

MASS PROPAGATION OF SOMATIC EMBRYO-DERIVED PLANTLETS OF  
YELLOW-POPLAR FOR FIELD TESTING

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Abstract. Somatic embryogenesis is generally regarded to be the most promising tissue culture regeneration system for mass propagation of desirable genotypes. However, few field tests employing somatic embryo-derived trees have been initiated, primarily due to inadequate numbers of somatic embryo-derived plantlets ("emblings"). In this study, we report the development of a system for large-scale production of yellow-poplar somatic embryos followed by high-frequency conversion, and its integration into a collaborative project to field test the tissue-cultured material. Embryogenic yellow-poplar suspension cultures were initiated from immature zygotic embryos resulting from controlled pollinations of trees growing in a University of Tennessee yellow-poplar breeding orchard near Knoxville, TN. Following testing of suspension cultured lines for their ability to produce somatic embryos and emblings, 9 lines were chosen for mass production. To produce synchronous populations of mature somatic embryos, proembryogenic masses (PEMs) of each line were size-fractionated and the desired fraction was cultured on filter paper overlaid on a plate of semisolid basal medium. Following 2 weeks of incubation under these conditions, mature somatic embryos were transferred to plates of a semisolid basal germination medium and shipped to cooperators at the University of Tennessee, Knoxville. Germinants were transferred to GA7 vessels containing plantlet development medium. Conversion rates averaged approximately 66 percent. Following up to 4 months in GA7 vessels, emblings were transferred to potting mix in planting containers and acclimatized in a misting greenhouse. Following 2 months of acclimatization, approximately 5500 emblings, representing 9 clones, were transferred to larger containers and placed in a shade house for further growth.

Keywords:———*Liriodendron tulipifera*, somatic embryogenesis, conversion

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## INTRODUCTION

Among the plant tissue culture techniques that have been applied to forest tree species, somatic embryogenesis, the asexual production of embryo-like structures, has received increasing attention in recent years. During this time, great progress has been reported in the initiation of embryogenic cultures of both coniferous and hardwood species (cf. reviews by Tulecke 1987, Wann 1989). Somatic embryogenesis is currently viewed as a tool with great potential to be applied for mass propagation of desirable genotypes of agronomic, horticultural and forest tree species. Compared to both conventional and other *in vitro* propagation systems, many embryogenic systems offer high multiplication rates, especially when they can be maintained as suspension cultures. Even more attractive economies of scale may be possible if bioreactor and continuous culture technologies can be applied to embryogenic systems (e.g. Styer 1987, Stuart et al. 1987). Furthermore, the products of these cultures are virtually complete propagules in themselves, requiring no separate shoot elongation or rooting steps to produce plantlets. This property of somatic embryos has opened the possibility that they may be adapted for direct delivery to greenhouse or field as "artificial seeds" (e.g. Redenbaugh et al. 1986; Kitto and Janick 1985; Gupta and Durzan 1987). These features of embryogenic systems serve to lower labor inputs relative to those required for other *in vitro* propagation methods.

Somatic embryogenesis has taken on an added level of importance with the advent of plant gene transfer technology. Several of the features of embryogenic systems make them amenable to gene transfer via both *Agrobacterium-Ti* plasmid-mediated and direct gene transfer techniques. For example, the capacity for embryogenic cultures of walnut (*Juglans regia*) to undergo cycles of repetitive somatic embryogenesis has been employed to produce transformed embryos and plantlets of that species, following *Agrobacterium-mediated* gene transfer (McGranahan et al. 1989, 1990). Recently, embryogenic suspension cultures of such agronomic species as maize and cotton have been transformed using a direct gene transfer technique known as microprojectile bombardment, in which DNA is delivered to plant cells via metal particles which are shot into them with a "gun" (Gordon-Kamm et al. 1990, Finer and McMullin 1990). Transformed plantlets of yellow-poplar (*Liriodendron tulipifera*) have also been regenerated following microprojectile bombardment of embryogenic suspension cultures (Wilde, Meagher and Merkle, submitted).

