests that there could be some meristem development, but observations of the serial sections verified the lack of a defined SAM. The class 2 embryos also appear to have cells that are more vacuolated than class 3 and class 4 embryos (Fig. 4.7). Since embryos lacking a well-defined radicle (class 1) were not numerous enough to include in the analysis, and all the other classes stipulated a well-defined radicle, the morphology of the radicle development was similar for classes 2, 3, and 4. Fig. 4.8a, 4.8b, and 4.8c show radicles from embryos of classes 2, 3, and 4, respectively.

American chestnut somatic embryos were also stained for starches, lipids, and proteins. The American chestnut zygotic embryo is known as a low fat, high starch product, with approximately 6% protein (Abrams and Nowacki 2008). Fig. 4.9 shows all three types of chemical staining and also a micrograph of TB staining for comparison. Starch (Fig. 4.9a) was clearly abundant in the embryos, as would be expected. However, the staining was negative for lipids (Fig. 4.9b) and proteins (Fig. 4.9c). Although analyses have indicated that American

chestnut zygotic embryos contain approximately 6% protein, that amount is either too little to detect with our method of protein staining, or was not present in the somatic embryo stages that we sampled.

Although the BSO treatments conducted in this experiment did not have a significant effect on the yield or development of American chestnut embryos, the histological examination of these embryos provided some useful information pertaining to embryo quality and storage product make-up. Knowing what embryo morphological form is associated with healthy SAM formation and a more normal growth pattern could help with the development of a more efficient somatic embryogenesis process. Excluding those embryos that will most likely not grow in to quality somatic seedlings and focusing on the higher quality material could help make the production of quality chestnut somatic seedlings more efficient.

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Table 4.1: Treatments tested in BSO experiment.

Liquid treatment in flasks (4 days)	Semi-solid treatment on plates	Number of plates per treatment
0*	0	2
0	0.01 mM	2
0	0.01 mM	2
0.01 mM*	0	2
0.01 mM	0.01 mM	2
0.01 mM	0.01 mM	2
0.1 mM *	0	2
0.1 mM	0.01 mM	2
0.1 mM	0.01 mM	2

*0, EDM with no BSO

*0.01mM, EDM with 0.01mM BSO

*0.1mM, EDM with 0.1mM BSO

Figures 4.1-4.9

Fig. 4.1a-c: Examples of morphological structure for each embryo class. **a**. class 4 (scale bar = 2mm) ; **b.** class 3 (scale bar = 2mm); **c**. class 2 (scale bar = 2mm).

Fig. 4.2: Total embryo yield per 1 g of starting material for each BSO treatment and each genotype. On the x-axis, the first number represents the liquid medium treatment and the second number represents the semi-solid medium treatment: 0, no BSO; 0.01, 0.01 mM BSO; 0.1, 0.1 mM BSO.

Fig. 4.3a-c: Proportional yields of each embryo class for each BSO treatment and genotype; **a**. AM54-1; **b**. CD322-21; **c**. AM58-1. On the x-axis, the first number represents the liquid medium treatment and the second number represents the semi-solid medium treatment: 0, no BSO; 0.01, 0.01 mM BSO; 0.1, 0.1 mM BSO.

Fig. 4.4a-b: Examples somatic embryos with well-developed taproots of genotype AM54-1 from two different BSO treatments. **a**. Liquid treatment EDM with 0.01 mM BSO and semi-solid treatment of standard EDM. **b**. Liquid treatment EDM with 0.1 mM BSO and semi-solid treatment of standard EDM.

Fig. 4.5a-c: Shoot apical meristem formation on a class 4 chestnut somatic embryo. a. Bar = 200 μm ; **b**. Bar = 200 μm; **c**. Bar = 100μm. The location of the cotyledon is denoted by the number 1 and the location of the hypocotyl is denoted by the number 2.

Fig. 4.6a-d: Different morphological types found among class 3 embryos. **a**. Class 3 embryo with a fused cotyledon bar = 500 μm ; **b**. Bar = 200 μm ; **c**. Class 3 embryo with separated cotyledons bar = 500 μm ; **d**. Bar = 200 μm. The location of the cotyledon is denoted by the number 1 and the location of the hypocotyl is denoted by the number 2.

Fig. 4.7a-c: Examples of class 2 chestnut somatic embryos. a. Bar = 500µm; b. Bar =500µm ; c. Bar = 200µm. The location of the cotyledon is denoted by the number 1 and the location of the hypocotyl is denoted by the number 2.

Fig. 4.8a – c: Root meristem development for embryos of each class. **a**. Class 2, bar = 200 µm; **b**. Class 3, bar = 100 µm; **c**. Class 4, bar = 200 µm

Fig. 4.9a – d: Chestnut somatic embryos stained for different storage products: **a.** Stained with IKI for starch, bar = 50 µm. **b.** Stained with Sudan black B for lipids, bar = 100 µm. **c.** Stained with Coomassie blue for proteins, bar = 50 µm. **d.** Stained with toluidine blue for structural form, bar = 20 µm.

