

Propagation of *Liriodendron* and *Magnolia* via Somatic Embryogenesis

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Introduction

In recent years, substantial progress has been made in the area of *in vitro* propagation and manipulation of horticultural and forest tree species. One *in vitro* regeneration system which has shown considerable promise for propagation of trees is somatic embryogenesis, the production of embryo-like structures from somatic cells (as opposed to zygotic embryogenesis, in which embryos are produced via the union of gametes). In many cases, somatic embryos can be germinated to form plantlets which resemble seedlings in their appearance and growth characteristics. Somatic embryos have a number of advantages over both conventional vegetative propagation systems and other *in vitro* propagation systems, such as micropropagation. Probably the most obvious advantage of somatic embryogenesis is the fact that the product is morphologically and physiologically similar to a zygotic embryo. Thus somatic embryos are virtually complete propagules in themselves, with embryonic roots, shoots, leaves, and, most importantly, the "program" to make a complete plant. Unlike other clonal propagation systems, therefore, no separate shoot elongation or rooting steps are required for plantlet

production. Another advantage of embryogenic systems for mass propagation is the fact that many such systems continue to proliferate via repetitive embryogenesis and thus are capable of producing virtually unlimited numbers of clonal embryos. The number of propagules is not constrained by the amount of available mother plant material, as with conventional methods. On the other hand, a major disadvantage of somatic embryogenesis for mass propagation of trees is the fact that, with few exceptions, all embryogenic systems established for these species to date have employed immature tissues (from seeds, zygotic embryos or seedlings) as explants. Thus, any plantlets regenerated from these cultures are of unknown genetic value. With respect to the Magnoliaceae, this means that desirable cultivars cannot yet be directly cloned via somatic embryogenesis.

During the past 7 years, our laboratory at the University of Georgia School of Forest Resources has concentrated on developing embryogenic regeneration systems for a number of species in the Magnoliaceae, including members of the genera *Magnolia* and *Liriodendron*. Although much of this research has been published in scientific journals, here I would like

to summarize it and bring it up to date for readers who have a special interest in the Magnoliaceae.

