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BRIEF COMMUNICATION

Somatic embryogenesis and plant regeneration in C*edrela fissilis*

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Abstract

Somatic embryos were obtained from immature zygotic embryos of *Cedrela fissilis* Well. (*Meliaceae*), after a culture period of 12 months, with regular subcultures every 6 - 8 weeks. Callus was developed on explants in 2 months on Murashige and Skoog (MS) medium containing 2,4 dichlorophenoxyacetic acid (2,4-D) or picloram (PIC). When the calli were transferred to fresh medium, embryogenic tissue appeared on MS + 45 μM 2,4-D, or 22.5 μM 2,4-D + 0.4 μM 6-benzyladenine (BA), or 20.7 μM PIC after 6 months. Sub-culture of embryogenic tissue in MS medium supplemented with 4.5 μM 2,4-D resulted in the differentiation into somatic embryos after further 4 months. Repeated secondary somatic embryogenesis was achieved by regular subculture on this medium. Maturation and conversion of somatic embryos into plantlets was achieved on MS medium without plant growth regulators and the conversion frequency was approximately 12.5 %. The plantlets were successfully acclimatized in pots with soil. Histological studies showed that somatic embryos had no detectable connection with the mother explants and that somatic embryos in advanced stages were bipolar with shoot and root apical meristems, they contained vascular system and showed typical characteristics of a somatic dicotyledonous embryo.

Additional key words: callus induction, growth regulators, secondary embryogenesis.

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Cedrela fissilis Vell. (*Meliaceae*) is an economically important tree up to 30 m tall grown in Latin America from North Argentina to Panama and Costa Rica. Although, plant regeneration through somatic embryogenesis or organogenesis has been accomplished in many forest species (*e.g*. Tisserat *et al*. 1979, Ammirato 1983, Thorpe *et al*. 1991, Dunstan *et al.* 1995), little work has been done with respect to the species of *Cedrela*. Whereas some works has been done in *Cedrela odorata* (Gerdas *et al*. 1998) only one micropropagation system based in *in vitro* culture of bud was reported in *Cedrela fissilis* (Da Costa Nunes *et al*. 2002).

Among the various *in vitro* methods, somatic embryogenesis is the method of choice, bringing certain advantages, such as the probable single-cell origin of the regenerants and consequently the propagules produced have fewer variations, a very high proliferation potential easily accessible for automation, and the possibility of conversion to artificial or synthetic seeds. Somatic embryogenesis has been documented in other *Meliaceae* members, neem (Su *et. al*. 1997, Murthy and Saxena 1998, Akula *et al*. 2003, Chaturvedi *et al*. 2004) and paradise tree (Vila *et al*. 2003), but not reported in this species. We report here, for the first time, a protocol for somatic embryogenesis and plant regeneration in *Cedrela fissilis* by *in vitro* culture of immature zygotic embryos.

Immature fruits were collected from *Cedrela fissilis* Vell. trees located in the Campus of the Facultad de Ciencias Agrarias (UNNE), Corrientes, Argentina in early February in 2004. Fruits were surface sterilized first by a 2 min immersion in 70 % ethanol followed by 20 min immersion in 3.5 % (m/v) sodium hypochlorite with two drops of *Tween*® and finally, were washed three times in distilled water. The explants (zygotic embryos of 1 - 2 mm in length) were extracted and cultured individually into 11 cm^3 glass tubes containing different media. All the media were composed of mineral salts, sucrose and vitamins according to Murashige and Skoog (1962; MS). The MS medium was supplemented with 9, 22.5, 45 and 90 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 8.9, 20.7, 41.4 and 82.8 μM picloram (PIC) and combinations of these compounds with 0.4 μM

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Abbreviations: BA - 6-benzyladenine; 2,4-D - 2,4 dichlorophenoxyacetic acid; MS - Murashige and Skoog; PIC - picloram.
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6-benzyladenine (BA). The same media were employed for subculture of globular calli. The pH of each medium was adjusted to 5.8 with either KOH or HCl prior the addition of 0.7 % agar (*Sigma*). Tubes were covered with aluminum foil and autoclaved at 1.46 kg cm^{-2} for 20 min. Then the tubes containing the explant were covered with *Resinite AF 50*® (*Casco S.A.C. Company*, Buenos Aires, Argentina) and incubated in a growth room at temperature of 27 ± 2 °C and 14-h photoperiod with a irradiance of 116 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

When embryogenic tissue appeared, pieces of them were cultured on MS medium supplemented with 4.5 μM 2,4-D. Approximately 10 - 15 mg fresh mass was cultured into 11 glass tubes containing 3 cm^3 of medium. The tubes were covered with *Resinite AF 50®* and were held in the same conditions as described above for callus induction. Ten tubes were used per treatment and each experiment was repeated three times.

Somatic embryos were transferred to MS medium without plant growth regulators for the conversion into plants. Somatic embryos converted into plants were scored and subsequently placed in pots containing soil and covered with plastic bags to maintain high humidity.