Fig. 4.1

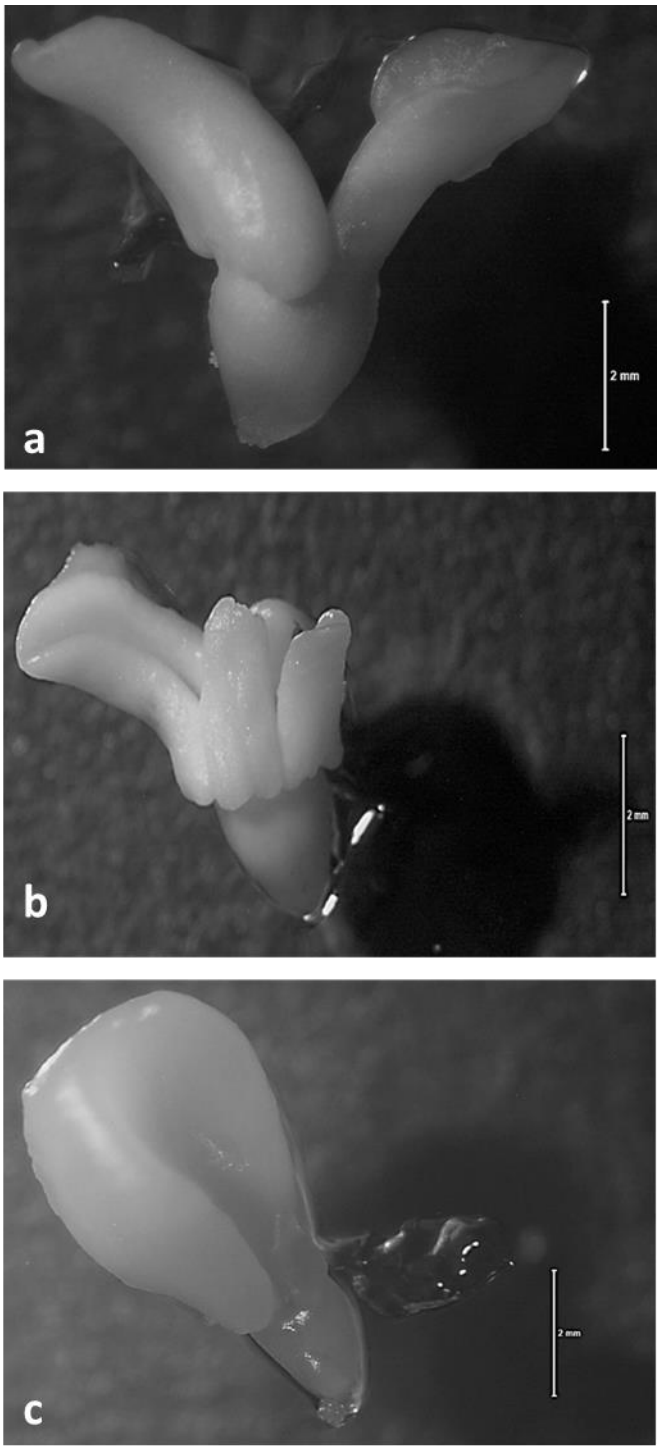


Fig. 4.2

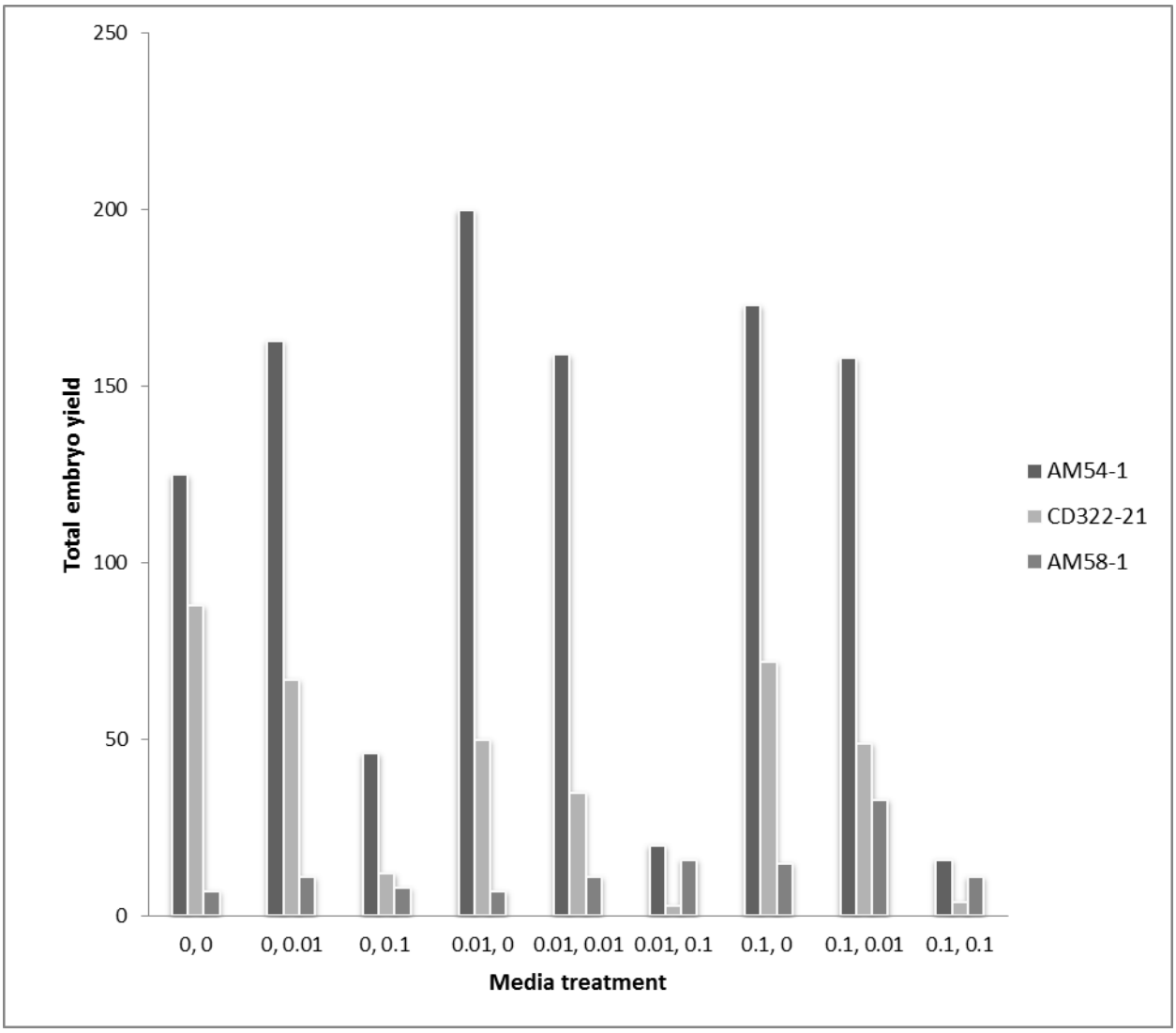

250
200
150
100
50
0
Total embryo yield
0, 0
0, 0.01
0, 0.1
0.01, 0
0.01, 0.01
0.01, 0.1
0.1, 0
0.1, 0.01
0.1, 0.1
Media treatment
AM54-1
CD322-21
AM58-1

