# IMPROVEMENT OF SCALED-UP SOMATIC SEEDLING PRODUCTION IN HYBRID SWEETGUM (*Liquidambar styraciflua* × *L. formosana*)

By

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## ABSTRACT

Some clones of hybrid sweetgum (*Liquidambar styraciflua* × *L.formosana*,), the hybrid of American sweetgum (*Liquidambar styraciflua*) and Chinese sweetgum (*L. formosana*), demonstrate increased vigor, accumulating biomass faster than elite native sweetgum. Somatic embryogenesis is a primary method to micropropagate hybrid sweetgum. Considerable progress in developing somatic embryogenesis as a mass clonal propagation system for hybrid sweetgum has been made in recent years, but labor costs remain the largest cost component of the system. Different bioreactors had been considered for their potential to improve the efficiency of the system and thus decrease labor costs by decreasing the total process time. Air-lift bioreactors have been successfully applied to improve growth efficiency in some woody species. Stirred-tank bioreactors can be helpful for optimizing proliferation protocol because culture conditions can be controlled. Using two genotypes of hybrid sweetgum (28-1-5A, 28-1-8), we tested

proliferation of suspension cultures of PEMs in airlift bioreactors and stirred-tank bioreactors, compared to culture in shaken flasks. Airlift bioreactors showed 12.6% and 104.2% faster growth rates in two genotypes compared to flasks. Thus, the use of airlift bioreactors has the potential to lower costs during the proliferation stage of this propagation system. Stirred-tank bioreactors were used to monitor the consumption of different sugars (sucrose, glucose and fructose) by the growing tissue, with differential use of different sugars being found during proliferation. Bioreactors with temporary immersion designs, such as the RITA®, have been applied to improve in vitro propagation of a number of woody species. We tested RITA®s for their potential to improve the production efficiency of high-quality hybrid sweetgum somatic seedlings in comparison to somatic seedlings from embryos germinated on semisolid medium in GA7 vessels. In hybrid sweetgum genotype 28-1-5A, RITA® units with 50 embryos had about 20% higher high-quality somatic seedling production frequency ($p<0.05$) and about 35% higher conversion frequency ($p<0.05$) than the GA7 vessels. In all genotypes we tested, somatic seedlings germinated in RITA® bioreactors had statistically higher survival frequencies than somatic seedlings germinated in the GA7 vessels.

INDEX WORDS: Hybrid sweetgum, Somatic embryogenesis, RITA®, Air-lift bioreactor, Stirred-tank bioreactor

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DEDICATION

To my grandparents and parents

Yongxi Lu, Chunhua Yang, Hui Xie and Xiaojun Lu

And my husband and lovely daughter

Ping Zhang and Margret Zhang.

I love you all!

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# CHAPTER I

## 1. INTRODUCTION AND LITERATURE REVIEW

Sweetgum (*Liquidambar styraciflua*) is one of the most important hardwood species in forests of the southern United States (Wright and Cunningham 2008). Sweetgums are large, deciduous trees, ranging from 25 to 40 meters tall. Under natural conditions, sweetgum grows in moist to wet acidic soils and is commonly found in swamps, near ponds and rivers. The tree is widely utilized for pulp, lumber, pallets, veneer, panels, and fuel wood by a large number of mills in the southern U.S. (Wright and Cunningham 2008). Many selected cultivars of sweetgum are used to enrich fall foliage color in urban environments (Wright and Cunningham 2008).

In addition to traditional usages, sweetgum is a candidate with high potential for short-rotation biomass energy plantations. In the 1980s, sweetgum was not regarded as a top choice for biomass energy plantations (Steinbeck and Skinner 1985; Tuskan 1998). However, continued advances in sweetgum silviculture and genetic improvement and the rise of varietal sweetgum make a strong case for reconsidering sweetgum as a short-rotation feedstock (Merkle and Cunningham 2011; Wright and Cunningham 2008).

In the research described here, efforts were made to improve sweetgum as a candidate for short-rotation biomass energy plantations by choosing hybrid sweetgum as the primary object of study. We attempted to enhance the productivity of hybrid sweetgum clones through optimizing the process of proliferating proembryogenic masses (PEMs) for the production of somatic embryos and by using temporary immersion bioreactors for somatic embryo germination, to produce large numbers of vigorous somatic seedlings.

### *1.1.1 Sweetgum genetic improvement*

Genetic improvement of sweetgum began in the 1960s. Both the North Carolina State University Hardwood Research Program and the Western Gulf Forest Tree Improvement Program contributed to the first cycle of genetic improvement of sweetgum and have made second-generation selections (Wright and Cunningham 2008). Currently, organizations such as ArborGen Inc. have access to seeds from the best of the second generation sweetgum to do further experiments (Wright and Cunningham 2008).

### *1.1.2 Hybrid sweetgum*

Sweetgum (*Liquidambar styraciflua*) has counterparts, including the Formosan sweetgum *(L. formosana*), native to eastern Asia, and *L. orientalis*, native to Turkey (Santamour 1972). *L. styraciflua* and *L. formosana* have been separated from each other by continental drift for at least 10 million years, but they remain interfertile (Santamour 1972). Vendrame et al. (2001a) produced several *L. styraciflua* x *L. formosana* hybrid clones via somatic embryogenesis. Some of the hybrid clones generated via somatic embryogenesis have demonstrated hybrid vigor, accumulating biomass significantly faster than elite native sweetgum in field tests (Merkle and Cunningham 2011)

### *1.1.3 In vitro propagation of sweetgum*

Sweetgum is amenable to several different methods of vegetative propagation, including rooted stem cuttings, root cuttings, adventitious shoot-based micropropagation, and somatic embryogenesis. Brown and McAlpine (1964) propagated 3-year-old seedlings and 20-year-old trees directly from root cuttings. Farmer Jr (1966) reported successful rooting of softwood sweetgum cuttings taken from root suckers. However, in vitro approaches for clonal propagation of sweetgum have been reported to be more efficient than conventional vegetative propagation

methods (Sommer 1981). Researchers have developed in vitro adventitious shoot-based propagation methods for sweetgum using a range of explants, including hypocotyl sections (Sommer 1981), juvenile shoot tips (Sutter and Barker 1985), and mature leaves and petiole segments (Brand and Lineberger 1988). Transgenic sweetgum trees were produced via *Agrobacterium*-mediated gene transfer by using leaf tissue derived from aseptic shoot tip cultures (Sullivan and Lagrimini 1993) and young leaf explants (Chen and Stomp 1992), and regenerated through adventitious shoot induction. Sommer and Brown (1980) regenerated sweetgum via somatic embryogenesis from seedling hypocotyl-derived callus. Merkle et al. (1998) described initiation of embryogenic sweetgum cultures from immature seeds by explanting them onto semisolid induction-maintenance medium (IMM) with 9.0 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 1.1 M benzyladenine (BA). Vendrame et al (2001a) later applied the seed-based protocol from Merkle et al. (1998) to produce embryogenic cultures of hybrids between *L. styraciflua* and *L. formosana*

The reports of somatic embryogenesis in sweetgum using seed or seedling tissues resemble those of the majority of the reports of SE in forest trees, in that they employed genetically unproven, immature tissues as explant material. This type of somatic embryogenesis makes use of 'pre-embryogenic determined cells' (Merkle et al. 1998). In this type of explant, the cells are in a stage close to the embryonic state and can directly replicate themselves when treated with a strong auxin, such as 2, 4-D. The drawback of this approach is that it cannot be used to clone proven elite trees, since it can only be used with seed or seedling tissues.
To avoid this shortcoming, researchers have attempted to induce somatic embryogenesis through 'induced embryogenic determined cells', using tissues from mature trees such as floral or inflorescence tissues as explant material. These routes are more difficult, since the explants

consist of differentiated cells that must undergo epigenetic changes to initiate somatic embryo production (Merkle et al. 1998). Floral and inflorescence tissues of a number of woody perennials have been successfully used as explant material for indirect somatic embryogenesis (Alemanno et al. 1996; Gingas 1991; Grönroos et al. 1989; Jörgensen 1989; Jörgensen 1992; Kiss et al. 1992; Lopez-Baez et al. 1993; Teixeira et al. 1994). Since 1997, a number of studies have reported sweetgum somatic embryogenesis using parts of staminate and pistillate inflorescences from mature trees, and demonstrated that induction frequency of embryogenesis was affected by genotype, explant type, and the duration of cold storage of explant material., Germination and conversion frequencies of infloresence-derived sweetgum somatic embryos were affected by genotype and the interaction between genotype and pre-germination cold treatment (Merkle et al. 1997; Merkle et al. 1998; Merkle and Battle 2000; Merkle et al. 2003). Merkle et al. (2010) reported inflorescence-derived embryogenic cultures of hybrid sweetgum, using the same methods used for *L. styraciflua*. The embryogenesis induction frequency of staminate inflorescences ranged from 1.6% to 12.8%, and varied with both clone and plant growth regulator (PGR) treatment. These induction frequencies were relatively low compared to those for *L. styraciflua* (Merkle et al. 2010).

### *1.1.4 Scale-up of hybrid sweetgum somatic embryo and somatic seedling production*

Dai et al. (2004) described a method of scaled-up production of hybrid sweetgum somatic embryos by growing suspension cultures of proembryogenic masses (PEMs) in shaken flasks of liquid IMM. Dai et al.'s (2004) protocol required the suspension cultured PEMs to be transferred to fresh IMM every 3 weeks, because of the exhaustion of the nutrients in the medium. PEM proliferation in shaken flasks is ultimately limited by the size of the container (Dai et al. 2004; Maene and Debergh 1985). For somatic embryo production, size-fractionated

PEMs were collected on filter paper using a Büchner funnel, and cultured on semi-solid development medium (DM), which was the same as IMM, but lacked 2,4-D and 6BA (Dai et al. 2004). Once plated, somatic embryo development and maturation required about one month. Using this method, Dai et al. (2004) reported the production of over 1000 hybrid sweetgum somatic embryos per 4000 PEMs of starting material. For germination, mature sweetgum embryos were incubated on semi-solid DM without casein hydrolyzate (CH) in GA7 vessels (Magenta Corp.) (Dai et al. 2004). About 45 days were required to produce sweetgum somatic seedlings using this method.

Dai et al.'s (2004) method for propagating hybrid sweetgum trees via somatic embryogenesis (hereafter referred to as the "standard method"), like most in vitro propagation methods, is highly labor-intensive. In micropropagation, labor generally accounts for 40–60% of the production costs (Etienne and Berthouly 2002). As a result, it has been concluded that commercial application of micropropagation would only be possible if new technologies are available to reduce the labor-intensive steps (Kitto 1997).

### *1.1.5 Bioreactors for in vitro plant propagation*

Over the past 20 years, bioreactors have shown promise as a way of improving the efficiency of micropropagation. There are several advantages of bioreactors over conventional methods for micropropagation. First, bioreactors can have increased working volume, which means many more somatic embryos or other propagules can be cultivated at one time. Second, in bioreactors, it is possible to closely control the cultural and physical environment for optimum growth, which means that many different treatments can be applied during the growth process (Tautorus et al. 1992). Finally, bioreactors can provide more oxygen, which can accelerate growth (Curtis 2005). Unfortunately, most bioreactor designs developed in the past are not

generally suitable for plant micropropagation, as they were not designed to meet the specific requirements of plant cells, such as sensitivity to shear forces, mechanical damage, and the foam formation characteristic of bubble-aerated bioreactors (Teisson et al. 1999).