Despite the great potential for embryogenic systems to be adapted for mass propagation and gene transfer for forest trees, there remain several major obstacles to be overcome before somatic embryogenesis can be integrated with operational tree breeding programs and begins to make a real contribution to applied forest tree improvement and/or reforestation. One major problem is that the majority of forest tree embryogenic systems have employed immature tissues as explants. For full advantage to be taken of propagation via this technique, systems using explants from mature, genetically-proven material will have to be developed. Until such systems are available, there is much information that can be gained by employing cultures derived from immature tissues, which are presently available. In addition, recent advances in cryopreservation of embryogenic cultures (e.g. Shillito et al. 1989) have made possible the long term storage of potential superior genotypes while somatic embryo-derived trees of the same clone are evaluated for field performance.

### Importance of field testing

One important question that can be addressed using available embryogenic material concerns the potential for somatic embryo-derived plantlets ("emblings") to be used operationally as propagules. In order to judge the true usefulness of these trees, their *ex vitro* survival and growth must be evaluated in greenhouse, nursery and field situations. As seedlings are currently the primary propagules used in forestry, they are the standard to which emblings must be compared. An important part of this comparison will be determining if the per unit costs of emblings are competitive with those of seedlings. In addition, since one of the presumed advantages of utilizing tissue culture propagation systems is the production of clonal plant material, a major consideration is embling performance with regard to clonal fidelity. Finally, as the reality of genetically-engineered trees draws nearer, information on field performance of somatic embryo-derived trees will be needed to establish a baseline to which to compare the genetically-engineered trees when they are tested in the field.

### Previous field studies of tissue culture-derived trees

Given the great progress with embryogenic systems for forest trees and the importance of information concerning the *ex vitro* performance of the trees, one might expect that a number of such tests would be underway to answer these questions. On the contrary, information on the field performance of forest tree emblings is currently unavailable. To date, the only report of a large-scale, *ex vitro* evaluation of somatic embryo-derived trees in North America has been that of Webster et al. (1990), in which 1200 emblings of interior spruce (*Picea glauca*, *Picea engelmannii*, and natural hybrids) were tested for nursery performance. They found that growth rates, final height, shoot and root morphology, and frost hardiness were similar for emblings and seedlings following the first growing season. Probably the woody perennial for which *ex vitro* performance of emblings has been most extensively tested is the oil palm (*Elaeis guineensis*). Field trials established by Unilever involved approximately 30,000 plantlets, representing at least 50 clones. (Choo 1990). Early results indicated that plantlets within a clone were highly uniform, especially for highly heritable characters (Corley et al. 1981).

Reports on field tests of forest trees derived from other tissue culture regeneration systems (micropropagation, organogenesis) indicate that plantlets derived via these techniques perform similarly to seedlings, although some characteristics of plantlets may differ from seedlings of the same age. Micropropagated plantlets of hardwoods including *Amelanchier*, *Betula*, *Populus*, *Quercus*, *Ulmus*, *Salix* and *Sorbus* have generally performed very well in the field (Ahuja 1987; Chalupa 1987; McCown and McCown 1987). Tissue culture derived plantlets of teak (*Tectona grandis*) actually outperformed seedling-derived plants when growth increments were compared for the first three years of a field test (Mascarenhas et al. 1987). However, special precautions may be necessary to prevent root circling and deformation in micropropagated hardwood transplants (McCown and McCown 1987). For example, sweetgum (*Liquidambar styraciflua*) plantlets derived from adventitious shoots and planted in a raised nursery bed suffered from poor root form which limited their usefulness for field establishment (Sommer et al. 1985).

