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Ex Vitro Conversion of Pyramid Magnolia Somatic Embryos

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Abstract. **Low conversion rates of somatic embryos and poor early growth of somatic embryo-derived plantlets of some forest trees may be related as much to prolonged maintenance in vitro as to basic developmental problems with the embryos. We tested ex vitro conversion as an alternative method for producing the rare North American pyramid magnolia (*Magnolia pyramidata* Bartram) plantlets from somatic embryos. Tissue cultures were initiated from immature seed explants of pyramid magnolia. Immature seeds collected from each of three trees formed proembryogenic masses (PEMs) following 7 to 10 weeks of continuous culture on semisolid medium containing 9.0 μM 2,4-D, 1.1 μM BA, and 1 g casein hydrolysate/liter. PEMs transferred to semisolid medium without plant growth regulators produced somatic embryos that germinated following transfer to the same medium without casein hydrolysate. Conversion frequency to plantlets was higher and plantlets were more vigorous when germinants were transferred directly to potting mix and grown in a humidifying chamber instead of being maintained in plantlet development medium in test tubes. Chemical names used: 2,4-dichlorophenoxyacetic acid (2,4-D); *N*-(phenylmethyl)-1*H*-purine-6-amine (BA).**

The pyramid magnolia is found in southwestern Georgia, northwestern Florida, southern Alabama, and southeastern Mississippi (Treseder, 1978). It is one of the rarest magnolias in North America and is highly desirable as a landscape tree due to its compact, pyramidal crown; fragrant, creamy-white flowers; and auriculate leaves (Gardiner, 1989; Treseder, 1978). Although magnolias can be propagated from seed and by several vegetative propagation techniques (Gardiner, 1989), this species has been described as difficult to propagate (Treseder, 1978). Other magnolias are amenable to propagation via somatic embryogenesis (Merkle and Watson-Pauley, 1993; Merkle and Wiecko, 1990). However, embryo conversion and early growth rates of somatic embryo-derived plants with some of these species have been disappointing. Problems with conversion and early growth following transfer to ex vitro conditions are common to somatic embryo-derived trees. In many reports dealing with tree somatic embryogenesis, if conversion is achieved, the number of plantlets recovered is low or not reported at all. At least for some species, the conversion step may fail because in vitro conditions are simply not suitable for postgerminative growth. For example, the inhibitory levels of ethylene that may accumulate in closed containers or agar-solidified medium may disrupt water relations. Thus, we believe it is possible that early transfer to ex vitro conditions would save the time and expense of transplanting, and result in healthier plants. Our objectives, therefore, were to 1) initiate embryogenic cultures of pyramid magnolia and 2) compare conversion and early growth of somatic embryo-derived pyramid magnolia plantlets maintained in vitro to those transferred to ex vitro conditions immediately following germination (radicle elongation).

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Materials and Methods

Due to the rarity of the species and limited fruit production, immature open-pollinated fruit (aggregates of follicles) were collected from trees growing in a small population in Bulter County, Ala., on two dates that we estimated to be 3 to 5 weeks postpollination. We based our collection time of explant material on the seed maturity stage that had previously produced the highest frequency of embryogenic cultures with other magnolia species (Merkle and Watson-Pauley, 1993; Merkle and Wiecko, 1990). Fruit were sampled from a single large (12 m) tree (Tree A) on 1 June and from a second large tree (Tree B) and a clump of three smaller trees ("Tree" C) on 11 June. Fruit were kept refrigerated (4C) until used and were washed with a 10% Roccal (National Laboratories, Montvale, N.J.) solution, rinsed with tap water, and dissected with a grafting knife to remove seeds. Then seeds were surface-disinfested using the following sequence: 70% ethanol for 20 sec, 10% Roccal for 1 min (repeating ethanol and Roccal steps once), full-strength bleach (5.25% sodium hypochlorite) for 5 min, sterile water rinse for 3 min, 0.01 M HCl rinse for 3 min, and three additional sterile-water rinses. Seeds were bisected longitudinally with a scalpel, and the halves were placed cut surface downward on semisolid yellow-poplar (*Liriodendron tulipifera* L.) induction medium (Merkle et al., 1990) in 60-mm plastic petri dishes (three seeds per dish). Induction medium contained 9.0 μM 2,4-D, 1.1 μM BA, 1 g casein hydrolysate (CH)/liter, and 40 g sucrose/liter. Thirty-nine, 114, and 120 seeds were cultured from Tree A, B, and C, respectively. At the time of culturing, the endosperm was liquid and zygotic embryos, if present, were too small to be detected with a dissecting microscope. Cultures were maintained in darkness at 22C and transferred to fresh medium after 1 month. Plates from which explants were transferred were retained and kept under the same culture conditions for observation. All plates were examined 2 months after culture initiation, and the number of explants producing proembryogenic masses (PEMs; Halperin, 1966) was recorded. After PEM production was scored, PEMs from each explant were transferred to individual plates of induction medium and maintained by transferring to fresh induction medium monthly thereafter.