### *1.1.6 Bioreactor types*

**Stirred-tank bioreactors**

Stirred-tank bioreactors (STBs) are the standard device for contemporary fermentation. Thousands of years ago, people began using fermentation to produce wines by converting the glucose found in various fruits, seeds, and tubers into alcohol. At that time, and even now, more conventional fermenters, like wood vessels or containers which do not have a stirring mechanism, were used for wine production. Recently, as the knowledge of bacteria and fungi has developed, more uses for fermenters have been developed, and a more advanced form of conventional fermenters—STBs—have been invented. Within STBs, environmental conditions (i.e. air flow rate, temperature, pH and dissolved oxygen level) and agitation speed can all be controlled. As a result, it is relatively easy to test for the optimal growth environment and maintain that environment for further study. Researchers began to use STBs to create a stable and an optimal environment for bacteria, fungi, and mammalian cells, to produce various products like recombinant proteins (e.g., antibodies, hormones and enzymes), and secondary metabolites like organic acids. For example, there are many FDA approved prescription drugs that are produced by STBs. These include the antihemophilic factors (ReFacto™) and Tenecteplase (TNKase™), which are purified glycoproteins; Trastuzumab (Herceptin™), a monoclonal antibody for treating breast cancer; and Etanercept,an immunoglobulin-TNF (tumor necrosis factor) receptor fusion protein for treating rheumatoid arthritis (Chu and Robinson 2001). Additionally, STBs are

used in mass-production of hyoscyamine (Hilton and Rhodes 1990) and penicillin (Ariyo et al. 1998). While STBs have been widely and successfully used in the pharmaceutical industry, cases in which STBs have been used for plant micropropagation are rare. In the research reported here, we tested the potential for STBs to be used to proliferate hybrid sweetgum embryogenic cells.

**Air-lift bioreactors**

Recently, because of increasing interest in the commercial application of micropropagation, bioreactors specifically designed for plant cells have been invented. An air-lift bioreactor (ALB) is one bioreactor design that has been tested for proliferation of embryogenic cultures. An ALB is a type of bioreactor that relies on simple construction. In ALBs, the contents are stored in a container and pneumatically agitated by a stream of air. In addition to the agitation, the air also functions as an oxygen resource and facilitates the exchange between the gas phase and the medium (Merchuk 1990). ALBs have been used to grow embryogenic cultures of various plant species, such as carrot (*Daucus carota*) (Teng et al. 1994), grape (*Vitis vinifera*) (Tapia et al. 2009) and cork oak (*Quercus suber*) (Jiménez et al. 2011). For the proliferation of sweetgum PEMs in the current study, ALBs were constructed following the protocol described by Kong et al. (2014) for American chestnut (*Castanea dentata*) embryogenic cultures.

**Temporary immersion bioreactors**

Another bioreactor design is the temporary immersion bioreactor (TIB). This type of bioreactor has been used for such applications as somatic embryo germination and axillary shoot multiplication. Like other bioreactor designs, TIBs provide much more uniform culturing conditions, an easier way to renew the medium without changing the container, complete renewal of the culture atmosphere by forced ventilation and larger volumes than those achievable

by culturing on semi-solid media. In addition, they also permit temporary (rather than continuous) contact between plant tissues and the liquid medium.

TIBs with different designs have been used for plant micropropagation studies. For example, an agitated, thin-film liquid TIB has been used for micropropagating *Hosta* (Adelberg 2005), *Hemerocallis spp* (Adelberg et al. 2007), and elephant ear *(C.esculenta)* (Adelberg and Toler 2004). They have also been used to test the antioxidant capacity of rhizomes of turmeric (*Curcuma longa L.*) (Adelberg and Cousins 2006). Air-lift-balloon type bubble bioreactors have been used for improving micropropagation of *Alocasia amazonica* (Jo et al. 2008), *Malus domestica* (Chakrabarty et al. 2007*)*, *Euphorbia millii* (Dewir et al. 2006a) and *Spathiphyllum cannifolium* (Dewir et al. 2006b). Plastic airlift bioreactors has been used to develop a scaled-up system for *Cucumis sativus L* (Konstas and Kintzios 2003) propagation and for in vitro multiplication of *Rubus chamaemorus L.*(Debnath 2007).

In the current research, we tested the RITA® TIB system for germination of hybrid sweetgum somatic embryos. The RITA® system is a TIB composed of a 1-liter vessel comprised of two compartments, a top one with the plant material and a bottom one with the liquid medium. Air pressure applied to the bottom compartment pushes the medium into the top one. In the top compartment, plant material is immersed as long as the pressure is applied. During the immersion period, air is bubbled through the medium, gently agitating the tissues and renewing the head space atmosphere inside the culture vessel, with the pressure escaping through outlets on the top of the apparatus (Etienne and Berthouly 2002).

The RITA® has shown great advantages in improving the micropropagation of several different species. In *Eucalyptus* micropropagation, a four- to six-fold increase in yield, in half the growth time, and hardier plantlets were achieved with the RITA® system compared with axillary

bud proliferation on semi-solid media (B. McAlister et al. 2005). In *Citrus deliciosa* (Cabasson et al. 1997), *Musa acuminata* (Escalant et al. 1994), *Psidium guajava* (Kosky et al. 2005), and *Hevea brasiliensis* (Etienne et al. 1997), the RITA® system improved both the quantity and quality of somatic embryos produced, increased germination rate and epicotyl emergence, and controlled or even eliminated hyperhydricity (Etienne and Berthouly 2002).

### *1.1.7 Testing bioreactors for improvement of hybrid sweetgum production*

Bioreactors of the types described above have the potential to greatly enhance different stages of plantlet production from forest tree embryogenic cultures, like those of hybrid sweetgum, from proliferation to somatic embryo production to germination. ALBs have already shown promise for accelerating the growth of cultures of some forest trees, such as cork oak (Jiménez et al. 2011) and chestnut (Kong et al. 2014) and, therefore, are likely to be highly adaptable for proliferating hybrid sweetgum PEMs. It is also possible that other bioreactor designs, such as stirred tank bioreactors, could be used to accelerate hybrid sweetgum PEM proliferation. To our knowledge, no previous studies have been published on growing forest tree embryogenic cultures in conventional fermenters, but carrot embryogenic cultures, which are similar to sweetgum PEMs, have been grown in fermenters (Shigeta et al. 1996). It also possible that bioreactors can not only be used for culture proliferation, but for somatic embryo production and maturation, as an alternative to accomplishing these steps on a semisolid medium, but there have been no such studies with forest tree embryogenic cultures to date. Until now, there has been no research concerning the germination of somatic embryos of any forest tree using TIBs such as the RITA® system. However, experiments using the RITA® system with other woody species, as mentioned above, have indicated that there is great potential for applying this approach to forest trees such as sweetgum.

The overall goal of the research described here was to test bioreactors as alternative approaches for scalable *in vitro* propagation for hybrid sweetgum via somatic embryogenesis. Specific objectives were to:

1. Test the potential of the RITA® TIB system to germinate mature hybrid sweetgum somatic embryos to produce somatic seedlings;
2. Make a morphological comparison between somatic seedlings produced by the RITA® system and somatic seedlings produced on a semisolid medium;
3. Compare proliferation of hybrid sweetgum PEMs produced in shaken flasks, air-lift bioreactors and fermenters;
4. Test the effect on growth rate of using different sugars and genotypes on sweetgum PEM production process via fermenters;

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# CHAPTER II

## 2. TESTING DIFFERENT BIOREACTOR CONFIGURATIONS TO IMPROVE HYBRID LIQUIDAMBAR SOMATIC EMBRYOGENESIS EFFICIENCY[1]

[1] Siran Lu, Paul R. Volny, David L. Blum and Scott A. Merkle*. To be submitted to *In Vitro Cellular & Developmental Biology – Plant*.

**Abstract**

Hybrid sweetgum (*Liquidambar styraciflua x L. formosana*) has great potential for use as a short-rotation biomass feedstock in the southeastern United States. Considerable progress in developing somatic embryogenesis as a mass clonal propagation system for hybrid sweetgum has been made in recent years, but labor costs remain the largest cost component of the system. In order to commercialize the hybrid sweetgum system, we need to overcome this barrier. Air-lift bioreactors have been successfully applied to improve growth efficiency in some woody species. Stirred-tank bioreactors have rarely been used in plant micropropagation, due to a number of constraints. However, this approach may meet the requirements of proliferating proembryogenic masses (PEMs), which characterize hybrid sweetgum cultures. Stirred-tank bioreactors can be helpful for optimizing a proliferation protocol, because the environment inside them can be controlled. Using two genotypes of hybrid sweetgum, we tested proliferation of suspension cultures of PEMs in airlift bioreactors and stirred-tank bioreactors, compared to culture in shaken flasks. Airlift bioreactors showed 12.6% and 104.2% faster growth rates in two genotypes, compared to flasks. Thus, the use of airlift bioreactors has the potential to lower costs during the proliferation stage of this propagation system.

## 2.1 Introduction

Sweetgum (*Liquidambar styraciflua*) is a fast-growing southern U.S. hardwood that has been increasingly used by the southern pulp and paper industry (Merkle and Nairn 2005). In recent years, genetic improvement and the rise of varietal sweetgum make the species a strong candidate for short-rotation feedstock for biomass energy (Merkle and Cunningham 2011; Wright and Cunningham 2008). Hybrid sweetgum (*Liquidambar styraciflua* x *L. formosana*) clones have demonstrated hybrid vigor, accumulating biomass significantly faster than elite native sweetgum in field tests (Merkle and Cunningham 2011).

Sweetgum somatic embryogenesis was first reported by Sommer and Brown (1980), who initiated cultures from seedling hypocotyls. Later, Merkle et al. (1998) initiated embryogenic sweetgum cultures from immature seeds. Embryogenic sweetgum cultures have also been initiated from parts of unexpanded staminate inflorescences from mature trees, Induction frequency was affected by genotype, explant type, and the length of time that explant material was held in cold storage. Germination and conversion frequencies of inflorescence-derived sweetgum somatic embryos were affected by genotype and the interaction between genotype and length of pre-germination cold treatment (Merkle et al. 1997; Merkle et al. 1998; Merkle and Battle 2000; Merkle et al. 2003). Embryogenic cultures of hybrid sweetgum have been initiated from immature seeds (Vendrame et al. 2001) and from unexpanded staminate inflorescences (Merkle et al. 2010).

Somatic embryogenesis has been considered to have great potential for large-scale micropropagation of superior sweetgum genotypes, because unlike other short-rotation hardwood biomass feedstock candidates (i.e., yellow-poplar and sycamore), which must be initiated from seeds with uncertain genotypes, embryogenic sweetgum cultures can be initiated from the tissues

of a mature tree (i.e., staminate inflorescences) (Merkle and Cunningham 2011), allowing direct propagation of superior genotypes.

Currently, sweetgum somatic embryogenesis technology remains in the laboratory stage. The system still requires substantial manipulation by humans, making cost per somatic seedling far higher than that of conventional seedlings (Merkle and Cunningham 2011). If methods for decreasing the labor costs of producing large batches of somatic seedlings can be identified, it will facilitate commercial production of clonal sweetgum planting stock. The stages of sweetgum somatic embryogenesis include culture initiation, proliferation of proembryogenic masses (PEMs), and somatic embryo development, maturation, germination and conversion. Among these steps, enhancing the proliferation of PEMs has very high potential for increasing the production scale and decreasing labor costs.

Bioreactors have shown promise as a way of improving micropropagation, especially somatic embryogenesis. First, bioreactors can increase working volume, which means many more embryogenic cells or somatic embryos can be cultivated at one time. Second, in bioreactors, it is possible to closely control the chemical and physical environment of the embryogenic cells or somatic embryos for optimum growth, which means that many different treatments can be applied during the growth process (Tautorus et al. 1992). Third, bioreactors can provide more oxygen for embryogenic cell proliferation (Curtis 2005).

An air-lift bioreactor (ALB) is one bioreactor design that has been tested for the proliferation of embryogenic cultures. In ALBs, the PEMs are incubated in a vessel of liquid medium and pneumatically agitated by a stream of air. In addition to the agitation, the air also functions as an oxygen resource and facilitates exchange between the gas phase and the medium (Curtis 2005). ALBs have been used to grow embryogenic cultures of various plant species,

including carrots (*Daucus carota*) (Etienne et al. 1997), grapes (*Vitis vinifera*) (Cabasson et al. 1997) and cork oak (*Quercus suber*) (Jiménez et al. 2011).