Fig. 4.3

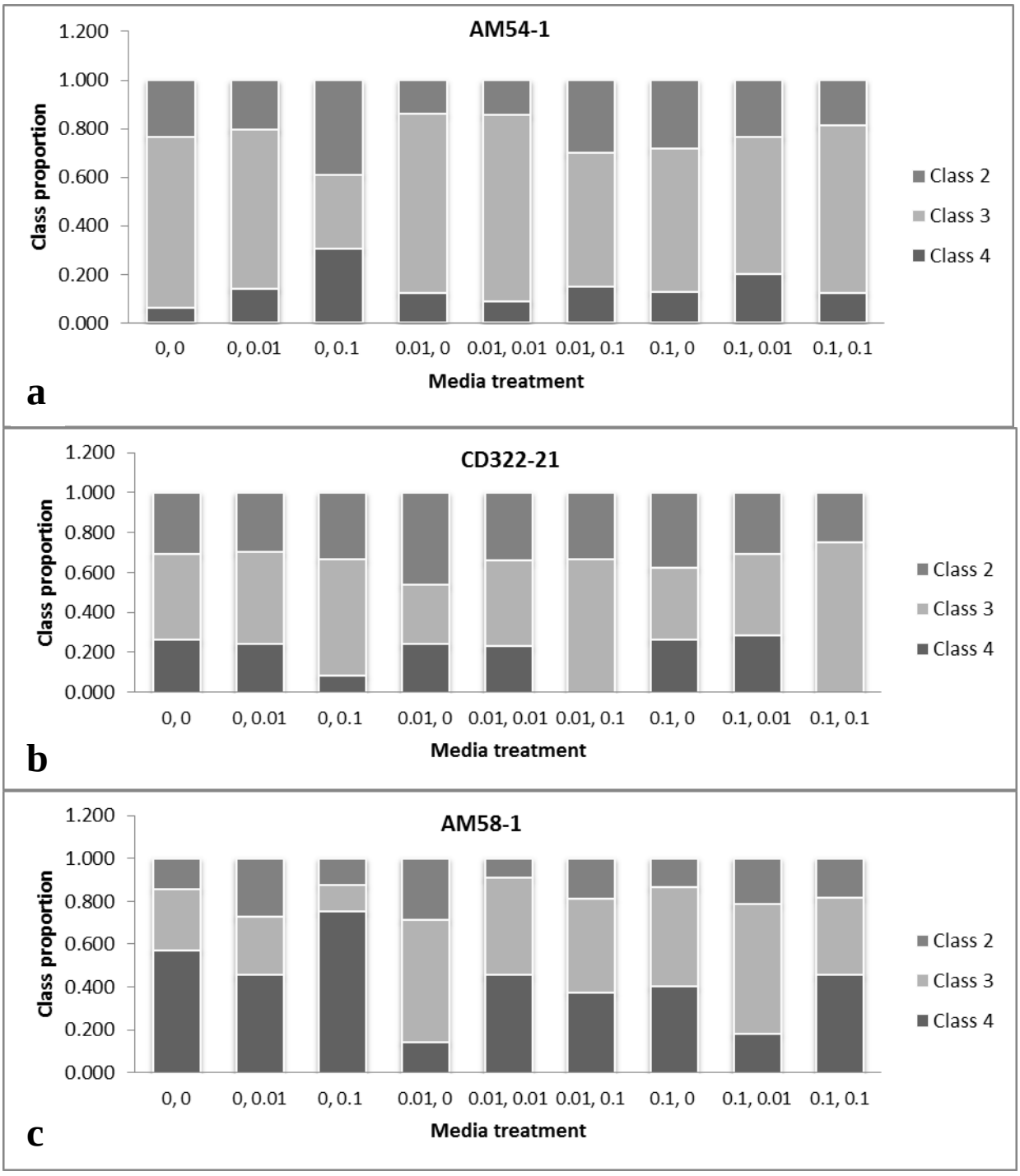

AM54-1
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Class proportion
0, 0
0, 0.01
0, 0.1
0.01, 0
0.01, 0.01
0.01, 0.1
0.1, 0
0.1, 0.01
0.1, 0.1
Media treatment
Class 2
Class 3
Class 4
a
CD322-21
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Class proportion
0, 0
0, 0.01
0, 0.1
0.01, 0
0.01, 0.01
0.01, 0.1
0.1, 0
0.1, 0.01
0.1, 0.1
Media treatment
Class 2
Class 3
Class 4
b
AM58-1
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Class proportion
0, 0
0, 0.01
0, 0.1
0.01, 0
0.01, 0.01
0.01, 0.1
0.1, 0
0.1, 0.01
0.1, 0.1
Media treatment
Class 2
Class 3
Class 4
c