When 100 to 200 mg of PEMs had been produced from each embryogenic culture, PEMs from each line were subcultured to semisolid, yellow-poplar basal medium (Merkle et al., 1990) in plastic petri plates to promote development of somatic embryos. The basal medium was the same composition as the induction medium, but without plant growth regulators. These cultures also were maintained in darkness at 22C. Five embryogenic lines displaying high-frequency embryo production were chosen for the conversion experiment. For each of these lines, 20 to 40 individual somatic embryos that appeared mature (Fig. 1E) were transferred to basal medium lacking CH and were cultured under cool-white fluorescent lights (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 16-h photoperiod. Germinants (embryos with elongating radicles) were given one of two conversion treatments to produce autotrophic plantlets. Our standard conversion treatment with other magnolia species was to transfer individual germinants to 20 ml yellow-poplar plantlet development medium (Merkle et al., 1990) in 25 × 150-mm test tubes. Tubes were maintained under cool-white fluorescent lights (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 16-h photoperiod. The alternative treatment was to transfer germinants directly to Peat-lite (Fafard, Agawam, Mass.) potting mix in Hillson Rootrainer (Spencer-Lemaire, Edmonton, Alberta, Canada) planting containers. To plant germinants, a 1-cm-deep hole was pushed into the potting mix with the tip of a Nalgene wash bottle. Then, holding the germinant by the hypocotyl with forceps, its root was inserted into the hole so that the hypocotyl and cotyledons remained above the surface. Then the potting mix was washed in around the root, using the wash bottle.

Containers were placed in a plexiglass humidifying chamber maintained at 100% rela-

tive humidity (RH) under cool-white fluorescent lights (180 µmol·m^{-2}·s^{-1}) with 16-h photoperiods. Humidity was maintained by adding deionized water to an 8-cm-deep layer of perlite in the bottom of the chamber. An atomizer (model 505; Defensor, Zurich, Switzerland) also was used when it was necessary to raise the RH rapidly (e.g., subsequent to opening the chamber for fertilizing). The atomizer was never operated for a period long enough to deposit water droplets on the plantlets. Germinants in containers were fertilized weekly with 1 ml Hoagland's solution (Hoagland and Arnon, 1950) supplemented with Murashige and Skoog's (1962) iron and minor salts. After 5 weeks on either plantlet development medium or potting mix, the percentage of germinants converting to plantlets was determined. To be scored as a plantlet, a germinant had to have produced at least one noncotyledon leaf. Relative plantlet vigor was assessed by counting the number of noncotyledon leaves produced, measuring the length of the largest leaf, measuring the total length of the root system, and counting the number of first-order lateral roots. Measurements were taken on at least four plantlets per clone for each of the two conversion treatments.

Results and Discussion

PEMs were present on some seed explants within 2 weeks of transfer to fresh induction medium (7 weeks after initial culture). When associated with the original explant, PEMs always appeared at the micropylar end of the seed where the zygotic embryo would be expected to develop (Fig. 1A). Thus, although we did not observe PEMs proliferating directly from zygotic embryos, there is evidence that they were derived from this source as opposed to other seed tissues. The proportion of explants that produced embryogenic cultures was between 30% and 40%, with an overall average of 35% (Table 1). In a few cases, all three seed explants on a plate produced PEMs.