Stirred-tank bioreactors (STBs) are the standard device for contemporary fermentation. STBs are advanced models of traditional fermenters, improved with the knowledge of bacteria and fungi in recent years. Within STBs, environmental conditions (air flow rate, temperature, pH, dissolved oxygen level, agitation speed) can all be controlled. As a result, it is relatively easy to identify the optimal growth environment and maintain that environment for further study. The working volume of STBs is also easy to manipulate.

Because of the advantage of STBs, they have been used for the commercialization of variable products, such as recombinant proteins (e.g., antibodies, hormones and enzymes), and secondary metabolites, like organic acids. Many FDA-approved prescription drugs are produced using STBs, including antihemophilic factors (ReFacto™) and Tenecteplase (TNKase™), which are purified glycoproteins; Trastuzumab (Herceptin™), a monoclonal antibody for treating breast cancer; and Etanercept, an immunoglobulin-TNF (tumor necrosis factor) receptor fusion protein for treating rheumatoid arthritis (Chu and Robinson 2001). Additionally, STBs are used in the mass-production of hyoscyamine (Hilton and Rhodes 1990) and penicillin (Ariyo et al. 1998).

Despite the fact that STBs are widely and successfully used by the pharmaceutical industry, reports of research in which STBs have been used for plant micropropagation are rare. Theoretically, STBs should be able to provide an optimal environment for embryogenic culture proliferation since the stirring speed, temperature, pH, and dissolved oxygen level can all be closely controlled. In the research reported here, we compared STBs, ALBs and shaken flasks for their relative abilities to proliferate hybrid sweetgum PEMs.

## 2.2 Material and methods

*Culture initiation and embryogenic culture lines*

Embryogenic cultures of two genotypes (28-1-5A, and 28-1-8) of hybrid sweetgum were used in this research. Hybrid sweetgum cultures were initiated and maintained as described by Merkle et al. (2010). Briefly, embryogenic cultures were initiated by culturing immature seeds on induction maintenance medium (IMM), which consisted of Blaydes' medium (Witham et al. 1971), with Brown's minor salts (Sommer and Brown 1980), iron supplements of Murashige and Skoog (1962), vitamins of Gresshoff and Doy (1972), 1 g/l casein hydrolysate (enzymatic; CH), 40 g/l sucrose and 2 mg/l 2,4-dichlorophenoxyacetic acid (2, 4-D).

PEMs of both genotypes were proliferated in ALBs and STBs versus the standard approach, in which they were proliferated in shaken flasks.

*Comparison of airlift bioreactors to shaken flasks*

ALBs were constructed and operated as described by Kong et al. (2014) for growing American chestnut (*Castanea dentata*) embryogenic cultures. Each ALB consisted of a glass bottle (250 ml NALGENE, USA) with a plastic cap (autoclavable, NALGENE, USA) fitted with two connectors (Nalge Nunc Barbed fitting, NALGENE, USA), and two in-line filters (autoclavable, NALGENE, USA) (Fig. 2.1). The ALB system included an air pump, a humidifier and multiple bioreactor vessels. These three components were connected with silicon tubing (1/4 × 3/8 and 1/8 × 3/8 in., NALGENE, USA) (Kong et al. 2014). Two ml settled cell volume (SCV) of hybrid sweetgum PEMs were inoculated into the 250 ml Pyrex® bottles containing 100 ml of liquid IMM. The air flow rate was adjusted to 300 ml $min^{-1}$, as measured by an airflow meter (GF2200, Gilmont Instruments USA). As the control treatment, 2 ml SCV of hybrid sweetgum PEMs were inoculated into 250 ml Erlenmeyer flasks containing a 100 ml of IMM. Flasks were

shaken at 110 rpm $min^{-1}$. The IMM was refreshed every week for six weeks by pipetting off the old medium, followed by addition of an equal quantity of fresh medium. For both ALBs and shaken flasks, the volume of PEMs was measured at each feeding with graduated pipettes and 5 ml, 10 ml, and 25 ml measuring cylinders. For both flasks and ALBs, cultured material was allowed to settle for 3 min, and the volume of settled tissue was recorded. All material was then poured back into culture vessels under sterile conditions. All cultures were incubated at 25±1 ℃ in the dark for six weeks.

*Testing of stirred tank bioreactors*

For testing growth in STBs, 6 ml SCV of hybrid sweetgum PEMs were inoculated into 1 L STBs (Model SR0700ODLS, DASGIP, Germany) containing 400 ml of liquid IMM. In the STBs, a 6-blade Rushton turbine impeller (46 mm in diameter) was installed; the STB vessel is made of borosilicate glass with a stainless steel headplate; a temperature probe, dissolved oxygen probe and pH probe were used to monitor the culture environment (Fig.2.2.B).

During the experiments, the impeller was rotated at 60 rpm and temperature was controlled at 25±1 ℃. The gassing flow rate was 3 lph of air and the dissolved oxygen level was monitored but not controlled, since the dissolved oxygen level remained at a high level by itself during the experiment. The pH level was monitored for most runs and was controlled at 5.6 for the last run. The vessel was covered by aluminum foil to ensure that all cultures were incubated in the dark.

To refresh the medium in the STBs, an aseptic replenishing system (Fig. 2.2.A and C) was constructed. The aseptic replenishing system consisted of glass bottles (2 L NALGENE, USA), sterile pump bottle rigs, and a peristaltic pump. This system was used to aseptically draw off spent medium above the cells and replenish with fresh sterile medium. IMM was refreshed by

the aseptic replenishing system two weeks after inoculation and every week afterwards for six weeks.

In another experiment, STBs containing IMM supplemented with 40 g/l sucrose (control), 40 g/l glucose or 40 g/l fructose were run using one genotype (28-1-5A) to test the effect of these different sugars on PEM proliferation. The IMM was refreshed by the aseptic replenishing system two weeks after inoculation and every week afterwards for six weeks.

Samples of the medium were collected from the STBs on a daily basis for analysis. All samples were analyzed using a SUPELCOGEL™ Ca HPLC Column. The column used a di H20 mobile phase, 20 µL injection, and 0.5mL/min flow rate. PEM volumes were measured after six weeks of incubation using 100 ml and 25 ml graduated cylinders. Then, the PEMs were dehydrated in a laboratory oven for ten days at 90 $^{o}$C and dry weights were measured.

*Experimental design and statistical analysis*

Experiments for comparison of ALBs to shaken flasks employed three 250 ml Pyrex® bottles as ALB vessels and three 250 ml Erlenmeyer flasks for each of the two genotypes, and the experiment was repeated three times. For STBs, genotype 28-1-5A was inoculated in IMM using sucrose for three runs, in IMM using glucose for one run, and in IMM using fructose for one run; genotype 28-1-8 was inoculated in IMM using sucrose for one run. Data were plotted using Microsoft Excel. For the STBs, the data collection system was the DASGIP Control software, and for HPLC, data were collected by ChemStation software. Effects of bioreactor type and different STB treatments on PEM proliferation were analyzed by ANOVA and Tukey's test of Minitab 17 (Minitab Inc., State College, PA).

## 2.3 Results and Discussion

*Productivity of different bioreactors*

Dai et al. (2004) grew hybrid sweetgum PEMs in shaken flasks of liquid IMM for six weeks, feeding once, before size-fractionating the suspensions on stainless steel sieves and culturing a selected size fraction on semi-solid medium to produce synchronous populations of somatic embryos. Here, we compared the growth rates of suspension cultures of hybrid sweetgum PEMs using the method reported by Dai et al. (2004), to growth rates in air-lift bioreactors and stirred-tank bioreactors.

For genotype 28-1-8, Fig. 2.3 shows that one week following inoculation, PEMs grown in flasks grew an average of 10.1% more than PEMs grown in the ALBs, although the difference was not significant ($p=0.076$). At week two, there was also no significant difference in the amounts of PEMs grown using the two methods. By week 3, however, PEM growth in ALBs was significantly higher than in shaken flasks ($p=0.027$), and this difference increased as time passed. At weeks 4 and 5, PEM growth in ALBs remained significantly higher than in shaken flasks ($p= 0.014$ and $p=0.0004$, respectively). After six weeks, PEM production in the ALBs was more than two-fold (204%) higher than PEM production in the shaken flasks ($p=0.004$).

For genotype 28-1-5A (Fig. 2.4), the situation was more complicated than for 28-1-8. At one week following inoculation, PEM growth in flasks was significantly higher than that in ALBs ($p=0.036$). In the following two weeks, there was no significant difference in the amount of PEMs grown by these two methods ($p=0.083$ and $p=0.814$, respectively). At week 4 and week 5, there were still no significant differences in the amounts of PEMs grown by the two methods ($p=0.715$ and $p=0.603$, respectively), even though Fig. 2.4 appears to show growth in the ALBs

is beginning to surpass that in the flasks. By the final week, PEM growth in the ALBs was significantly higher than in shaken flasks ($p= 0.022$).

Based on these results, there appears to be a differential growth response between the ALBs and flasks, depending on genotype, although we did not test this interaction. However, by the end of the growth period, for both genotypes, PEM growth in ALBs was significantly higher than that in shaken flasks.

*Proliferation in stirred-tank bioreactors*

Three experiments were run to test growth of hybrid sweetgum PEMs in STBs. In the first experiment, 6 ml SCV of PEMs from genotype 28-1-5A proliferated to 44 ml SCV in STBs after 6 weeks, and the color of the PEMs produced was yellow mixed with brown. In the second experiment, 6 ml SCV of PEMs from the same genotype proliferated to 39.2 ml SCV and 6 ml SCV of PEMs from genotype 28-1-8 proliferated to 47.3 ml SCV in the STBs.

In the third experiment, we tested different sugars as carbon and energy resources for their effects on proliferation of 6 ml SCV of PEMs from genotype 28-1-5A. In the STB with sucrose, 6 ml SCV of PEMs from genotype 28-1-5A proliferated to 55.5 ml (dry weight = 7.1 g) in STBs, while with glucose as the carbon source, the same amount of starting material proliferated to 51 ml (dry weight = 4.2 g), and PEMs cultured with fructose as the carbon source proliferated to only 19.3 ml (dry weight = 2.2 g), The fructose treatment gave PEMs with a dark brown color, which may indicate that the PEMs became inactive (Figure 2.5).

Medium samples taken daily from the three experiments were analyzed for sugar content. In the first two experiments, which only employed sucrose as a carbon source in the medium, most of the sucrose in the medium was converted to glucose and fructose within the first three

days of culture (data not shown). Most glucose was consumed within 14 days following culture inoculation, while the level of fructose remained almost unchanged. As a result, we hypothesized that hybrid sweetgum PEMs may use glucose as their major carbon and energy resource. Fructose may not be useful in the proliferation process.

Based on the observations described above, in the third experiment, we tested glucose and fructose as carbon sources in the medium, in comparison to sucrose. Interestingly, the dry weight of PEMs grown with sucrose was roughly equal to the dry weight of PEMs grown with glucose plus the dry weight of PEMs grown with fructose. Fig. 2.6a (results for STB with fructose) shows the same changes in sucrose, glucose and fructose levels described above for the first two experiments. Figs. 2.6b and 2.6c indicate that in the STB with glucose, the amount of glucose gradually decreased over time between feedings, while the amount of fructose dropped slightly at the end of the period between feedings.

Our results provide evidence that ALBs can be a good method to accelerate proliferation of hybrid sweetgum embryogenic cultures. With the same initial cell density and medium volume, ALBs produced twice the volume of PEMs produced by the flasks for one hybrid sweetgum genotype, although the difference between ALBs and flasks was much less for the other tested genotype.