Fig. 4.4

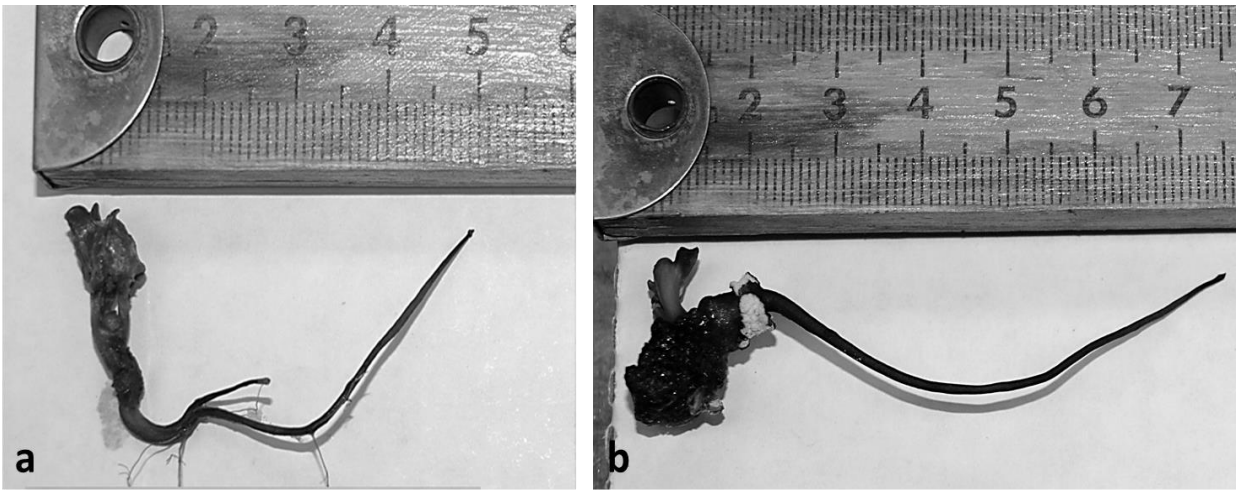


Fig. 4.5