Histology was performed according to Gonzalez and Cristóbal (1997). Samples of calli alone and calli with embryos were fixed in FAA (formaline, acetic acid, 70 % ethanol; 5:5:90), dehydrated with a solution for histological dehydratation *BIOPUR*®, followed by paraffin embedding as described by Johansen (1940). The embedded material was then sectioned at 8 - 10 μm thick serial sections. These sections were mounted on glass slides and stained with Safranin (C.I. 50240)-Astra blue (Luque *et al.* 1996) and observed under a light microscope.

Among the different induction media used, it was possible to find proliferation of globular callus after 2 months in culture in eleven of them. However, when these calli were subcultured to fresh media, embryogenic tissue could be distinguished after 6 months only in three media (Table 1). The embryogenic callus was yellowish, nodular and friable (Fig. 1*A*). The initiation of embryogenic tissue was very slow, and in the majority of the media, the explants turned brown without embryogenic response. However, between 30 and 56.7 % of calli

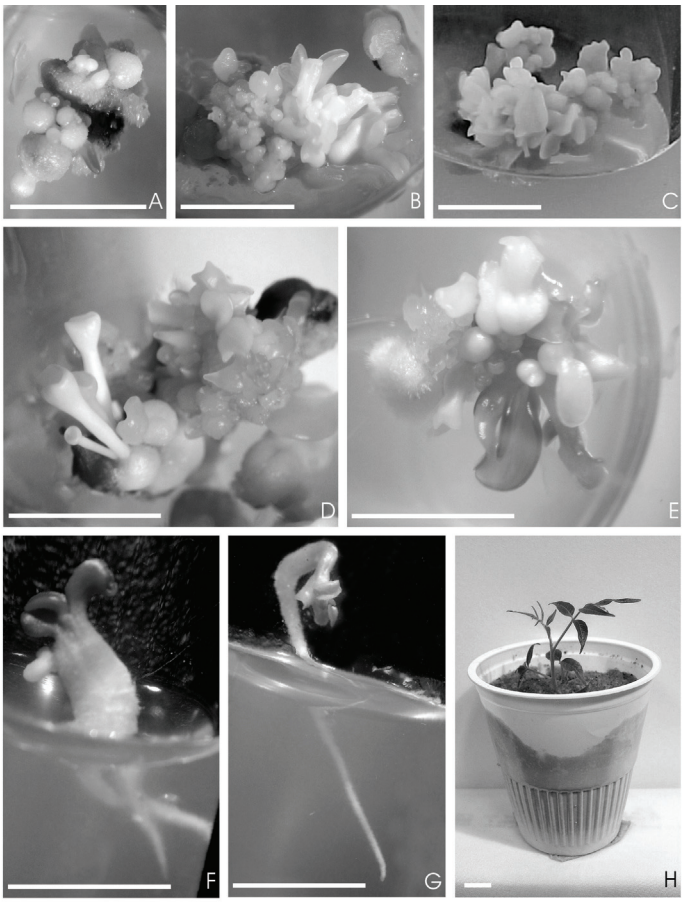

Fig. 1. Somatic embryogenesis and plant regeneration from culture of zygotic embryos of *Cedrela fissilis*: *A* - embryogenic callus; *B* - somatic embryos induced on MS medium supplemented with 4.5 μM 2,4-D; *C* - secondary somatic embryos at various developmental sages; *D,E* - morphological variations of regenerated somatic embryos; *F* - germination of fused embryos; *G* - germination of a single somatic embryo cultured on MS; H - regenerated plantlet. *Bar* = 5 mm.

Table 1. Morphogenic response of immature zygotic embryos of C*edrela fissilis* cultured on MS media supplemented with different plant growth regulators determined after 2 and 6 month of culture and after further 4 months of culture in MS + 4.5 μM 2,4-D. Means ± SE, *n* = 30.

Growth regulators [μM]	2 months globular callus [%]	6 months embryogenic tissue [%]	4 months number of embryos
2,4-D 9	3.3 ± 3.3	0	0
2,4-D 22.5	6.6 ± 3.3	0	0
2,4-D 45	20.0 ± 0.6	56.7 ± 3.3	16.0 ± 1.5
2,4-D 90	10.0 ± 5.8	0	0
2,4-D 9 + BA 0.4	3.3 ± 3.3	0	0
2,4-D 22.5 + BA 0.4	13.3 ± 3.3	30.0 ± 5.8	9.3 ± 1.2
2,4-D 45 + BA 0.4	6.7 ± 3.3	0	0
PIC 8.9	3.3 ± 3.3	0	0
PIC 20.7	10.0 ± 5.8	36.7 ± 3.3	14.0 ± 0.6
PIC 82.8	3.3 ± 3.3	0	0
PIC 82.8 + BA 0.4	3.3 ± 3.3	0	0

produced embryogenic tissue on media supplemented with 45 μM 2,4-D, or 22.5 μM 2,4-D + 0.45 μM BA, or 8.9 μM PIC (Table 1). After initiation, the embryogenic tissue rapidly proliferated. Somatic embryos appeared on the embryogenic callus after four months, when embryogenic tissue was transferred to MS with 4.5 μM 2,4-D (Fig. 1*B*, Table 1). In general, the absence or reduction of plant growth regulators led to the development and differentiation of somatic embryos or their conversion into plantlets (Merkle 1995, Hu *et al*. 2008, Kumar *et al*. 2008, Yang *et al.* 2008). The requirement of 2,4-D for somatic embryogenesis of *Cedrela fissilis* is coincident to that obtained with many other species (*e.g.* Ammirato 1983, Wendt dos Santos *et al.* 2008) including woody species (Dunstan *et al*. 1995).

