Somatic Embryogenesis and Organogenesis in *Magnolia dealbata* Zucc. (Magnoliaceae), an Endangered, Endemic Mexican Species

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Abstract. Plants of *Magnolia dealbata* were regenerated from zygotic embryos through somatic embryogenesis and direct organogenesis. Medium and incubation conditions were determining factors for the development of morphogenetic responses. Photoperiodic exposure was a limiting factor in the general development of the explants, and incubation in darkness allowed their development. The highest formation of shoots per responding explant were obtained on woody plant (WP) medium supplemented with 13.3 μM or 22.2 μM 6-benzylaminopurine (BA) in combination with 2.26 μM or in absence of 2,4-dichlorophenoxyacetic acid (2,4-D) from which 2.5 shoots per explant were induced. Subcultures on WP medium, supplemented with polyvinylpyrrolidone (PVP) 40,000 1 g·L⁻¹ avoided necrosis of explants. Somatic embryos were formed in 85% of explants cultivated on WP medium with 2,4-D (2.3 μM or 4.5 μM); 20% induced indirect embryogenesis and 65% formed direct somatic embryogenesis. The plants were transferred to soil to acclimatize under greenhouse conditions, achieving 90% survival. Somatic embryo conversion to plantlets was obtained with subculture on WP basal medium without growth regulators. In vitro culture can play a key role in the propagation and conservation of this endangered species.

Magnolia dealbata is a species not too well-known, considered extinct until 1977 when it was rediscovered (Vovides and Iglesias, 1996). It is endemic to Mexico and its distribution is limited to six populations with few individuals, and few or no renewals, in the cloud forests of south-central Mexico, between 1400 to 2000 m above sea level (Hernández-Cerda, 1980). Each population is made up of six to 10 isolated trees, except for the population in Veracruz (Mexico), which consists of about 80 adult individuals (Gutiérrez and Vovides, 1997). This species is included in the Red List (Hilton-Taylor, 2000) and is endangered (Norma Oficial Mexicana, 2002) as a result of alterations to its habitat due to cattle ranching and agriculture. Its seeds are recalcitrant, they lose their viability very early, and have low germination percentages; additionally, the species has a limited capacity to regenerate vegetatively (Vovides and Iglesias, 1996). Each adult tree is able to produce new stump sprouts after they have been cut, but only one or two principal stems will survive (Corral-Aguirre

and Sánchez-Velázquez, 2006). On a few occasions, vegetative reproduction through cuttings and layering have been tried without success (Iglesias, pers. comm.).

M. dealbata is a timber-yielding species, appreciated as an ornamental plant, although its bark, but especially its flowers, are used to treat heart ailments in traditional medicine (Gutiérrez and Vovides, 1997). No attempts have been made to cultivate it, with the exception of the germination tests that were carried out at the Francisco Javier Clavijero Botanical Garden in Veracruz (Vovides and Iglesias, 1996). It is therefore necessary to develop a methodology that can provide the efficient propagation of this species for its conservation and sustainable use. In the past couple of decades, plant tissue culture has demonstrated its usefulness as an instrument in the propagation of endangered species (Chávez et al., 1998; Fay, 1994; Mata et al., 2001a, b), because it offers the possibility of propagating plants at higher rates than those obtained through traditional procedures. It also allows for ex situ conservation actions to be carried out in combination with in situ actions.

There are some reports about in vitro culture of a few species of the Magnoliaceae family, which include in vitro germination for *M. grandiflora* and *M. soulangiana* (Le Page-Dégivry, 1970), callus formation for

Liriodendron tulipifera (Stefaniak and Wozny, 1983), and a few magnolia hybrids (Biedermann, 1987); organogenesis in *M. grandiflora* (Tobe, 1990) and *M. soluan-geana* (De Proft et al., 1985); embryogenesis in *L. tulipifera* (Merkle and Sommer, 1986; 1991; Merkle et al., 1990), *M. virginiana*, *M. fraseri*, and *M. acuminata* (Merkle and Wiecko, 1990), and *M. macrophylla* and *M. pyramidata* (Merkle and Watson-Pauley, 1993, 1994). However, to our knowledge, no in vitro studies (organogenesis and somatic embryogenesis) on *M. dealbata* have been conducted.