Among coniferous forest species, large numbers of micropropagated trees of such species as redwood (*Sequoia sempervirens*) and radiata pine (*Pinus radiata*) are being produced for field planting (Boulay 1990; Thorpe et al. 1991). In the United States, the North Carolina State University Project on Tissue Culture established 16 field plantings of loblolly pine (*Pinus taeda*) plantlets derived from adventitious buds (Amerson et al. 1988). In these tests, loblolly pine plantlets were significantly smaller than seedlings after 1 and 2 years of growth, suggesting that the plantlets experienced an adaptation period to field conditions. However, by the fourth year, growth increments were similar for the plantlets and seedlings. For unknown reasons, plantlets displayed lower rates for fusiform rust infection than seedlings, and their morphology was more mature than similarly aged trees of seedling origin. Douglas-fir (*Pseudotsuga menziesii*) plantlets displayed a similar lag in height growth compared to seedlings, although annual growth increments were similar (Ritchie and Long 1986).

Although lack of resources may have prevented initiation of field tests of somatic embryo-derived trees in some cases, it is likely that the primary factor limiting the establishment of such tests is that most forest tree embryogenic systems reported to date are simply not capable of high frequency production of emblings exhibiting *ex vitro* survival and growth. For example, in one of the few reports to provide quantitative data on conversion of forest tree somatic embryos to plantlets, Becwar et al. (1989) calculated the rate of overall efficiency of plantlet recovery from a Norway spruce (*Picea abies*) embryogenic system to be only 0.5%. Although conversion rates for conifer somatic embryos continue to improve with new techniques (e.g. Roberts et al. 1990; Krogstrup 1990), most systems still must be vastly improved before large populations of clonal plantlets are available for testing. Here, we describe the development of an embryogenic regeneration system for the hardwood forest tree yellow-poplar (*Liriodendron tulipifera*) to a stage where thousands of somatic embryo-derived trees can be routinely produced for *ex vitro* establishment. Using this system, we have recently initiated a project that integrates tissue culture research and practical tree improvement to test somatic embryo-derived trees, derived from select germplasm, for early growth and survival during acclimatization, greenhouse, nursery and field phases.

#### INITIATION OF YELLOW-POPLAR EMBLING TESTS

##### Establishment of embryogenic yellow-poplar cultures

Collaboration between laboratories at the University of Georgia (UGA) School of Forest Resources, the University of Tennessee (UT) Department of Forestry, Wildlife and Fisheries, UT Botany Department, and the Tennessee Division of Forestry (TDF) was begun in 1987, when controlled pollinations were conducted by UT and TDF personnel among superior yellow-poplar selections at the University of Tennessee's yellow-poplar seed orchard near Knoxville, TN. Collections of developing aggregates of samaras were made at bi-weekly intervals throughout the summer and shipped via overnight mail to UGA. Breeding and staged collection of immature samaras were repeated in 1988. By initiating cultures from embryos obtained from the samaras at different developmental stages, it was ascertained that the optimal stage of embryo development for explanting to obtain embryogenic cultures was the globular to early heart stage, which occurred

approximately 8 weeks postpollination (Sotak et al., in press). In 1989, this information allowed us to make a single collection of control-pollinated seeds for culturing, resulting in production of the majority of the embryogenic lines used in this study.

Embryogenic yellow-poplar cultures were initiated following procedures outlined in Merkle and Sommer (1986). Briefly, following surface sterilization, samaras were dissected and immature zygotic embryos and endosperm were explanted onto a modified Blaydes (Witham et al. 1972) induction medium containing Murashige and Skoog's (1962) iron, Brown's minor salts (Sommer and Brown 1980), Gresshoff and Doy's (1972) vitamins, 40 g/l sucrose, 1 g/l casein hydrolysate (CH), 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.25 mg/l benzyladenine (BA). Usually within 2 months after explanting on induction medium, zygotic embryos produced proembryogenic masses (PEMs), which continued to proliferate as long as left on this medium. Embryogenic suspension cultures were initiated by inoculating PEMs into 50 ml Erlenmeyer flasks containing 20 ml of liquid induction medium. Suspension cultures were maintained on a gyratory shaker at 90 rpm.