The results also demonstrate the STBs have some potential to be applied for production of hybrid sweetgum PEMs, although their potential utility for this purpose has some problems. We could not directly compare the productivity of the STBs to those of the ALBs and shaken flasks, since they were not part of the same experiment. Unlike the ALBs and flasks, it was not possible to measure growth in the STBs on a weekly basis due to risk of contamination, so only final SCVs of PEMs after six weeks were measured. In addition, the volume of the STBs and of

the medium used in the STBs were four times those of the ALBs and flasks, and the initial amount of PEMs inoculated into the STBs was three times the volume of PEMs inoculated into the ALBs and flasks. However, based on the fact that the STBs were inoculated with three times the SCV of PEMs that was used to inoculate the ALBs and flasks, some rough comparisons (Table 2.1) can be made by multiplying the final SCVs of the ALBs and flasks after six weeks of growth by three. For genotype 28-1-5A, the mean SCV of PEMs produced by STBs was 46.33 ml, while the mean SCV of PEMs produced by shaken flasks was 43.5 ml (14.5 x 3), and the mean SCV of PEMs produced by ALBs was 49.02 ml (16.34× 3). Thus, the mean SCV of PEMs produced by STBs was less than the mean SCV of PEMs produced by ALBs, but greater than the mean SCV of PEMs produced by shaken flasks. For genotype 28-1-8, the mean SCV of PEMs produced by STBs was 47.3 ml SCV, while the mean SCV of PEMs produced by shaken flasks was 35.55 ml (11.85× 3), and the mean of SCV of PEMs produced by ALBs was 72.6 ml (24.2× 3). So, as was the case for genotype 28-1-5A, the mean SCV of PEMs produced by STBs was less than the mean SCV of PEMs produced by ALBs but greater than the mean SCV of PEMs produced by shaken flasks.

PEMs produced by STBs were a darker brown color than PEMs produced in the flasks or ALBs (Fig.2.7). The browning may be the consequence of the more violent agitation and shear forces generated by the impeller. Although we did not test this, the browning might be reduced by decreasing the RPM of the impeller or by using a different impeller design. STBs did have an advantage over the ALBs and shaken flasks in that conditions inside the vessels could be closely monitored and controlled, if desired. The HPLC results regarding changes in the levels of sucrose, glucose, and fructose during the culture cycle indicated that glucose in the medium is used by the hybrid sweetgum PEMs, while fructose in the medium remained relatively unused.

However, sucrose remains the most suitable way to provide carbon and energy for hybrid sweetgum embryogenic culture proliferation, due to its low price and its ability to support PEM growth.

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**Table 2.1.** Rough comparison of productivity (ml SCV) of shaken flasks, ALBs and STBs

| Genotype | 28-1-8 | 28-1-5A |
|---|---|---|
| Shaken flasks[1] | $11.85\times 3 = 35.33$ | $14.5\times 3 = 43.5$ |
| ALBs[1] | $24.2\times 3 = 72.6$ | $16.34\times 3 = 49.02$ |
| STBs[2] | 47.3 | 46.33 |

[1]The initial amount of PEMs inoculated into shaken flasks and ALBs was 2 ml.

[2]The initial amount of PEMs inoculated into STBs was 6 ml.

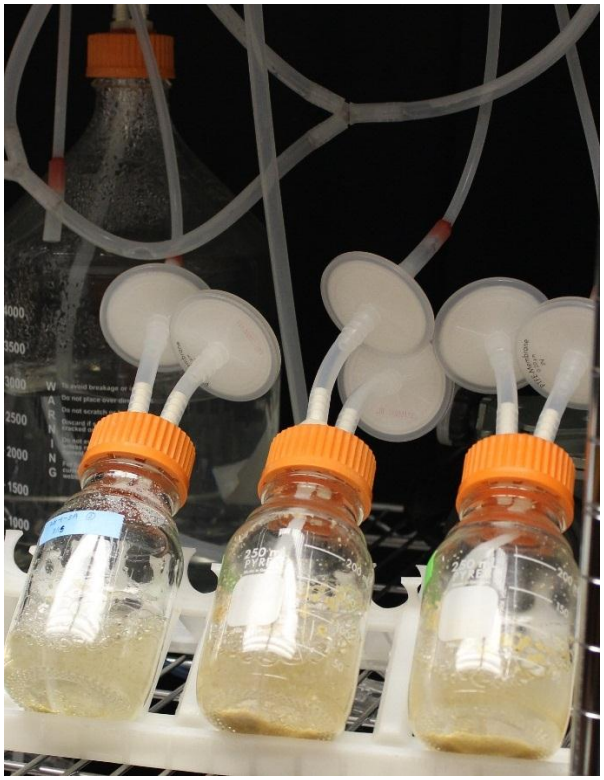


**Figure 2.1.** Construction of ALBs used for hybrid sweetgum PEMs proliferation. Culture initiation amount was 2 ml SCV.

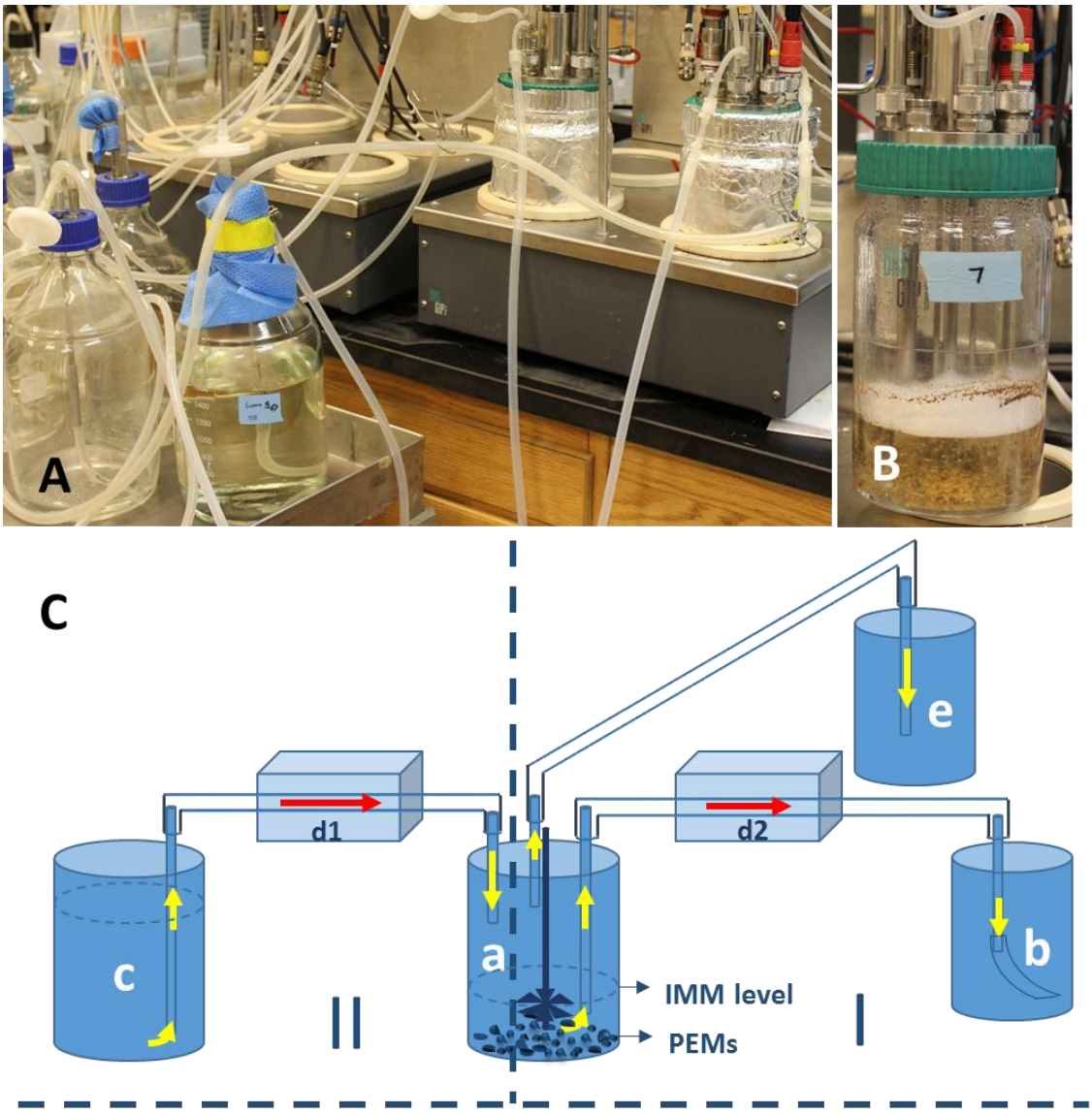



**Figure 2.2**. STBs and the aseptic replenishing system used to refresh the medium in the STB. A. set-up of the STBs and aseptic replenish system in the lab. STBs are covered with aluminum foil to exclude light; B. A STB with hybrid sweetgum PEMs proliferating inside; C. Diagram of the aseptic replenish system, a. STB vessel. b. Waste bottle. c. Bottle of IMM stock. d. Peristaltic pump e. Air bottle, to keep air pressure normal. Components in the 'I' half of the diagram were run first to remove all old medium above PEMs, then the flow between A and B was stopped; components in the 'II' half of the diagram were run to supply fresh medium into the STB vessel.

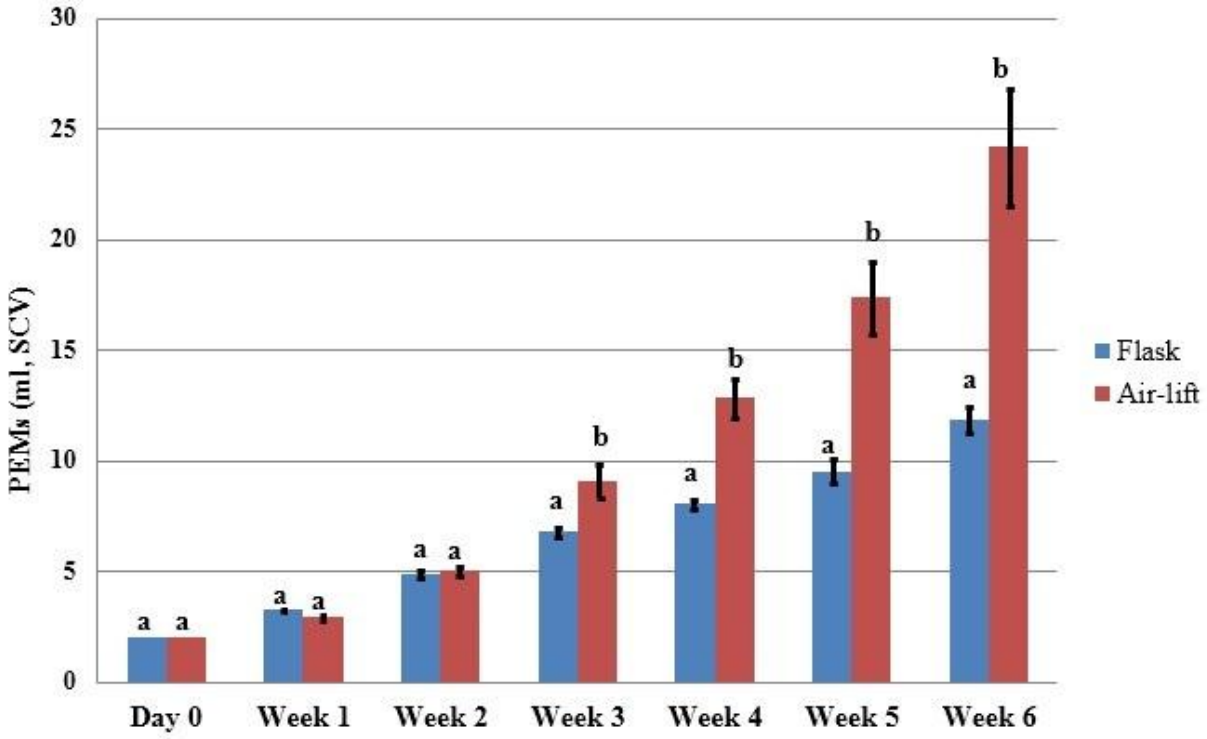



**Figure 2.3.** Comparison of PEM proliferation between flasks and ALBs for hybrid sweetgum genotype 28-1-8. Culture initiation amount was 2 ml SCV. Values are the mean ± SE of four vessels. Means with different letters at each time point are significantly different according to Tukey's test ($P<0.05$)

