GENETIC TRANSFORMATION OF SWEETGUM (*LIQUIDAMBAR STYRACIFLUA* L.) PROEMBRYOGENIC MASSES BY MICROPROJECTILE BOMBARDMENT

by

Michael Jason Walsh

(Under the Direction of Scott Arthur Merkle)

ABSTRACT

To date, transformation of sweetgum from microprojectile bombardment of embryogenic cells has not been reported. Growth of sweetgum (*Liquidambar styraciflua*) proembryogenic masses (PEMs) cultures was characterized while improving yields of size-fractionated PEMs for microprojectile bombardment. Transformation and selection parameters for PEMs were identified. Pre-bombardment osmotic conditioning of PEMs with 0.5 M equimolar mannitol and sorbitol produced the highest transient beta-glucuronidase (GUS) expression from 3 lines. Post-bombardment recovery periods tested for PEMs prior to initiation of selection revealed that initiating selection of PEMs immediately following bombardment allowed for the identification and recovery of transformed PEMs, while longer recovery periods did not. Stable integration of the pBI 426 transgene was confirmed by Southern analysis. A protocol from this work could be applied to embryogenic cell cultures to produce transgenic sweetgum.

INDEX WORDS: Genetic transformation, Microprojectile bombardment, Sweetgum, Proembryogenic masses, Embryogenic cells, GUS, NPTII, Thesis

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B.S., Tuskegee University, 1994

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2003

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The University of Georgia
December 2003

DEDICATION

To my mother for doing her best, under difficult circumstances, to raise a boy of wonder into a man of character.

To my wife Dionne for her patience, support, and understanding throughout my graduate program.

To my son Evan and future children, I hope to give you the loving home and direction so that you may lead fulfilling lives no matter what the endeavor.

To Dr. C.S. Prakash for introducing me to the field of plant transformation research and enriching my academic development through patient guidance.

To myself for learning to understand my ability and talents while forgiving my limitations and shortcomings.

ACKNOWLEDGEMENTS

I would like to thank the following people for their technical and peer support through different phases of my work: Gisele Andrade, P.J. Battle, Mandy Beggs, Jeff Dean, Myoung Kim, Peter Lafayette, Paul Montello, Joe Nairn, Rodney Robichaud, and Clayton Rugh.

Thanks must go to Sarah Covert for use of her lab, and the members of her lab for their help and technical assistance.

Thanks to my committee members Wayne Parrott and Hazel Wetzstein for thorough review of my manuscript and insightful criticism of my work.

Thanks to the following people for administrative support during my graduate training: Maureen Grasso, Arnett Mace Jr., and Rosemary Wood.

The following people and organizations must be thanked for their personal and academic support while giving me opportunities to broaden my research training: P.K. Biswas, Bob Buchanan, Walter Hill, Pablo Jordan, Peter Palukaitis, Errol Rhoden, Eric Richards, The Summer Research Opportunity Program through The Ohio State University, The Undergraduate Research Program through Cold Spring Harbor Laboratory, The Cornell Plant Science Center Undergraduate Research Program, Lawrence Berkeley Laboratory Science and Engineering Research Semester through the Department of Energy, NASA, and The National Science Foundation.

Lastly, and with great appreciation and gratitude, I thank Scott Merkle for his enduring patience even when I had lost my own. He has done a marvelous work in seeing me through my graduate program.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The life cycles of long-lived, slow-maturing forest trees make the application of traditional breeding techniques to tree improvement a time consuming and difficult process. In addition, traditional breeding is limited to modifying traits already present within the species or closely related, hybridizing species. Techniques for the transfer of engineered genes to plant tissues bypass some of the adversities associated with traditional plant improvement strategies (Dean et al., 1997). Several genes have been engineered for introduction into plant genomes and are available to aid tree improvement programs. These include genes for resistance to herbicides (Comai et al., 1985), insects (Lacey and Goettel, 1995), and fungal diseases (Terakawa et al., 1997). Genes are also available for altered floral morphology (Mandell and Yanofsky, 1995), altered lignin composition (Halpin et al., 1994) and remediation of heavy metal pollution (Cunningham et al., 1995). Many of these genes are now in transgenic trees and some of these trees are taking on new roles in the study of plant physiology (Herschbach and Kopriva, 2002; Pena and Seguin, 2001).

Several transformation protocols based on either *Agrobacterium tumefaciens* (Horsch et al., 1985) or microprojectile bombardment (Klein et al., 1988) have been developed to create plants with engineered genes since the first reports on plant transformation two decades ago (Davey et al., 1980; De Framond et al., 1983; Hoekama et al., 1983; Zambryski et al., 1983). Alternative protocols for transformation of plant cells using biological (Draper et al., 1988; Meyer et al., 1992; Potrykus, 1991) or physical (Kaeppler et al., 1992; Paszkowski et al., 1984) methods have also been described.

In the *Agrobacterium*-mediated transformation approach, explants to be transformed are infected with a compatible strain of *Agrobacterium tumefaciens* harboring a vector with the gene of interest and co-cultured on plant regeneration medium for a short period. During this time, the

virulence genes in the bacteria are induced, the bacteria bind to the plant cells around the wounds of the explant, and the gene transfer process occurs. After this event, tissues are transferred to medium containing selection agents to kill the bacteria and to inhibit growth from untransformed plant cells. Plants regenerated via organogenesis or somatic embryogenesis from these tissues potentially contain the introduced gene of interest.

Microprojectile bombardment or biolistics has become a popular method for transforming plants that were once considered recalcitrant to *Agrobacterium*-mediated transformation (Christou, 1996; Morikawa et al., 1994; Sanford et al., 1993). In biolistic transformation, small, high-density particles (microprojectiles) are accelerated to high velocity by a gas-driven apparatus such that they have sufficient momentum to penetrate plant cell walls and membranes to deliver a vector with the gene of interest into the nuclei of the bombarded cells. The bombarded cells are allowed to recover over a short period before being cultured on a selection medium to inhibit growth from non-transformed cells. Plants regenerated by organogenesis or somatic embryogenesis from these transformed cells potentially contain the introduced gene of interest. While the first biolistic devices required a high degree of optimization for controlled bombardments, current devices are easily optimized for increased control over the bombardment conditions (Kikkert, 1993; McCabe and Christou, 1993; Sautter, 1993). Other biolistic devices based on particle inflow (Vain et al., 1993a), compressed gas (Oard, 1993), and electric discharge (McCabe et al., 1988) have also been used for plant transformation research. Advances from biolistic gene transfer have allowed for transformation of monocots (Fromm et al., 1990) and woody plants (McCown et al., 1991), species that were once considered recalcitrant to *Agrobacterium*-mediated transformation.

Agrobacterium tumefaciens-mediated transformation of woody plants

A diverse number of woody plants have been transformed by *A. tumefaciens* since the first demonstrations of stable transformation of *Populus* (Fillatti et al., 1987) and walnut (McGranahan et al., 1988). Woody plants transformed using this approach to date include silver birch (Keinonen-Mettala et al., 1998), larch (Levee et al., 1997), pecan (McGranahan et al., 1993), hybrids of aspen and poplar (De Block, 1990), apple (James et al., 1989), grapevine (Mullins et al., 1990), avocado (Cruz-Hernandez et al., 1998), Ohio buckeye (Trick and Finer, 1999), *Allocasuatina verticillata* (Franche and al., 1997), sweet orange (Cervera et al., 1998), Japanese persimmon (Gao et al., 2000), and some conifers (Merkle and Dean, 2000). The diverse species of woody plants transformed by *A. tumefaciens* has demonstrated the potential for applying this transformation approach to almost any woody plant species.

Various explants and culture material from woody plants have been used as target material for *A. tumefaciens*-mediated transformation. Somatic embryos (McGranahan et al., 1993; McGranahan et al., 1988), embryogenic masses (Levee et al., 1997), stem nodes (Keinonen-Mettala et al., 1998), leaves (Fillatti et al., 1987; James et al., 1989), and stems (De Block, 1990) have all been employed to produce transgenic plants using this method.

Microprojectile-mediated transformation of woody plants

The creation of transgenic woody plants by microprojectile bombardment was first demonstrated in hardwoods with *Populus* hybrids (McCown et al., 1991) and in conifers with *Picea glauca* (Ellis et al., 1993). Other forest species transformed using this approach include *Liriodendron tulipifera* (Wilde et al., 1992), *Picea mariana* (Charest et al., 1996), and *Pinus radiata* (Walter et al., 1998). In addition, several non-forest tree woody plants have been

transformed via this method, including *Vitis* (Kikkert et al., 1996), cranberry (Serres et al., 1992), papaya (Fitch et al., 1990) and rose (Marchant et al., 1998).

Tissues used as target material for biolistic transformation of woody plants include embryogenic suspension cultures (Charest et al., 1996; Finer and McMullin, 1990; Kikkert et al., 1996; Wilde et al., 1992), protoplast-derived cells (McCown et al., 1991), nodules (Kim et al., 1999; McCown et al., 1991), stems (McCown et al., 1991), somatic embryos at different developmental stages (Charest et al., 1996; Ellis et al., 1993), and embryogenic calli or tissue (Marchant et al., 1998; Walter et al., 1998).

Gene transfer to plant cells capable of somatic embryogenesis is considered by many authors to be the best and most economical approach for large-scale production of transgenic trees and other woody plants (Ellis et al., 1993; Gupta et al., 1991; McGranahan et al., 1988; Merkle et al., 1990; Wilde et al., 1992). Transformation of woody plant species by microprojectile bombardment of embryogenic suspension cultures is a promising approach because it reduces the likelihood of regenerating chimeras, by allowing selection pressure to be applied stringently to isolated cells and cell clusters in liquid culture (Merkle et al., 1990; Wilde et al., 1992). Microprojectile bombardment of embryogenic suspension cultures has proven successful for the production of transgenic plants of several plant species. Transgenic plants of cotton (Finer and McMullin, 1990), maize (Gordon-Kamm et al., 1990) soybean (Finer and McMullen, 1991), yellow-poplar (Wilde et al., 1992), rice (Ayres and Park, 1994), grape (Kikkert et al., 1996), cassava (Schöpke et al., 1996), black spruce (Charest et al., 1996), and wheat (Bommineni et al., 1997) were regenerated from bombarded embryogenic cell suspensions.

Manipulations for improving microprojectile-mediated transformation

The introduction of engineered genes into plants requires matching tissue culture and transformation protocols for recovering transgenic plants from cells containing the introduced transgenes (reviewed by Ritchie and Hodges, 1993). While it was important to the success of earlier biolistic transformation research to characterize the physical conditions of the biolistic process (e.g. helium pressure, macrocarrier travel distance), the current success of biolistic transformation has come to rely more on the manipulations of cell cultures throughout the transformation protocol (Russell et al., 1992). Culture variables that have been shown to have an effect on transformation frequency include growth phase of cell cultures, osmotic conditioning of cells, choice of selectable marker genes and selection agent, and handling of bombarded material for selection.

Growth phase of cell cultures

DNA replication associated with plant cells in a state of exponential growth or in the M phase of the cell cycle has been shown to give the highest transformation frequencies from microprojectile bombardment. The increased transformation rates are thought to be due to integration of transgenes into replicating DNA through different types of recombination events (Makarevitch et al., 2003) and greater access to the genome by the microprojectiles in the absence of a nuclear membrane during M phase (Hazel et al., 1998; Iida et al., 1991; Sanford et al., 1993). Methods to measure growth rates of cell suspensions have been developed in order to identify the period of exponential growth during the culture cycle, which has been reported to be the optimal period for highest frequency transformation for both *Agrobacterium* co-cultivation (An, 1985) and microprojectile bombardment (Paszty and Lurquin, 1987).

Osmotic conditioning

The influence of osmotic conditioning on the frequency of plant cell transformation by microprojectile bombardment has been investigated for rice (Alfonso-Rubi, 1999), *Picea abies* (Clapham et al., 1995), maize (Vain et al., 1993b), and tobacco (Russell et al., 1992). Osmoticum has also been used with chloroplast transformation by microprojectile bombardment (Ye et al., 1990).

Osmotic agents used for conditioning cells for transformation by microprojectile bombardment include mannitol (Armaleo et al., 1990; Shark et al., 1991), sorbitol (Armaleo et al., 1990), sucrose, myo-inositol (Clapham et al., 1995), and raffinose (Kandler, 1982; Russell et al., 1992). Osmotic conditioning can also be carried out through partial drying of the target tissue (Finer and McMullen, 1991; Finer and McMullin, 1990). Enhanced transient expression and stable transformation from microprojectile bombardment of plant cells conditioned by osmotic agents is believed to result from plasmolysis of the cells, which reduces cell damage by preventing extrusion of the protoplasm from bombarded cells due to low cell turgor pressure. Plasmolysis also makes the vacuole less of an obstruction to the microprojectiles penetrating the nucleus, by reducing the volume of vacuole (Armaleo et al., 1990; Sanford et al., 1993).

Armaleo et al. (1990) were first to show that high sugar molarity in the plating medium was required for optimal transformation efficiency from microprojectile bombardment of *S. cerevisiae*. Their work also found a synergistic effect of sorbitol and mannitol together that was most pronounced when each was present at a concentration of 0.75 M.

The timing and duration of plant cell exposure to osmotic conditioning can affect the frequency of transformation by microprojectile bombardment. Embryogenic cells of *Zea mays* cultured on medium consisting of equal molarities of sorbitol and mannitol 4 hours prior to and

16 hours after bombardment resulted in a 2.7-fold increase in transient ß-glucuronidase (GUS) expression and a 6-8-fold increase in stably transformed maize clones (Vain et al., 1993b). Pre-bombardment osmoticum exposure alone resulted in a 43% increase in transient expression, while a post-bombardment exposure alone had no effect on transient expression. An extended pre-bombardment osmotic conditioning of 48 hours led to cells that were less responsive to transformation, with transient GUS expression lower than the controls. The authors believed the alteration was due to the osmotic adjustment (Turner and Jones, 1980) or reduction of cell proliferation (Handa et al., 1983).

The type and combination of osmotic agents as well as the concentration of osmoticum used in conditioning cells for bombardment also influence transformation frequency. Russell et al. (1992) demonstrated that the addition of 0.25 M raffinose or 0.125 M mannitol and 0.125 M sorbitol to the bombardment medium increased the frequency of transient GUS expression seven-fold, while higher concentrations had no enhancing or even an inhibitory effect on transient expression. A ten-fold increase in stable transformation occurred when cells cultured on medium containing 0.25 M of either raffinose or mannitol and sorbitol were brought to a lower osmoticum concentration through a stepwise concentration reduction, thereby avoiding cell injury from possible osmotic shock. Osmotic conditioning of non-embryogenic plant cells in medium containing mannitol and sorbitol resulted in a 7-10 fold increase in stable transformation (Russell et al., 1992). Optimal transformation of tobacco cells was achieved when the bombardment medium was supplemented with 0.2 -0.4 M osmoticum (Sanford et al., 1993). An increased molar concentration of sorbitol from 0 to 1.5 M in the bombardment medium resulted in a seven-fold increase in transformation efficiency of *Bucillus megaterium* (Shark et al., 1991). However, the greatest number of transformants was produced using a treatment combining 1 M

sorbitol with 0.75 M mannitol. Transformation efficiency from chloroplasts increased as the concentration of osmoticum was increased (Ye et al., 1990). A 1.0 M equimolar sorbitol/mannitol treatment gave the highest increase (20-fold) in transformation efficiency. However, at 1.5 M equimolar mannitol/sorbitol, transformation efficiency was significantly reduced. High osmotic conditions that are inhibitory, but not lethal to plant cell cultures, have been shown to reduce background growth from non-transformed cells during selection (Russell et al., 1992).

Selectable marker genes and selection agents

Numerous selectable marker genes are available for transformation research in plant species (Draper and Scott, 1991; Gruber and Crosby, 1993; Ritchie and Hodges, 1993). Some of these markers have been used in a limited number of plants, while others have been utilized throughout a wide range of plants.

The *bar* gene encodes the enzyme phosphinothricin acetyltransferase, which confers resistance to the herbicide Bialaphos (phosphinothricin). This selectable marker gene is used mostly with major cereal crops such as corn (Fromm et al., 1990; Gordon-Kamm et al., 1990), barley (Wan and Lemaux, 1994), oat (Somers et al., 1992), and wheat (Vasil et al., 1992). The gene *als* encodes the enzyme acetolactate synthase for resistance to the herbicide chlorsulforon. This gene has found limited use in the development of plant transformation protocols. In one of the first published reports of corn transformation, Fromm et al. (1990) used *als* as one of the selectable markers. The gene *hpt* encodes for the enzyme hygromycin phosphotransferase and confers resistance to the antibiotic hygromycin (Waldron, 1985). This gene has been used in the transformation of several plant species including rice (Christou et al., 1991), sweetgum (Kim et al., 1999), and cotton (Finer and McMullin, 1990).

The most widely used selectable marker gene for recovering transformed plants is *nptII* (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983). This gene encodes for the enzyme neomycin phosphotransferase, which confers resistance to the aminoglycoside antibiotics neomycin, geneticin (G418), paramomycin, and kanamycin. These antibiotics interfere with translation in prokaryotic and eukaryotic cells and are inactivated by phosphorylation reactions mediated by neomycin phosphotransferase. An added benefit of using *nptII* is that its expression can be quantified with an ELISA enzyme assay in plants (Charest et al., 1996; Marchant et al., 1998; Wilde et al., 1992). The *nptII* gene has been used with numerous dicot species and some monocot species (Bowen, 1993). Initial reports of woody plant transformation describe the use of *nptII* as a selectable marker gene for transformed material selected with kanamycin, geneticin, or paramomycin. Kanamycin has been commonly used to select cells with the *nptII* gene and was used in the selection of stable transformants following microprojectile bombardment in *Vitis* species (Kikkert et al., 1996), black spruce (Charest et al., 1996), *Populus* species (McCown et al., 1991), yellow-poplar (Wilde et al., 1992), eucalyptus (Serrano et al., 1996), and white spruce (Ellis et al., 1993). Wide-spread use of kanamycin for selection of transformed tissue has presented some situations where use of this antibiotic may either fail to stringently select against escapes (Schöpke et al., 1996; Walter et al., 1998), adversely affect morphogenesis (Everett et al., 1987; Mullins et al., 1990), or induce gene silencing (Schmitt et al., 1997). These problems have led researchers to test alternative antibiotics, selectable marker genes or protocols for handling various bombarded cells or tissues during selection.

Post-bombardment handling of target material

For some systems, relatively straightforward post-bombardment protocols have given good transformation frequencies. Examples of these systems include biolistic transformation of yellow-poplar (Wilde et al., 1992); rose (Marchant et al., 1998) and *Pinus radiata* (Walter et al., 1998) using fractionated embryogenic cells, embryogenic suspension cultures, and embryogenic tissue, respectively. However, other systems have proven to be much less straightforward, requiring researchers to customize the ways in which various bombarded cells or tissues are handled during selection. In many cases, the handling must be fine-tuned due to the importance of applying selection at levels that are non-lethal yet inhibitory to proliferation of non-transformed cells. This level of selection avoids excessive cell death and negative effects on regenerative competence of transformed cells (McCown et al., 1991; Mullins et al., 1990). Thus, modifications to standard protocols were needed to transform nodules and stems of *Populus* hybrids (McCown et al., 1991), somatic embryos of *Picea glauca* (Ellis et al., 1993), cotyledonary somatic embryos and embryonal masses of *Picea mariana* (Charest et al., 1996), nodules of sweetgum (Kim et al., 1999), zygotic embryos of *Eucalyptus globulus* (Serrano et al., 1996), and embryogenic cells of *Vitis* spp. (Kikkert et al., 1996). Several different approaches have been taken to handle bombarded material during selection, some to overcome adverse interactions between selection agents and the material used for bombardment, and others adapted to the nature of the tissue culture protocol used with bombardment.