This study was done to develop an efficient protocol of in vitro propagation from zygotic embryos of *M. dealbata*, to determine the requirements needed to induce multiple shoots and somatic embryos, and to promote their development and establishment under greenhouse conditions.

Materials and Methods

Plant material and disinfection

Mature fruits were harvested from 15 *M. dealbata* individual trees in Veracruz, Mexico (Fig. 1A). Undamaged seeds were rinsed in running tap water for 10 min and then received an initial disinfection with sodium hypochlorite (6% active chlorine) at 30% (volume/volume) for 30 min, after which they were rinsed three times with sterile distilled water. The exocarp was eliminated mechanically, and the remainder of the seeds were washed with a detergent for 20 min and surface sterilized with 70% ethanol for 2 min, followed by 30% (volume/volume) sodium hypochlorite for 20 min. The seeds were rinsed three times with sterile distilled water under aseptic conditions. With the help of a stereoscopic microscope, embryos were excised and placed in two different culture media.

Induction media

The basal media used were woody plant (WP) (Lloyd and McCown, 1980) and modified Blaydes' medium (BM), consisting of major salts of Blaydes' medium, Brown's minor salts, iron of Murashige and Skoog (MS); Gresshoff and Doy's vitamins, and casein hydrolysate (enzymatic) (1 g·L⁻¹) (Merkle and Sommer, 1991). Different concentrations of 6-benzylaminopurine (BA; 0, 4.4, 8.9, 13.3, and 22.2 μM) with 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 0.5, 2.3, and 4.5 μM) were tested in all possible combinations (induction media). Two embryos were sown per container (125-mL baby food jar) with 10 repetitions. The induction time was 60 d. The explants were then subcultured on WP without growth regulators containing 1 g·L⁻¹ polyvinylpyrrolidone (PVP) and 1 g·L⁻¹ activated charcoal. Explants were subcultured every 30 d.

Rooting

Shoots at least 0.5 cm were individualized and subcultured again on WP basal medium without growth regulators, to which was added 1 g·L⁻¹ activated charcoal to induce rooting.

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Fig. 1. (A) Mature plant of *Magnolia dealbata*. (B) Direct Organogenesis from zygotic embryos cultivated in WP plus BA 13.3 μM . (C) Adventitious shoots before individualization. (D) Rooting of shoots in WP plus activated charcoal (1 $\text{g}\cdot\text{L}^{-1}$). (E) Somatic embryos from zygotic embryos of *M. dealbata* in globular stage cultivated in WP plus 2,4-D (2.3 μM). (F) Somatic embryos in heart stage. (G) Somatic embryos in torpedo and cotyledonary stages. (H) Direct somatic embryo proliferation in WP. (I) Conversion of somatic embryos to plants (different stages). (J) Plants from somatic embryos ready for ex vitro culture. Plants with secondary embryos in the stem base (arrow). (K) Plants obtained from organogenesis and somatic embryogenesis after 6 months in soil. Bar = 3 mm, double bar = 3 cm.

The pH of all media was adjusted to 5.7 with 0.1 N HCl and 0.1 N NaOH before the addition of Phytigel (2.7 $\text{g}\cdot\text{L}^{-1}$). The media were autoclaved at 105 kPa, 120 °C for 15 min.

Cultures with induction media were incubated at 25 ± 1 °C, half of them in the dark and the rest under a 16-h photoperiod and a

photonic flux of 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. After induction, cultures in WP without growth regulators were incubated under the 16-h photoperiod.

Germination and secondary somatic embryo proliferation

The development of a root or shoot was considered proof of embryo germination.

Four hundred fifty somatic embryos, either in the torpedo or cotyledonary stage from the cultures incubated in the dark were subcultivated on WP basal medium with activated charcoal 1 $\text{g}\cdot\text{L}^{-1}$, and the percentage of germination and malformations were registered. The experiment was repeated three times. The embryos were incubated with the photoperiod described earlier.