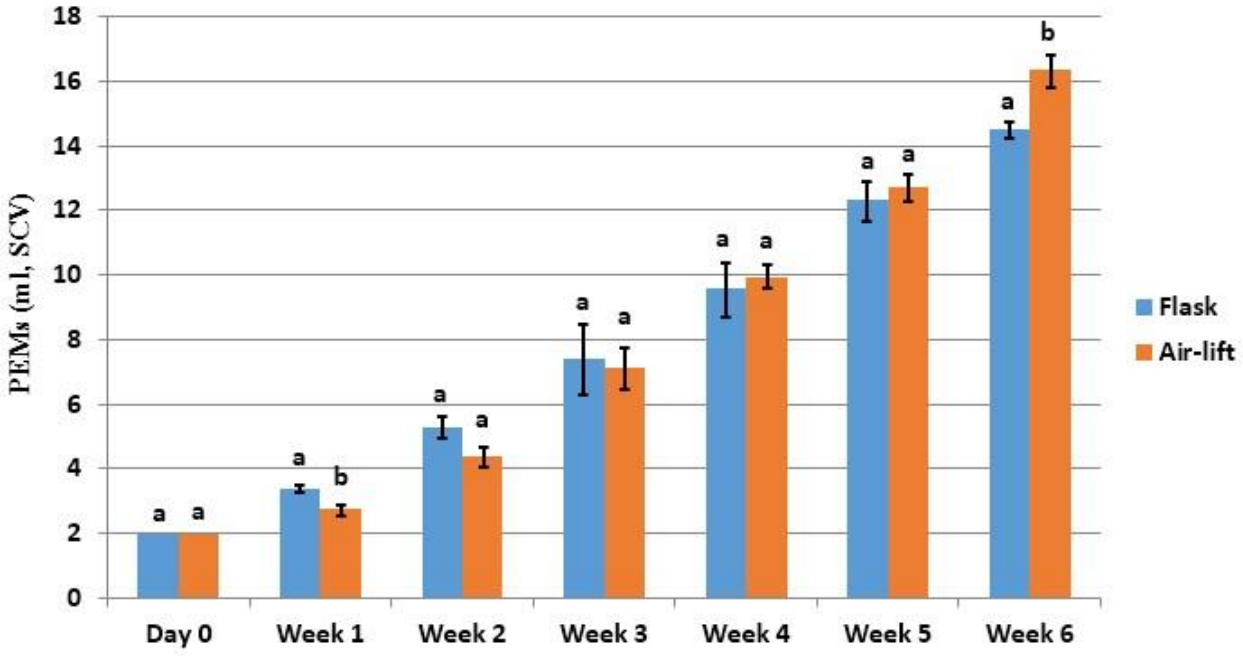



**Figure. 2.4.** Comparison of PEM proliferation between flasks and ALBs for hybrid sweetgum genotype 28-1-5A. Culture initiation amount was 2 ml SCV. Values are the mean ± SE of 5 vessels. Means with different letters at each time point are significantly different according to Tukey's test ($P<0.05$)

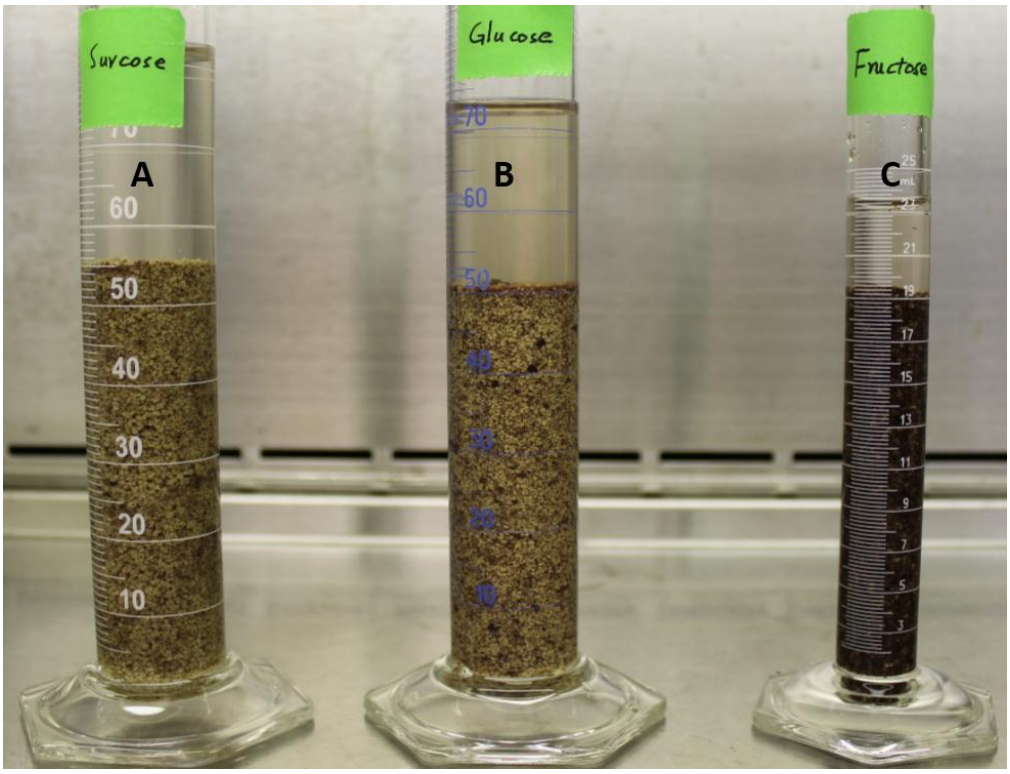
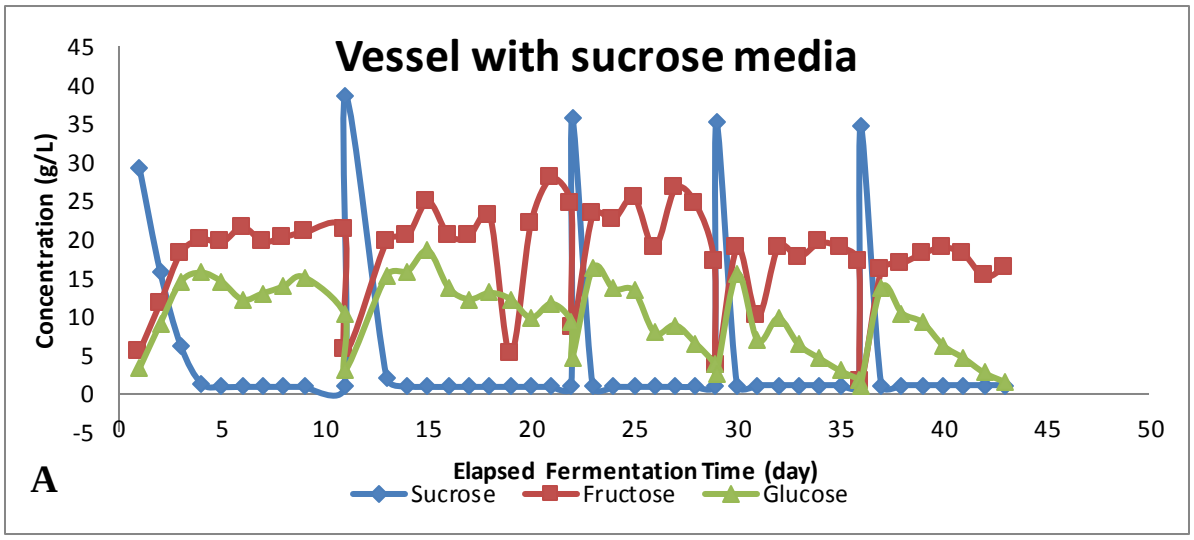
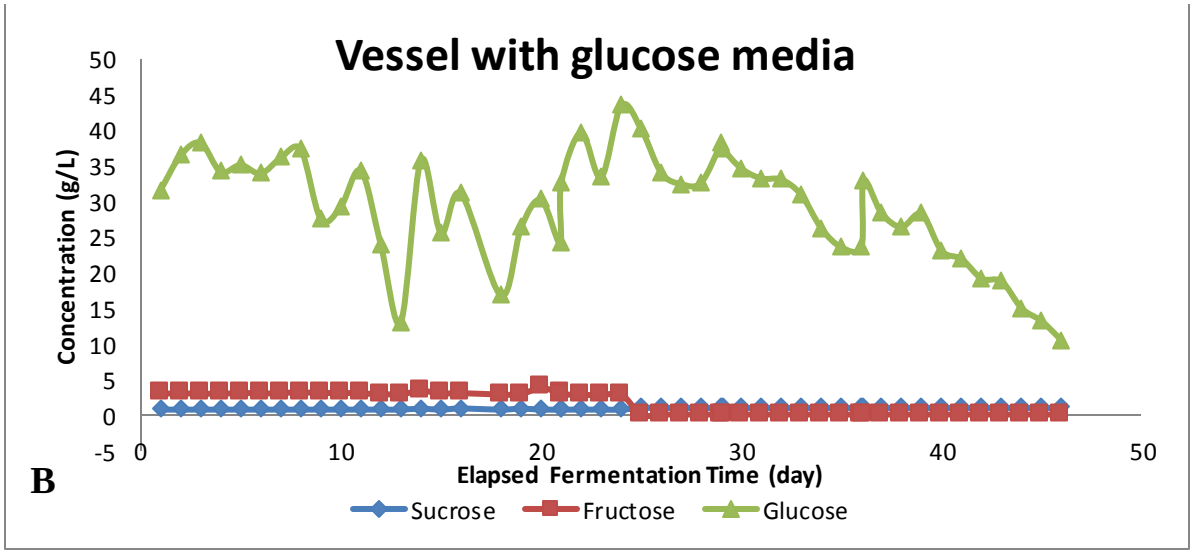



**Figure. 2.5.** Hybrid sweetgum PEMs grown in suspension culture in the STBs for six weeks with different sugars used as carbon sources. A. PEMs grown in medium with sucrose; B. PEMs grown in medium with glucose; C. PEMs grown in medium with fructose.


Vessel with sucrose media
Concentration (g/L)
45
40
35
30
25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
40
45
50
Elapsed Fermentation Time (day)
Sucrose
Fructose
Glucose
A


Vessel with glucose media
Concentration (g/L)
50
45
40
35
30
25
20
15
10
5
0
-5
0
10
20
30
40
50
Elapsed Fermentation Time (day)
Sucrose
Fructose
Glucose
B

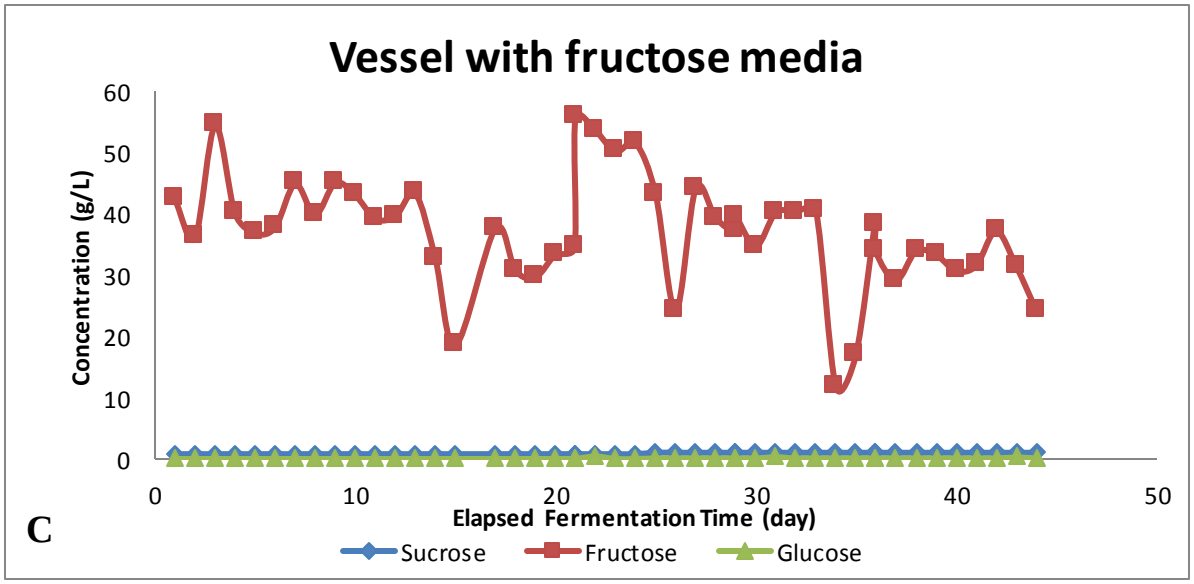

Vessel with fructose media
Concentration (g/L)
60
50
40
30
20
10
0
0
10
20
30
40
50
Elapsed Fermentation Time (day)
Sucrose
Fructose
Glucose
C

**Figure. 2.6.** Use of sugars by hybrid sweetgum PEMs grown in STBs. Cultures were fed with fresh medium every two weeks. A. PEMs grown in IMM with sucrose as carbon source; B. PEMs grown in IMM with glucose as carbon source; C. PEMs grown in IMM with fructose as carbon source.