For microprojectile-mediated transformation of *Liriodendron tulipifera*, bombarded proembryogenic masses from suspension cultures were cultured on an induction medium for two days before transfer to an induction medium containing kanamycin (Wilde et al., 1992). After 5 to 6 weeks of culture on selection medium, kanamycin-resistant microcalli were individually

transferred to plates of the selection medium. Suspension cultures were initiated from GUS-positive microcalli growing on selection medium. Suspensions were grown in a liquid induction medium containing half the concentration of kanamycin used for the initial selection of cells, which was sufficient to inhibit the growth of non-transformed suspensions. Somatic embryogenesis was induced in cell cultures from suspension cultures and mature embryos were converted to plantlets that were transferred to soil. No special handling of cells was needed for the recovery of kanamycin-resistant proembryogenic masses, as these small cell clusters, obtained by size-fractionation of the suspensions, apparently allowed for direct contact with the selection medium, resulting in efficient transformation.

Embryogenic suspension cultures were also bombarded for transformation of rose (Marchant et al., 1998). In this case, embryogenic calli were transferred to medium with or without osmoticum one hour post-bombardment. Embryogenic calli maintained on medium with osmoticum were transferred to the same medium without osmoticum 24 hours post-bombardment. After an additional 24 hours of culture, calli from both post-bombardment treatments were transferred to medium containing a high concentration of kanamycin and subcultured to fresh selection medium every 14 days. Calli surviving four cycles of subculture on selection medium were used to produce embryos that were matured and germinated under kanamycin selection.

A similar approach for initiating selection was used with embryonal masses of *Pinus radiata* (Walter et al., 1998). Bombarded filter papers with embryonal masses were transferred to selection medium containing geneticin three days following bombardment. After 6 to 8 weeks of selection following bombardment, centers of embryogenic tissue growth were detected. Resistant tissue on filters was transferred to fresh selection medium at two- to three-week

intervals. Selected tissue was maintained in an undifferentiated state or allowed to undergo development to form "bullet" stage embryos by transferring embryonic tissue to medium lacking growth regulators. Selection with geneticin was maintained throughout all early stages of embryo development until the maturation stage. Unlike reports for transformation of *Picea* species (see below), the transformation of *Pinus radiata* used embryonal masses in a manner similar to embryogenic cells from *Liriodendron tulipifera*, thus avoiding the necessity of manipulating embryos from later developmental stages. This approach was adopted because unlike *Picea* somatic embryos, de-differentiation of *Pinus* somatic embryos is difficult to achieve. Furthermore, protocols that give rise to mature somatic embryos in pine species require several steps, and the yield of mature somatic embryos is lower than that reported for *Picea* species.

In contrast to the reports summarized above, biolistic transformation with other types of embryogenic cultures or tissues has been characterized by problems requiring modifications to standard protocols. In the first report on transformation of a hardwood tree by microprojectile bombardment, protoplasts, nodules and stems of hybrid poplar were used as target material for bombardment by a particle acceleration device (McCown et al., 1991). Bombarded disks containing protoplasts were cultured without selection until substantial cell division was observed. Selection using kanamycin was then applied. Once microcalli were observed, the disks were moved to semi-solid medium containing twice the concentration of kanamycin initially used for selection. In order to maintain stringent selection, these plates were flooded with water containing the same concentration of kanamycin as used in the semi-solid medium, maintaining a film of kanamycin solution over the disks and medium surface. Green calli that emerged were removed to separate plates. For bombarded nodule and stem cultures, all plates

were cultured in the dark for 48 hours. Once new growth was observed, the plates were flooded with kanamycin solution. The liquid film created by flooding the plates was supplemented or replaced with kanamycin-water to maintain the film over the partially submerged tissue in order to maintain inhibition of growth from non-transformed cells. Green microcalli or shoot primordia that appeared were transferred to fresh plates. In this case, the flooding of plates with kanamycin solution was critical to the recovery of kanamycin-resistant tissue.

In the first report describing transformation of a conifer species, somatic embryos of *Picea glauca*, following particle bombardment, were cultured for 14 days on non-selective medium before embryos were transferred to selection medium containing 5 µg/ml kanamycin for six weeks (Ellis et al., 1993). After this initial kanamycin treatment, bombarded embryos with associated embryogenic callus or proembryos were transferred to medium without kanamycin to allow the callus to proliferate for several weeks, after which calli were screened on a medium with 10µg/ml kanamycin. This regime, combined with a very low kanamycin level (5 µg/ml) for initial selection, allowed the production of stably transformed callus lines from which transgenic plants were regenerated, but also resulted in a very high (95%) frequency of escapes. Even so, this work became the basis for transformation of other conifers.

Bombarded somatic embryos of *Picea mariana* (Charest et al., 1996) were placed on a medium to re-induce secondary somatic embryogenesis for 1 week in the absence of selection, followed by transfer to medium containing kanamycin at a concentration that inhibited most secondary embryogenesis in control non-bombarded cotyledonary somatic embryos. For bombarded embryonal masses, kanamycin was increased to a concentration that could inhibit embryonal growth. During selection, pieces of embryonal masses from both somatic embryos and embryonal masses were used to screen for stable transformation by GUS histochemical

assay. Embryonal masses with GUS positive sectors were used to initiate suspension cultures in medium containing very low levels of kanamycin.

Microprojectile bombardment of embryogenic suspensions for bombardment was also used for the transformation of *Vitis* cells (Kikkert et al., 1996). One day following bombardment, these cells, which were pre-conditioned on osmoticum, were cultured on medium lacking osmoticum to reduce the osmotic pressure on the cells. Selection for kanamycin-resistant cells began two days following bombardment using selection media containing a range of kanamycin concentrations to identify the optimal concentration for selecting bombarded cells. Initial selection of bombarded cells used a low concentration of kanamycin to select for transformed cells while allowing for embryos to develop from them. After at least six weeks of culture, masses of embryogenic cells were transferred to selection medium containing a higher concentration of kanamycin. These cells were transferred to fresh selection medium every 6 to 8 weeks. Cells on selection medium containing activated charcoal began producing embryos 14 to 15 weeks after bombardment. Additional embryos continued to form from cells on selection over a 1-year period. In a second experiment, bombarded cells were treated to reduce osmoticum and placed on selection medium as described above, but using two higher concentrations of kanamycin. The cells in this experiment were transferred to fresh selection medium every 6 to 8 weeks. Cells began producing embryos 29 weeks after bombardment. The longer period required for embryos to form in the second experiment was attributed in part to the higher initial level of kanamycin used for selection.

A prolonged culture period between bombardment and initiation of selection was needed for production of transformed material from bombarded nodule cultures of sweetgum (Kim et al., 1999). Bombarded nodules were cultured on non-selective medium for 1 week before transfer to

medium with hygromycin. Hygromycin was chosen as a selection agent because Dr. M.K. Kim (Diversa Corp., San Diego, CA, personal communication) observed high natural resistance of sweetgum nodule cultures to kanamycin (unpublished data). The concentration of hygromycin was increased at the third week of selection and again at the eighth week of selection. Nodules remained on selection for 3 months before checking hygromycin-resistant nodules for the presence of the transgene.

An even longer post-bombardment recovery of tissue before initiating selection was used for transformation of *Eucalyptus globulus* (Serrano et al., 1996). Bombarded segments from zygotic embryos of *E. globulus* were cultured on non-selective medium for 2 months to regenerate shoots and calli prior to transfer to medium with kanamycin. The authors believed this two-month culture period on non-selective medium was needed to allow the cells to divide and to produce sectors that would proliferate easily under selection.

In one report on transformation of *Picea abies* (Clapham et al., 2000), cells on filter papers were transferred to selection medium 2, 5, or 8 days after bombardment. Results from this experiment showed no marked effect on the period between bombardment and the onset of selection. Therefore, an 8-day culture period between bombardment and initiation of selection was adopted. Bombarded filter paper disks with osmoticum-conditioned embryogenic cells were transferred to a medium with osmoticum and cultured for eight days to recover turgor before transfer to the same medium containing the herbicide Basta. Fifteen days after bombardment, the cells were transferred to fresh selection medium and maintained on selection with monthly transfer to fresh medium. Embryogenic tissue resistant to Basta appeared 2-4 months after bombardment.

Sweetgum tissue culture and transformation

Sweetgum (*Liquidambar styraciflua*) has been genetically engineered via *Agrobacterium tumefaciens*-mediated transformation using leaves as target tissue, followed by adventitious shoot regeneration from the leaves or from nodules produced from the leaf callus. The species has also been transformed using microprojectile bombardment of nodule cultures. To date, there have been no reports on the transformation of sweetgum embryogenic cells and production of transgenic plants using microprojectile bombardment.

Sweetgum is a bottomland hardwood common to the southeastern United States (Kormanik, 1990). It is one of the most important commercial hardwoods used for pulpwood in the production of paper products (Brand and Lineberger, 1992). Paper companies are showing increasing interest in sweetgum for use in short rotation plantations, as accessible natural stands of sweetgum and other hardwoods become less abundant (Brand and Lineberger, 1992). Sweetgum is also a popular tree for use in ornamental horticulture (Brand and Lineberger, 1992). Its form, fall color, summer foliage, and ability to provide shade are characteristics that make it attractive for this purpose.

While sweetgum is a valuable tree for the paper and horticultural industries, improvement of the tree would be welcome. For the paper industry, establishment of sweetgum plantations is hampered during the first three to five years by slow seedling growth and competition from weeds. Slow seedling growth requires expensive cultivation practices to reduce competition from weeds in order to promote acceptable survival rates until vertical growth begins (Kormanik, 1986). The horticulture industry uses numerous commercial sweetgum cultivars that are selected for form, leaf variegation, fall color, fruitlessness, and cold hardiness (Santamour and McArdle, 1984). Selection for fruitlessness is especially desirable because the spiny fruit are unsightly and

are considered a litter problem. For both industries, disease and insects can potentially pose problems to sweetgum. Leader dieback, blight, and twig canker in sweetgum are caused by *Botryosphaeria ribis.* Two leaf-feeding insects, the forest tent caterpillar (*Malacosoma disteia*) and luna moth (*Actias luna*), damage sweetgum foliage (Baker, 1972).

Several methods are available for in vitro propagation of sweetgum including: (1) axillary shoot proliferation (Sutter and Barker, 1985), (2) organogenesis (Brand and Lineberger, 1988; Kim et al., 1997; Sommer and Brown, 1980; Sutter and Barker, 1983; Sutter and Barker, 1985) and (3) somatic embryogenesis (Merkle et al., 1998; Sommer and Brown, 1980). In vitro sweetgum cultures have been initiated from many different explants including: juvenile shoot tips (Sutter and Barker, 1985), seedling hypocotyl segments (Kim et al., 1997; Sommer, 1981; Sommer and Brown, 1980), lateral buds from mature trees (Brand and Lineberger, 1988), leaf and petiole segments (Brand and Lineberger, 1988), immature seeds (Merkle et al., 1998), and staminate and pistillate inflorescences (Merkle and Battle, 2000).

Micropropagation

Axillary shoot proliferation is currently the most reliable and established method for producing large numbers of superior genetically identical clones (Brand and Lineberger, 1988). Shoot production was obtained from sweetgum shoot explants from actively growing seedlings or shoot tips and dormant bud explants from mature trees cultured on woody plant medium (WPM; (Lloyd and McCown, 1980)) containing 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA) (Sutter and Barker, 1985). Shoot tips taken from mature trees required initial frequent transfers to fresh medium at 2-3 day intervals for the first month of culture to prevent death of explants. Up to 8 months of culture were required to establish actively growing cultures. Shoots derived from both “juvenile” material and from mature trees required up to 6

weeks and 12 months of culture, respectively, on WPM containing indole-3-butyric acid (IBA) for rooting. Axillary shoots have also been rooted in liquid medium (Lee et al., 1988). Shoot proliferation from juvenile material in liquid medium has been reported (Sommer et al., 1985), but attempts to repeat the use of liquid culture for shoot proliferation failed to produce the previously reported results (Brand and Lineberger, 1992).

Organogenesis

Adventitious shoot regeneration in sweetgum has been demonstrated using hypocotyls cultured on media containing high BA/NAA ratios (Sommer, 1981; Sommer et al., 1985) and on semi-solid media containing high N^6- (2-isopentyl) adenine (2ip) / 3-indolyl-acetic acid (IAA) ratios followed by culture in liquid medium of the same composition (Sommer et al., 1985). An alternative protocol employing a combination of thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) also induced adventitious shoot production from sweetgum hypocotyl explants (Kim et al., 1997). A modification of this protocol was later used to initiate nodule cultures, which could be proliferated in suspension culture with TDZ and 2,4-D, and which were capable of producing adventitious shoots (Kim et al., 1999). Shoot organogenesis and plant regeneration were also obtained from leaves and petiole segments from mature phase sweetgum trees, cultured on a medium containing BA with or without NAA (Brand and Lineberger, 1988). Lateral buds from mature trees were used to initiate axillary shoot proliferation cultures that provided aseptic petiole and leaf explants for the experiments.

Somatic embryogenesis

Somatic embryogenesis has great potential for efficiently producing large numbers of propagules (Merkle et al., 1990) and is the culture method of choice for gene transfer research with a number of forest species (Ellis et al., 1993; McGranahan et al., 1988; Wilde et al., 1992).

Sweetgum embryogenic cultures have been initiated from immature seeds (Merkle et al., 1998), seedling hypocotyls (Sommer and Brown, 1980), male inflorescence tissues (Merkle et al., 1997; Merkle et al., 1998) and female inflorescence tissues (Merkle and Battle, 1999).

Sommer and Brown (1980) first reported somatic embryogenesis in sweetgum from cultures initiated from seedling hypocotyls. Callus from the hypocotyls cultured on semisolid medium containing BA and NAA produced torpedo stage embryoids when maintained in suspension culture on medium without BA and NAA. The embryoids were capable of producing elongated shoots that formed plants resembling seedlings when cultured on semisolid medium without BA and NAA. The torpedo stage embryoids also formed secondary embryoids. The highest proliferation of embryoids occurred from cultures initiated on semisolid medium containing 0.1mg/l NAA and 0.5mg/l BA and transferred to medium without plant growth regulators. However, other NAA and BA combinations also led to embryogenesis, and thus no clear indication could be found for a specific combination of plant growth regulators for inducing embryogenesis.

Staminate sweetgum inflorescences were used to produce embryogenic cultures capable of making embryos that germinated and converted to plantlets (Merkle et al., 1997). Expanding buds containing staminate inflorescences were collected from seven source trees on a weekly basis over a four-week period that ended when the staminate inflorescences had expanded out of the buds. Contamination and damage from the disinfestation treatment used to initiate cultures caused loss of all explants except those from the first of the four collections. Media supplemented with 0.01 to 1 mg/l TDZ produced embryogenic cultures from the least expanded staminate inflorescences. Inflorescences from only one of the seven source trees produced embryogenic cultures. Embryogenic cultures were produced from both continuous culture on

TDZ or a one-week pulse on medium with TDZ, followed by culture on basal medium. Continuous culture on medium containing 0.01 mg/l TDZ led to a high frequency of repetitive embryogenesis and also produced structures resembling proembryogenic masses (PEMs).

In a follow-up study, both inflorescence tissues and immature seeds were studied for induction of somatic embryogenesis (Merkle et al., 1998). Staminate inflorescence explants on semisolid medium with 0.01 mg/l TDZ gave rise to somatic embryos that appeared as globular structures. Mixtures of PEMs and somatic embryos also occurred in some cultures with 0.01 or 0.1 mg/l TDZ. PEMs from these cultures could be selectively cultured to produce pure cultures of PEMs. Source tree, collection date, and cultural treatment all affected induction of embryogenesis. The bulk of the embryogenic cultures produced were from inflorescences collected during the first week of the study in January, when buds were completely dormant. Treatments with a continuous exposure to a consistent level of TDZ were more effective than treatments where the explant was pulsed with one level of TDZ prior to transfer to basal medium or a lower level of TDZ. Only cultures continuously exposed to TDZ produced PEMs. The highest frequency of induction from seed explants occurred with a two-week pulse on semi-solid medium containing 0.5 mg / l 2,4-D and 0.2 mg/l BAP. Many of the seed-derived embryogenic cultures proliferated as PEMs (Merkle et al., 1998). Induction of embryogenesis from seeds was not affected by the source tree or by cultural treatment. Induction frequency from seed explants was also related to collection week, with earlier stages of embryo development producing a higher frequency of embryogenic cultures than later stages.

In the most recent study, male inflorescences, female inflorescences, and leaves from dormant buds of three sweetgum source trees were tested for induction of somatic embryogenesis (Merkle and Battle, 1999). Embryogenic cultures were obtained from all of the

trees tested, although frequency of embryogenesis induction was again influenced by genotype. Explant type also influenced induction of embryogenic cultures. Staminate inflorescences had the highest potential for embryogenesis induction, with 100% of the inflorescence explants from one source tree producing embryogenic cultures. Female inflorescences were also capable of producing embryogenic cultures, while leaves were not. There was a significant difference in induction of embryogenic cultures when the plant growth regulator treatments were grouped as explants continuously cultured on medium containing NAA, NAA with TDZ or no plant growth regulators. Medium with NAA alone resulted in the highest induction of repetitively embryogenic cultures and also produced PEMs at a frequency of 29% from male and female inflorescences.

***Agrobacterium tumefaciens*-mediated genetic transformation**

The current *A. tumefaciens*-mediated transformation protocols available for introducing engineered genes into sweetgum are based on adventitious shoot regeneration from leaves (Sullivan and Lagrimini, 1993) or nodule cultures (Chen and Stomp, 1992) to recover transgenic plants. Chen and Stomp (1992) developed a regeneration system for sweetgum in which nodule cultures were established from leaf pieces and the nodules were used to regenerate shoots. Using this protocol, leaf pieces were inoculated with *A. tumefaciens* and cultured on semi-solid medium to promote callus formation in the presence of antibiotics for controlling *Agrobacterium* growth and selecting transformed cells. Callus formation from the leaf edges was visible in 4-6 months of culture and callus was used to establish nodule cultures in liquid WPM containing BA, NAA, and 40 mg/ml kanamycin. Nodules 2.5mm and greater in diameter growing in liquid selection medium and plated onto semi-solid WPM containing BA and 40 mg/ml kanamycin generated shoots following two months of culture. Transformed shoots were rooted under kanamycin

selection in semi-solid WPM containing NAA, and plantlets were transplanted to a soiless growth medium.