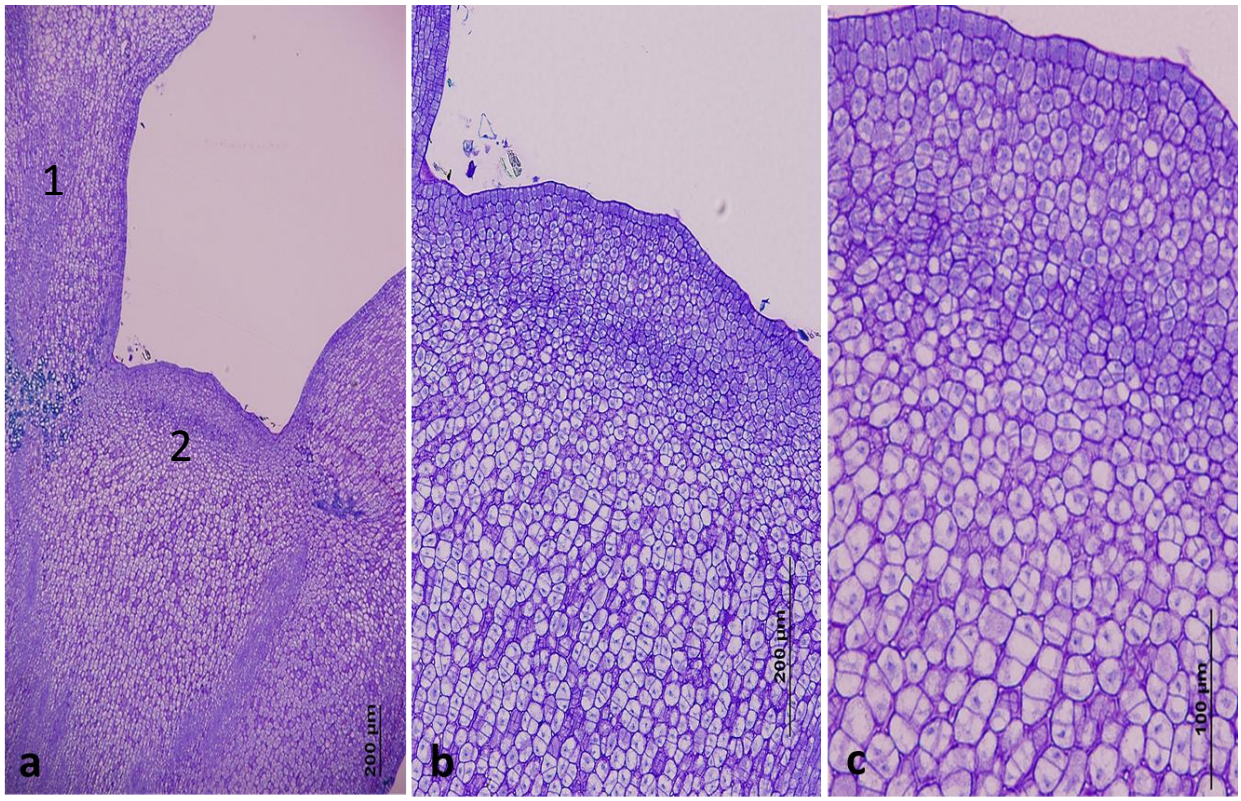


Fig. 4.6

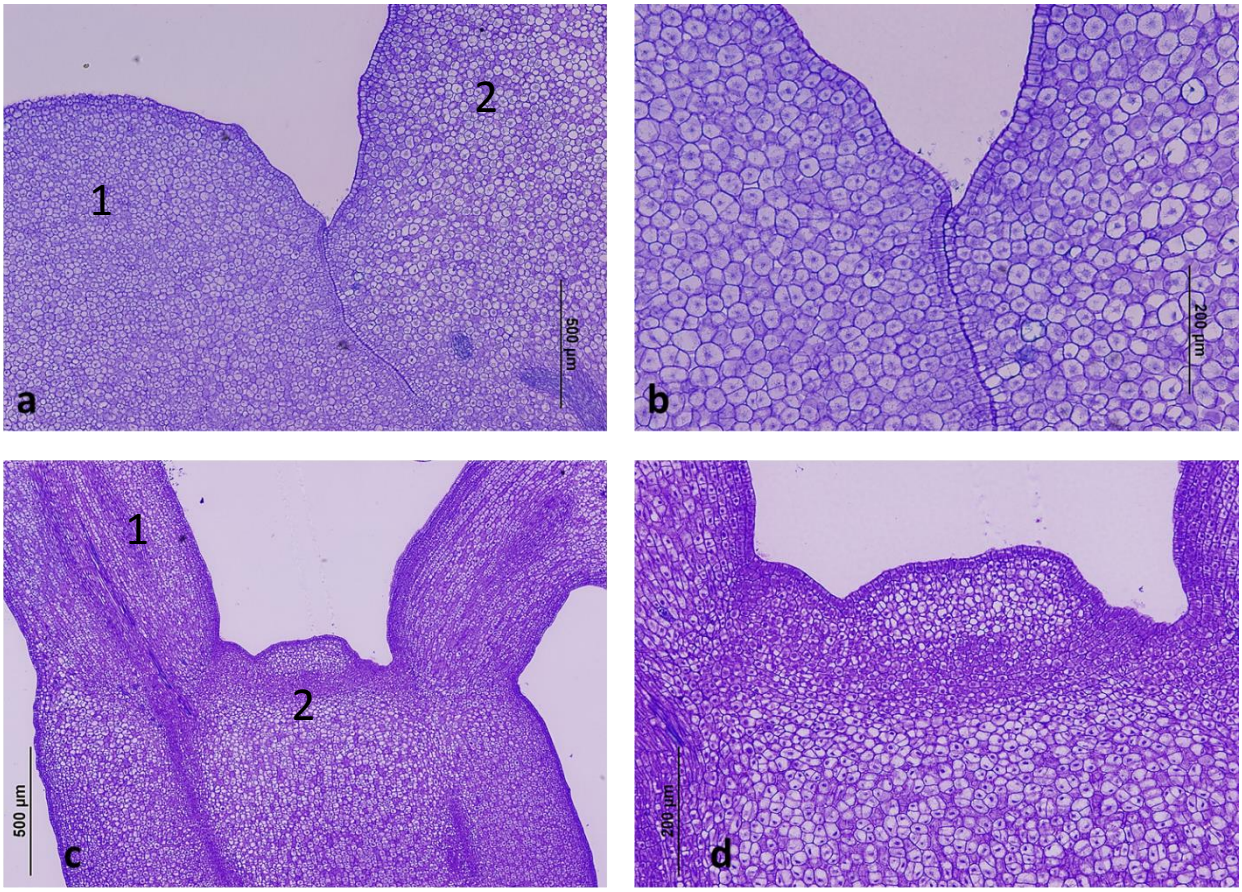