Unexpectedly, PEMs appeared on the medium 2 to 3 weeks after the explants had been transferred from some of the plates on which the explants were originally cultured to plates of fresh induction medium. We believe that either the zygotic embryo or microscopic populations of embryogenic cells were probably left behind when the explants were transferred and continued to proliferate to produce PEMs. The appearance of these PEMs on the plates following removal of the explants was so rapid that we were presented with the possibility that compounds produced by the senescent explants would inhibit proliferation of PEMs if they were not separated from the explant. We attempted to test the inhibitory effects of continuous culture of the entire explant on PEM proliferation by splitting the cultures from each tree into two groups. In one of the groups, any PEMs that had appeared within 7 weeks were separated from the explant and placed directly on the medium, and in the other group, PEMs were allowed to remain on the explant. No obvious differences in PEM proliferation could be detected between the two groups after an additional 4 weeks (data not shown). However, the inhibitory effects of the senescent explant tissues possibly could have had more of an impact when the populations of embryogenic cells were smaller.

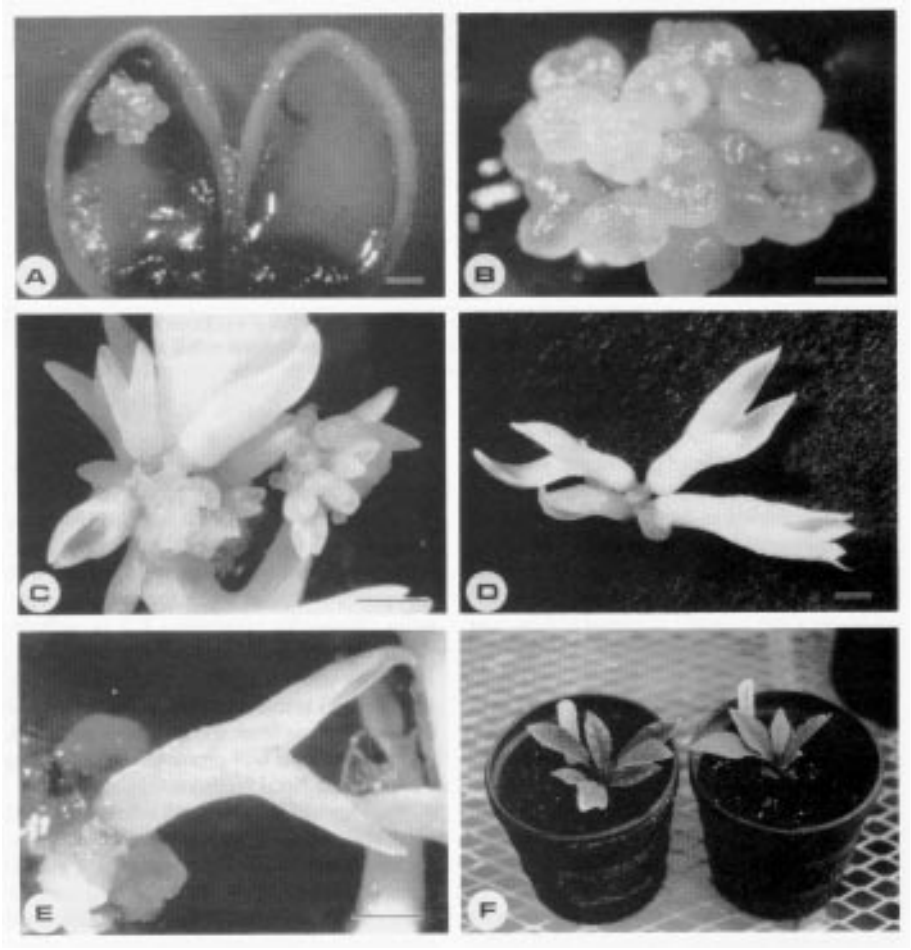

Fig. 1. Somatic embryo and plantlet production in pyramid magnolia. (**A**) Proliferation of proembryogenic masses at micropylar end of longitudinally bisected pyramid magnolia seed following 6 weeks of culture on induction medium. (**B**) Cluster of globular-stage somatic embryos 3 weeks after transfer of proembryogenic masses to basal medium. (**C**) Cluster of somatic embryos at various stages of development. (**D**) Cotyledon-stage somatic embryos with different cotyledon configurations. (**E**) Mature, cotyledon-stage, somatic embryo with cylindrical cotyledons. (**F**) Somatic embryo-derived plantlets following transfer to soil and acclimatization to greenhouse conditions. Bar in A–E equals 1 mm.