Sullivan and Lagrimini (1993) infected cut leaves from aseptically propagated plants with an *A. tumefaciens* strain harboring binary vectors for either *Bacillus thuringiensis* (*B.t.*) toxin (Carozzi et al., 1992), GUS (Jefferson et al., 1987), or tobacco anionic peroxidase (Lagrimini, 1990). After co-cultivation, leaf explants transferred to non-selective WPM containing 2.5 mg/l BA and cefotaxime generated callus along the cut surfaces before transfer to WPM containing kanamycin and cefotaxime. Transgenic shoots arose from kanamycin-resistant callus cultured on selection medium. Presence of the *B.t.* endotoxin and tobacco anionic peroxidase genes in transformed shoots was confirmed by Southern analysis. Gene expression from transgenic shoots with *uidA* was quantified through fluorometric assay and showed GUS activity of 3.5 to 13-fold higher than non-transformed tissue. Rooted transgenic shoots containing the tobacco anionic peroxidase gene were further analyzed by Dowd et al (1998).

Microprojectile-mediated genetic transformation

Kim et al. (1999) developed a regeneration protocol for sweetgum in which nodule cultures were established from seedling hypocotyls and proliferated in liquid medium containing TDZ and 2,4-D. The nodules regenerated shoots that could be rooted ex vitro. Using this protocol, synchronized, proliferating nodules of 0.38 mm to 1.2 mm in diameter were bombarded with a plasmid harboring genes for hygromycin resistance (Waldron, 1985) and GUS (Jefferson et al., 1987). Following bombardment, nodules were transferred to non-selective semisolid medium for 1 week, followed by transfer to semisolid medium containing hygromycin B. Nodules proliferating after 3 months of selection expressed the GUS gene. A total of 13 independent lines of hygromycin resistant nodules were recovered. Integration of the GUS

transgene was confirmed by Southern blot analysis of DNA from nodules and shoots from the single regeneration-competent transgenic line. Most of the shoots from this line were GUS-positive in their stems and expanding leaves during culture, but some shoots from GUS-positive, hygromycin- resistant nodules were GUS-negative. Eleven GUS-positive transgenic shoots were rooted ex-vitro.

To date, no microprojectile bombardment transformation protocol for sweetgum embryogenic cultures has been reported. A microprojectile bombardment protocol for transformation of sweetgum embryogenic cells and production of transgenic plants would offer a method for rapidly introducing several gene constructs and characterizing their expression, leading to genetically improved sweetgum trees for commercial goals or work with phytoremediation. Thus the goal of the study described here was to develop a protocol for the transformation of embryogenic sweetgum cultures based on transformation by microprojectile bombardment. To accomplish this goal, we examined the growth phase of suspension cultures to optimize the timing of bombardment, and tested different types and levels of selection agents for inhibiting growth from non-transformed cells, pre-bombardment osmoticum conditioning of cells, and the duration of the recovery period for bombarded cells prior to application of selection.

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CHAPTER 2

GENETIC TRANSFORMATION OF SWEETGUM (*LIQUIDAMBAR STYRACIFLUA* L.) PROEMBRYOGENIC MASSES BY MICROPROJECTILE BOMBARDMENT[1]

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ABSTRACT

Conditions for transformation and selection of sweetgum (*Liquidambar styraciflua*) pro-embryogenic masses (PEMs) were identified. Sub-lethal levels of kanamycin for selection of PEMs were determined for three sweetgum culture lines. Testing of different pre-bombardment osmotic conditioning treatments of one PEM cell line on semi-solid medium indicated that 0.5 M equimolar mannitol/sorbitol produced the highest transient expression of the reporter gene beta-glucuronidase (GUS). In a follow-up experiment, the same level of mannitol/sorbitol promoted high transient GUS expression from three bombarded sweetgum PEM lines. Tests of post-bombardment PEM culture recovery periods prior to initiation of selection revealed that PEMs transferred to selection medium containing 50µg/ml kanamycin immediately following bombardment allowed for the identification and recovery of transformed PEMs, while longer recovery periods did not. Stable integration of the *gus:nptII* transgene from pBI 426 was confirmed by Southern analysis.

INTRODUCTION

Sweetgum is a bottomland hardwood common to the southeastern United States (Kormanik, 1990). It is one of the most important commercial hardwoods used for pulpwood in the production of paper products (Brand and Lineberger, 1992). Paper companies have shown interest in sweetgum for use in short rotation plantations, as accessible natural stands of sweetgum and other hardwoods become less abundant (Brand and Lineberger, 1992). Sweetgum has few insect and disease problems, but slow growing saplings can be overgrown by competing vegetation during plantation establishment, requiring costly management practices to control the competing species (Baker, 1972; Kormanik, 1986). Sweetgum is also a popular tree for use in

ornamental horticulture (Brand and Lineberger, 1992). Its form, fall color, summer foliage, and ability to provide shade are characteristics that make it attractive for this purpose. Several sweetgum cultivars are selected for form, leaf variegation, fall color, fruitlessness, and cold hardiness (Santamour and McArdle, 1984). Selection for fruitlessness is especially desirable because the spiny fruit are unsightly and are considered a litter problem.

While conventional breeding has achieved substantial improvement of some forest trees, the long life cycles of most forest species make breeding with traditional techniques an extended process. Traditional breeding is also limited to the modification of traits present within the species or closely related, hybridizing species. Techniques for the transfer of foreign genes to regenerative plant tissues can bypass some of the problematic bottlenecks associated with traditional plant improvement strategies (Dean et al., 1997). Biotechnology-based techniques used for improving major agricultural crops with engineered genes are now being applied to the improvement of forest trees. Sweetgum has been genetically engineered using *Agrobacterium tumefaciens*-mediated transformation of leaf sections, followed by production of adventitious shoots, either from the leaf explants, or from nodule cultures derived from leaf explants (Chen and Stomp, 1992; Sullivan and Lagrimini, 1993). Hybrid sweetgum was transformed via co-cultivation of *Agrobacterium* with embryogenic cultures (Dai, 2003). The tree has also been transformed using microprojectile bombardment of nodule cultures (Kim et al., 1999).

Embryogenic suspension cultures are considered by several authors to be potentially the best and most economical approach for large-scale production of transgenic trees (Gupta et al., 1991; McGranahan et al., 1988; Merkle et al., 1990). Microprojectile bombardment of embryogenic cultures has been used to transform many woody plant species, including yellow-

poplar (Wilde et al., 1992), radiata pine (Walter et al., 1998), various spruce species (Bommineni et al., 1997; Charest et al., 1996; Ellis et al., 1993) and rose (Marchant et al., 1998).

Protocols have been established for initiating sweetgum embryogenic cultures from mature (Merkle et al., 1997; Merkle and Battle, 1999; Merkle et al., 1998) and immature (Merkle et al., 1998) tissues. To date, however, there have been no published reports on the transformation of sweetgum via microprojectile bombardment of embryogenic cells. In this study, conditions for microprojectile-mediated transformation of embryogenic sweetgum cultures were established. Levels of osmoticum for pre-bombardment conditioning of embryogenic cells and sensitivity of embryogenic cells to antibiotics were determined. Identifying the length of post-bombardment culture prior to initiating selection was the most critical parameter to the success of recovering transformed embryogenic cells.

MATERIALS AND METHODS

PEM Culture lines and culture conditions

Three embryogenic sweetgum PEM lines were used in this study. The protocol used for initiation of these cultures was described previously (Merkle et al., 1998). Briefly, one line, designated 7851-7B, was initiated during the summer of 1994 from immature seeds supplied by International Paper Company (Bainbridge, GA, USA). Two additional lines, SLS5-8I and SLS9-7IV, were initiated during the summer of 1995 from immature seeds collected from two trees growing on the University of Georgia (Athens, Georgia, USA) campus. Cultures were initiated and maintained on semi-solid induction-maintenance medium (IMM; see below for medium details), on which they proliferated as proembryogenic masses (PEMs). Approximately 0.5 g fresh weight of PEMs was used to initiate suspension cultures, which were cultured in 50 ml of

liquid IMM in 125 ml Erlenmeyer flasks. Embryogenic cell suspensions were maintained on a gyratory shaker at 130 rpm under cool white fluorescent light at 100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ with a 16-h photoperiod at 27° C. PEM suspensions were subcultured on 21 day cycles.

Media

All media were based on woody plant medium ((WPM) Lloyd and McCown, 1980). IMM was WPM supplemented with 0.1 g/l myo-inositol, 1.0 g/l casein hydrolysate (enzymatic; CH), 30 g/l sucrose, 2 mg/l 2,4-D, and 0.25 mg/l 6BA, with pH adjusted to between 5.6 and 5.7. Semi-solid media used in testing selection agents and osmoticum levels were prepared using IMM with 8 g/l Phytagar (Gibco, Life Technologies, Grand Island, NY), added after the medium pH was adjusted. Media were autoclaved for 20 minutes at 115 psi prior to use. Media for experiments testing the inhibition of PEM proliferation by kanamycin were prepared by adding filter-sterilized kanamycin to autoclaved IMM that had cooled to 65° C to obtain the desired concentration. Media for the osmotic conditioning experiments (see below) were also prepared using IMM. Medium pH was measured after the osmoticum for each treatment was dissolved.

PEM size fractionation

PEMs from suspension culture were size fractionated by sieving through a 140 μm pore size stainless steel screen and collecting on a 38 μm pore size screen to isolate PEMs ranging from 38 to 140 μm in diameter. PEMs collected on the 38 μm screen were back-washed into empty flasks using IMM without plant growth regulators, to generate suspensions in which cell clumps could be counted. PEM counts in these fractionated suspensions were performed in a 60 x 10 mm plastic Petri plate using an inverted microscope. PEMs were counted for three 30 μl drops per fraction. The average count from the three drops was used to calculate the volume of fractionated PEM suspension required to obtain the desired density of PEMs for collection on

filter paper disks prior to microprojectile bombardment. Ten thousand PEMs were collected onto a black 42 mm filter paper disk, as was previously described for transformation of yellow-poplar (Wilde et al., 1992), unless stated otherwise. PEMs were collected on filter disks using a glass microanalysis filter holder assembly (Fisher Scientific), by pipetting the volume of fractionated suspension required to obtain 10,000 PEMs and washing the PEMs with approximately 20 ml of IMM without plant growth regulators, under mild vacuum. PEMs collected on filters were overlaid onto the semi-solid medium treatments (see below) and plates were sealed with Parafilm.

Determination of PEM sensitivity to selection agent

Five levels of kanamycin (0, 10, 25, 50, and 100 μg/ml) were tested for their ability to inhibit proliferation of PEMs from the four embryogenic sweetgum lines. Media for the experiment were prepared as described above. A cell plating system described by Horsch and Jones (1980) was used in these experiments to allow for accurate PEM fresh weight measurements. Briefly, PEM suspensions, 7 to 16 days from their last transfer to fresh medium, were size-fractionated and PEMs were collected on filters as described above. This filter with PEMs was overlaid onto semi-solid medium treatment plates having a 50 mm filter disk in direct contact with the selection medium surface. Plates were sealed with Parafilm and cultured in the dark at 27° C. PEM fresh weights were estimated by removing the filter with PEMs, weighing it in a sterile culture plate, and subtracting the weight of the filter paper with PEMs from the weight of the same size filter paper without PEMs. Observations were taken at weekly intervals beginning after 24 hrs of culture on selection treatments. Five observations were taken over a 4-week period. The experiment was carried out in a completely randomized design with a factorial arrangement of treatments. There were three replications per treatment.

DNA vector and bombardment conditions

The plasmid pBI 426 (kindly provided by Dr. W.L. Crosby, Plant Biotechnology Institute, Saskatchewan, Canada; Fig. 1) was selected for this work based on reports by Russell et al. (1992), Kikkert et al. (1996), and Charest et al. (1996). The 6.3 Kbp plasmid has a translational fusion between the GUS and the NPTII coding region (Datla and Hammerlindl, 1991) under the control of a double 35S promoter (Kay et al., 1987) linked to a translational enhancer from alfalfa mosaic virus (Datla et al., 1993). Gold microprojectiles (average diameter 1 μm) were prepared according to the protocol described by Sanford et al. (1993). DNA was precipitated onto microprojectiles at 1 μg per mg, respectively. Five microliters of the microcarrier suspension containing 1 μg of DNA were loaded onto a macrocarrier and accelerated towards the target with the PDS-1000/He biolistics device (Bio-Rad; Hercules, California, USA). Plates of cells were placed 9 cm from the microcarrier launch assembly and were bombarded twice. The following conditions were used for the bombardments: acceleration of microcarriers with 1100 psi rupture disks, 1 cm distance between the rupture disk and the macrocarrier launch point, 16 mm macrocarrier flight distance, and sample chamber evacuated to 25 - 28 in Hg.

Osmotic conditioning treatments

Two experiments were conducted to test pre-bombardment conditioning of PEMs with osmoticum for its potential to improve transformation frequency. The first experiment compared 0, 0.25, 0.5, 0.75, and 1M equimolar concentrations of mannitol/sorbitol using a single culture line (SLS 9-7IV). The experiment was conducted with fractionated PEMs from 3-day old suspension cultures that were cultured in 250 ml flasks containing 25 ml of medium. This experiment was repeated three times. The second experiment tested three PEM lines (7851-7B,

SLS 5-8I, and SLS 9-7IV) conditioned on either 0 or 0.5 equimolar osmoticum treatments. This experiment was repeated twice with suspension cultures transferred to fresh medium 10 days prior to fractionation. White filter disks with 10,000 PEMs per filter were used. PEM filters were transferred to osmoticum treatment plates and cultured 20 to 24 hr prior to bombardment. Three days following bombardment, PEM filters were cut in half, and PEMs on one half of each filter were assayed for transient GUS expression. The remaining half-filters from each treatment were transferred to IMM for 4 days, after which time they were transferred to IMM supplemented with 50 μg/ml kanamycin. PEMs were cultured on selection medium for 8-12 weeks.

The first experiment was set up as a completely randomized design while the second experiment was set up as a 3 X 5 factorial. Both experiments used five replications per treatment. Data were analyzed by analysis of variance and Duncan's multiple range test using SAS (SAS Institute, Inc.).

Post-bombardment PEM selection for transformants

The experiment compared four post-bombardment recovery periods prior to initiating selection. Recovery periods were 0, 3, 5, or 7d of culture on plates of semi-solid IMM prior to transfer to IMM containing 50μg/ml kanamycin. The experiment was conducted with size-fractionated PEMs collected from 10-day old suspension cultures. Fractionated PEMs from lines SLS 5-8I and SLS 9-7IV were collected onto filter disks at 10,000 PEMs per filter. Filters with PEMs were placed onto semi-solid IMM containing 0.5 M osmoticum consisting of 0.25 M of mannitol and 0.25 M sorbitol and incubated for 20 to 24 hours prior to bombardment.

Following bombardment and recovery, PEMs were cultured under selection on semi-solid medium containing 50 mg/L kanamycin, with transfer to fresh selection medium every 4

weeks. After 8 - 12 weeks, PEM colonies displaying resistance to kanamycin were individually transferred to 50 ml flasks containing 12.5 ml of liquid IMM with 25 μg/ml kanamycin. One clump of PEMs (approximately 50 mg) was used to inoculate each flask of selection medium. PEMs were cultured under selection for 6 to 8 weeks, with subcultures at 14 d intervals. The experiment was set up as a 2 X 4 factorial, completely randomized design, with 5 replications per treatment. The experiment was conducted twice, although the second replication differed from the first in that PEMs were collected for bombardment from 3-day old suspension cultures rather than 10-day old cultures.

Analysis of transformants

Histochemical detection of GUS expression with the substrate X-gluc followed the protocol of Jefferson et al. (1987). Transient GUS activity in bombarded PEMs was initially assessed 3 d following bombardment. PEMs were incubated 24 hrs in X-gluc solution at 37° C before scoring. Each blue area was considered as one GUS expressing focus, as described by Russell et al. (1992). The number of GUS foci per 10,000 PEMs was used as a measure of transient GUS expression. PEMs cultured under selection for over 8 weeks were also assessed for GUS activity as an indication of stable transformation.

Genomic DNA for PCR and Southern analysis was extracted from transformed PEMs maintained in suspension cultures using the method of Lassner et al. (1989). Samples from multiple extractions were pooled for use with Southern analysis. Putatively transformed lines were screened by PCR using 100 ng of genomic DNA from transformed and non-transformed lines and 10 ng of pBI 426 plasmid as positive control per 25 μl reaction. Primers nptII 162 (5'-CGG TGC CCT GAA TGA ACT-3') and nptII 795 (5'-TCA GAA GAA CTC GTC AAG AAG G-3') were used to amplify a 631 bp fragment from nucleotide 2024 to nucleotide 2655 in the

nptII region of the *gus-nptII* fusion gene. The conditions for amplification consisted of an initial denaturation step at 94° C for 4 min, followed by 30 cycles at 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min. The final extension was at 72° C for 10 min, after which the reaction mixture was held at 4° C. The amplification products were loaded directly onto a 1% agarose gel and resolved by electrophoresis.

Blots for Southern analysis were performed as described by Ausubel et al. (1993). DNA from transformed lines was digested with (5 units/µg) of the restriction enzyme *Eco*RI overnight and resolved on a 0.8 % agarose gel at 93 V for 3 hours. Blots were probed using the 631 bp amplified fragment of the *nptII*-coding region from pBI 426 prepared using the DECAprime™ random priming DNA labeling kit (Ambion Inc.).

Somatic embryo development from PEMs

PEMs were cultured on semi-solid embryo development medium in 60 x 10 mm plastic Petri plates. Embryo development medium consisted of basal WPM supplemented with 0.1 g/l myo-inositol, 1.0 g/l CH, and 30 g/l sucrose. PEM suspension cultures were size-fractionated four days after subculture to fresh medium, as described above, except that basal liquid embryo development medium was used for washes instead of IMM. Fractionated PEMs were collected on black paper filter disks at 1000 PEMs per disk. Disks with PEMs were overlaid on the surface of semi-solid embryo development medium. Plates were sealed with Parafilm and cultured in the dark at 27° C for six weeks before checking for embryo development.

RESULTS AND DISCUSSION

Inhibition of PEM growth by kanamycin

Kanamycin was tested for its ability to inhibit growth of three sweetgum PEM lines. Growth for lines 7851-7B, SLS 9-7IV and SLS 5-8I (data not shown) was effectively inhibited by kanamycin at 50 and 100 µg/ml (Fig. 2). There was a clear difference in growth between the control and tested levels by three weeks of culture. Kanamycin at 10 µg/ml and 25 µg/ml allowed growth from line 7851-7B while kanamycin at 10 µg/ml allowed growth from line SLS 9-7IV. Growth rate differences among the 3 lines were evident, even with no kanamycin (Fig. 3). After the second week, line SLS 9-7IV had the highest growth rate (0.19 g/wk), followed by line 7851-7B (0.14 g/wk), and line SLS 5-8I (0.04 g/wk).