#### Early attempts at somatic embryo production and conversion

When PEMs were transferred to solid or liquid basal medium (same as induction medium but lacking growth regulators), somatic embryos developed from them. However, the majority of these embryos failed to complete the normal sequence of development to the point where they resembled mature zygotic embryos. Instead, most were malformed, often with fused cotyledons. These malformed embryos became swollen, followed by radicle elongation (germination), callus formation or secondary embryo production. Germination was only rarely accompanied by apical development. Due to this pattern of development, overall conversion rates of yellow-poplar somatic embryos were below 1%. However, some emblings were produced by transferring well-formed embryos to a test tubes containing a Risser and White's (1964) plantlet development medium with 2% sucrose and no growth regulators. Following 2 months of growth on this medium, emblings were transferred to a peat/vermiculite potting mix, acclimatized to ambient conditions in a humidifying chamber and grown in the greenhouse. During 1988-90, approximately 450 potted yellow-poplar emblings derived from Tennessee material were delivered to UT and TDF cooperators. Three hundred of these emblings, along with 100 check seedlings were planted on the Ames Plantation, a UT Experiment Station near Memphis, TN, in March, 1991. Another 135 emblings were planted at the East Tennessee State Nursery (TDF) adjacent to a full-sib yellow-poplar seedling test in May, 1991. However, it was recognized that much larger populations of emblings would be needed to conduct a meaningful test.

#### Improvements in embryo conversion

In order to make the embryogenic cultures useful for mass propagation, conversion frequency had to be raised substantially. Treatments that were tested to improve conversion are described in detail in Merkle et al. (1990). One promising technique employing size fractionation and treatment with abscisic acid produced roughly synchronous populations of well-formed, apparently mature embryos in suspension culture. However, conversion rates of these suspension-cultured embryos remained below 1%. Therefore, an alternative protocol was

adopted in which suspension-cultured PEMs were size-fractionated and the desired fraction was immediately plated on semisolid medium (Figure 1).