PEMs began to produce clusters of globular somatic embryos within 1 month following transfer to basal medium (Fig. 1B). Globular embryos continued to develop through heart and torpedo stages on basal medium (Fig. 1C), resulting in cotyledon-stage embryos within another month (Fig. 1D). Mature embryos often had multiple or fused cotyledons, and even if only two cotyledons were present, they were often cylindrical instead of flattened (Fig. 1E). Cotyledon-stage embryos did not germinate on basal medium with CH. However, 2 to 3 weeks subsequent to transfer to basal medium without CH and incubation in the light, an average of 56% of these embryos had germinated (Table 2), and cotyledons had greened.

Preliminary attempts to convert germinants to plantlets by transfer to yellow-poplar plantlet development medium in test tubes produced only a few chlorotic plantlets with weak root elongation. Therefore, we divided germinants from five clones into two groups and compared conversion frequency and vigor of germinants grown in vitro to that of germinants planted in potting mix and maintained in the humidifying chamber. Conversion frequency of germinants after 5 weeks on potting mix was almost double that obtained in vitro (Table 2). Furthermore, plantlets grown from germinants planted in potting mix were more vigorous than plantlets of the same age grown in vitro (Figs. 2A and 2B); their leaves were >3 times larger and root systems >9 times longer

Table 1. Results of culture initiation from immature seeds from three *Magnolia pyramidata* trees.

	Seeds		Embryogenic cultures	
Tree	Cultured	Surviving[z]	No.	% SD
A	39	39	13	33.3
B	114	99	38	38.4
C[y]	120	84	28	33.3
Total	273	222	79	35.0 (2.4)

[z]Seeds in cultures that did not become contaminated.
[y]Tree C was actually a group of three small trees growing adjacent to each other.

Table 2. Conversion response of somatic embryos from five embryogenic *Magnolia pyramidata* clones.

		In vitro					Potting mix				
			Roots		Leaves			Roots		Leaves	
Clone	% Germination[z]	% Conversion[y]	No.[x]	Length (mm)[w]	No.	Length (mm)[v]	% Conversion	No.	Length (mm)	No.	Length (mm)
A4	45.5	72.7	1.0	11.5	1.5	6.5	100	3.6	95.8	4.6	22.3
A10	40.6	42.9	1.0	5.4	1.9	6.5	83	2.8	78.3	4.0	21.0
A12	64.0	44.4	1.0	8.2	1.9	6.0	100	6.7	104.3	4.4	23.3
B14	66.7	25.0	1.0	3.0	1.0	3.0	100	4.0	71.2	4.5	25.8
B24	63.0	55.5	1.1	17.1	2.0	10.2	100	4.1	73.1	2.6	24.2
Mean	56.0	49.0	1.0	9.0	1.7	6.4	96.6	4.2	84.5	4.0	23.3
SE	5.4	9.0	0.0	2.5	0.2	1.1	3.4	0.7	6.6	0.4	0.8

[z]Percentage of somatic embryos that germinated (elongated radicles) after 3 weeks on basal medium without casein hydrolysate.
[y]Percentage of germinants that produced plantlets with at least one noncotyledon leaf after 5 weeks.
[x]Mean number of first-order lateral roots (1 = taproot only).
[w]Mean overall length of root system.
[v]Mean length of largest leaf.

(Table 2). Plantlets from germinants grown in potting mix also averaged more than twice as many noncotyledon leaves and 4 times as many first-order lateral roots as in vitro plantlets (Table 2). The striking impact on number of first-order lateral roots is of particular interest because this variable is related to first-year field survival of tree seedlings (Kormanick, 1986).

Once three to four new leaves were produced by the plantlets in the humidifying chamber, RH was lowered to ambient levels over ≈2 weeks by gradually opening the doors of the chamber. Following this acclimatization step, plantlets were transferred to the greenhouse and repotted in 4.5-liter pots, where growth continued (Fig. 1F).