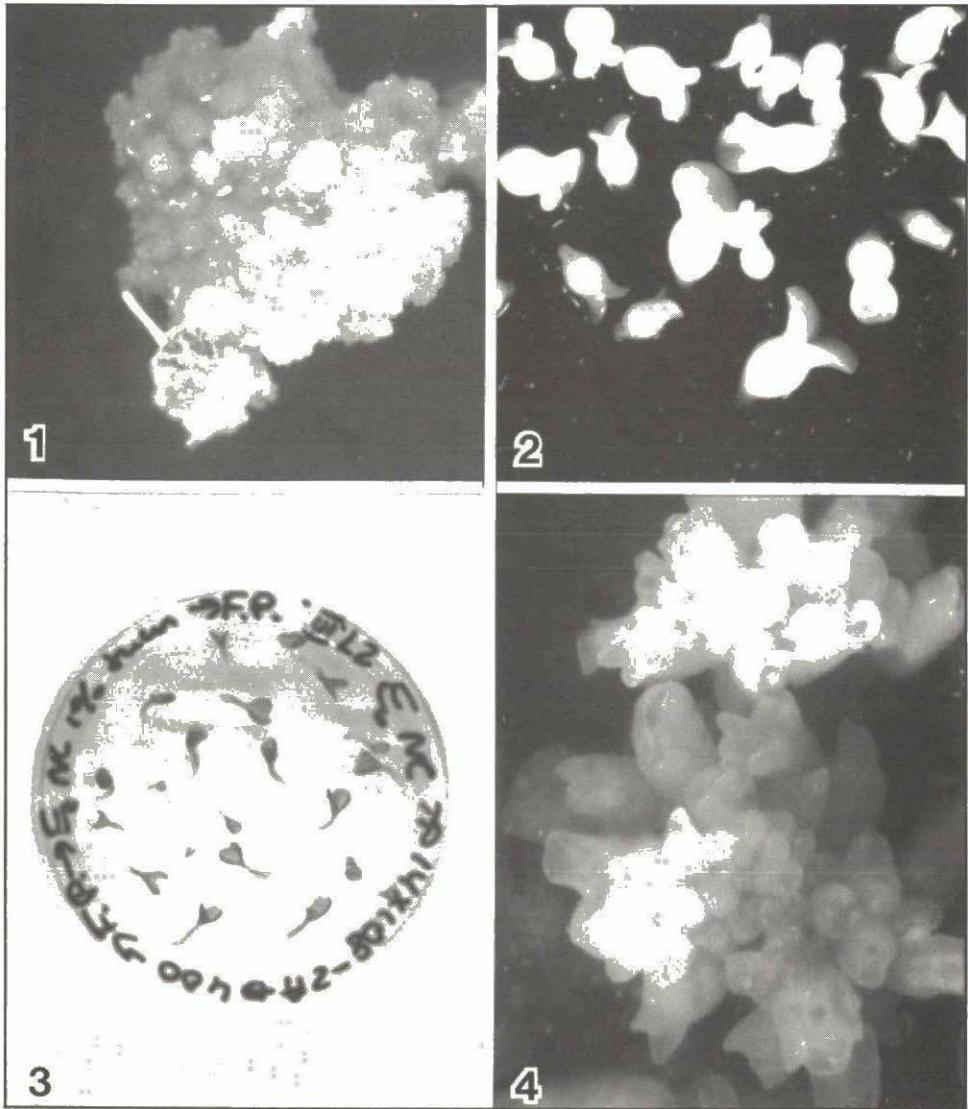
Liriodendron research

We first reported somatic embryogenesis in yellow-poplar (also known as tulip-poplar; *Liriodendron tulipifera*) a number of years ago (Merkle and Sommer 1986), and have since optimized the system so that we can routinely initiate new cultures and produce large numbers of embryos and plantlets (Merkle *et al.* 1990; Sotak *et al.* 1991). We have found that the best explants for initiating embryogenic cultures of this species are immature zygotic embryos. Briefly, to initiate cultures, unopened aggregates of samaras are collected approximately 8 weeks following pollination and broken apart into individual samaras using a grafting knife. Then, the samaras are surface sterilized by immersing them in a sequence of chemical solutions, including undiluted Clorox. Following surface sterilization, samaras are dissected under aseptic conditions and the immature embryos and endosperm are removed together and placed on a Blaydes' (Witham *et al.* 1971) induction medium containing 2 mg/l of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/l of the cytokinin benzylaminopurine (BA), 40 g/l sucrose and 1 g/l casein hydrolysate (CH), and solidified with 8 g/l Phytagar. Usually within 1-2 months following explanting, the embryos produce what appears to be a rapidly-growing, pale yellow callus. However, upon closer examination, the "callus" is actually seen to be organized into clumps of very

early-stage embryos, called proembryogenic masses (PEMs; Figure 1). Proliferation of PEMs can be maintained for years by transferring them to fresh induction medium every 3-4 weeks.

In order to initiate embryo production, PEMs are transferred from induction medium to basal medium, which is the same formula as induction medium, minus the plant growth regulators. The removal of auxin from the medium allows the PEMs to continue their development through the remaining stages of embryogeny. Usually within 4-6 weeks following transfer to basal medium, embryos emerge from the PEMs. They follow the classic stages of zygotic embryo development, passing through globular, heart and torpedo stages. Then, the embryos are transferred to a plantlet development medium, based on a formula by Risser and White (1964), for germination and early growth. Usually, plantlet development to a stage with 3 or 4 leaves takes 2 months. Then plantlets are transferred to a peat-vermiculite potting mix in Hillson-type Roottrainers (Spencer-Lamaire) and placed in a humidifying chamber for acclimatization. During the next 8-10 weeks, as the humidity in the chamber is gradually lowered to ambient conditions, the plantlets are fertilized once a week with a modified Hoagland's (Hoagland and Arnon 1950) solution. Following acclimatization, plantlets are moved to the greenhouse for further growth.