Kanamycin has been reported to be an effective selection agent for sweetgum transformation by others (Chen and Stomp, 1992; Sullivan and Lagrimini, 1993), but Dr. M.K. Kim (Diversa Corp., San Diego, CA, personal communication) observed high natural resistance of sweetgum nodule cultures to kanamycin. Natural resistance to kanamycin has been reported in *Pinus radiata* (Walter et al., 1998), *Picea mariana* (Charest et al., 1996), and *Manihot esculenta* (Schöpke et al., 1996). A preliminary sensitivity experiment with sweetgum PEMs indicated that kanamycin, geneticin and paramomycin were all capable of inhibiting growth at 25 µg/ml or higher and thus could be used for selection of PEMs following bombardment (data not shown). The identification of sub-lethal levels of kanamycin is important for allowing bombarded cells to integrate the introduced gene while preventing non-transformed embryogenic cells from over-growing the medium. Another important reason for using sub-lethal levels of kanamycin was to prevent extensive cell death from non-transformed cells which release

phenolics and other metabolites that can potentially inhibit growth from transformed cells (McCown et al., 1991).

Osmotic conditioning of PEMs

Two experiments were conducted to determine the effect of pre-bombardment osmotic conditioning of PEMs on transformation frequency. The first experiment compared equimolar mannitol/sorbitol concentrations at 0, 0.25, 0.5, 0.75, and 1 M for enhancing transformation frequency from PEM line SLS 9-7IV. In the second experiment, three PEM lines (7851-7B, SLS 5-8I, and SLS 9-7IV) were conditioned on 0 or 0.5 M mannitol/sorbitol treatments to test for improved transformation frequency across multiple lines.

PEMs from line SLS 9-7IV conditioned on 0.5 M mannitol/sorbitol had a significantly higher ($p<0.0001$) average number of transiently expressing GUS foci than did PEMs of this line conditioned on any other level of osmoticum (Fig. 4). In addition to the 0.5 M treatment, the 0.25, 0.5, and 0.75 M treatments all had significantly ($p<0.0001$) higher GUS expression than the control. There was no significant ($p<0.0001$) difference between the 1 M treatment and the control or between the 0.25 and 0.75M mannitol/sorbitol treatments. Figure 4 indicates that transformation of PEMs from this line improved with the addition of up to 0.5 M mannitol/sorbitol to the culture medium, but levels greater than 0.5 M mannitol/sorbitol gave a diminished transformation frequency, suggesting that no more than 0.5 M mannitol/sorbitol should be used for conditioning of sweetgum PEMs for bombardment. Similar results were reported for myo-inositol by Clapham et al. (1995). Pre-bombardment osmotic conditioning of sweetgum embryogenic cells alone was sufficient for improving transient expression, while still allowing these cells to grow when transferred to selection medium. This is consistent with Vain

et al. (1993) who also reported an increase in transient expression from pre-bombardment treatment of cells with osmoticum.

Methods for handling osmotically conditioned cells after bombardment using a stepwise reduction of osmoticum (Russell et al., 1992) or a post-bombardment culture of cells on medium containing osmoticum (Marchant et al., 1998; Rosillo et al., 2003; Vain et al., 1993) prior to initiating selection were not tested with embryogenic sweetgum cells. While it is possible that such variants on our treatments would have further improved transient GUS expression, we believe the level of the transient expression obtained using the 0.5 M pre-bombardment treatment indicated a high likelihood that stable integration would follow.

The second osmotic conditioning experiment tested three lines conditioned on media containing 0.5 M mannitol/sorbitol or no osmoticum. For all lines, significantly ($p<0.0001$) higher numbers of transient GUS expressing foci resulted when PEMs were cultured on medium containing 0.5 M mannitol/sorbitol than when they were cultured on medium without osmoticum (Fig. 5). There was also a significantly higher ($p<0.01$) number of transient GUS foci from line 7851-7B conditioned on 0.5 M osmoticum, compared to the other lines. This result indicates an interaction between the lines and osmoticum treatments. Despite the high levels of transient expression observed in this experiment, no kanamycin-resistant PEM colonies were obtained following 8-12 weeks on selection medium.

Conditioning of cells on a medium that contains osmoticum has been shown to be an important component for some biolistic transformation protocols. Enhanced transient expression and stable transformation from microprojectile bombardment of plant cells conditioned by osmotic agents is believed to result from plasmolysis of the cells, which reduces cell damage by preventing extrusion of the protoplasm from bombarded cells due to low cell turgor pressure.

Plasmolysis also makes the vacuole less of an obstruction to the microprojectiles penetrating the nucleus, by reducing the volume of vacuole (Armaleo et al., 1990; Sanford et al., 1993).

Post-bombardment PEM culture for selection of transformants

Recovery periods of 0, 3, 5, and 7 days on IMM without kanamycin between bombardment and initiation of selection were tested for lines SLS 9-7IV and SLS 5-8I. Following the recovery period, PEMs were transferred to the same medium containing 50 μg/ml kanamycin for selection of resistant PEMs. Of the four post-bombardment recovery periods tested in both replications of the experiment, only the 0 d recovery period allowed for identification of potentially kanamycin-resistant PEMs after 8 weeks of culture (Fig. 6). Potentially kanamycin-resistant PEMs could not be identified from the 3, 5, and 7 d periods, as growth from bombarded PEMs covered the medium surface.

PEMs from line SLS 9-7IV transferred to selection immediately following bombardment produced several colonies showing initial resistance to the selection medium containing 50 μg/ml kanamycin after 8 weeks of culture. However, only a small proportion of these PEM colonies maintained kanamycin resistance following an additional 4 weeks of culture on fresh selection medium, resulting in an overall transformation efficiency below 0.1 percent (Table 1). Bombarded PEMs from line SLS 5-8I transferred to selection immediately following bombardment also produced several colonies showing initial kanamycin resistance, with even fewer PEMs maintaining kanamycin resistance with additional culture on fresh selection medium (Table 1). When this experiment was repeated with fractionated PEMs from 3-day old suspension cultures and 4 replicate plates per treatment, only two kanamycin resistant PEM lines were recovered from one plate in this experiment. As was the case in the first replication of the experiment, both stably-transformed colonies arose from material given 0 d of recovery

following bombardment prior to selection (data not shown). PEMs from kanamycin-resistant cultures were transferred to liquid proliferation medium with 25 μg/ml kanamycin for additional, stringent selection in suspension culture based on the work by Wilde et al. (1992).

The use of 50 μg/ml kanamycin, as determined from earlier selection experiments, was important for testing initiation of selection as short as 0 days following bombardment because previous reports indicated the importance of applying selection at an inhibitory level for cell proliferation that is non-lethal. This avoids excessive cell death and possible detrimental effects on morphogenetic competence of transformed cells (McCown et al., 1991; Mullins et al., 1990). Problems with kanamycin selection and target material used with bombardment have occurred in work with cotyledonary somatic embryos and embryonal masses of *Picea mariana* (Charest et al., 1996), embryogenic cells of *Vitis* sp. (Kikkert et al., 1996), somatic embryos of *Picea glauca* (Ellis et al., 1993), and nodules and stems of *Populus* hybrids (McCown et al., 1991). To our knowledge the use of immediate selection of bombarded embryogenic cells to recover kanamycin- resistant cell lines has not been reported previously. From our results, we do not have an understanding of the interaction between cells conditioned on osmoticum and the possible effect on selection. The recovery period lengths between bombardment and initiation of selection for this and previous bombardment experiments were based on the growth curves from our cell lines cultured on semisolid medium. In one report on adventitious shoot formation from sweetgum leaves and petioles, Brand and Lineberger (1988) identified increased shoot formation by wounding explants. It may be the case that these cell lines were stimulated to grow from wounding associated with penetration from microprojectiles during bombardment and this cell growth allowed the cells to outgrow selection.

Analysis of transformants

GUS assays of eight putatively transformed embryogenic sweetgum lines recovered from the 0 day post-bombardment treatment and grown in suspension culture under selection identified seven that were GUS-positive and one that was GUS-negative (Fig. 7). While it is possible that the GUS-negative line could have been an escape, the identification of a kanamycin-resistant cell line that was GUS negative following microprojectile-mediated transformation has occurred previously in work with *Liriodendron tulipifera* (Wilde et al., 1992), *Vitis* (Kikkert et al., 1996), and *Pinus radiata* (Walter et al., 1998). One of the benefits to using PEMs with transformation research is the reduced chance of recovering chimeras (Wilde et al., 1992). An individual transformed cell under selection produces more transformed cells, each having the potential to regenerate a transgenic plant. The potential for chimeras from PEMs can be further reduced by stringent selection applied to putatively transformed PEMs through suspension cultures. Recovering a kanamycin-resistant, but GUS-negative line containing a gene fusion driven by one promoter for expression of GUS and NPTII could be due to scrambling of genomic DNA in the *gus* region of the gene fusion through illegitimate recombination (Makarevitch et al., 2003).

DNA isolated from all eight kanamycin-resistant embryogenic cell lines screened in the GUS assay was tested for the presence of the *gus-nptII* transgene by polymerase chain reaction (PCR). Primers for the *nptII* region of the gene fusion were designed to amplify a fragment 631 bp in length. The presence of the *nptII* gene was confirmed in all eight kanamycin-resistant lines, including the GUS-negative line, whereas the non-transformed line and PCR mix controls did not show a band for the amplified *nptII* fragment (Fig. 8).

DNA from the lines screened for the presence of *nptII* by PCR was analyzed for transgene integration by Southern blot analysis (Fig. 9). DNA was cut with *Eco*RI, which has a unique restriction site just 3' to the NOS- T sequence of the gene construct, to reveal integration patterns of the plasmid in the genome of transformed cells. Hybridization of the *nptII* probe to *Eco*RI-digested genomic DNA showed that the plasmid pBI426 had integrated into the sweetgum genome at multiple sites in five out of the eight lines. No hybridization of the probe to the non-transformed sweetgum line occurred. Three lines had fragments that appeared to migrate to the same location in the gel as fragments produced from the plasmid controls. We found this result to be unusual and believe it may have occurred due to the samples not having migrated far enough through the gel to show separation of bands between the transgenic lines and positive controls. Three kanamycin-resistant lines (two of which were also GUS-positive) did not show signal from hybridization of the probe. Absence of signal from these lines and faint signal from other lines could be due to inconsistent quantities of DNA used for analysis from quantification of DNA by spectrophotmeter.

PEM suspensions grown in liquid IMM with 25 μg/ml kanamycin were size-fractionated and plated onto embryo development medium in the absence of kanamycin, in an effort to regenerate somatic embryos and recover transgenic plants. Unfortunately the lines used for bombardment experiments had lost their capacity to make embryos over the extended period they had been in culture by the time transformed PEMs were obtained. Thus, neither the transgenic lines nor the wild-type source lines from which they were derived were capable of producing somatic embryos or plants by the time transgenic material had been produced.

We have identified a selection agent and concentration, a pre-bombardment osmoticum conditioning treatment, and a post-bombardment selection method for the transformation and

recovery of transgenic embryogenic cultures of sweetgum. While our embryogenic cell lines lost their ability to produce somatic embryos, we believe a protocol based on our findings could be applied to embryogenic cell cultures of sweetgum to produce transgenic plants.

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Table 1. Recovery of kanamycin resistant (kan^r) PEMs from selection initiated 0 days after bombardment for the first recovery period experiment.

Line	Number of PEMs Bombarded	Emerging Kan^r PEMs	PEMs Maintaining Kan^r
SLS 9-7IV	50,000 (5 Plates)	23 (0.046 %)	11 (0.022 %)
SLS 5-8I	50,000 (5 Plates)	22 (0.046 %)	2 (0.004%)

Figure 1. Restriction site map for the gene fusion of pBI 426. The sites are unique to the construct and size of each component is labeled. The bold bar represents the region of gus::npt fusion amplified by PCR and hybridized to with labeled PCR product during Southern blot analysis.

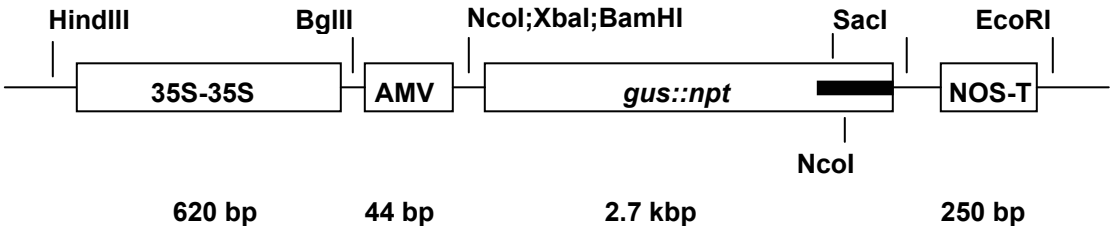
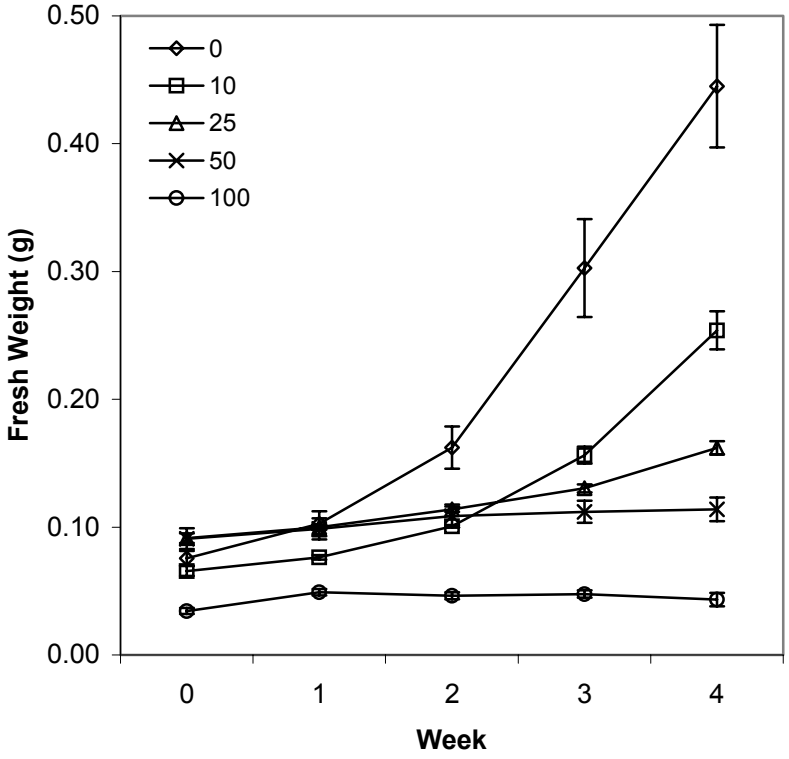

HindIII
BglII
NcoI;XbaI;BamHI
SacI
EcoRI
35S-35S
AMV
gus::npt
NOS-T
NcoI
620 bp
44 bp
2.7 kbp
250 bp

Figure 2. Growth inhibition curves for two embryogenic sweetgum culture lines on media with 5 levels of kanamycin. Top, line 7851-7B. Bottom, line SLS 9-7IV. Bars represent standard error.


0.50
0.40
0.30
0.20
0.10
0.00
Fresh Weight (g)
0
10
25
50
100
0
1
2
3
4
Week

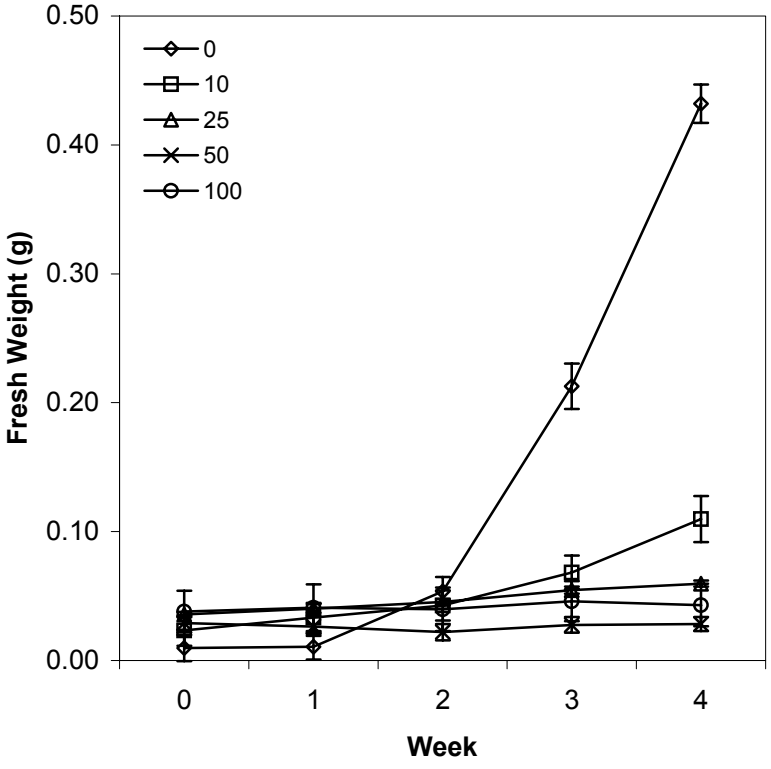

0.50
0.40
0.30
0.20
0.10
0.00
Fresh Weight (g)
0
10
25
50
100
0
1
2
3
4
Week

Figure 3. Comparison of growth between three embryogenic sweetgum lines cultured on semi-solid induction-maintenance medium. Bars represent standard error.

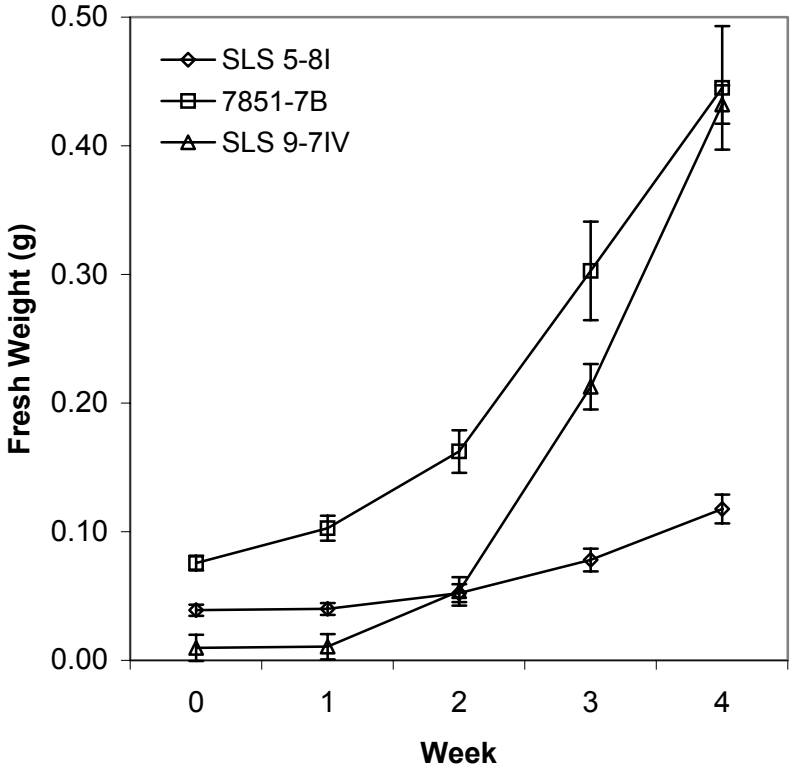

0.50
0.40
0.30
0.20
0.10
0.00
Fresh Weight (g)
SLS 5-8I
7851-7B
SLS 9-7IV
0
1
2
3
4
Week

Figure 4. Transient GUS expressing foci from embryogenic sweetgum line SLS 9-7IV cultured on induction-maintenance medium with five levels of osmoticum before bombardment. Bars represent standard error.