Transfer to soil

Regenerated plantlets from organogenesis and embryogenesis were rinsed in running tap water to eliminate the culture medium; planted in plastic pots containing a soil mixture of leaf litter, loam, and perlag (1:1:1); and placed under greenhouse conditions that averaged 30 °C with a high relative humidity (80% to 90%). Initially, a relative humidity of 90% was maintained by covering the pots with plastic domes. These were removed gradually until an atmospheric humidity of 50% after 30 d was reached. When the plants reached 10 cm or more they were planted in 5" plastic bags with the same soil mixture.

Statistical analysis

For statistical significance, the data of shoots per explant were analyzed using analysis of variance, and the treatments were discriminated by the LSD test at the 5% level.

Results and Discussion

The effect of incubation conditions on embryo development was significant. Cultures exposed to a photoperiod exhibited necrosis and did not present any morphogenetic response, whereas embryos cultivated in the dark developed callus, adventitious shoots, and somatic embryos in the different treatments. In embryogenic cultures of *Liriodendron* spp., some of the genotypes showed a tendency to oxidation under light; therefore it was considered preferable to maintain them in the dark (Merkle and Sommer, 1986).

Most of the embryos in the treatments with BM suffered from severe oxidation and lethal necrosis. Only in those treatments with high BA concentrations (13.3 μM and 22.2 μM) and low or null concentrations of auxins was this situation reduced, and it was possible to obtain 2.3 shoots per responding explant. In other treatments, even with little oxidation, the number of shoots per explant ranged from zero to less than one. BM has been the most widely used basal medium for the induction of morphogenetic responses in other species of the Magnoliaceae family, although the conditions under which morphogenesis occurs were not clear (Merkle and Sommer, 1986; Merkle et al., 1990; Merkle and Watson-Pauley, 1994).

The embryos of *M. dealbata* were sensitive to light. Most of them turned necrotic by the third day of incubation. Those grown in darkness suffered no negative effects and began to grow. In most cases, growth became apparent after the fifth day of culture, when most embryos became swollen (hydrated)

and, depending on the treatment, a few elongated and acquired a yellowish green color (those in the presence of the cytokinin). Others became swollen in the region of the hypocotyl and maintained a pale-yellow color (those exposed to 2.3 to 4.5 μM 2,4-D).

Direct organogenesis

In general, the percentage of explants with response was higher in WP medium than in BM. In the former, a few treatments (60% to 69%) of explants responded with the formation of shoots. In contrast, in BM, only the treatment with added BA 22.2 μM 22% of explants presented some kind of response. For the remaining treatments, the percentage of response was null or very low (data not shown). On the other hand, the response in explants cultivated on WP only with 2,4-D, or in combination with BA, demonstrated formation of somatic embryos and a null response to shoot formation (Table 1). In general, the average number of shoots per explant was less than or equal to one. Nevertheless, in the treatments with BA 13.3 μM in combination with 0.5 μM 2,4-D, and BA 22.2 μM alone, or with 0.5 μM 2,4-D, more than one shoot per explant was achieved (Table 1). After 30 d of culture on WP induction medium, embryo growth was evident, and nodular structures formed at the base of the plumule. Upon transfer to a medium lacking growth regulators, these nodules developed further into adventitious shoots that elongated their stem and expanded their leaves soon after.

Analyzing only the responsive explants, by the end of 120 d, the highest formation of shoots per responding explant was 2.5, achieved in WP medium with 13.3 μM BA in combination with 2.3 μM 2,4-D (Fig. 1B), but in this treatment only 5.6% of the explants formed shoots. This was followed by the treatment added with 22.2 μM of BA

in combination with 2,4-D 0.5 μM , where 46.7% of the explants formed shoots, and with 13.3 μM of BA in combination with 2,4-D 0.5 μM , which formed 2.3 shoots per responding explant in 55.6% of responding explants. Lower cytokinin concentrations only promoted the germination of some of the embryos. Necrosis was an important factor that affected the development of the explants in all treatments; 40% suffered total necrosis. Callaway (1994) mentioned that the main difficulty in the propagation of magnolias was the high content of phenolic compounds that can be excreted to the culture media and inhibit growth, making frequent subculturing necessary.