Although the method outlined above has resulted in the production



- Figure 1. Yellow-poplar proembryogenic masses growing on induction medium (14X).
- Figure 2. Mature yellow-poplar somatic embryos following size fractionation and growth for three weeks on filter paper overlayed on semisolid basal medium (14X).
- Figure 3. Germinating yellow-poplar somatic embryos one week following transfer from filter paper to basal medium lacking casein hydrolysate.
- Figure 4. Clumps of sweetbay magnolia somatic embryos (14X)

of hundreds of yellow-poplar plantlets, some modifications of the technique have been necessary in order to "scale-up" embryo and plantlet production to the level necessary for large scale field testing of somatic embryo-derived trees. This scaling-up was made possible by the fact that yellow-poplar PEMs can be maintained and manipulated in suspension culture. Once cultures initiated on semi-solid medium have produced a few grams of PEMs, the PEMs are placed in a liquid version of the induction medium described above and maintained in Erlenmeyer flasks on a gyratory shaker. We found that we can initiate synchronous somatic embryo development from these suspension cultured PEMs by size-fractionating them on stainless steel screens and collecting the desired fraction on a piece of filter paper. Then, the filter with PEMs is placed directly on semisolid basal medium. Within a few weeks, a synchronous population of somatic embryos develops on the filter paper (Figure 2). Once the embryos are moved to semisolid basal medium without the intervening layer of filter paper, they green and germinate vigorously (Figure 3).

In collaboration with Dr. Scott Schlarbaum and Dr. Otto Schwarz at the University of Tennessee and Mr. Russell Cox at the Tennessee Division of Forestry, we have used the method described above to produce over 5000 yellow-poplar plantlets representing 8 clones for field testing in Tennessee. These tests will determine how well somatic embryo-derived yellow-poplar trees grow in comparison to seedling-derived trees from the same

full-sibling families. In addition, evaluations will be made of the clonal fidelity of the somatic embryo-derived trees with regard to growth, form, etc. We believe that our experiment will be the first large-scale field test of somatic embryo-derived trees of any species in the United States.

One application of our embryogenesis protocol which we are currently testing is the propagation of hybrid *Liriodendron* trees derived from crossing *L. tulipifera* with the Chinese tuliptree, *L. chinense*, a native of mainland China. Seedling-derived hybrid *Liriodendron* trees have been reported to be heterotic with regard to growth rate and to produce orange flowers. Currently these hybrid trees are rare in the United States because very few Chinese tuliptrees of flowering age are available in this country. We initiated cultures from immature seeds resulting from controlled pollinations of yellow-poplar flowers with Chinese tuliptree pollen. Unopened flowers containing the pollen were kindly provided by Dr. Chris Sacchi, who collected them from a Chinese tuliptree growing at the Orland E. White Arboretum at the University of Virginia's Blandy Experimental farm. Pollinations were performed by Dr. Scott Schlarbaum at the University of Tennessee's yellow-poplar breeding orchard near Knoxville, Tennessee. We have recovered a number of cultures which appear to be embryogenic, and expect to regenerate plantlets during the next few months. Cultures and plantlets will be tested using DNA markers to determine if they are truly hybrids.