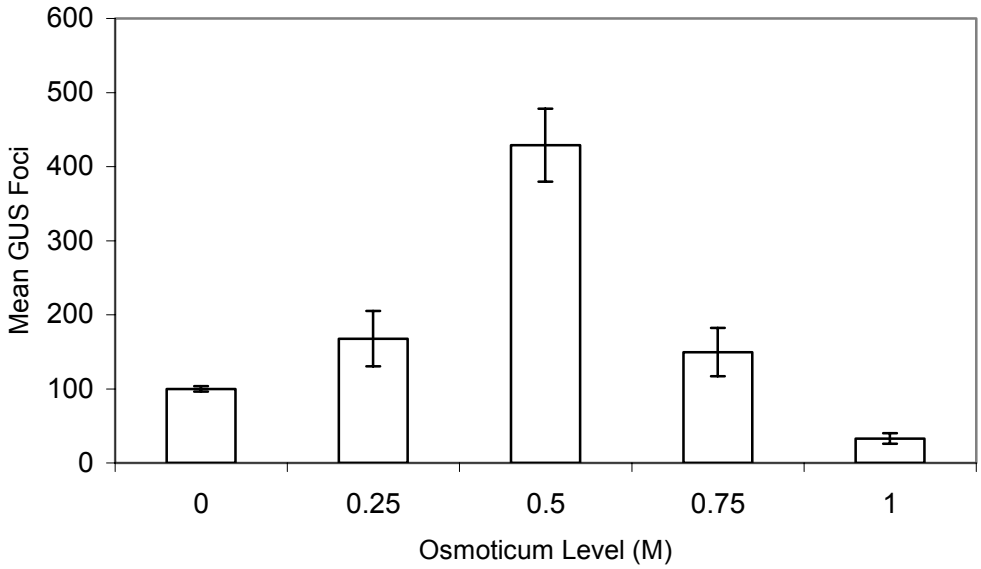
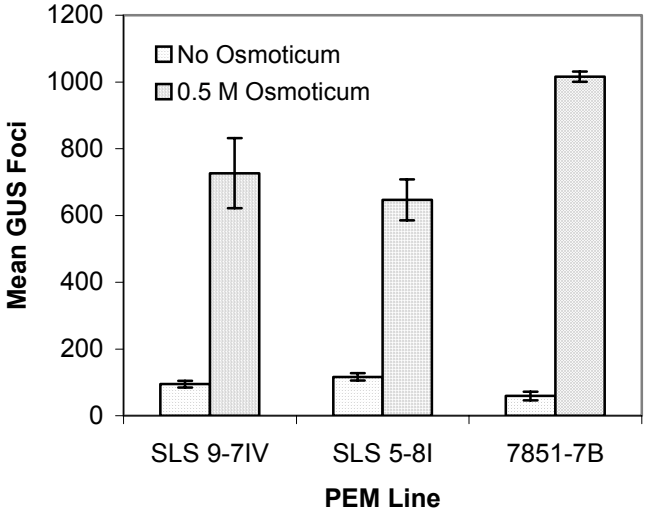

600
500
400
300
200
100
0
Mean GUS Foci
0
0.25
0.5
0.75
1
Osmoticum Level (M)

Figure 5. Transient GUS expression from 3 embryogenic sweetgum lines cultured on induction-maintenance medium with or without 0.5 M osmoticum. Top, mean number of GUS staining foci. Bars represent standard error. Bottom, sweetgum PEMs on filters expressing GUS.


1200
1000
800
600
400
200
0
No Osmoticum
0.5 M Osmoticum
Mean GUS Foci
SLS 9-7IV
SLS 5-8I
7851-7B
PEM Line

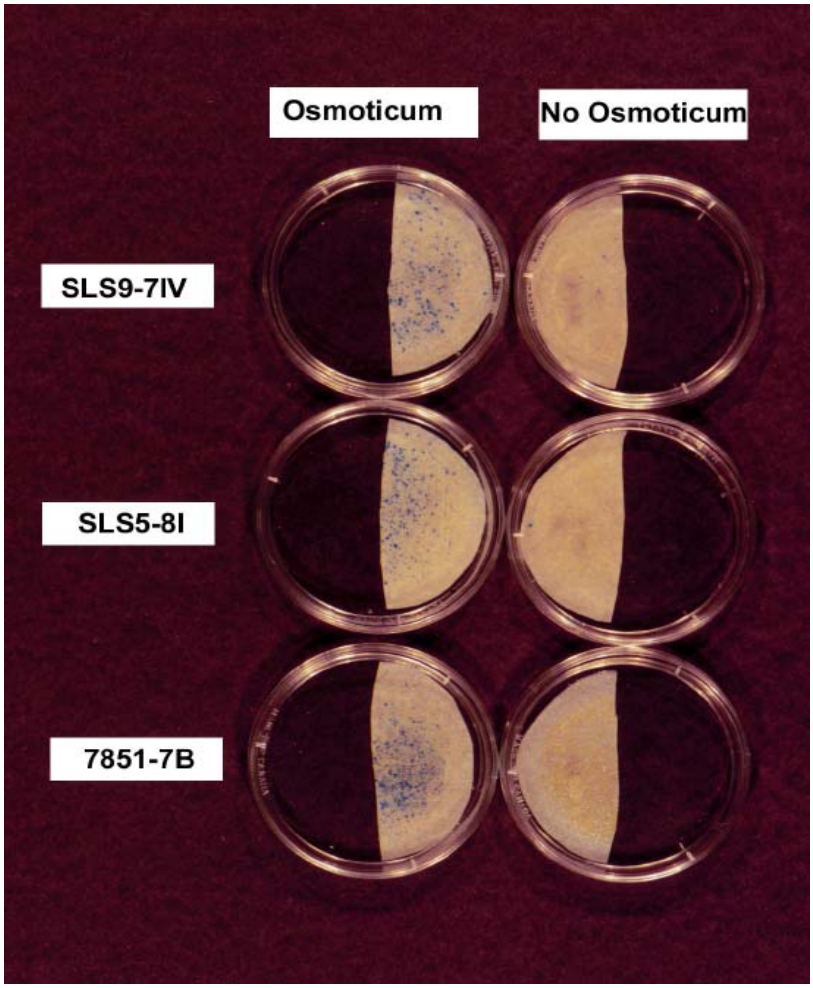

Osmoticum
No Osmoticum
SLS9-7IV
SLS5-8I
7851-7B

Figure 6. Relative growth of two sweetgum PEM lines after 10 weeks of culture on kanamycin selection medium. Bombarded PEMs were allowed to recover with culture on semi-solid IMM for 0, 3, 5, or 7 days before transfer to kanamycin selection medium.

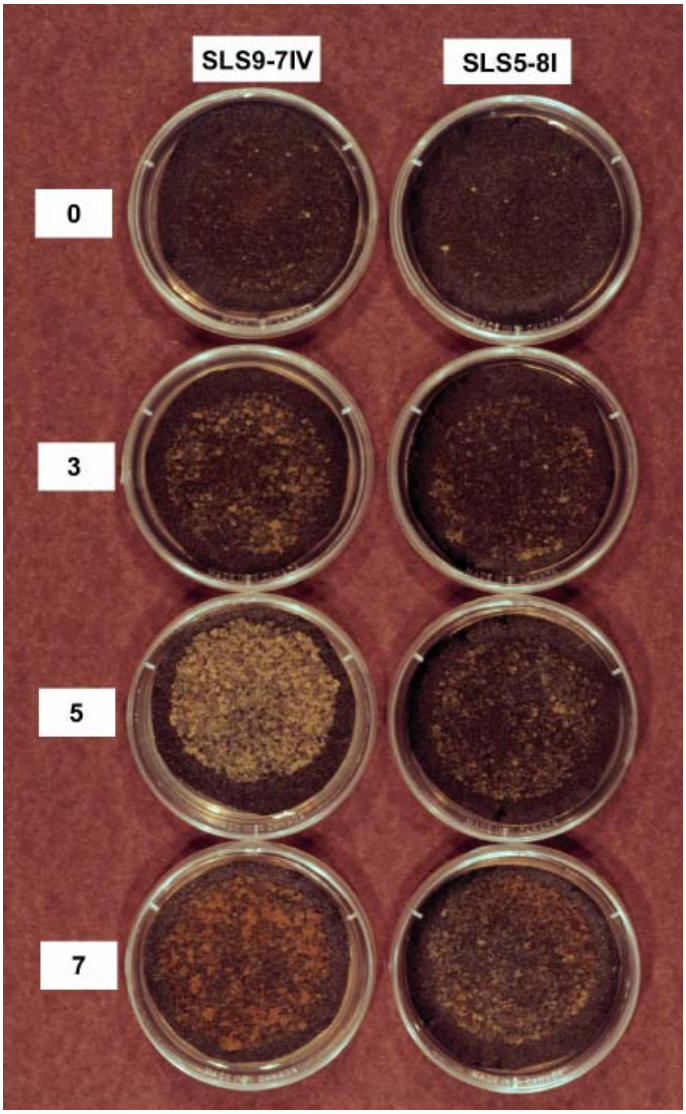

SLS9-7IV
SLS5-8I
0
3
5
7

Figure 7. Long-term GUS expression from PEMs of transformed embryogenic sweetgum lines. Wells: (1) Line SLS9-7IV-05-01; (2) Line SLS9-7IV-05-02; (3) Line SLS9-7IV-04-01; (4) Line SLS9-7IV-04-06; (5) Line SLS5-8I-24-01; (6) Line SLS5-8I- 24-04; (7) Line SLS9-7IV- 05-06; (8) Line SLS9-7IV-01-01.

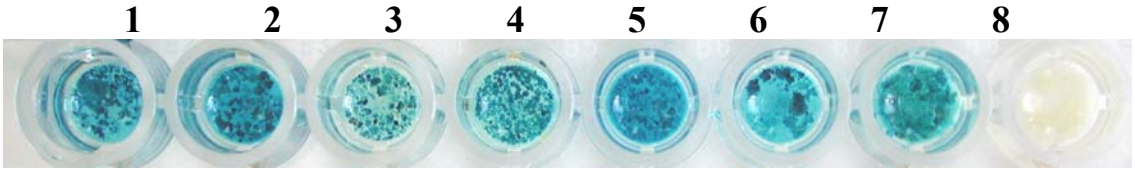

1
2
3
4
5
6
7
8

Figure 8. Ethidium bromide gel of 631 bp PCR fragments amplified from *nptII* region of the gene fusion in seven GUS-positive (lanes 4 to 10) and 1 GUS-negative (SLS9-7IV-01-01, lane 3) embryogenic sweetgum lines. Lanes: (1) Molecular marker ladder; (2) Positive control from *Eco*RI digested pBI 426; (11) Non-bombarded sweetgum line; (12) Negative PCR control. Arrows next to bands of the marker ladder: (top) 750 bp, (bottom) 500 bp.

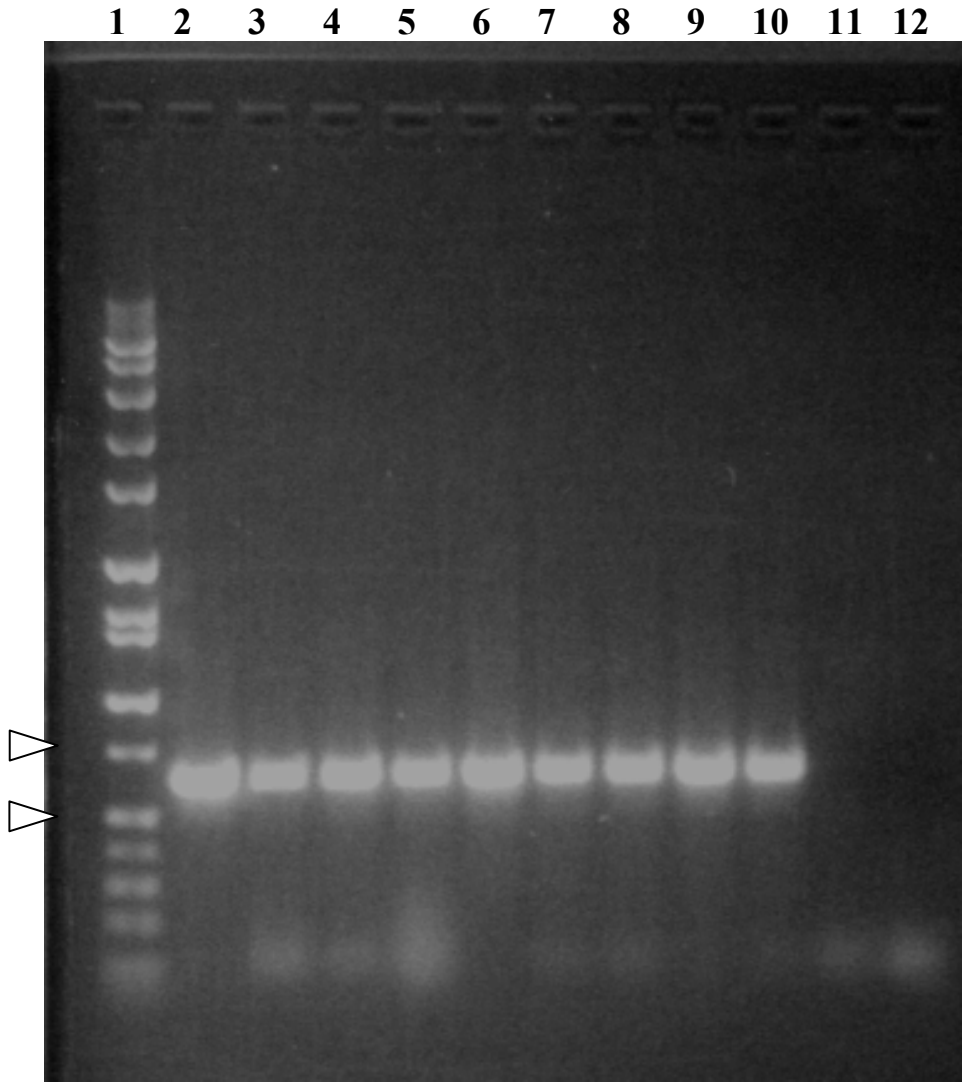
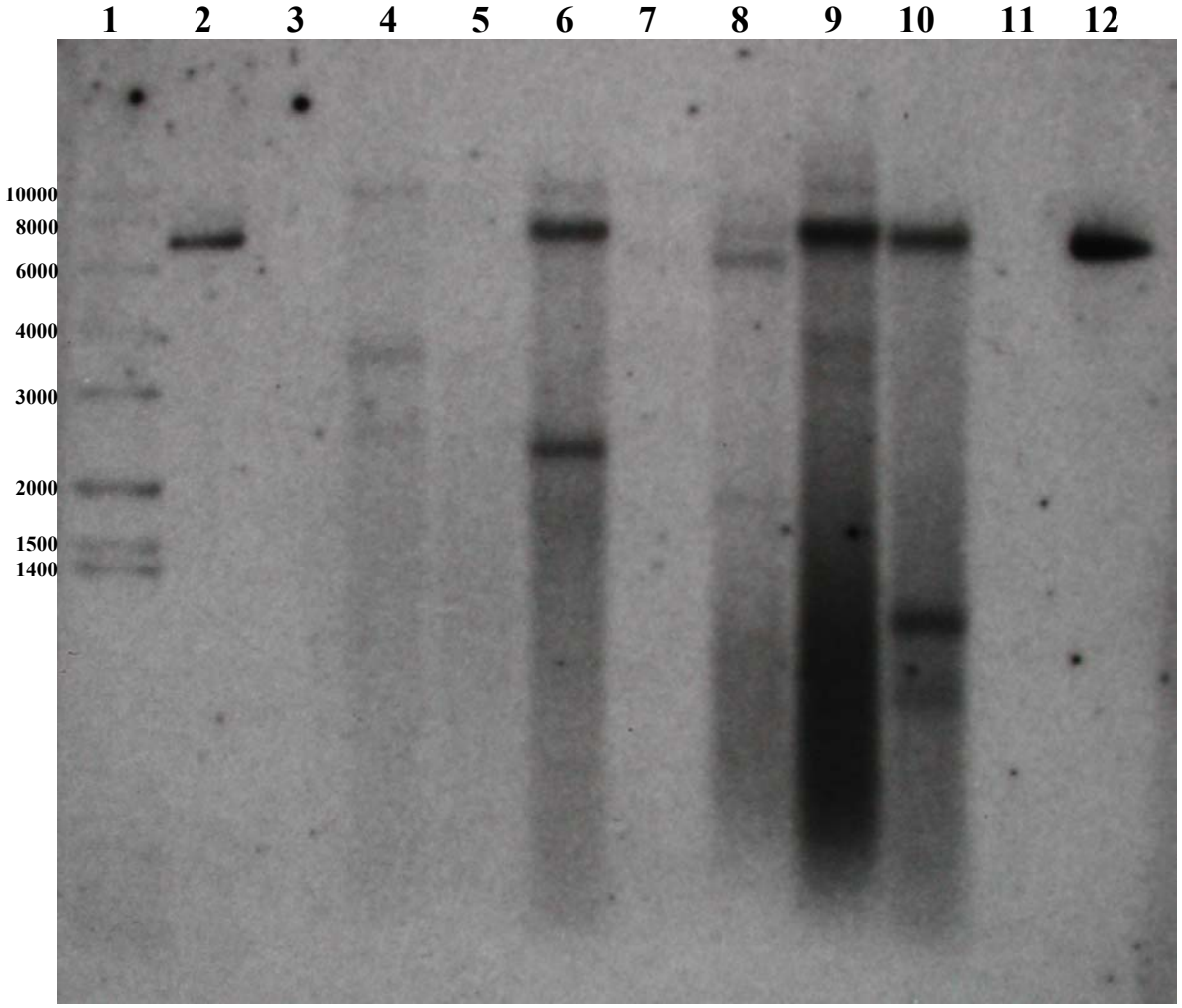

1 2 3 4 5 6 7 8 9 10 11 12

Figure 9. Southern blot from transformed embryogenic sweetgum lines. All samples and controls were digested with *Eco*RI. Lanes: 1) Molecular marker ladder; 2) pBI 426 plasmid as positive control; 3) Line SLS9-7IV-01-01; 4) Line SLS9-7IV-04-01; 5) Line SLS9-7IV-04-06; 6) Line SLS9-7IV- 05-06; 7) Line SLS5-8I-24-01; 8) Line SLS5-8I- 24-04; 9) Line SLS9-7IV-05-01; 10) Line SLS9-7IV-05-02; 11) Non-bombarded sweetgum line; 12) pBI 426 positive control. The marker ladder band sizes are labeled as base pairs.