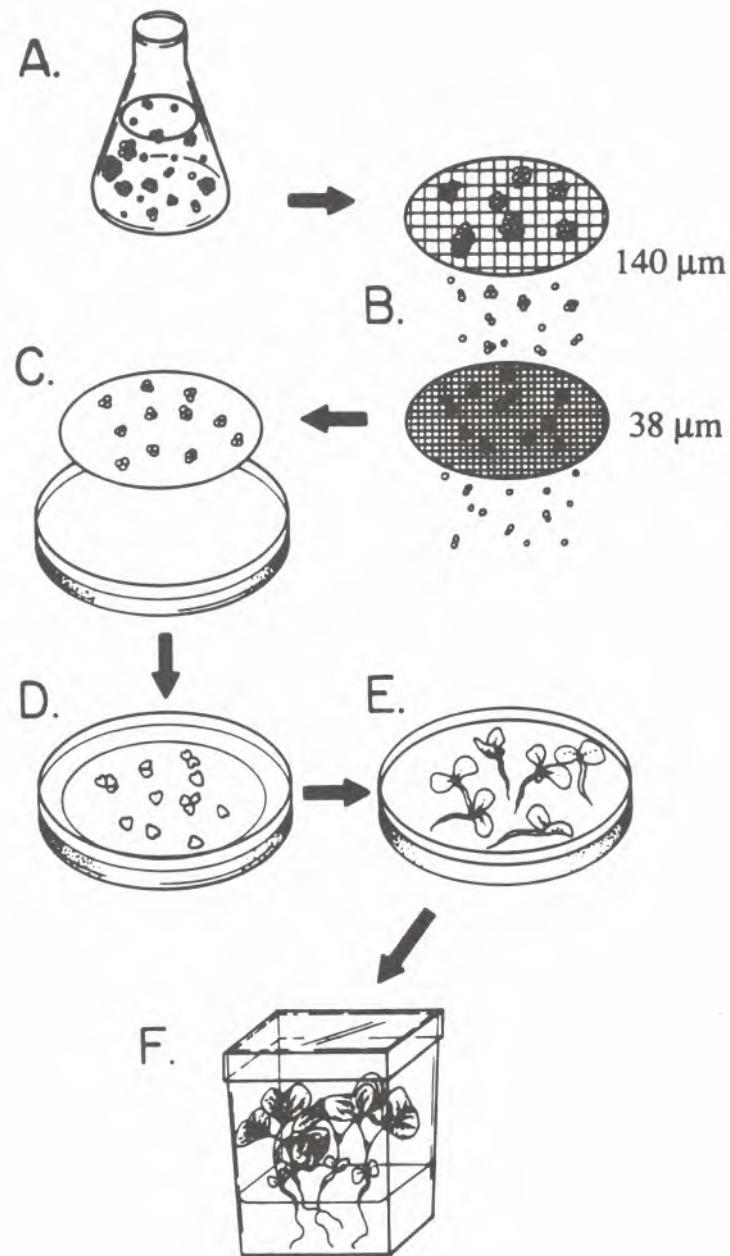


Figure 1. Fractionation/plating protocol used to produce yellow-poplar somatic embryos and emblings. A. Embryogenic suspensions are grown in liquid induction medium. B. PEMs are sieved on a 140 μm stainless steel screen and the fraction passing through is resieved on a 38 μm screen. C. PEMs remaining on the 38 μm screen are collected on filter paper and plated on basal medium. D. Somatic embryos develop synchronously from PEMs, maturing within 14 days. E. Mature embryos are transferred to basal medium without CH to promote germination. F. Germinants are transferred to plantlet development medium in GA7 **vessels**. (Figure from Parrott et al., in press).

In this protocol, two weeks following transfer to fresh induction medium, 1 g of PEMs was sieved on a 140  $\mu$ m stainless steel screen and the fraction passing through was resieved on a 38  $\mu$ m screen. The fraction remaining on the 38  $\mu$ m screen was rinsed with basal medium and then backwashed from the screen onto a single layer of filter paper in a Buchner funnel. PEMs were again rinsed with basal medium while under mild vacuum, which served to spread PEMs into a single layer. When excess liquid medium had been drawn off, the filter paper with PEMs was placed on semisolid basal medium and incubated under fluorescent light (16 hr/day) at 30° C. As PEMs developed into somatic embryos on the filter paper, mature torpedo-stage embryos with well-developed cotyledons were selected and transferred to petri plates containing germination medium, which was the same as basal medium, but without CH. This modification of the basal medium was made following our discovery that CH inhibited somatic embryo germination, probably by raising the osmotic potential of the medium, and that eliminating it promoted vigorous germination and cotyledon greening within 1 week (Merkle et al. 1990). Using this method, a 60-70% synchronous population of embryos could be produced within 2 weeks following plating of PEMs, and following transfer to plantlet development medium, an average of 32% of these somatic embryos converted to emblings (Merkle et al. 1990).

#### Scale-up and somatic embryo production for field testing

Approximately 20 embryogenic suspension cultures were tested for ability to produce embryos and emblings. Based on data from this test, 9 embryogenic clones were chosen for scale-up. The selected clones displayed mature embryo conversion rates of up to 100% and overall conversion rates of up to 85% (Table 1). Amount of PEMs available for embryo production from a clone at a given time was increased by growing suspensions of the selected lines in 80 ml of induction medium in 250 ml Erlenmeyer flasks on a gyratory shaker. Cultures were maintained by inoculating fresh medium with approximately 2 g of PEMs every 3 weeks. Synchronous populations of embryos of the 9 clones chosen for scale-up were produced using the same size fractionation/plating method described above. Mature embryos were transferred to germination medium in 60 x 15 mm petri plates, 25 embryos per plate.