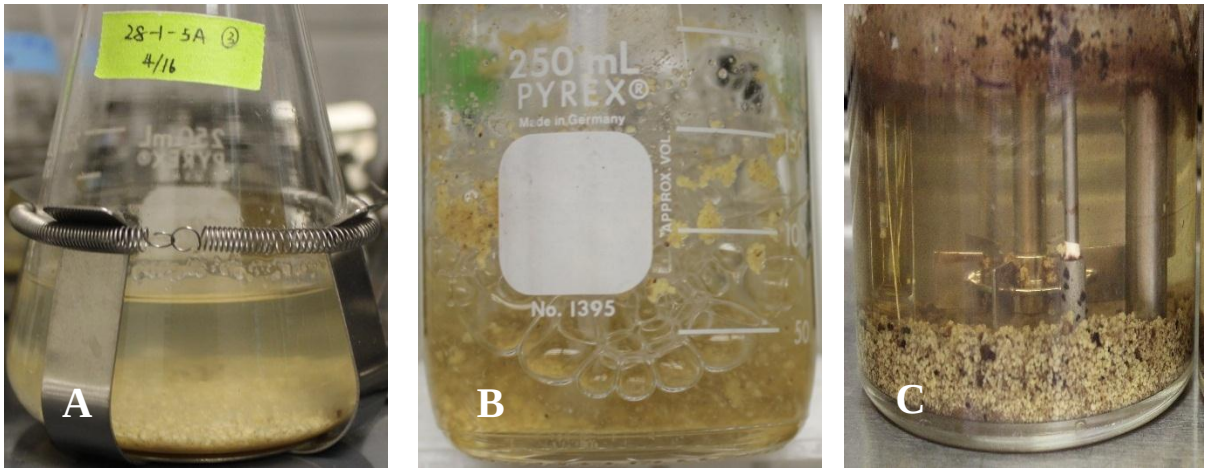



**Figure. 2.7.** Appearance of hybrid sweetgum PEMs grown in suspension culture in different vessels. A. PEMs grown in a shaken flask; B. PEMs grown in an ALB; C. PEMs grown in a STB.

# CHAPTER III

# 3. ENHANCING HYBRID LIQUIDAMBAR SOMATIC SEEDLING PRODUCTION USING A TEMPORARY IMMERSION BIOREACTOR[2]

[2] Siran Lu and Scott A. Merkle. To be submitted to *Trees - Structure and Function*.

**Abstract**

Fast-growing hybrid southern hardwood trees should make excellent material for woody biomass production in the Southeastern U.S., if elite clones can be identified and efficiently propagated. We have enhanced the potential of sweetgum (*Liquidambar styraciflua*) as a biomass species by generating hybrids between the tree and its Chinese relative, *Liquidambar formosana*, and propagating the most promising clones via somatic embryogenesis. Some of the hybrid clones have already demonstrated superior biomass productivity compared to elite *L. styraciflua* trees. However, production of somatic seedlings from these clones remains labor-intensive. Bioreactors, specifically temporary immersion designs, such as the RITA®, have been applied to improve in vitro propagation of a number of woody species. We tested RITA®s for their potential to improve the production efficiency of high-quality hybrid sweetgum somatic seedlings. In one tested genotype, a RITA® with 50 somatic embryos had about 35% higher conversion frequency ($p<0.05$) and about 20% higher high-quality somatic seedling production frequency ($p<0.05$) than somatic embryos germinated on semisolid medium in GA7 vessels. In all genotypes we tested, somatic seedlings produced in RITA® bioreactors had higher survival frequency than somatic seedlings produced on semisolid medium in GA7 vessels.

## 3.1 Introduction

Today, fossil fuels still have a dominant status in the world energy market. However, they are non-renewable and not eco-friendly. To solve this dilemma, people are pursuing energy choices that are affordable, reliable, diverse, safe, and environmentally acceptable to replace fossil fuels. Wind, solar, water, geothermal, biomass, and nuclear are alternatives now being researched to replace fossil fuels. Among them, biomass is one of the most promising alternative energy resources, due to its self-renewable nature and its potential to improve air quality and develop rural economies (http://www.energy.gov/).

Over the past several decades, sweetgum (*Liquidambar styraciflua*) has been considered as a potential candidate for short-rotation biomass energy plantations (Merkle and Cunningham 2011; Wright and Cunningham 2008). This fast-growing hardwood species, native to the southern U.S., is already widely utilized for pulp, lumber, pallets, veneer, panels, and fuelwood by a large number of mills in the southern U.S. (Wright and Cunningham 2008). Hybrids between *L. styraciflua* and the Chinese sweetgum, *L. formosana* (Figure 3.1) have exhibited hybrid vigor, producing biomass more rapidly than the parent species (Merkle and Cunningham 2011). Somatic embryogenesis has been reported for hybrid sweetgum (Vendrame et al. 2001), and a suspension culture-based system has been described which is capable of producing large numbers of hybrid sweetgum somatic seedlings (Dai et al. 2004). The methods reported to date for propagating hybrid sweetgum trees via somatic embryogenesis, like most *in vitro* propagation methods, are highly labor-intensive, which raises per-propagule production costs. In micropropagation, labor generally accounts for 40–60% of the production costs (Etienne and Berthouly 2002). As a result, it has been concluded that commercial application of

micropropagation would only be possible if new technologies are available to reduce the labor-intensive steps (Kitto 1997).

Over the past 20 years, bioreactors have shown promise as a way of improving micropropagation. Unfortunately, most bioreactor designs developed in the past are not generally suitable for plant micropropagation, as they were not designed to meet the specific requirements of plant cells, which are sensitive to shear forces, mechanical damage, and the foam formation characteristic of bubble aerated bioreactors (Teisson et al. 1999). Recently, because of the increasing requirements of the commercial application of micropropagation, bioreactors specifically designed for plant cells have been developed. A temporary immersion bioreactor (TIB) is one bioreactor design that has been tested for production and germination of somatic embryos. This type of bioreactor provides much more uniform culture conditions, an easier way to renew the medium without changing the container, complete renewal of the culture atmosphere by forced ventilation and larger volumes than culturing on semi-solid media. In addition, they also permit temporary contact between the plant tissues and the liquid medium.

The RITA® system is a TIB composed of a 1-L vessel comprised of two compartments, a top one with the plant material and a bottom one with the medium. Air pressure applied to the bottom compartment pushes the medium into the top compartment. In the top compartment, plant material is immersed as long as the pressure is applied. During the immersion period, air is bubbled through the medium, gently agitating the tissues and renewing the head space atmosphere inside the culture vessel, with the pressure escaping through outlets in the top of the apparatus (Etienne and Berthouly 2002)

The RITA® has shown great promise in improving the micropropagation of several different species, including some woody species. In *Eucalyptus* micropropagation, a four- to six-

fold increase in yield in half the growth time and hardier plantlets were achieved with the RITA® system compared with axillary bud proliferation on semi-solid media (B. McAlister et al. 2005). In *Citrus deliciosa* (Cabasson et al. 1997), *Musa acuminate* (Escalant et al. 1994), *Psidium guajava* (Kosky et al. 2005), and *Hevea brasiliensis* (Etienne et al. 1997), the RITA® system improved both the quantity and quality of the somatic embryos produced, increased germination rate and epicotyl emergence, and controlled or even eliminated hyperhydricity (Etienne and Berthouly 2002).

TIBs may have the potential to greatly enhance different stages of plantlet production from forest tree embryogenic cultures, like those of hybrid sweetgum, including somatic embryo production, maturation, germination and conversion. To our knowledge, there have been no studies comparing these steps of somatic seedling production between TIBs and semisolid medium with forest tree embryogenic cultures to date. The objective of this study was to test the potential of the RITA® temporary immersion bioreactor system to enhance conversion (somatic seedling production) from mature hybrid sweetgum somatic embryos.

## 3.2 Materials and Methods

*Culture initiation and somatic embryo production for experiments*

Somatic embryos of five genotypes of hybrid sweetgum (*Liquidambar styraciflua* x *L.-formosana*), 28-1-5A, 28-1-5B, 28-1-8, 4-8-4 and 4-8-2B, were used to test the germination potential of the RITA® bioreactors versus germination on semi-solid medium, although all five genotypes were not used in each experiment. Hybrid sweetgum embryogenic cultures were initiated following the protocol of Vendrame et al. (2001). Briefly, embryogenic cultures were initiated by culturing immature hybrid sweetgum seeds on induction maintenance medium (IMM) which included Blaydes' medium (Witham et al. 1971), with Brown's minor salts (Sommer and Brown 1980), iron supplements of Murashige and Skoog (1962), vitamins of Gresshoff and Doy (1972), 1 g/l casein hydrolysate (enzymatic; CH), 40 g/l sucrose and 2 mg/l 2, 4-D. Production of somatic embryos from embryogenic suspension cultures followed the method described by Dai et al. (2004). Approximately 0.5 g of PEMs was inoculated into 125 ml Erlenmeyer flasks containing 30 ml IMM. Erlenmeyer flasks were incubated in the dark at 25 ℃ on a rotary shaker at 110 rpm for two culture cycles (3 weeks per cycle). After proliferation, PEMs (37-152 mm in diameter) were collected using stainless steel Cellector® sieves (Bellco Glass). The collected PEMs were re-suspended in liquid embryo development medium (EDM; IMM without PGRs), then collected on filter paper disks (4.25 cm in diameter) using a Büchner funnel under mild vacuum, and cultured along with the filters in 60 x 15 mm plastic Petri plates on semisolid EDM in the dark at 25 ˚C to produce somatic embryos. After 4 weeks, mature somatic embryos with well-formed cotyledons were collected and given a pre-germination cold treatment at 8 °C for at least 8 weeks.

*Tests of RITA® bioreactors*

The operation of the RITA® system (CIRAD, France) was briefly described above. Three different inoculation densities of mature somatic embryos per RITA® (50, 100 and 200) were tested. Each RITA® unit was filled with 150 ml of liquid germination medium (GM; EDM without CH; Dai et al. 2004). The RITA® system was fed every 14 days by pouring out the old medium and refilling it using a pipette. The immersion duration and frequency were set to one minute every 12 hours by using a Leviton LT113 digital timer to control a Tetra Whisper 60 gallon aquarium air pump (Spectra Brands Inc., Blacksburg VA). As the control, 16 mature somatic embryos were incubated on 150 ml GM gelled with 8g $L^{-1}$ Phytoblend (Caisson) in GA7 vessels (Magenta Corp.) and were moved to GA7s with a fresh medium every 14 days. We limited the GA7s to 16 mature somatic embryos to provide a similar area per somatic embryo in the GA7 (3.71 $cm^2$ per embryo) to that available in the RITA® unit with 50 embryos (5.09 $cm^2$ per embryo) and 100 embryos (2.54 $cm^2$ per embryo).