1
2
3
4
5
6
7
8
9
10
11
12
10000
8000
6000
4000
3000
2000
1500
1400

CHAPTER 3

EXPERIMENTS TO CHARACTERIZE GROWTH AND SELECTION OF SWEETGUM (*LIQUIDAMBAR STYRACIFLUA* L.) PROEMBRYOGENIC MASSES

INTRODUCTION

A tissue culture protocol allowing targeted cells to integrate introduced genes, survive selection, and rapidly regenerate transformed plants is one of the most important components in creating transgenic plants (White, 1993). Cell suspension cultures have become routine target material for the biolistic transformation approach to create transgenic plants. Cell suspensions in early log-phase have been shown to give the highest rates of transformation compared to cells in mid log-phase, which displayed dramatically lower rates of transformation (An, 1985). Synchronized cells bombarded at M phase of the cell cycle had the highest amount of GUS expression (Iida et al., 1991). Newly subcultured embryogenic cells had high mitotic activity from the second to the sixth day of growth in suspension culture and produced higher GUS expression than cells bombarded at other times in the culture period (Hazel et al., 1998).

Initial experiments to characterize parameters for transformation of sweetgum (*Liquidambar styraciflua*) proembryogenic masses (PEMs) used lines maintained in suspension culture as PEMs that were size-fractionated and plated using a protocol originally established for yellow-poplar (*Liriodendron tulipifera*) embryogenic suspension cultures (Merkle et al., 1990). When size-fractionated on stainless steel screens to generate a population of small cell clusters for initial selection experiments, this culture protocol yielded PEMs within the desired size range, but in numbers too low to expect useful numbers of transformation events. Furthermore, culture growth characteristics necessary for targeting PEMs at the optimal phase of growth were unknown for the embryogenic sweetgum lines. Described in this chapter are experiments conducted to characterize growth of sweetgum embryogenic suspensions while improving yields of size-fractionated PEMs to provide target material for microprojectile bombardment. Growth of sweetgum PEMs in suspension culture was characterized by conducting a growth curve study.

In early growth curve experiments, the method described by Chou (1987) for generating growth curves for yellow-poplar suspension cultures was tested. This method proved unsatisfactory for the objectives of the current study. Therefore, a more reliable method for quantifying PEM growth in suspension culture was devised. This method avoids the use of centrifugation for fresh weight and packed cell volume observations.

MATERIALS AND METHODS

Embryogenic culture lines and culture conditions

Four embryogenic sweetgum lines were used in this study. Protocols used for initiation of these cultures were described in Merkle et al. (1998). Briefly, two lines, designated 7851-7A and 7851-7B, were initiated in the summer of 1994 from seeds supplied by International Paper Company (Bainbridge, GA, USA). Two additional lines SLS 5-8I and SLS 9-7IV, were initiated in the summer of 1995 from seeds collected from a tree growing on the University of Georgia (Athens, Georgia, USA) campus. These lines, which proliferated as PEMs, were maintained in suspension culture by inoculating 0.5 g fresh weight of cells into 125 ml Erlenmeyer flasks containing 50 ml of induction-maintenance medium (IMM; see below for medium details). Embryogenic cell suspensions were cultured on a gyratory shaker at 130 rpm under cool white fluorescent lights at 100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ with a 16-h photoperiod at 27° C. PEM suspensions were subcultured on 21 day cycles.

Culture media

Media were based on woody plant medium ((WPM) Lloyd and McCown, 1980) unless stated otherwise. WPM-IMM, which was used for culture proliferation, was WPM supplemented with 0.1 g/l myo-inositol, 1.0 g/l casein hydrolysate (CH; enzymatic), 30 g/l sucrose, 2 mg/l 2,4-

dichlorophenoxyacetic acid (2,4-D), and 0.25 mg/l 6-benzyladenine (6BA), with pH adjusted to between 5.6 and 5.7. Semisolid medium used in testing selection agents were prepared using WPM-IMM with 8 g/l Phytagar (Gibco, Life Technologies, Grand Island, N.Y.) added after the medium pH was adjusted. Media were autoclaved for 20 minutes at 115 psi prior to use.

Culture medium for improved growth of sweetgum PEMs in suspension culture

Embryogenic sweetgum lines were originally maintained in suspension culture in B-IMM, an induction-maintenance medium based on a modified Blaydes' (Witham et al., 1971) medium, which was used to grow embryogenic yellow-poplar cultures by Merkle (1986). Briefly, this medium contained Blaydes' macro salts, Brown's micro salts Sommer and Brown (1980), iron of Murashige and Skoog (1962) and vitamins of Gresshoff and Doy (1972). As was the case for the WPM-IMM, this medium was supplemented with 0.1 g/l myo-inositol, 1.0 g/l CH, 30 g/l sucrose, 2 mg/l 2,4-D, and 0.25 mg/l 6BA, with pH adjusted to between 5.6 and 5.7. Four PEM lines (SLS 5-8I, SLS 9-7IV, 7851-7A, and 7851-7B) were compared between two media for growth rate. The lines used were 17 days old at the time of initiation. Each line was cultured in suspension using 50 ml of either B-IMM or WPM-IMM and 125 ml flasks that were weighed with caps on before initiation of the experiment. Three replicate flasks for each line and medium treatment combination were prepared to give a total of 24 experimental units. Settled cell volume (scv) and fresh weight (fw) were used to measure growth of cell suspensions for the growth curve study.

The scv of inoculum was measured in a 1.5 ml microcentrifuge tube. The tube contained 0.5 ml of medium to which PEMs from suspension culture flasks were added until the medium volume was displaced to the rim of the tube. Tubes were marked where PEMs had settled and the scv was determined later by measuring the volume of water required to fill the tube to the

mark. Approximately 1.0 ml scv of cells was used as inoculum. The fresh weight (fw) of PEM inoculum was measured for each line by removing the medium above the settled PEMs to the culture flask of that line and weighing the PEMs inside the tube. The tube weight was subtracted from the tube containing settled PEMs to give cell fw. Approximately 1.0 g fresh weight of cells was used as inoculum.

Fresh weight observations were made by removing media from the pre-weighed treatment flasks containing PEMs to labeled 50 ml screw cap centrifuge tubes and weighing the PEMs inside the capped flask. Settled cell volume observations were made by pouring media from the labeled tubes into their respective flasks of PEMs and pouring the re-suspended PEMs back into the tube to allow them to settle over a 10 minute period. Tubes were marked where the PEMs had settled for later measurement. The PEMs were returned from the tubes to their respective flasks. Observations were made at three day intervals over a 21 day period. The experiment was repeated three times.

Growth of PEMs on two week suspension culture cycles with two flask sizes

Growth rates for PEM lines SLS 5-8I, SLS 9-7IV, and 7851-7B were compared between two Erlenmeyer flask sizes (125 ml and 250 ml) over a 2-week culture period. The experiment was set up as a 2 x 3 factorial experiment with 3 replications per treatment. Flasks containing 50 ml of proliferation medium were inoculated with approximately 2.5 to 3.0 ml scv of PEMs. An inoculum corresponding to 2.5 ml scv was determined by measuring the displacement volume of PEMs using 50 ml conical bottom centrifuge tubes. Observations of scv were recorded at initiation and at 3 d intervals for the 15 d culture period. At total of 6 observations were made. Observations of scv were performed as described above.

Medium to flask volume ratios for producing fine suspension cultures of PEMs

Two medium-to-flask volume ratio treatments were tested for their effects on producing fine PEM suspension cultures. Treatments for this experiment were created by adding 50 and 25 ml of proliferation medium to 125 and 250 ml flasks for 2:5 and 1:10 medium-to-flask volume ratios, respectively. All treatments were inoculated with 2.5 ml scv of PEMs from line SLS 9-7IV using the method described above. Size-fractionation of PEM suspensions was used to determine the fineness of the PEMs in the cultures from the two treatments after a 2-week culture period. PEMs from suspension culture were size-fractionated by sieving suspensions through a 140 μm pore size stainless steel screen and collecting on a 38 μm pore size screen to isolate PEMs ranging from 38 to 140 μm in size. PEMs collected on the 38 μm screen were back-washed into empty 250 ml flasks using up to 45 ml of basal WPM, and the volume was adjusted to a final volume of 50 ml to generate suspensions in which PEMs could be counted. Five 30 μm samples from each tube of PEM fraction were pipetted into an empty 60 mm plastic Petri dish and counted. The experiment was conducted as a completely randomized design with 3 replications per treatment and was repeated once.

Identification of selection agents and levels for inhibiting PEM growth

Two experiments were conducted to determine selection agents and concentrations suitable for inhibiting proliferation from non-transformed sweetgum PEMs. The first experiment compared five levels of kanamycin, paramomycin, and geneticin for inhibiting proliferation from PEM line SLS 5-8I. Kanamycin was tested at 0, 50, 100, 150, and 200 μg/ml, while geneticin and paramomycin were tested at 0, 25, 50, 75, and 100 μg/ml. A follow-up experiment was conducted to test five levels of kanamycin, paramomycin, and mercuric chloride ($HgCl_2$) for inhibiting proliferation of the same PEM line. Kanamycin was tested at 0, 10, 25, 40, and 50

μg/ml, paramomycin was tested at 0, 5, 10, 15, and 25 μg/ml, and $HgCl_2$ was tested at 0, 10, 25, 40, and 50 μmol. B-IMM, prepared as described above, was supplemented with selection agents for both experiments. Filter-sterilized selection agents were added to autoclaved B-IMM cooled to 65° C to obtain the desired concentrations.

PEMs from suspension culture were size-fractionated by sieving 4-week old suspensions through a 140 μm pore size stainless steel screen and collecting on a 38 μm pore size screen to isolate PEMs ranging from 38 to 140 μm in size. PEMs collected on the 38 μm screen were back-washed into empty flasks using B-IMM without growth regulators to generate suspensions in which cell clumps could be counted. PEM counts in these fractionated suspensions were performed in a 60 mm plastic Petri dish using an inverted microscope. PEM counts were made for three 30 μl drops per fraction. An average from the three drops was used to calculate the volume of fractionated PEM suspension required to obtain the desired density of PEMs for collection on filter paper disks. PEMs were collected onto 40 mm black filter disks using a glass microanalysis filter holder assembly (Fisher Scientific) by pipetting the volume of fractionated suspension required for obtaining 2500 PEMs and washing the PEMs with approximately 20 ml of B-IMM without plant growth regulators under mild vacuum. The double filter paper plating method described by Horsch and Jones (1980) was used in these experiments to allow for accurate PEM fresh weight measurements. Following this method, the 40 mm black filter papers with PEMs were overlaid onto 60 mm plates of semi-solid medium treatment having a 50 mm white filter disk in direct contact with the medium surface. Plates were sealed with Parafilm and cultured in the dark at 27° C. Fresh-weights of PEMs were determined by removing the PEM filter, weighing it in a sterile culture plate, and subtracting the weight of the PEM filter from the weight of the same size filter paper without PEMs.

For both experiments, observations were taken at weekly intervals beginning after 24 h of culture on selection treatments. Nine observations were taken over an 8-week period for both experiments. Each experiment was carried out in a completely randomized design with three replications per treatment.

RESULTS AND DISCUSSION

Culture medium for improved growth of sweetgum PEMs in suspension culture

The most noticeable difference in fresh weight between the two tested media was with lines 7851-7A and SLS 9-7IV (Fig. 10), while lines 7851-7B and SLS5-8I showed no difference in fresh weight between the two media (Fig. 11). Line 7851-7A had a slightly higher rate of growth on WPM-IMM (0.11 g per day) than on B-IMM (0.09 g per day) while line SLS 9-7IV had a considerably higher rate of growth on WPM-IMM (0.30 g per day) than on B-IMM (0.19 g per day). Lines 7851-7B and SLS 5-8 grown in B-IMM had growth rates of 0.16 and 0.11 g of PEMs per day, respectively, while these same lines grown in WPM-IMM had growth rates of, 0.17, and 0.12 g of PEMs per day respectively. Line SLS 9-7IV began to show considerably faster growth on WPM-IMM compared to B-IMM after day 9 of culture, while line 7851-7A began to show faster growth on WPM-IMM compared to B-IMM after day 3.

Based on settled cell volume (scv), lines 7851-7A, SLS 5-8I, and SLS 9-7IV had higher rates of growth in WPM-IMM than in B-IMM (Figs. 12 and 13). Lines 7851-7A, SLS 5-8I, and SLS 9-7IV cultured in WPM-IMM had growth rates of 0.17, 0.20, and 0.30 ml PEMs per day respectively, while growth rates from these same lines cultured on B-IMM were 0.13, 0.18, and 0.22 ml PEMs per day, respectively. Line 7851-7B had a similar rate of growth on WPM-IMM (0.18 ml per day) as for B-IMM (0.19 ml per day). Interestingly, most of these lines

demonstrated the fastest growth on both media between day 0 and day 3 of culture. However line 7851-7A cultured in B-IMM had the highest growth rate period between day 3 and day 6 of culture. Line SLS 9-7IV displayed two periods of rapid growth in both media. The rapid growth rates in B-IMM occurred between day 0 and day 3 and between day 9 and day 12. The rapid growth rates in WPM-IMM occurred between day 0 and day 3 and between day 12 and day 15.

Growth of PEMs on two week suspension culture cycles in two flask sizes

In order to allow more cultures to be available for experiments, the culture period of PEM suspensions was changed from 21 days to 14 days. This change required identifying the point during the 14 day culture period where the highest rate of growth occurred. Additionally, a comparison of flask sizes containing the same volume of medium was tested in the 14 day culture period for improving growth PEM lines.

For all lines tested, the greatest difference in growth rate between the 250 ml flask and the 125 ml flask over a 14 day culture period, as determined by scv, occurred after day 9 of culture (Figs. 14 and 15). All lines had faster growth in 250 ml flasks than in 125 ml flasks. Lines SLS 9-7IV and SLS 5-8I cultured in 250 ml flasks both grew at 0.6 ml scv per day over the 15 day culture period. Growth rates for these lines were higher than for line 7851-7B cultured in the 250 ml flask which grew at a rate of 0.3 ml scv per day over the same period. The highest growth rates for each line cultured in 250 ml flasks occurred between days 12 and 15 of the culture period. During this period line SLS 9-7IV had the highest rate of growth at 0.9 ml scv per day, followed by line SLS 5-8I which grew at a rate of 0.8 ml scv per day. Line 7851-7B had the lowest rate of growth at 0.4 ml scv per day between days 12 and 15. The increase in PEM proliferation with suspension culture in 250 ml flasks could be due to higher dissolved oxygen in

the medium from the larger medium surface area and headspace volume for diffusion of gases, leading to increased respiration from cells of the PEMs.

Medium to flask volume ratios for producing smaller PEMs in suspension cultures

Based on the observation from the previous experiment that PEMs cultured in the larger flasks appeared to grow as finer suspensions that those cultured in the smaller flasks, an experiment was conducted to test the ratio of medium volume to flask volume for its effect on PEM size in suspension cultures. PEMs of line SLS 9-7IV ranging from 38 to 140 μm in diameter were collected by size-fractionation at the end of a 2 week culture period and counted to determine the effect of medium and flask volume on promoting the formation of smaller PEMs. Results are shown in Fig. 16. The amount of fine-fraction PEMs produced from suspension cultures grown in the 250 ml flasks containing 25 ml of medium (1:10 medium to flask volume ratio) was greater than for cultures grown in the 125 ml flasks containing 50 ml of medium (2:5 medium to flask volume ratio). The results from this experiment seem to support the trend observed from the results in the experiment described above. Culture of sweetgum PEMs in suspension using a high medium-to-flask ratio can produce a higher proportion of PEMs that are of the size range desirable for transformation by particle bombardment.

Identification of selection agents for inhibiting growth of PEMs

Two experiments were conducted to determine selection agents and concentrations suitable for inhibiting growth from PEMs of non-transformed sweetgum line SLS 5-8I. The first experiment compared five levels of three antibiotics, while the second experiment compared five levels of two antibiotics and mercuric chloride ($HgCl_2$) for inhibition of PEM growth.

Inhibition of PEM growth was observed for the three antibiotics tested after four to six weeks of culture when the minimum levels of 50 μg/ml for kanamycin or 25 μg/ml for

paramomycin and geneticin were compared with the control (Fig. 17). PEM growth was completely inhibited by higher levels of these antibiotics. Thus, there were no differences in PEM growth among the higher levels of kanamycin, paramomycin and geneticin. It was apparent from this experiment that the levels of these antibiotics were too high for identifying a level that would begin to inhibit growth of sweetgum PEMs. Therefore it was desirable to test lower levels of kanamycin and paramomycin in a follow up experiment.

In the second experiment, kanamycin, paramomycin and $HgCl_2$ were all capable of inhibiting PEM growth after six weeks of culture as seen between the control and the levels of selection agents tested (Figs. 18 and 19). Inhibition of PEM growth by kanamycin was observed when levels of 10 µg/ml or higher were compared with the control. Differences with inhibition of PEM growth occurred between the 10 µg/ml level and all higher levels after 7 weeks of culture. However, there were no differences in PEM growth among the 25, 40 and 50 µg/ml kanamycin treatments. Inhibition curves for paramomycin indicated that 10, 15, and 25 µg/ml all inhibited PEM growth compared to the control, with no differences in inhibition among these treatments, but, as indicated in Fig. 18, only the 25 µg/ml level completely inhibited PEM proliferation. Mercuric chloride did not allow PEM growth to occur at any of the levels tested except for the control. There was no difference in PEM growth among $HgCl_2$ treatments tested. The results seem to indicate minimum levels of 25 µg/ml kanamycin and 25 µg/ml paramomycin are required for inhibiting growth from non-transformed PEMs, while a minimum level of $HgCl_2$ could not be identified. In follow-up experiments to test $HgCl_2$ for inhibiting PEM growth, however, growth from a higher plating density (10,000 per plate) of PEMs could not be inhibited at the same or higher levels of $HgCl_2$ tested (data not shown).

While kanamycin has been used to select sweetgum transformed by T-DNA (Chen and Stomp, 1992; Sullivan and Lagrimini, 1993), Dr. M.K. Kim (Diversa Corp., San Diego, CA, personal communication) observed high natural resistance of sweetgum nodule cultures to kanamycin. Natural resistance to kanamycin has been reported in graminaceous plants (Hauptmann et al., 1988; Potrykus et al., 1985) and in woody plants, including *Pinus radiata* (Walter et al., 1998), *Picea mariana* (Charest et al., 1996), and *Manihot esculenta* (Schöpke et al., 1996). In the current experiments, suitable levels of kanamycin, paramomycin, and geneticin for selection against growth of non-transformed sweetgum PEMs after bombardment could all be determined.

The methods described here for improved suspension culture and selection of sweetgum PEMs may be useful for transformation of the tree. From these experiments, the best protocol for culture of PEMs by suspension culture would use 25 ml of WPM-IMM medium in a 250 ml flask and a two week culture period. Fine PEMs from these suspensions would be obtained by fractionation for use in bombardment or selection experiments at 3 to 6 or 9 to 12 days after subculture. Selection of bombarded PEMs for the recovery of transformed material could be accomplished using at least 25 µg/ml of kanamycin or paramomycin. Results from some of these experiments were applied to experiments described in Chapter 2. However, only one biolistic experiment utilized PEMs from suspension cultures based on the improvements described here. Selection experiments described in this chapter served as the starting point for the selection experiment described in Chapter 2. From the work described in this chapter, additional experiments were conducted that led to a protocol for recovery of transformed sweetgum PEMs via microprojectile bombardment.