From this small-scale study, we concluded that pyramid magnolia responded well to the protocols we developed for embryogenic culture initiation and somatic embryo production with other North American magnolia species. Although we did not test a range of seed development stages for their potential to initiate embryogenic cultures, the average proportion of explants that produced embryogenic cultures (35%) obtained in this study using 3- to 5-week-old, postpollination seeds was as high or higher than we obtained with the optimum explant stages for sweetbay magnolia (*Magnolia virginiana* L.) or yellow-poplar (Merkle and Wiecko, 1990; Sotak et al., 1991). As with the other systems, PEM development into somatic embryos was promoted by their release from the influence of 2,4-D, and germination was promoted by removing CH from the basal medium.

Problems of low conversion frequency and weak early plantlet development were significantly alleviated by direct transfer of germinants to potting mix and growth in a humidifying chamber. Following acclimatization and removal from the humidifying chamber, plantlets responded well to greenhouse conditions. However, because we had no seedling-derived trees with which to compare our plantlets, we could not compare early growth of our plantlets to that of seedlings. Somatic embryos of other woody angiosperms also may respond to accelerated transfer to ex vitro conditions. Preliminary results with hybrid *Liriodendron* somatic embryo germinants, for example, indicate that this approach may improve early growth of these plantlets as well (data not shown).

The practice of growing somatic embryo-derived plants in vitro may be a holdover from traditional axillary shoot-based micropropagation, in which multiple in vitro steps for shoot elongation and rooting are often necessary before transfer ex vitro. Depending on species, additional in vitro manipulation of mature somatic embryos may simply be unnecessary and may actually inhibit development once germination is underway. Thus, for germinating somatic embryos of some species, exposure to appropriate ex vitro conditions is superior to in vitro culture for conversion and early plantlet development. The ability of germinated somatic embryos to respond rapidly to transfer ex vitro may have positive implications for their use as a type of synthetic seed under conditions where RH can be closely controlled.

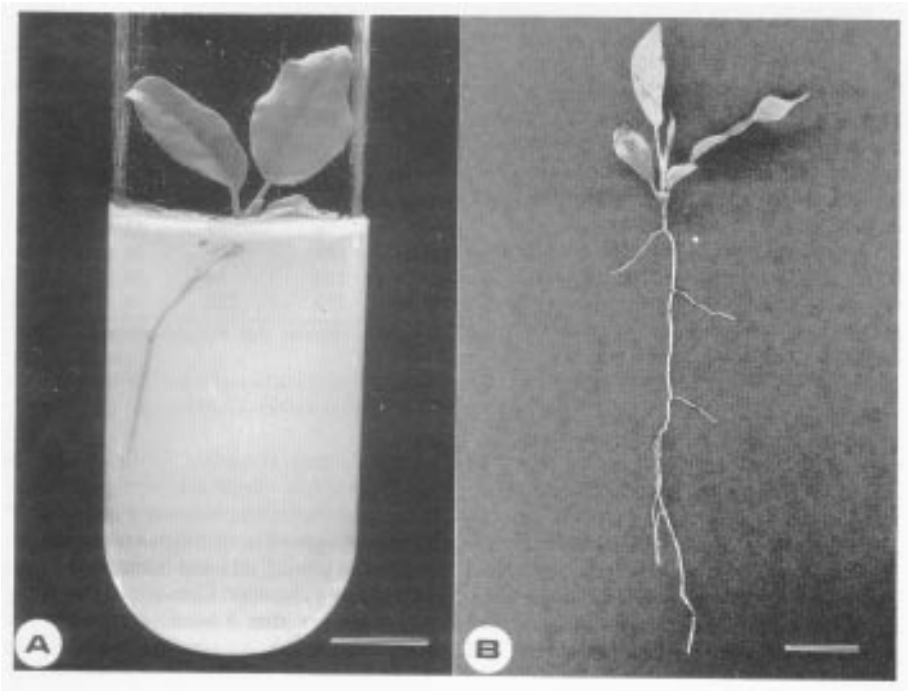

Fig. 2. Somatic embryo-derived plantlets of pyramid magnolia. (**A**) Five-week-old plantlet derived from germinant grown in plantlet development medium. Bar equals 1 cm. (**B**) Five-week-old plantlet from germinant grown in potting mix in a humidifying chamber. Note branching root system. Bar equals 2 cm.

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