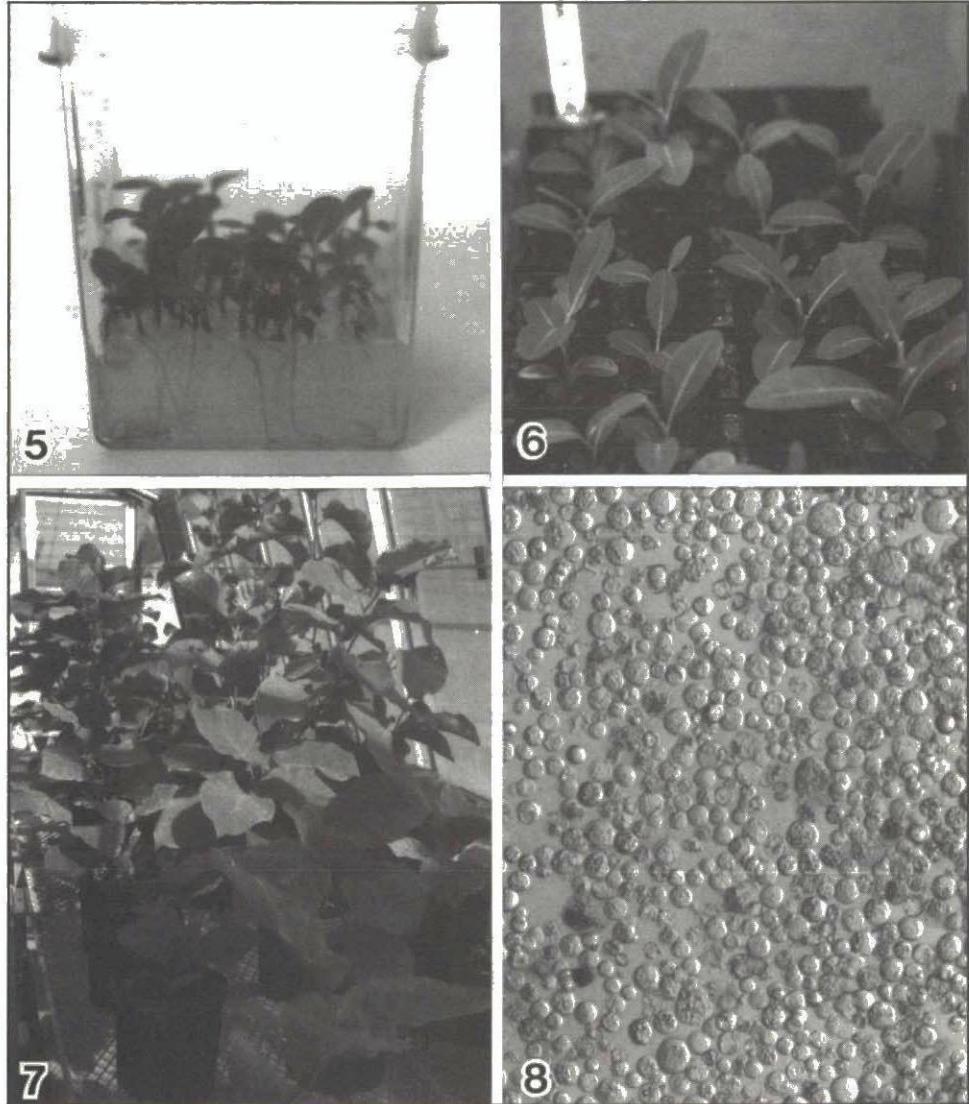


Figure 5. Germination and early growth of sweetbay magnolia somatic embryos on plantlet development medium.

Figure 6. Somatic embryo-derived sweetbay magnolia plantlets growing in humidifying chamber following transfer to potting mix.

Figure 7. Somatic embryo-derived yellow cucumber tree plantlets growing in greenhouse.
Figure 8. Protoplasts isolated from embryogenic yellow cucumber tree suspension culture (150X).

Although beyond the scope of this summary, we have employed embryogenic yellow-poplar cultures for a number of other plant biotechnological experiments besides mass propagation, including protoplast culture (Merkle and Sommer 1987) and gene transfer (Wilde *et al.*, in press).

Magnolia research

We have adapted the method used to produce embryogenic cultures of yellow-poplar to induce somatic embryogenesis in a number of *Magnolia* species (Merkle and Wiecko 1990). To date we have produced somatic embryos of four species—sweetbay magnolia (*M. virginiana*), Fraser magnolia (*M. fraseri*), yellow cucumber tree (*M. cordata*), and bigleaf magnolia (*M. macrophylla*). Plantlets have been produced from somatic embryos of each species except bigleaf magnolia.

To initiate embryogenic cultures of these species, developing fruits (aggregates of follicles) are collected during the late spring or early summer, depending on the species, and the immature seeds are dissected from the fruits using a grafting knife. Then the seeds are surface sterilized using the same method as with the yellow-poplar samaras. Following surface sterilization, seeds are bisected longitudinally and the two halves are placed cut surface downward on yellow-poplar induction medium. Usually within 6-8 weeks following explanting, either somatic embryos or PEMs are seen proliferating from the embryo end of the explanted seed halves on the medium.