RITA®s and GA7 vessels were incubated in a temperature-controlled room (24± 1°C) under cool white fluorescent light (16 hours per day; 35 µmol $m^{-2}$ $s^{-1}$) for 45 days, after which the somatic seedlings were removed from in vitro conditions. Somatic seedlings were planted in standard 4 inch nursery pots with Fafard 3B potting mix and moved to a Plexiglas hardening-off chamber with 16 hours of daylight and a light intensity of 165 µmol $m^{-2}s^{-1}$. After 8 - 10-weeks of acclimatization, somatic seedlings were moved to the greenhouse.

*Experimental design and statistical analysis*

The numbers of converted somatic embryos (i.e. those that produced somatic seedlings) in the RITA®s and GA7s were counted after 45 days of incubation. During the acclimatization process, the number of surviving somatic seedlings was counted every seven days, and after five months of

growth in the greenhouse, the number of surviving plantlets was recorded and the shoot lengths and the diameters of shoots at 4 inches above the root collar were measured for each plantlet.

The experiment was conducted four times. In the first run, somatic embryos from genotype 28-1-5B were inoculated in the RITA® bioreactors at two different densities (50 and 100), with one RITA® unit per density, and three GA7 vessels with somatic embryos were used as controls. In the second run, somatic embryos from genotype 4-8-4 were inoculated in the RITA® bioreactor at two different densities (50 and 100), one RITA® unit per density was used, and three GA7 vessels were used as controls. In the third run, somatic embryos from genotype 28-1-5A were inoculated in the RITA® bioreactors at three different densities (50, 100, and 200), three RITA® units per density were used, and three GA7 vessels were used as controls. In the fourth run, genotypes 28-1-5A, 28-1-8, and 4-8-2B were inoculated in the RITA® bioreactors, with 100 somatic embryos per RITA® unit, three RITA® units per genotype were used, and three GA7 vessels per genotype were used as controls.

Somatic seedlings from genotype 28-1-5A, 28-1-5B and 4-8-4 were used for evaluating the effects of the different germination environments on plantlet growth during and following acclimatization. For genotype 28-1-5A, 98 somatic seedlings from GA7 vessels, 64 somatic seedlings from the 50 embryos per RITA® units, 48 somatic seedlings from the 100 embryos per RITA® units, and 45 somatic seedlings from the 200 embryos per RITA® units were potted; for genotype 28-1-5B, 24 somatic seedlings from GA7 vessels, 18 somatic seedlings from the 50 embryos per RITA® units, and 32 somatic seedlings from the 100 embryos per RITA® units were potted; for genotype 4-8-4, 15 somatic seedlings from GA7 vessels, 19 somatic seedlings from the 50 embryos per RITA® units, 21 somatic seedlings from the 100 embryos per RITA® units were potted. Somatic seedlings were initially potted at a density of four per 4 inch pot. Following acclimatization, some plantlets were selected to be repotted singly into 1 gal pots and moved to the greenhouse. In all, 8 plantlets from

GA7 vessels and 25 plantlets from the RITA® bioreactors from genotype 28-1-5B were moved to the greenhouse.

The following data were collected and plotted using Microsoft Excel and analyzed by ANOVA, Fisher's test and Tukey's test using Minitab 17 (Minitab Inc., State College, PA).

$$\text{Conversion frequency (ConF)} = \frac{Number\ of\ converted\ somatic\ embryos}{total\ embryos} \text{ x } 100\%$$

$$\text{High-quality somatic seedling frequency (HQF)} = \frac{Number\ of\ good\ seedlings}{total\ embryos} \text{ x } 100\%$$

$$\text{Survival frequency} = \frac{Number\ of\ surviving\ somatic\ seedlings}{total\ somatic\ seedlings} \text{ x } 100\%$$

Conversion refers to all somatic embryos that converted to somatic seedlings, including those with short roots, weak shoots, poorly expanded leaves, or other problems. High-quality somatic seedlings were those with good leaf expansion, strong shoots, and extensive roots. They could have single shoots or multiple shoots. Survival frequency refers to those somatic seedlings that completed acclimatization. Only high-quality somatic seedlings were potted.

## 3.3 Results and Discussion

*Effect of vessel type and density on somatic embryo germination*

In the RITA® bioreactors, germination of the somatic embryos began around seven days following inoculation, when the embryos changed color from light yellow to shiny green. In the GA7 vessels, germination of the somatic embryos began around eleven days following inoculation.

Table 3.1 shows the comparison among conversion frequencies and frequencies of high-quality somatic seedlings for the somatic embryos inoculated into the RITA® bioreactors at different densities and those inoculated into the GA7 vessels for two genotypes. For genotype

28-1-5A, analysis of variance results indicated that both conversion frequency ($p=0.024$) and the frequency of high-quality somatic seedlings ($p=0.002$) differed significantly among the treatments. The conversion frequency for RITA®s with 50 embryos was higher than those of all other treatments. However, the conversion frequencies of RITA®s with 100 embryos and RITA®s with 200 embryos were not statistically different from the conversion frequency of the control treatment. The frequency of high-quality somatic seedlings (HQF) produced in RITA®s with 50 embryos was higher than the GA7 control, although no higher than for the RITA®s with the other two inoculation densities, which, in turn, were not higher than the control. Thus, both the highest conversion frequency (97.56%) and the highest frequency of high-quality somatic seedlings (53.20%) were obtained with embryos in the RITA® units inoculated with 50 embryos per unit. For genotype 4-8-4, there was not enough data for analysis of variance since there was only data from a single run with this line. After the second run of the RITA® comparison experiment was conducted, the PEMs of this line became inactive and no longer produced somatic embryos. However, the percentage data presented in Table 3.1 for this line, while not analyzed, indicate that the RITA®s gave higher conversion frequency and productivity of high-quality somatic seedlings, supporting the results obtained with 28-1-5A.

For both genotypes, the somatic seedlings from the RITA® system were morphologically similar to those from the GA7 vessel. No hyperhydricity was observed, as was also reported by (Cabasson et al. 1997) for *Citrus deliciosa* and by (Kosky et al. (2005)) for *Psidium guajava L.* somatic seedlings. After 45 days of growth in vitro, hybrid sweetgum somatic seedlings grown in the RITA® bioreactors were larger and more vigorous than the somatic seedlings germinated in the GA7 vessels. Figs 3.1A and 3.1C show the shoots of somatic seedlings on semisolid medium in a GA7 and in a RITA®, respectively. Although measurements were not taken at this

point, shoots of somatic seedlings grown in the RITA® system had more leaves and appeared to have longer internodes compared to the shoots of somatic seedlings on semisolid medium in GA7 vessels. Figs. 3.1A and 3.1B show the roots of somatic seedlings in gelled medium and in the RITA®s, respectively. Roots of somatic embryos germinated in the RITA® system appeared longer than those of somatic embryos germinated in GA7 vessels, and the roots of somatic embryos germinated in the RITA® system had multiple branches while those of somatic embryos germinated in GA7 vessels were mostly unbranched. The difference between embryos germinated in the RITA® system and those germinated in GA7 vessels may be explained by the much larger space provided by the RITA® system and air exchange between the RITA® system and outside environment. The gel matrix may also play a negative role in shoots and roots growth in the GA7s, since the resistance provided by the gel may contribute to slower growth of roots, lead to slower development of shoots. Also, in the RITA® system, a large reservoir of liquid IMM was available to the somatic seedlings, perhaps allowing improved mineral nutrition to the somatic seedlings. Improved absorption by the whole plant, which is covered with a thin film of medium, could have resulted in better shoot and root growth in the RITA® system.

*Somatic seedling survival frequency, height and diameter*

Following 45 days of growth in vitro, the high-quality somatic seedlings from the RITA®s and the GA7s were potted and moved to a hardening-off chamber (Figure 3.2). After 11 weeks of acclimatization, they were moved to the greenhouse (Figure 3.3).

Table 3.2 shows survival frequencies of the somatic seedlings following acclimatization. For genotype 28-1-5A, analysis of variance results indicated that survival frequencies of somatic seedlings varied among the different treatments ($p=0.032$). Survival frequencies of somatic seedlings grown in RITA®s at all three densities were significantly higher than those for somatic

seedlings grown in GA7s, although survival rates did not differ among the different densities tested in the RITA®s (Table 3.2). Over 91% of the somatic seedlings from the RITA®s sown with 100 embryos survived acclimatization, while only 70% of the somatic seedlings grown in GA7s survived.

For genotype 28-1-5B, somatic seedling survival frequencies also varied among the different treatments ($p=0.003$). Similar to the results with 28-1-5A, survival frequencies of somatic seedlings grown in RITA®s at both tested densities were significantly higher than those for somatic seedlings grown in GA7s, and survival rates did not differ between the two densities tested in the RITA®s (Table 3.2). About 90% of the somatic seedlings from the RITA®s grown with 50 embryos survived acclimatization, while only 33.33% of the somatic seedlings grown in GA7s survived.

Finally, for genotype 4-8-4, survival frequency also varied among the tested treatments ($p=0.001$). Survival frequencies of somatic seedlings grown in RITA®s at both tested densities were significantly higher than those for somatic seedlings grown in GA7s, although survival rates did not differ between the two densities tested in the RITA®s (Table 3.2). About 79% of the somatic seedlings from the RITA®s grown with 100 embryos survived acclimatization, while only 33.33% of the somatic seedlings grown in GA7s survived.

Almost 100% of the 28-1-5B hybrid sweetgum somatic seedlings moved to the greenhouse were still alive after 5 months. At this time, the shoot length and the diameter of shoot at 4 inches above the root collar were measured for each tree. The means of height and diameter are shown in Table 3.3. There was no significant difference between the average shoot lengths or the average shoot diameters between somatic seedlings from the control treatment and those from the RITA® system.

*Testing of additional genotypes*

A final experiment compared conversion frequencies between gelled medium and RITA®s sown with 100 somatic embryos, for two hybrid sweetgum genotypes that had not previously been tested, along with a previously tested genotype. Table 3.4 shows the conversion frequencies and high-quality somatic seedling frequencies of all three genotypes for the two treatments. In this experiment, no significant differences in ConF or HQF were found for genotypes 28-1-5A or genotype 4-8-2B. However, while the ConF for genotype 28-1-8 did not differ between the two treatments, the HQF was higher for the somatic seedlings from the GA7 vessels than those from the RITA® bioreactors. Thus, there is some evidence that not all hybrid sweetgum genotypes may benefit from germinating somatic embryos in a RITA® at least at relatively high density.

Based on our results, we can conclude that hybrid sweetgum somatic embryos germinated in RITA® bioreactors can obtain a higher ConF than the embryos germinated in the GA7 vessels (Table 3.1), but only for the relatively low sowing density of 50 embryos per unit. Of the densities we tested in the RITA®s, we found that 50 embryos per RITA® unit was optimal, because it had both the highest ConF and the highest frequency of high-quality somatic seedlings. Further experiments to optimize somatic embryo sowing density to gain high-quality hybrid sweetgum somatic seedlings might show that decreasing the number of sown embryos below 50 per RITA® unit would improve somatic seedling quality further. Alternatively, if the high-quality somatic seedlings could be harvested every week starting at 35 days of growth, more high quality somatic seedlings might ultimately be produced per RITA®, rather than harvesting all of the somatic seedlings on the same day, since this approach would make more space available for the

remaining somatic seedlings. In all three genotypes, hybrid sweetgum somatic seedlings inoculated in the RITA® bioreactor obtained a higher survival frequency than the hybrid sweetgum somatic embryos inoculated in the GA7 vessels.