#### Plantlet development and acclimatization

Plates of embryos on germination medium were carefully packed and shipped via overnight mail to cooperators at UT. During February and March, 1990, over 1000 embryos of each of the 9 clones were produced and shipped. Upon arrival at UT, germinants were aseptically transferred to GA7 vessels (Magenta Corp.) containing 100 ml of semisolid plantlet development medium. Twenty-five germinants were "planted" in each GA7 vessel and grown under fluorescent light at 22° C, where they completed conversion to emblings. Following 2-3 months of growth in GA7 vessels, emblings were removed from *in vitro* conditions and transferred fir cell Cone-tainers™ containing a peat/vermiculite potting mix. Previously, when somatic embryo-derived emblings were hardened-off at UGA, numbers were small enough so that acclimatization could be handled in a small chamber where humidity was maintained by an atomizer. However, the acclimatization of thousands of emblings required a much larger facility with controlled humidity.

Table 1. Somatic embryo production and conversion performance for Tennessee clones to be used in field test

Clone	Embryos/g PEMs (S.E.)	Mature/g PEMs (S.E.)	Conversion Mature	Conversion Overall	Emblings/g PEMs
14X108	850 (430)	720 (390)	88	74	630
1X10	370 (50)	220 (30)	88	52	190
10X1	850 (180)	740 (190)	96	84	710
4X12	950 (240)	720 (230)	58	44	420
4X10	350 (140)	230 (70)	96	63	220
5X39	220 (20)	150 (40)	100	68	150
5X7	350 (20)	310 (20)	96	85	300
7X14	800 (110)	630 (140)	64	50	400
10X4	280 (60)	200 (70)	100	71	200
Mean	560 (90)	440 (80)	87	66	360

Therefore, a 25 X 25 ft greenhouse bay at UT was modified by installing a fogging system to maintain various levels of humidity. Transfer of emblings from GA7 vessels to potting mix was performed in the greenhouse with the fogging system running to prevent desiccation of the emblings. Emblings were grown under fog for 2-3 months, during which time they were fertilized periodically with commercial plant fertilizer. Beginning approximately 2 months following initial potting, emblings that had produced new leaves and extensive root systems were transplanted to 45 cu.in. root-trainers containing the same potting mix, and transferred to a standard greenhouse. Root-trainers were not used initially due to limited space in the fogging bay of the greenhouse. Transplanting continued into early October.

During acclimatization using the fogging system at UT, yellow-poplar emblings failed to display the vigorous growth we had observed when emblings were acclimatized using the atomizer-driven humidifying chamber at UGA. Although an experiment is currently underway to determine the cause of the slow growth, it is likely that uneven distribution of moisture by the fogging system resulted in excessive moisture being deposited on developing leaves of some emblings, while others did not receive sufficient moisture. Nevertheless, of the approximately 8700 emblings transferred to *ex vitro* conditions, approximately 5500 (63%) were successfully hardened-off and transferred to the shadehouse,



where they set buds during autumn, 1990. Over 99% of these emblings successfully overwintered and broke dormancy during April, 1991, continuing seedling-like growth and development. At this stage, well-defined phenotypic differences could be observed among clonal blocks of emblings, similar to those seen among blocks of full-sibling seedling families. Current plans call for these emblings to **be** grown for another season in the shadehouse and planted in the field during early spring, 1992, **on** several UT Experiment Station sites. Full-sibling seedlings of some of the same families will be included as check plots.

#### Labor commitment

The various activities in the mass propagation effort were carefully monitored to aid in estimating labor commitments for this type of research. The production of approximately 12,000 somatic embryos by the UGA laboratory required about 150 person-hours. At UT, transferring the germinants to GA7 vessels and subsequently from GA7 vessels to Cone-tainers was estimated at 265 person-hours. An additional 130 person-hours were committed to transplant emblings from Cone-tainers to root-trainers, making the total labor commitment 545 person-hours. Dividing this number by the total number of emblings in soil generated by the experiment produces a statistic of 6 minutes of labor per embling. Field establishment of yellow-poplar emblings is projected to take 9 minutes per embling, based on estimates obtained in spring, 1991, for site preparation and planting full-sibling yellow-poplar seedling genetic tests. Therefore the total labor commitment for expansion of existing embryogenic cultures for mass production of somatic embryos to field establishment of emblings will be approximately 15 minutes per embling. This figure does not include time allocated for breeding activities, initial culture establishment or greenhouse/shadehouse maintenance.