Although 4.5 μM 2,4-D was used in various treatments, the callus formed was very small because of the extensive necrosis of the tissues. Rzedowski (1981), using Nitsch and Nitsch (1969) media supplemented with 2.3 μM 2,4-D and 4.6 μM kinetin, described the development of callus, roots, and shoots in various *Liriodendron* species. He mentions that after 7 to 8 weeks, the callus turned brown, and the roots changed to a darker color and ceased growing. The micropropagation of *M. grandiflora* by organogenesis was reported using WP medium supplemented with BA (Tobe, 1990). De Profit and colleagues, (1985) reported the micropropagation of *M. soulangeana* through direct organogenesis using Lepoivre medium (Quoirin and Lepoivre, 1977) with 4.4 μM BA, 2.5% sucrose, and 0.6% Difco Bacto agar.

Subculturing into media lacking growth regulators and supplemented with PVP (1 g·L⁻¹) diminished the necrosis effects and allowed for the development of the adventitious shoots. Because of this, oxidation was not a lethal factor and shoots 5 cm in length could be obtained 5 months after the initiation of the cultures (Fig. 1C). PVP and other

compounds like ascorbic acid, citric acid, L-norleucine, and activated charcoal, among others, have been widely used to try to control the oxidation of tissues cultivated in vitro (George, 1993).

Rooting

Individualized adventitious shoots (0.5–1.0 cm in length) continued to develop on WP supplemented with 1 g·L⁻¹ activated charcoal and without plant growth regulators. After two to three subcultures, when they reached a height of 4 to 5 cm, roots formed spontaneously (Fig. 1D). This represented a great advantage, because in most cases it is necessary to induce rooting either in vitro or ex vitro through the application of auxins. Maene and Debergh (1985a, b) found that 4-cm-long shoots of *M. soulangeana* rooted easier than those measuring 2 cm.

Somatic embryogenesis

The induction of somatic embryos was associated only with treatments that used 2.3 μM and 4.5 μM 2,4-D, without BA (Table 1), in which 85% of zygotic embryos produced somatic embryos. When auxin concentrations were lower or were combined with any concentration of BA, somatic embryos were not observed. In contrast to our study, Merkel and Watson-Pauley (1993) cultivating immature zygotic embryos of *M. macrophyllum* in yellow poplar induction medium with 9.0 μM 2,4-D, 1.1 μM BA, 40 g·L⁻¹ sucrose, and 1 g·L⁻¹ hydrolyzed casein, obtained percentages of embryos that were null or very low, only in one explant that previously had formed callus, formed a cluster of somatic embryos. Using the same media Merkle and associates (1993) cultured immature seeds (embryos and endosperm) of a *Liriodendron* hybrid and obtained, in 15.6% of the explants, the formation of either somatic embryos or proembryogenic masses (PEMs).

Eighty-five percent of the zygotic embryos provided two types of responses when on WP medium supplemented only with 2.3 μM or 4.5 μM of 2,4-D. Twenty percent formed a yellowish translucent and compact callus that originated somatic embryos, and 65% developed direct somatic embryos. The remaining percentage did not respond or oxidized completely.

Nearly 20% of the embryos formed a yellowish embryogenic callus of a compact, translucent appearance and exhibited fast growth. After subculture on growth regulator free media, the callus differentiated areas with small nodular structures that turned out to be PEMs that gave rise to somatic embryos in the globular phase (Fig. 1E). These continued to develop through the heart (Fig. 1F) and torpedo stages, and after 30 d more (90 d after culture initiation), they elongated and gave way to the cotyledonary phase, which after a month under illumination acquired a green color (Fig. 1G).

A number of these PEMs began to oxidize, taking on a dark-brown color. In the following subcultures, the surface of the

Table 1. Direct organogenesis from mature embryos of *M. dealbata* cultivated in WP induction media for 60 d and subcultivated in WP basal medium. Results 120 d after the initiation of the cultures.