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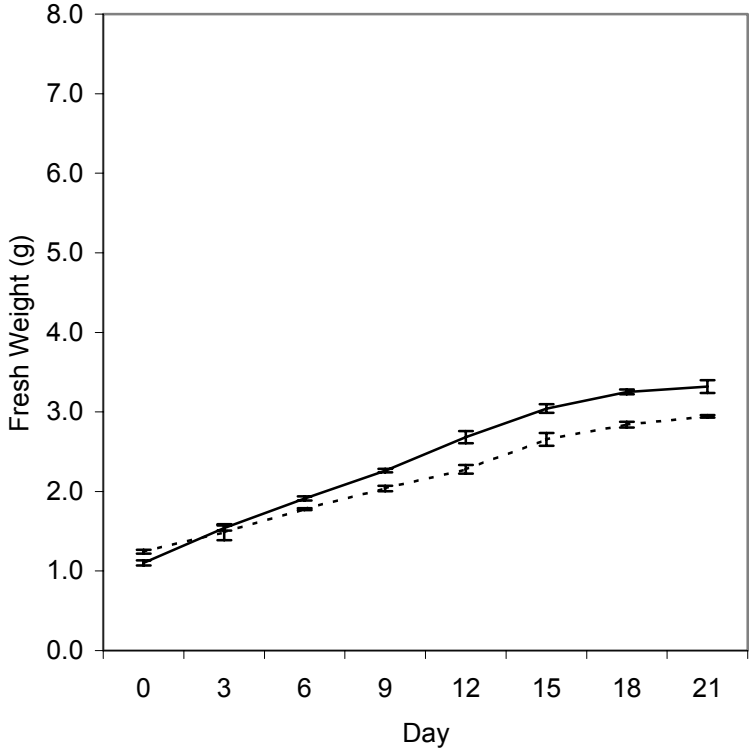
Figure 10. Fresh weight measurements of growth for two embryogenic sweetgum lines in two media, B-IMM (broken line) or WPM-IMM (solid line). Top, line 7851-7A. Bottom, line SLS9-7IV. Bars represent standard error.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Fresh Weight (g)
0
3
6
9
12
15
18
21
Day

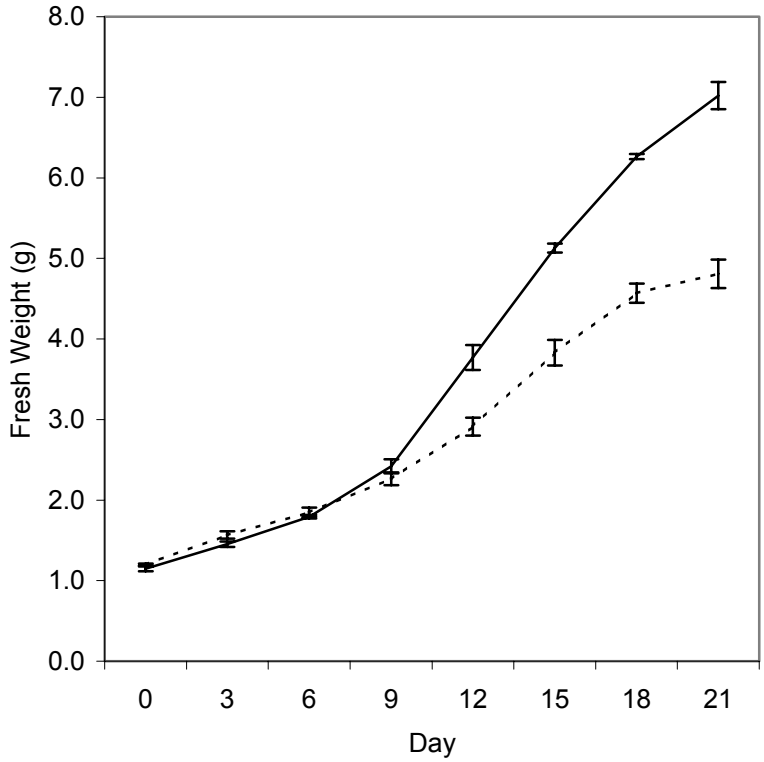
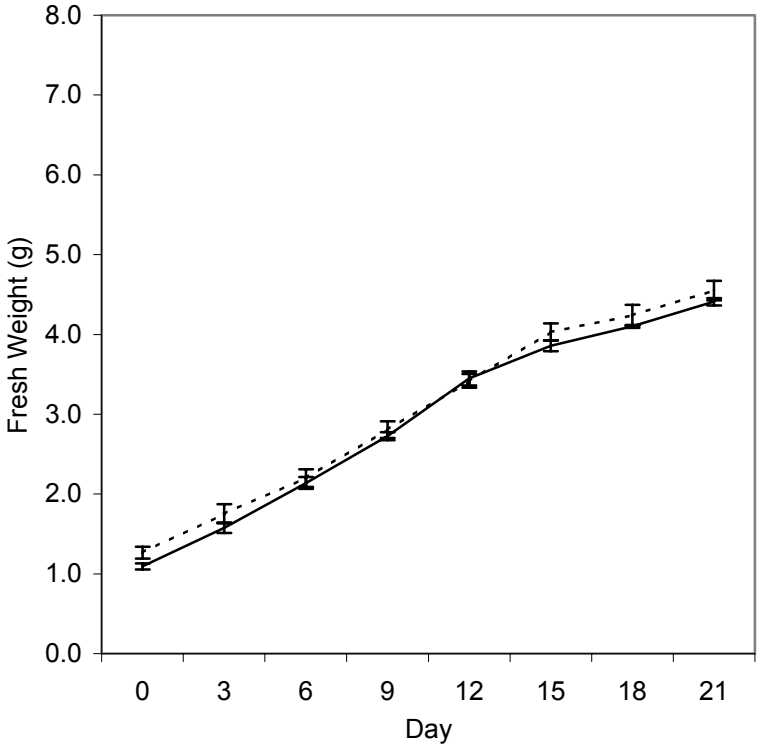

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Fresh Weight (g)
0
3
6
9
12
15
18
21
Day

Figure 11. Fresh weight measurements of growth for two embryogenic sweetgum lines in two media, B-IMM (broken line) or WPM-IMM (solid line). Top, line 7851-7B. Bottom, line SLS5-8I. Bars represent standard error.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Fresh Weight (g)
0
3
6
9
12
15
18
21
Day

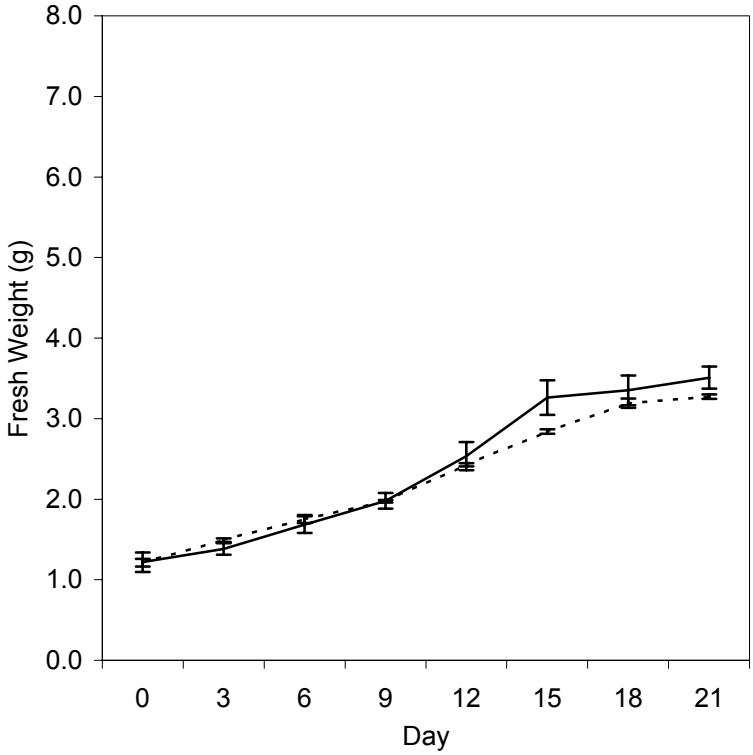
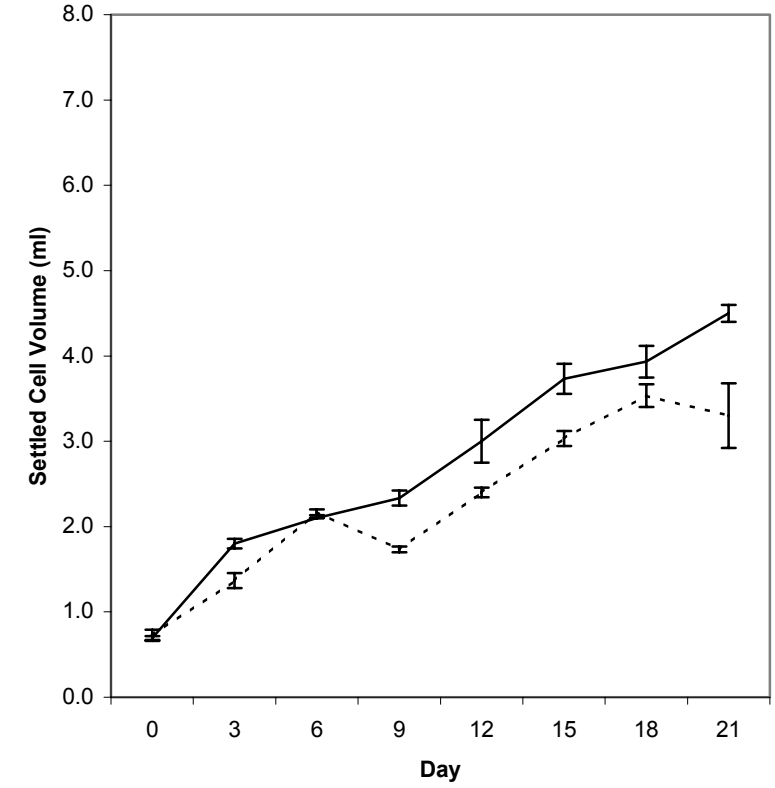

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Fresh Weight (g)
0
3
6
9
12
15
18
21
Day

Figure 12. Settled cell volume measurements of growth for two embryogenic sweetgum lines in two media, B-IMM (broken line) or WPM-IMM (solid line). Top, Line 7851-7A. Bottom, line 7851-7B. Bars represent standard error.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Settled Cell Volume (ml)
0
3
6
9
12
15
18
21
Day

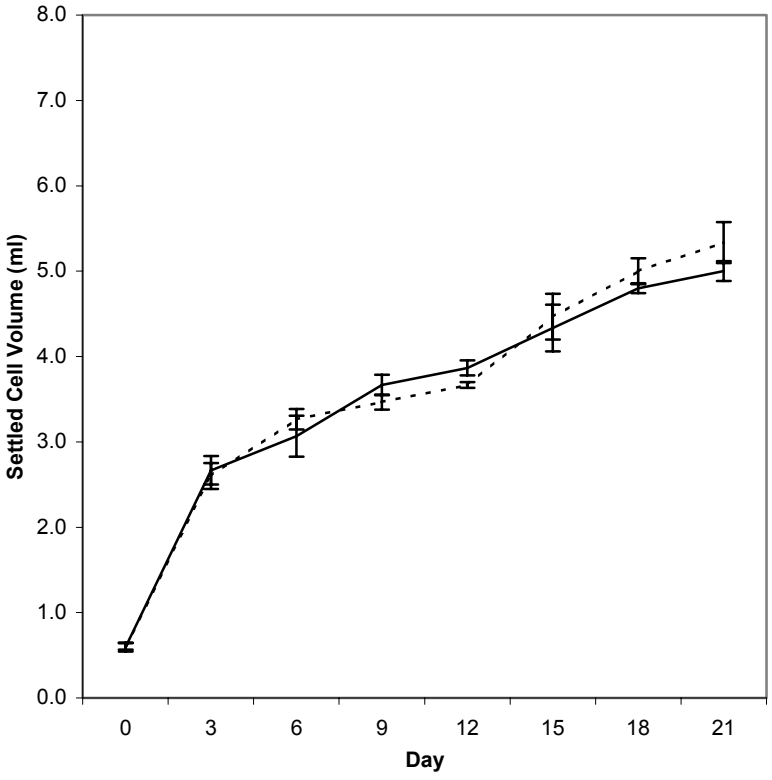
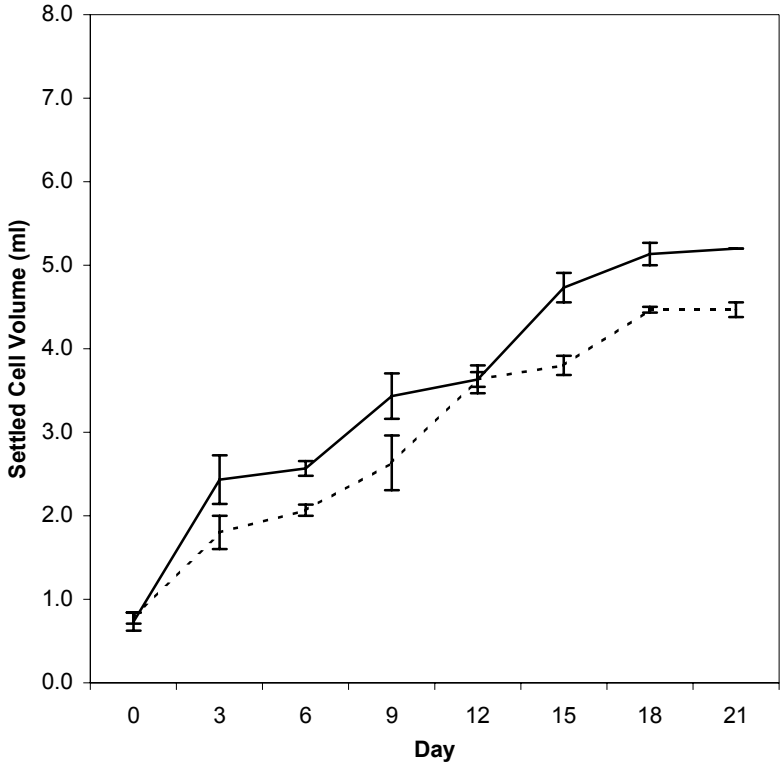

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Settled Cell Volume (ml)
0
3
6
9
12
15
18
21
Day

Figure 13. Settled cell volume measurements of growth for two embryogenic sweetgum lines in two media, B-IMM (broken line) or WPM-IMM (solid line). Top, line SLS5-8I. Bottom, line SLS9-7IV. Bars represent standard error.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Settled Cell Volume (ml)
0
3
6
9
12
15
18
21
Day

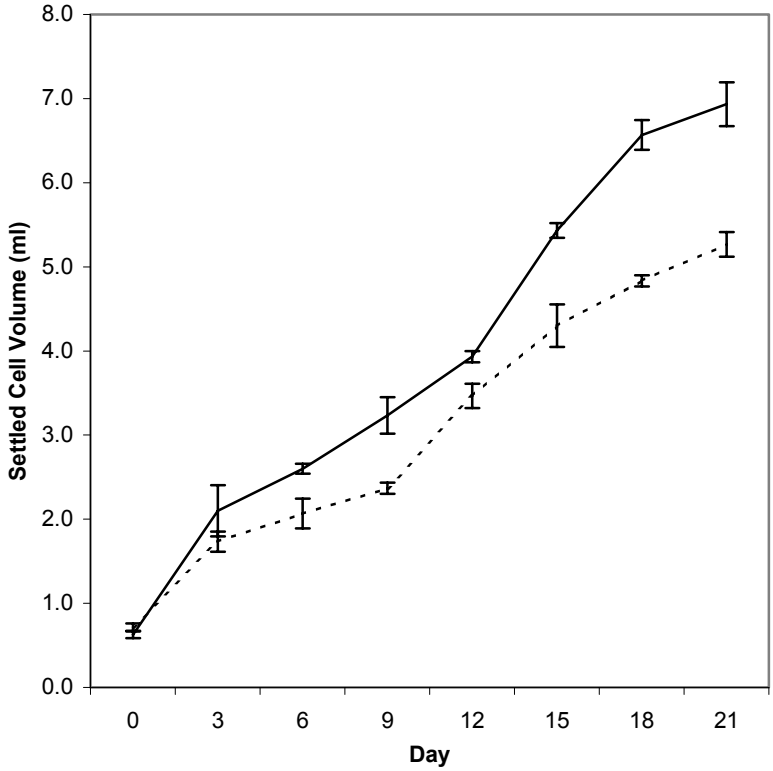
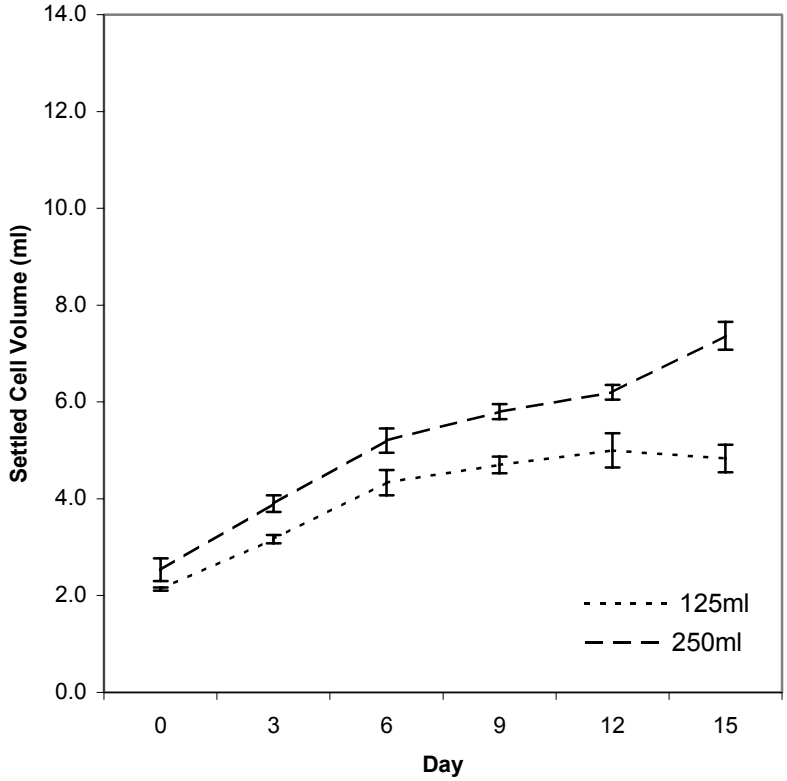

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Settled Cell Volume (ml)
0
3
6
9
12
15
18
21
Day

Figure 14. Growth curves for cultures on 2-week cycles using two vessel sizes. Top, line 7851-7B. Bottom, line SLS 9-7IV. Bars represent standard error.