The RITA® appears to have some promise as a tool for in vitro propagation of hybrid sweetgum via somatic embryogenesis. The reason for the higher quality somatic seedlings from the RITA®s may be at least partially the result of air exchange every 12 hours, which refreshes gases such as $O_2$ and $CO_2$. As mentioned earlier, the somatic embryos inoculated in the RITA® germinated more rapidly than those inoculated in the GA7 vessel. This phenomenon may due to the diluted ethylene concentration by gas exchange. Huxter et al. (1981) reported that ethylene in high concentration will inhibit shoot primordium formation in early culture stages of tobacco callus, and Ma et al. (1998) reported that an ethylene precursor, 1-aminocyclopropane-l-carboxylic acid ( ACC ), will delay root emergency and increase callus formation in shoot cultures of an apple cultivar. By the immersion mechanism, the whole plant surface is in contact with the GM and can absorb nutrients from it, but only a thin layer of GM remains on the plant tissue surface, which will not inhibit gas exchange and helps to diminish hyperhydricity. Also, the much larger inside space of the RITA® compared to the GA7 vessel may be the reason the somatic seedlings inoculated in the RITA® system grew faster than the somatic seedlings inoculated in the GA7 vessel.

Even though the final experiment, which compared RITA®s sown with 100 somatic embryos to GA7s, showed similar ConF and HQF, the RITA® bioreactor could still be considered to be more efficient that the GA7 vessel at this density. We could produce many more somatic seedlings from one RITA® bioreactor than from one GA7 vessel, even if the HQF and ConF were similar, because of the RITA®'s larger volume. For example, there were 100 somatic

embryos inoculated in the RITA® bioreactor, while 16 somatic embryos were inoculated in the GA7 vessel, which is close to the maximum we could fit onto the surface of the gelled medium. Thus, we could get many more high-quality somatic seedlings from the RITA® bioreactor than those from the GA7 vessel with similar HQF. As noted above, further manipulation of the somatic seedlings during the process (e.g. removing the larger somatic seedlings during the process to allow less developed somatic seedlings additional space to expand), may make it possible to inoculate the RITA®s with higher densities of somatic embryos while maintaining high levels of somatic seedling quality.

## 3.4 Acknowledgments

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**Table 3.1.** Comparison of conversion frequency (ConF) and high-quality somatic seedling frequency (HQF) or hybrid sweetgum somatic embryos inoculated into RITA®s at three densities and onto semisolid medium in GA7 vessels.

| Genotype | | GA7 vessel (control) | RITA® with 50 embryos | RITA® with 100 embryos | RITA® with 200 embryos |
|---|---|---|---|---|---|
| 28-1-5A[1] | HQF[2] (%) | 24.31b | 53.20a | 25.17 b | 24.67 b |
| | ConF[2] (%) | 61.11b | 97.56 a | 83.83 a b | 82.33 a b |
| 4-8-4[3] | HQF (%) | 59.25 | 76 | 81 | NA |
| | ConF (%) | 74.07 | 100 | 99 | NA |

[1]Values represent the means of 9 vessels for control treatment, 5 vessels with 50 embryos per RITA® unit, 6 vessels with 100 embryos per RITA® unit, 3 vessels with200 embryos per RITA® unit.

[2]Frequencies that do not share a letter are significantly different according to Tukey's pairwise comparisons.

[3]There were 3 vessels for the control treatment, 1 vessel with 50 embryos per RITA® unit, and 1 vessel with100 embryos per RITA® unit.

**Table 3.2.** Survival frequency (%) of hybrid sweetgum somatic seedlings inoculated in the RITA® system and in the GA7 vessels.

| Vessel treatments / Genotypes | GA7 vessel (control) | RITA® with 50 embryos | RITA® with 100 embryos | RITA® with 200 embryos |
|---|---|---|---|---|
| 28-1-5A[1,4] | 75 b | 90.63 a | 91.67 a | 89.58 a |
| 28-1-5B[2,4] | 33.33b | 90a | 75a | NA |
| 4-8-4[3,4] | 33.33b | 68.33a | 79.17a | NA |

[1]Value represent the means of 25 pots with somatic seedlings from the control treatment, 16 pots with somatic seedlings from RITA®s with 50 embryos, 12 pots with somatic seedlings from RITA®s with 100 embryos, and 12 pots with somatic seedlings from RITA®s with 200 embryos.

[2] Value represent the means of 6 pots with somatic seedlings from the control treatment, 5 pots with somatic seedlings from RITA®s with 50 embryos, and 8 pots with somatic seedlings from RITA®s with 100 embryos.

[3] Values represent the means of 4 pots with somatic seedlings from the control treatment, 5 pots with somatic seedlings from RITA®s with 50 embryos and 6 pots with somatic seedlings from RITA®s with 100 embryos.

[4]Frequencies that do not share a letter are significantly different according to Fisher pairwise comparisons.

**Table 3.3.** Height and diameter of hybrid sweetgum somatic plantlets after five months' growth in the greenhouse (from genotype 28-1-5B)

| | GA7 vessel (control) | RITA® |
|---|---|---|
| Height (inches) | 7.891±0.799 | 8.275±0.421 |
| Diameter (inches) | 0.094±0.008 | 0.134±0.016 |

**Table 3.4.** Conversion frequency (ConF) and high-quality somatic seedling frequency (HQF) of hybrid sweetgum somatic seedlings inoculated in the RITA® system and in the GA7 vessel

| Genotypes | | GA7 vessel | RITA® with 100 embryos |
|---|---|---|---|
| 28-1-5A[1] | ConF[2] (%) | 79.17a | 83a |
| | HQF[2] (%) | 33.33a | 30a |
| 28-1-8[3] | ConF[2] (%) | 80.56a | 88.3a |
| | HQF[2] (%) | 52.78a | 29b |
| 4-8-2B[4] | ConF[2] (%) | 74.07a | 93.67a |
| | HQF[2] (%) | 37a | 42a |

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[1] Values represent the means of 3 vessels for the control treatment and 3 vessels with 100 embryos per RITA® unit.

[2]Frequencies that do not share a letter are significantly different according to Tukey pairwise comparisons within genotypes.

[3] Values represent the means of 3 vessels for the control treatment and 3 vessels with 100 embryos per RITA® unit.

[4]Values represent the means of 3 vessels for the control treatment and 3 vessels with 100 embryos per RITA® unit.

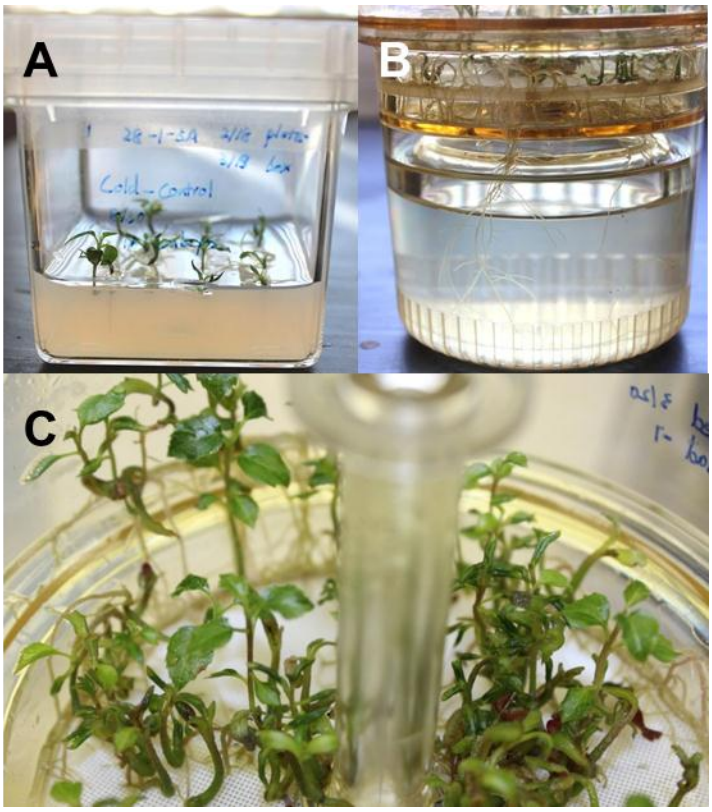


**Figure 3.1.** Somatic seedlings of hybrid sweetgum.

A. Embryos germinated on semi-solid medium in a GA7 vessel showing short shoots and short, unbranching roots. B. Long, branching root systems of embryos germinated in a RITA®. C. Shoots of embryos germinated in a RITA® showing long internodes.

**Figure 3.2.** Somatic seedlings of hybrid sweetgum genotype 28-1-5A a few weeks following potting in the hardening-off chamber.

**Figure 3.3.** Somatic seedlings of hybrid sweetgum genotype 28-1-5A just after repotting in the greenhouse.

**Figure 3.4.** Somatic seedlings of hybrid sweetgum genotype 28-1-5B following a season of growth in the shade house.

# CHAPTER IV

## 4. CONCLUSIONS

The research described in the previous chapters details our efforts to improve somatic embryogenesis of hybrid sweetgum using bioreactor technology. Our goal was to test new protocols that could be applied to scale-up production of clonal hybrid sweetgum planting stock to be grown as a short-rotation woody biomass feedstock. We expect these approaches could be very helpful for large-scale production of clonal hybrid sweetgum planting stock at a lower cost per propagule and with a shorter propagation time than other approaches.

ALBs show great potential for being a more efficient proliferation method than the conventional proliferation approaches on semisolid medium or in suspension culture using shaken flasks. Our results indicated that an ALB with a simple design and operation can greatly increase the production of hybrid sweetgum PEMs, which can in turn be used to produce large numbers of synchronous somatic embryos.

While the use of STBs did not increase the productivity of the embryogenic suspension cultures over that of ALBs or shaken flasks in our test, the ability to closely monitor and control conditions in this type of bioreactor makes it a valuable tool for investigating approaches to optimize the growth environment in the proliferation vessel. Another problem of STBs was the PEMs produced by it were yellow mixed with brown, which may indicate they have less vigor than PEMs produced by the other two methods. Further experiments could test whether PEMs produced by STBs have a similar ability to produce somatic embryos to the material produced in flasks or ALBs.

The RITA® TIB system is a very promising method that has the potential to significantly decrease labor costs in the somatic seedling production process. Fifty embryos per RITA® unit appeared to be the optimal density in our study for production of high-quality somatic seedlings. This density probably provides the necessary growth space and adequate nutrition for each somatic embryo, compared to one hundred embryos per RITA® unit or two hundred embryos per RITA® unit. Experiments to further optimize density for hybrid sweetgum somatic embryo germination in the RITA® bioreactor should probably test densities close to fifty embryos per RITA® unit. During the experiment, we observed a factor that probably affected growth space in the RITA® unit. In the early growth stage (before the somatic seedling roots adhere to the filter), the position of every hybrid sweetgum somatic embryo was changed by the bubbles produced by medium feeding every twelve hours. As a result of this movement, most somatic embryos settled unevenly on one side of the bubbles into a small space, greatly decreasing the growth space per somatic embryo. If there was a way to diminish the influence of the air movement, it may be very helpful to increase the conversion frequency in the RITA® unit. We tried to address this issue by slanting the RITA® units at an angle of 30 degrees on the lab shelf to try to counteract the effect of the uneven bubbling, but this action had no effect. Somatic seedlings of all three genotypes produced by the RITA® units had significantly higher survival frequency than somatic seedlings produced in the GA7 vessels. There was no significant difference among different RITA® densities on somatic seedling survival. These results indicated that somatic seedlings produced in the RITA®s are more likely to survive than those produced in GA7 vessels.

Our experiments with bioreactors applied for hybrid sweetgum somatic embryogenesis have shown that it is possible to improve hybrid sweetgum somatic seedling production by employing different bioreactors. We believe commercialization of hybrid sweetgum somatic

seedlings will make sweetgum a stronger candidate for biomass energy plantations and will help woody biomass become more widely used for energy.