Fig. 4.7

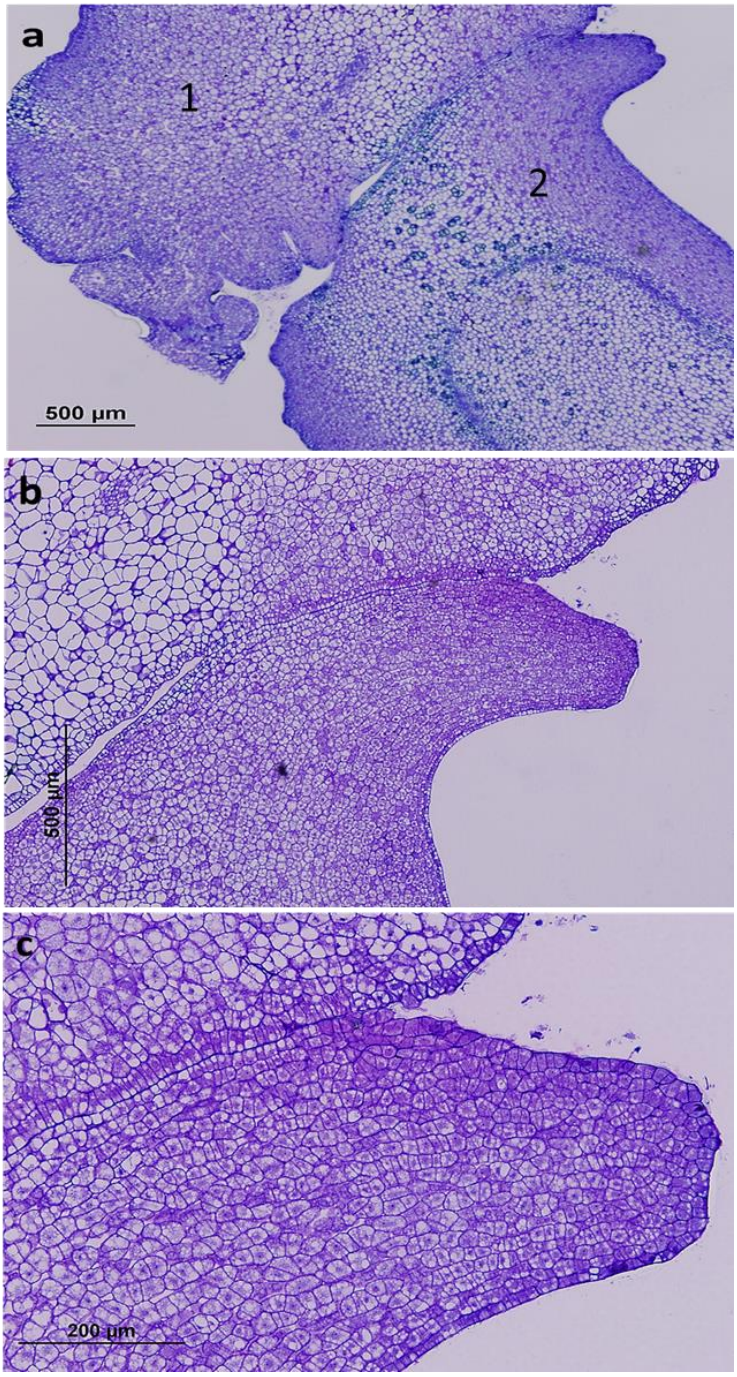


Fig. 4.8