Plant growth regulators (μM)		Percentage of responsive explants	Avg. of shoots per explant
BA	2,4-D		
0	0	69.1	1.0 $\pm$ 0.0 b
0	0.5	0	0 a
0	2.3	85	0 a ^z
0	4.5	85	0 a ^z
4.4	0	69.6	1.1 $\pm$ 0.3 b
4.4	0.5	23.8	1.3 $\pm$ 0.7 b
4.4	2.3	9.1	1.0 $\pm$ 0.0 b
4.4	4.5	0	0 a
8.9	0	66.7	1.0 $\pm$ 0.0 b
8.9	0.5	0	0 a
8.9	2.3	0	0 a
8.9	4.5	0	0 a
13.3	0	47.2	1.8 $\pm$ 1.1 c
13.3	0.5	55.6	2.3 $\pm$ 1.0d e
13.3	2.3	2.8	1.0 $\pm$ 0.0 b
13.3	4.5	8.3	1.0 $\pm$ 0.0 b
22.2	0	76.2	2.1 $\pm$ 1.4 cd
22.2	0.5	46.7	2.4 $\pm$ 1.3 e
22.2	2.3	0	0 a
22.2	4.5	4.8	1.0 $\pm$ 1.0 b

^zResponse obtained: somatic embryogenesis.

Different letters within columns indicate significant difference at $P \leq 0.05$.

BA, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid.

PEMs not in contact with the medium re-initiated a whitish growth in some areas, that later developed into somatic embryos, although they were fewer in number than in the callus that did not oxidize, the differentiation of the somatic embryos was obtained by subculturing on medium without growth regulators and under photoperiod a 16-h. Nevertheless, a certain number of them developed directly or indirectly in the induction media. Similar results have been reported for *M. macrophylla* and *M. pyramidata* (Merkle and Watson–Pauley, 1993,1994), and for *Liriodendron tulipifera* and *L. chinense*, where immature zygotic embryos were cultivated in BM supplemented with 2,4-D 9.0 μ M, BA 1.1 μ M, 20 g·L⁻¹ sucrose, and 1 g·L⁻¹ hydrolyzed casein (Merkle and Sommer, 1991).

Sixty-five percent of zygotic embryos produced direct somatic embryos. Initially they swelled and acquired a whitish yellow color. After 30 d, the formation of multiple nodules could be observed (small globular structures), which gave the embryos a rugose appearance. After 60 d, these nodules appeared as small protuberances that later consolidated to somatic embryos in the globular phase. The development of the cultures was asynchronous, as evidenced by the presence of somatic embryos in the globular, torpedo, and cotyledonary phases in the same explant. The asynchrony of embryogenic cultures is a frequent process (Ibaraki et al., 2000) that has been reported in various species of cycads (Chávez et al., 1998), conifers (Bomal and Tremblay, 1999; Miguel, et al., 2004), coffee (Etienne–Barry et al., 1999), *Quercus suber* (Hernández et al., 2003), cacti (Moebius–Goldamer et al., 2003; Torres–Muñoz and Rodríguez–Garay, 1996), and *Tilia cordata* (angiosperm) (Kärkönen, 2000).

The subculture of the explants to basal medium allowed for a faster differentiation of somatic embryos in the torpedo and cotyledonary phases and, in some cases, increased the number of somatic embryos from 30 to 100 (Fig. 1H). Some broke loose from the mass and began to germinate, whereas others induced the formation of new secondary embryos, mainly from the radicle zone. There were no apparent differences between either of the 2,4-D concentrations used.

Germination of somatic embryos

Germination was achieved by subculturing the somatic embryos in WP basal medium. Only 9.3% did not become plantlets, and a high percentage of the somatic embryos (40.7%) resembled mature zygotic embryos, that is, they presented a bipolar structure with two cotyledons and no difficulties during germination and plantlet development. Germination (conversion) began with the cotyledons becoming light green, the development of a radicle, and a noticeable increase in the size of the somatic embryo. Some reached 2 cm in length (Fig. 1I). Somatic embryos developed faster and the green color became more intense after were transferred

under illumination. They soon progressed to the formation of leaves; however, even in this state, the formation of secondary embryos was observed at the stem base (Fig. 1J), although to a lesser degree, without having a noticeable effect on the development of the plantlet. After 5 months, most plantlets had reached 6 cm in height and had developed various pairs of leaves (Fig. 1J).