Although the protocol and

medium used to initiate embryogenic cultures of *Liriodendron* and *Magnolia* species are almost identical, the characteristics of somatic embryogenesis in the two genera differ. Furthermore, each *Magnolia* species appears to respond to culture conditions in a slightly different manner from the other species in the genus. The optimal stage for initiating sweetbay magnolia cultures, for example, is only 3 weeks postanthesis (versus 8 weeks postanthesis for yellow-poplar). Also, as shown in Figure 4, sweetbay magnolia cultures do not proliferate as PEMs on induction medium, instead proliferating via direct repetitive embryogenesis (i.e. new somatic embryos are formed directly from older somatic embryos with no intervening callus). Following a few initial subcultures on induction medium, they actually grow best by subculturing monthly to fresh basal medium instead of induction medium. Apparently 2,4-D is not necessary for this species to continue to produce somatic embryos.

Fraser magnolia and yellow cucumber tree are more similar to yellow-poplar in that they produce PEMs on induction medium. Once PEMs are transferred to basal medium, somatic embryos are produced. However, even though these two species produce PEMs on induction medium, unlike yellow-poplar cultures, they are difficult to maintain as embryogenic suspension cultures. Thus propagation of *Magnolia* species via somatic embryogenesis will remain more labor intensive and fewer numbers of plantlets will be produced per

culture than for yellow-poplar.

Once somatic embryos of the *Magnolia* species are produced, they can be germinated on the same plantlet development medium as the yellow-poplar somatic embryos (Figure 5). However, early development of plantlets is usually slow compared to yellow-poplar plantlets. This slower initial growth may be due to less than optimal culture conditions for the magnolias, or it is possible that seedlings of these species simply grow more slowly than yellow-poplar seedlings.

Yellow-poplar is, after all, one of our fastest-growing eastern hardwood trees. Usually, magnolia plantlets require about 3 months to develop to the point where they are ready to be transferred *ex vitro* to potting mix in Hillson-type Roottrainers (Figure 6). Magnolia plantlets are hardened off for 2-3 months in the humidifying chamber prior to being moved to the greenhouse.

Preliminary greenhouse, nursery and field performance of somatic embryo-derived magnolia plantlets has been promising. To date, hundreds of sweetbay magnolias from 5 independent clonal lines have been hardened off, grown in the greenhouse to 12 to 18 inches, and passed on to local nurseries in north Georgia. The few I have planted personally have grown well to date and one of these produced a single flower during its first season in the ground. The first Fraser magnolia and yellow cucumber-tree plantlets were planted this fall. Somatic embryo-derived Fraser magnolias grew slowly in the greenhouse, possibly due to inadequate cooling. It remains to be seen how these trees

will grow in the Athens, Georgia vicinity, since Fraser magnolia is a mountain species. Yellow cucumber-tree plantlets responded well to greenhouse culture, with many adding over 1 meter of growth during their first season (Figure 7). Since they are native to the Athens vicinity, we expect them to continue to perform well following outplanting.

Of the magnolias tested, yellow cucumber-tree has proven to be the most amenable to initiation of suspension cultures, although these can only be maintained for a few months at a time. However, even the ability to grow these cultures for a short time gives us the potential to isolate and culture protoplasts of this species, as has been accomplished for yellow-poplar. We have isolated protoplasts from yellow cucumber-tree suspension cultures (Figure 8), but have not successfully cultured them to the PEM stage to date. If protoplasts can be successfully isolated and cultured from *Magnolia* species, we will have the opportunity to experiment with the creation of somatic hybrids between these species via protoplast fusion. Thus there is the possibility that magnolia hybrids which are currently unobtainable via sexual hybridization might be generated using this technology.

In conclusion, our research has demonstrated that members of the Magnoliaceae are highly amenable to *in vitro* propagation and manipulation via the process of somatic embryogenesis. Using immature seed explants, cultures can be initiated that are capable of

producing thousands of clonal plantlets, as well as providing vehicles for such applications as protoplast culture and gene transfer. A substantial barrier to the application of this technology remains in that embryogenic cultures of these species cannot currently be initiated from mature, genetically-proven material. With this problem in mind, we will continue to investigate methods for initiating embryogenic cultures from mature tissues such as flower parts. In the meantime, our current embryogenic systems provide an alternative means for propagating rare hybrids and those members of the family characterized by low seed production.

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