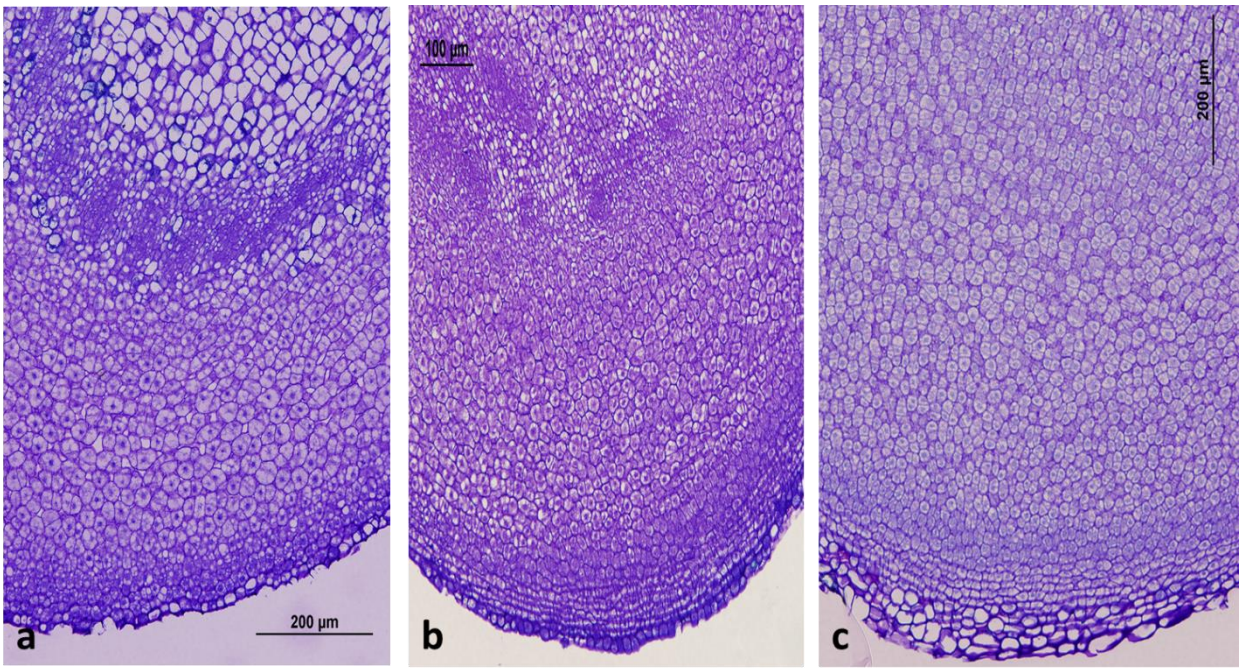
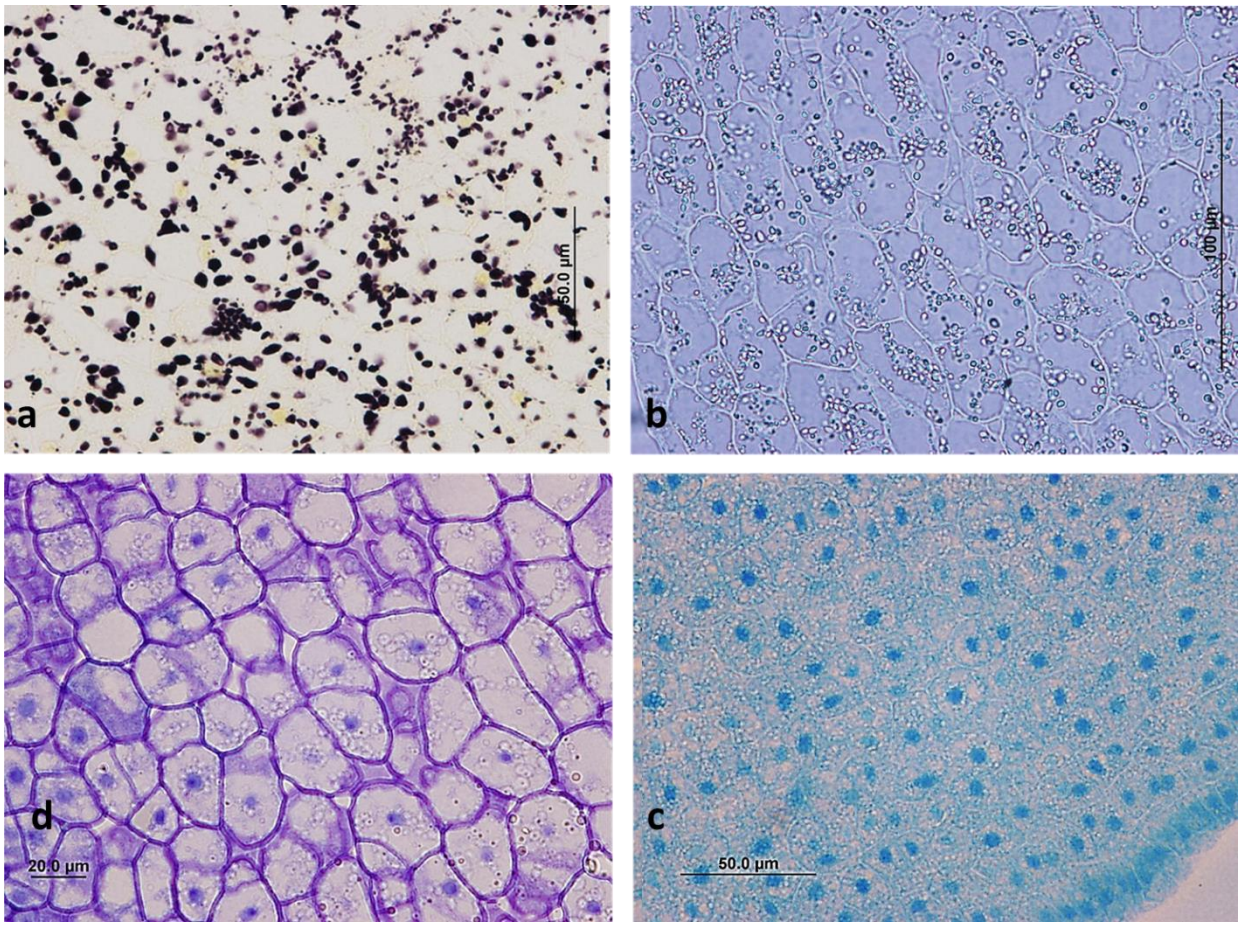