Subsequently new somatic embryos appeared on the embryogenic tissue and repeated secondary somatic embryogenesis was achieved by regular subculture (Fig. 1*C*). Some calli produced more than 35 embryos and did not lose embryogenic potential for more than 1 year if they were regularly subcultured on fresh medium at intervals of 8 weeks. The embryos generally developed two cotyledons, although some had tubular or horn shaped cotyledons, some had long hypocotyls and vestigial cotyledons (Fig. 1*D*), some had one cotyledon (Fig. 1*E*), and others had fused multiple hypocotyls (Fig. 1*F*). Similar abnormalities were described in others woody species (Nanda and Rout 2003, Rodriguez and Wetzstein 1994, 1998).

In this embryogenic system the initiation of embryogenic tissue was slow in comparison with those used immature or mature zygotic embryos of other *Meliaceae* species (Su *et al*. 1997, Murthy and Saxena 1998,Vila *et al*. 2003). For somatic embryogenesis in coffee or Christmas cactus, a culture period 1 year or more was necessary (Sreenath *et al*. 1995, Al-Ramamneh *et al*. 2006), while in elm or *Lycium barbarum* only 3 - 6 month was required (Corredoira *et al*. 2002, Hu *et al*. 2008).

Simultaneously with the transfers of embryos to MS with 4.5 μM 2,4-D, groups of somatic embryos were separated and transferred to MS without plant growth regulators. These somatic embryos produced green cotyledons, a plumule and a main root without callus formation (Fig. 1*G*). The conversion frequency of somatic embryos into plantlets was approximately 12.5 %. Plantlet formation occurred within 20 - 25 d of

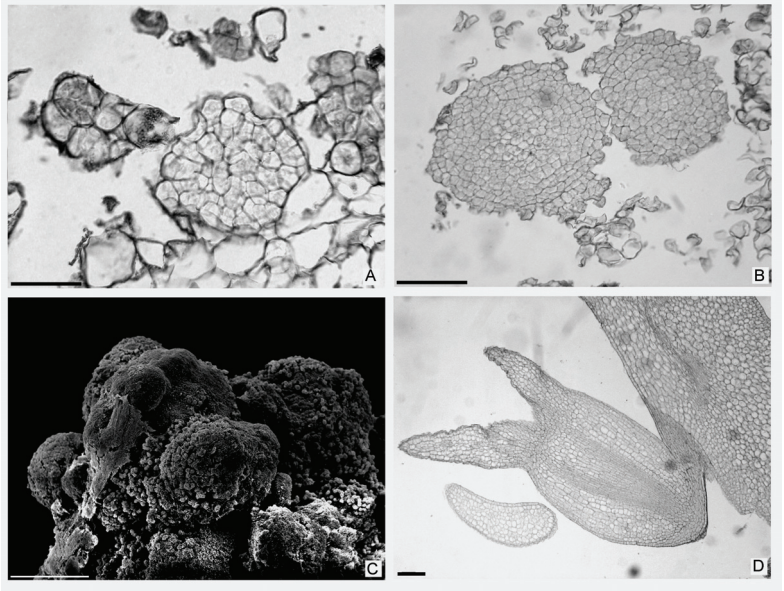

Fig. 2. Light and SEM micrograph of somatic embryos derived from immature zygotic embryos of *Cedrela fissilis*: *A,B* - globular somatic embryos, *bar* = 50 μm (*A*) and 100 μm (*B*); *C* - SEM micrograph showing a callus with nodular structures (*bar* = 500 μm); *D* - bipolar somatic embryo exhibiting shoot and root apical meristems (*bar* = 100 μm).

culture, more rapidly than in many other species (*e.g*. Yang *et al*. 2008). The plantlets were transferred to pots with soil for acclimatization. About 85 % of the plants survived when were maintained under mist in a green-house (Fig. 1*H*).

Histological studies revealed that early stages of somatic embryo formation are associated with cell division on the surface of the calli (Fig. 2*A*). This meristematic activity led to the development of globular structures composed of small cells (Fig. 2*B*). These structures formed many nodular embryos (Fig. 2*C*). Further differentiation led to the formation of somatic embryos characterized by evident bipolarity. They were without vascular connection with the callus and showed typical characteristics of a somatic dicotyledonous embryos (Fig. 2*D*).

In conclusion, although the induction and conversion of somatic embryos on *Cedrela fissilis* has been achieved, further research is still required regarding their efficiency in the induction time of embryogenic tissue. However, despite of this obstacle the protocol described here may be suitable for clonal propagation and genetic transformation of *Cedrela fissilis*.

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