A total of 50.1% of somatic embryos presented some anomaly: 26% presented fused cotyledons, 6.3% developed only one cotyledon, 15.1% formed three cotyledons, and 2.7% developed four cotyledons. These malformations of the somatic embryo did not impede their conversion into plantlets with a normal morphology. They developed a stem and leaves in a similar manner to those somatic embryos with two cotyledons or similar to the zygotic embryos. Unlike our research, higher percentages of somatic embryo malformations have been reported in different species belonging to the Magnoliaceae family. Merkle and Sommer (1986, 1991) reported that 90% of the somatic embryos in *Liriodendron* spp. presented no apical development. A total of 36.6% of the somatic embryos in *L. tulipifera* converted into plantlets, but only after trying various treatments (Merkle et al., 1990). The majority of embryos of *M. macrophylla* had some malformation, and only those with two to four cotyledons germinated in basal medium without plant growth regulators (Merkle and Watson–Pauley, 1993). Embryogenic cultures of *M. pyramidata* (Merkle and Watson–Pauley 1994) presented alterations similar to those found in *M. dealbata*. They obtained germination in 56% of the somatic embryos, which increased to 96.6% after the process was carried out using a potting mix in which the somatic embryos developed better, producing more leaves and roots.

The fact that 85% of zygotic embryos obtained from open pollinated seeds from different trees produced somatic embryos, indicates that there are no genetic barriers that could impede the obtaining of this kind of response. Merkle and Sommer (1991) emphasize this same observation for *Liriodendron* spp.

No additional growth regulators or transfer to a different media was required to achieve conversion, indicating that *M. dealbata* has a high potential for propagation through somatic embryogenesis. Other studies have reported the use of different culture media and the application of growth regulators to induce embryo maturation and germination (Litz et al., 1998). Cold or desiccation treatments, or the addition of gibberellic acid (GA₃) has been used to break dormancy in zygotic embryos. Likewise, osmoticums and abscisic acid have been used to prevent precocious germination and to promote the normal maturation of the embryos (Merkle and Wiecko, 1990). This is an important finding, because it is well-known that if the purpose of the type of research such as ours is the preservation of wild genotypes, the application of growth regulators should be limited whenever possi-

ble to increase the probability of genetic stability (George, 1993).

The regenerative potential of the somatic embryo has been maintained for more than 1 year by subculturing, and it increases when placed in liquid media in the presence of 2,4-D (2.3 μ M or 4.5 μ M; data not shown). After being subcultivated on solid WP basal medium under illumination for 2 months, somatic embryos germinated immediately, became green, developed a radicle, elongated their cotyledons, and later formed leaves. The somatic embryos incubated in the dark formed PEMs that did not develop any further nor germinated, confirming the photomorphogenic role of light for *M. dealbata*.

Ex vitro culture

After 120 d, 90% of the plantlets (obtained from organogenesis and somatic embryogenesis) survived under greenhouse conditions, increased their size, and presented an appearance similar to the seedlings obtained from seed germination (Fig. 1K). Rzedowski (1981) mentioned that the regenerated *L. tulipifera* plants died after they were transferred to soil.

Different magnolia species have been propagated mainly by seed. Because rooting of shoots has been difficult to achieve, vegetative propagation has been less successful, as in *M. denudata* (Callaway, 1994), *M. pyramidata* (Merkle and Watson–Pauley, 1994) and *M. dealbata* (Vovides and Iglesias, 1996). The development of somatic embryos has been obtained less frequently, and the establishment of plantlets in soil has been even more infrequent. In *M. dealbata*, acclimatization to ex vitro conditions was successful. For this reason, the results obtained in this research may be useful as an efficient regeneration protocol for plant production to be used in future investigations that seek the reintroduction of this species to its natural habitat. It is known that direct organogenesis and embryogenesis maintain a greater genetic stability than other routes of regeneration like indirect organogenesis and embryogenesis (George, 1993). The plants obtained can also be used as ornamental and timber-yielding plants, and can supply the traditional medicine market. With this protocol, we are contributing to the conservation of this valuable Mexican species.

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