Fig. 4.9



CHAPTER 5

CONCLUSION

As detailed in this thesis, over three years of culture initiations, we tested the effects of CC genome proportion on the success of SE induction using our standard protocol for culturing AC and, subsequently, protocols based on a published protocol for SE in European chestnut. Additionally, to test the efficacy of bioreactors, we compared the tissue growth process of embryogenic suspension cultures from initial suspension to maximum growth potential using air-lift bioreactors and shaken flasks and investigated germination efficiency of American chestnut somatic embryos using RITA® TIBs. Finally, in order to analyze the effects that BSO has on American chestnut embryo yield, quality, and conversion, different levels of the compound were added to maturation media and we compared the resulting embryos to those produced using the standard protocol for American chestnut SE.

Embryogenic culture initiation

One important conclusion provided by the culture initiation experiment was that there is substantial evidence that the standard protocol for initiating embryogenic cultures from American chestnut seeds can be applied for initiating embryogenic cultures of B3F3 hybrids, thereby providing the potential to clonally propagate the most desirable blight-resistant hybrid backcross material from conventional chestnut breeding programs. However, the standard protocol for inducing somatic embryogenesis in American chestnut was not successful for the Chinese chestnut or for F1, B1, or B2 hybrids. Thus, it appears that there may be some threshold of Chinese chestnut genome proportion, beyond which the standard SE induction protocol is not

effective. Subsequent research testing alternative SE protocols by introducing a medium with both a lower auxin potency (NAA instead of 2,4-D), and supplemented with cytokinin (BA) led to the successful production of somatic embryos from Chinese chestnut and non-repetitive embryogenic cultures from B2 and F1 cultures. We believe that clonal propagation of the best hybrid backcross chestnut material will play an important role in the program for chestnut restoration. While we demonstrated that the protocol developed for American chestnut is applicable to B3F3 material, as well as other hybrid chestnuts, it is essential that continued progress be made in the improvement of the chestnut somatic embryogenesis system if it is to be applied on an operational level for TACF's restoration program.

Bioreactor comparison

The comparison of suspension culture techniques showed that the use of air-lift bioreactors had a significant, positive impact on the growth efficiency of embryogenic chestnut cultures compared to shaken flasks. Because of the effectiveness of bioreactors in increasing output of tissue volume in this study, this method of suspension culture should be applicable to help advance the use of SE for the mass propagation of both transgenic and hybrid backcross American chestnut with blight resistance genes. Additionally, RITA TIBs should be the subject of further American chestnut SE studies to assess their capability of advancing the SE germination process to a more viable level.

Embryo quality treatments with BSO

Although the BSO treatments conducted in this experiment did not have a significant effect on the yield or development of American chestnut embryos, the histological examination of these embryos provided some useful information pertaining to embryo quality and storage product make-up. Knowing what embryo morphological form is associated with healthy SAM

formation and a more normal growth pattern could help with the development of a more efficient somatic embryogenesis process. Excluding those embryos that will most likely not grow in to quality somatic seedlings and focusing on the higher quality material, could help make the production of quality somatic seedlings more efficient.