14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Settled Cell Volume (ml)
0
3
6
9
12
15
Day
125ml
250ml

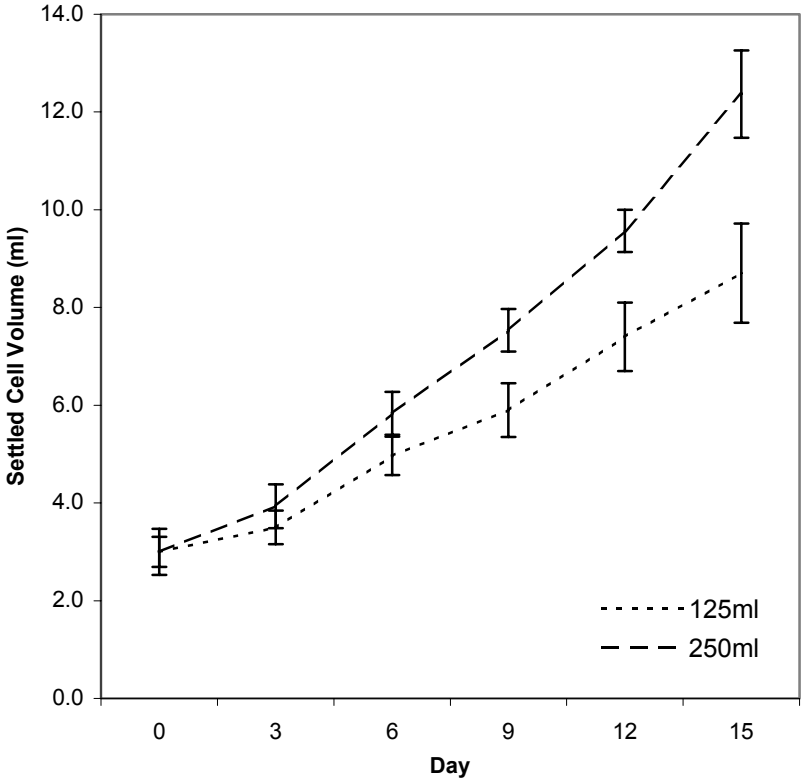

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Settled Cell Volume (ml)
0
3
6
9
12
15
Day
125ml
250ml

Figure 15. Growth curves for line SLS 5-8I on 2-week culture cycles using two vessel sizes. Bars represent standard error.

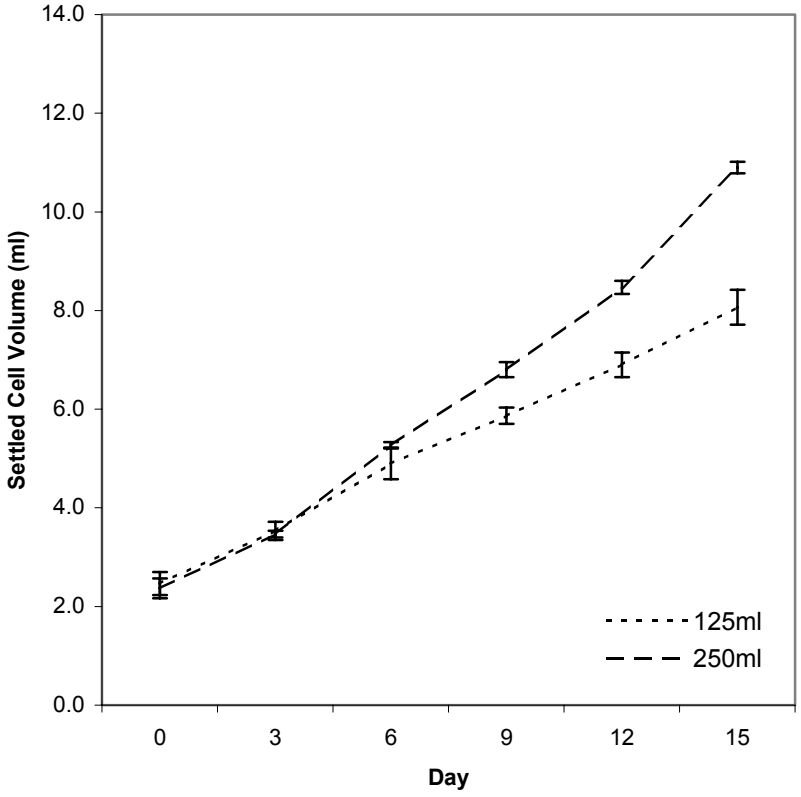

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Settled Cell Volume (ml)
0
3
6
9
12
15
Day
125ml
250ml

Figure 16. Effect of medium-to-flask volume on yield of PEMs less than 140 μm in diameter. Bars represent standard error.

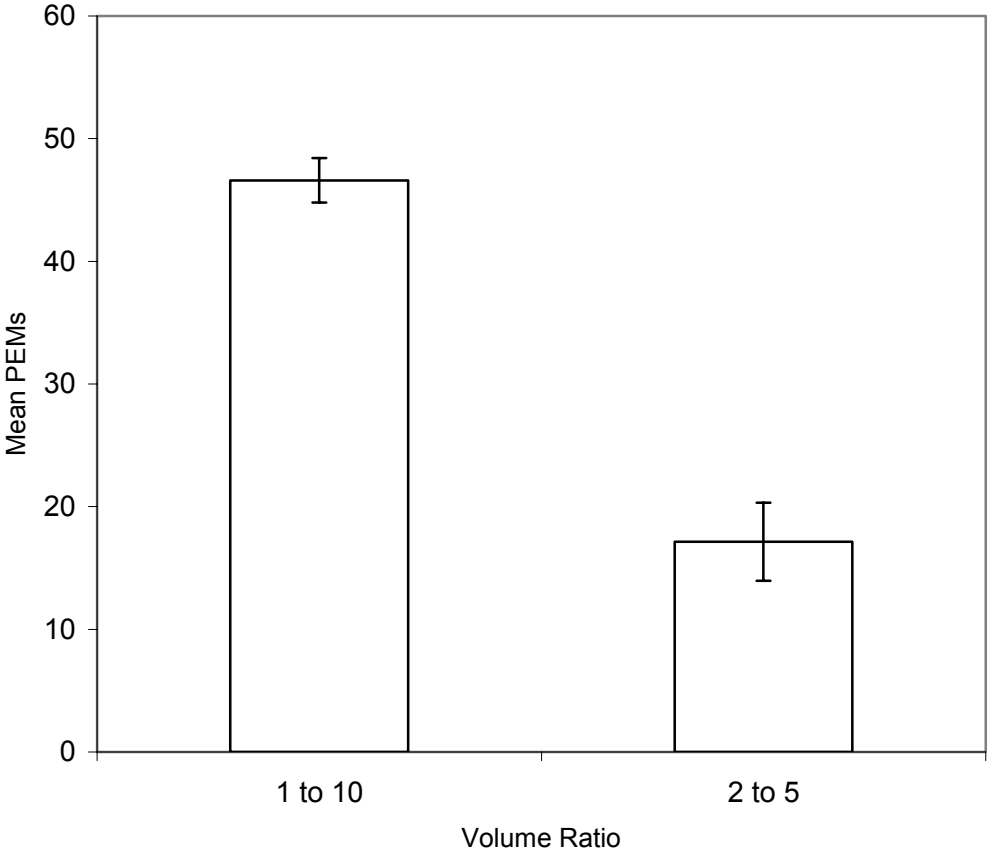
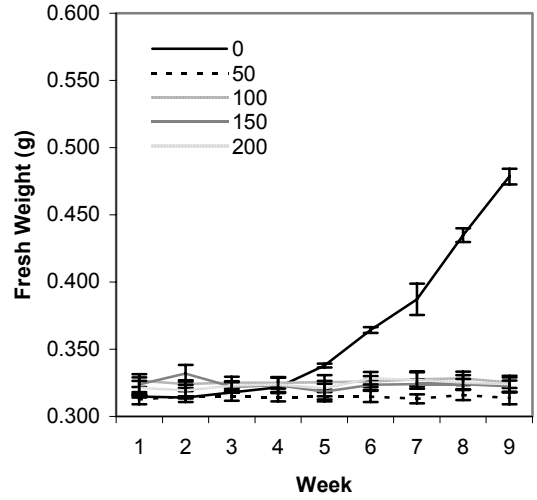
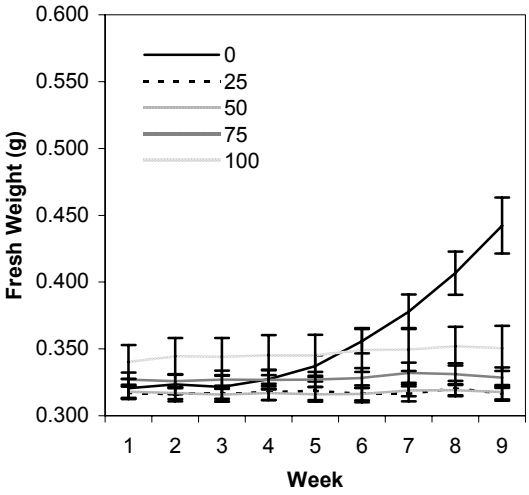

60
50
40
30
20
10
0
Mean PEMs
1 to 10
2 to 5
Volume Ratio

Figure 17. Effect of different concentrations of three antibiotics on inhibition of growth of PEM line SLS 5-8I. Top, kanamycin. Middle, paramomycin. Bottom, geneticin. Bars represent standard error. Antibiotic concentrations are in µg/ml.


0.600
0.550
0.500
0.450
0.400
0.350
0.300
Fresh Weight (g)
0
50
100
150
200
1 2 3 4 5 6 7 8 9
Week


0.600
0.550
0.500
0.450
0.400
0.350
0.300
Fresh Weight (g)
0
25
50
75
100
1 2 3 4 5 6 7 8 9
Week

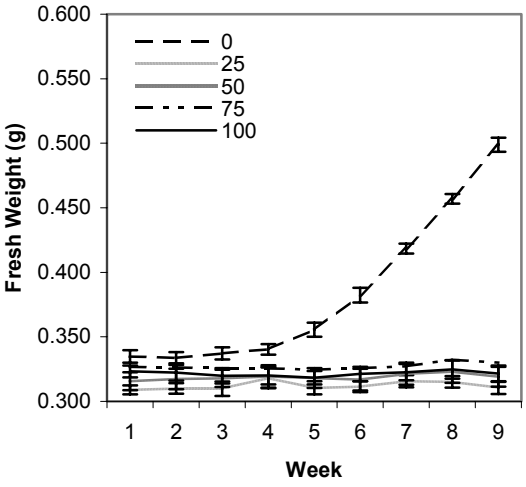
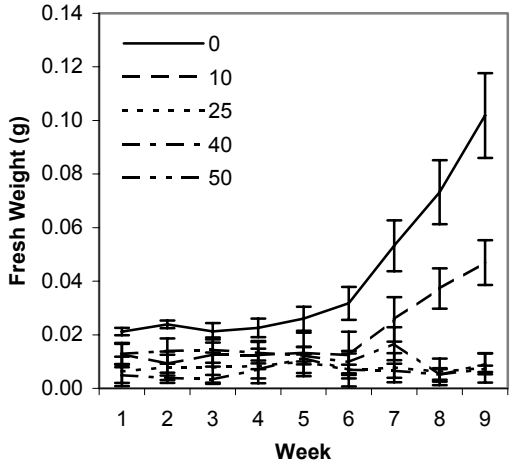
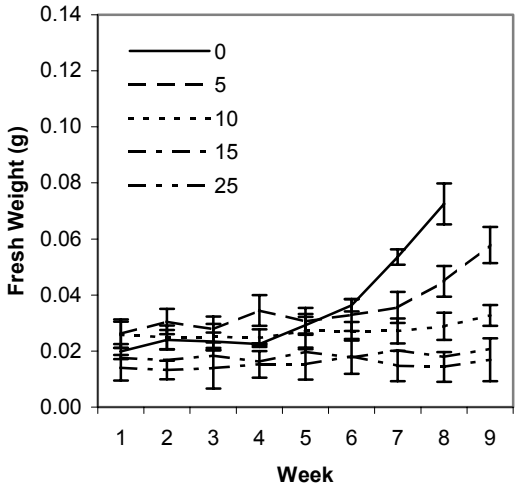

0.600
0.550
0.500
0.450
0.400
0.350
0.300
Fresh Weight (g)
0
25
50
75
100
1 2 3 4 5 6 7 8 9
Week

Figure 18. Effect of different concentrations of antibiotics and mercuric chloride on inhibition of growth from PEM line SLS 5-8I. Top, kanamycin. Middle, paramomycin. Bottom, mercuric chloride. Bars represent standard error. Antibiotic concentrations are in μg/ml and mercuric chloride concentration is μM.


0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Fresh Weight (g)
0
10
25
40
50
1 2 3 4 5 6 7 8 9
Week


0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Fresh Weight (g)
0
5
10
15
25
1 2 3 4 5 6 7 8 9
Week

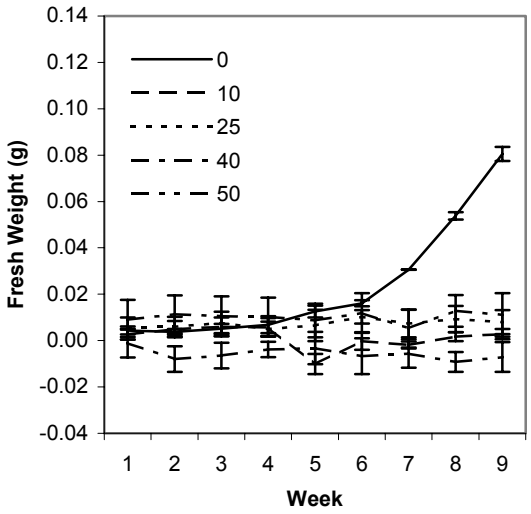
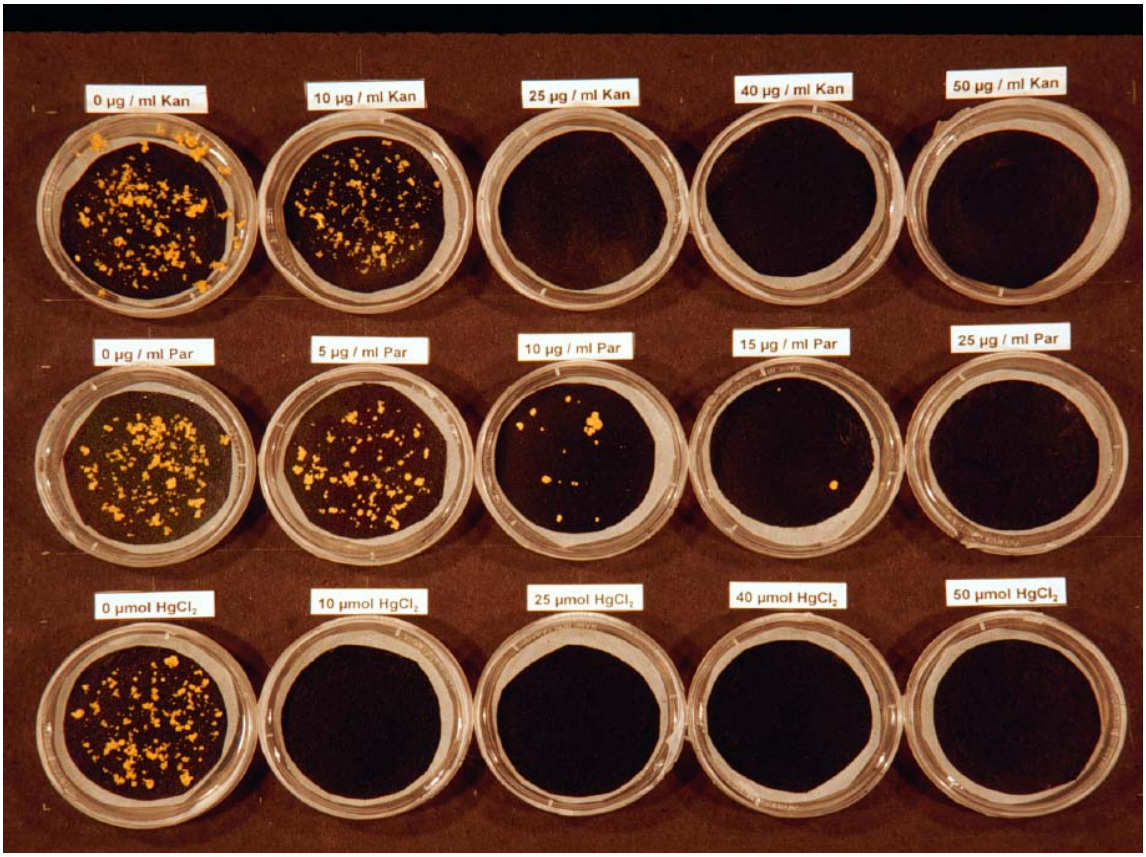

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
Fresh Weight (g)
0
10
25
40
50
1 2 3 4 5 6 7 8 9
Week

Figure 19. Plates of PEMs from line SLS 5-8I showing the effect of different concentrations of antibiotics and mercuric chloride on inhibition of PEM growth.


0 µg / ml Kan
10 µg / ml Kan
25 µg / ml Kan
40 µg / ml Kan
50 µg / ml Kan
0 µg / ml Par
5 µg / ml Par
10 µg / ml Par
15 µg / ml Par
25 µg / ml Par
0 µmol HgCl2
10 µmol HgCl2
25 µmol HgCl2
40 µmol HgCl2
50 µmol HgCl2

CHAPTER 4

CONCLUSION

Sweetgum (*Liquidambar styraciflua*) has been genetically engineered via *Agrobacterium tumefaciens*-mediated transformation and by using microprojectile bombardment. To date, there have been no reports on the transformation of sweetgum embryogenic cells and production of transgenic plants using microprojectile bombardment. While sweetgum is a valuable tree for the paper and horticultural industries, improvement of the tree would be welcome. The introduction of engineered genes into plants requires matching tissue culture and transformation protocols for recovering transgenic plants from cells containing the introduced transgenes. The current success of biolistic transformation has come to rely more on the manipulations of cell cultures throughout the transformation protocol. Culture variables reported to have an effect on transformation frequency include growth phase of cell cultures, osmotic conditioning of cells, choice of selectable marker genes and selection agent, and handling of bombarded material for selection.

Methods identified for improved suspension culture and selection of sweetgum PEMs were useful for transformation of this tree. From these methods, the best protocol for culture of PEMs by suspension culture would use 25 ml of WPM-IMM medium in a 250 ml flask and a two week culture period. Fine PEMs from these suspensions would be obtained by fractionation for use in bombardment or selection experiments at 3 to 6 or 9 to 12 days after subculture. Selection of bombarded PEMs for the recovery of transformed material could be accomplished using at least 25 μg/ml of kanamycin or paramomycin. Some of these results were applied to follow up experiments that identified selection agent and concentration, pre-bombardment osmoticum conditioning, and a post-bombardment selection method for the transformation and recovery of transgenic embryogenic cell cultures from sweetgum. Testing of different pre-bombardment osmotic conditioning treatments of one PEM cell line on semi-solid medium

indicated that 0.5 M equimolar mannitol/sorbitol produced the highest transient expression of the reporter gene β-glucuronidase (GUS). In a follow-up experiment, the same level of mannitol/sorbitol promoted high transient GUS expression from 3 bombarded sweetgum PEM lines. Tests of post-bombardment PEM culture prior to initiation of selection revealed that PEMs transferred to selection medium containing 50μg/ml kanamycin immediately after bombardment allowed for the identification and recovery of transformed PEMs, while longer recovery periods did not. While our embryogenic cell lines lost their ability to produce somatic embryos we, believe a protocol based on our findings could be applied to competent embryogenic cell cultures of sweetgum to produce transgenic plants.