Two factors may decrease the labor/embling estimate. Restricted greenhouse space dictated the use of Cone-tainers for the acclimatization process. The 3 cu.in. Cone-tainers employed were too small to grow yellow-poplar emblings to the size required for field establishment, necessitating transfer of the emblings to 45 cu.in. root-trainers. This transplanting step, requiring 130 person-hours, can be eliminated given sufficient greenhouse space, reducing the laboratory-through-acclimatization labor from 6 minutes per embling to 4.5 minutes per embling. In addition, we believe the percentage of emblings successfully hardened-off to greenhouse conditions could realistically be raised to at least 80%, based on results obtained using the atomizer-driven humidifying chamber at UGA. Based on this higher success rate, labor could be reduced to 3.6 minutes/embling. This estimate approaches the time required to transplant a yellow-poplar seedling from a germination tray to root-trainer for containerized production.

#### FUTURE FIELD TEST PROJECTS

Although the current experiment has demonstrated large scale clonal propagation of yellow-poplar is possible using somatic embryos, data on the actual field performance of these trees will not be available for some years. Furthermore, because the embryogenic cultures used in this experiment were originally initiated for other purposes, our design was limited and did not include a number of critical features needed to determine the true potential of

these propagules to substitute for seedlings. A major problem was the unavailability of yellow-poplar seedlings of the same age as the somatic embryo-derived trees from each of the families from which the embryogenic cultures were derived. Thus, we could not make quantitative comparisons of field performance between the two types of propagules. Another consideration is the fact that the cultures from which the emblings were derived were cultures that were initiated in 1987, 1988 and 1989. Thus some of the lines were over 3 years old when embryos were produced from them, while others were less than one year old. As embryogenic cultures become habituated to growth in suspension, somatic embryos capable of conversion often are produced at lower frequencies. It is also likely that such characteristics as clonal fidelity of emblings deteriorate over time. Therefore, we do not believe that a rigorous quantitative test of field performance of yellow-poplar emblings can be accomplished with the available material.

To lay the foundation for a test that will accomplish the goals outlined in the introduction, we have designed and initiated a new study. Controlled pollinations conducted in the UT yellow-poplar seed orchard during May, 1991 will provide seeds with which to initiate embryogenic cultures and seedling populations from at least 5 full-sib families. We expect to obtain at least 4 embryogenic cultures from each family for a total of 20 embryogenic lines. Synchronous populations of somatic embryos will be produced from each line, germinated, transferred to pots and acclimatized as described above. Emblings will be established in replicated, incomplete block field planting designs, along with seedlings from the same 5 full-sib families. Ten-tree plots will be used and each treatment (clonal line or seedling family) will be represented by 4 replications. This experimental design is intended to answer the following questions: (1) How stable is the performance of emblings within individual clones versus that of seedlings within individual families?, and (2) Overall, are emblings significantly different from seedlings in performance?

#### CONCLUSIONS

Although embryogenic regeneration systems for forest trees are expected to become a major source of clonal material for mass propagation and gene transfer purposes, currently very few systems are capable of producing large numbers of usable propagules for these applications. Once these systems begin to reach their potential, a major question will be how well trees derived from somatic embryos fulfill their role in providing plantations of superior trees. We have demonstrated that a collaborative research program between plant tissue culture laboratories and applied tree improvement programs can be established to begin to provide the answer to this question. Preliminary results from our project indicate that production-scale clonal propagation of hardwood forest trees is possible by combining traditional tree breeding, a high frequency embryogenic system and a standard hardening-off treatment. Whether or not the resulting emblings will provide a viable alternative to seedlings will not be known for some years.

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