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Induction of somatic embryogenesis and plant regeneration in the tropical timber tree Spanish red cedar [*Cedrela odorata* L. (Meliaceae)]

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Abstract Spanish red cedar (*Cedrela odorata* L.) is a tropical timber tree native to the Americas from southern Mexico to northern Argentina. Commercial plantations are scarce and, consequently, natural populations are overexploited. Traditional propagation practices for the establishment of large-scale plantations have had limited success in this species due to the relative scarcity of seeds, its broad genetic diversity and the lack of domesticated varieties. In vitro clonal propagation provides an effective method to overcome this situation and increase the yield of commercial plantations through the rapid multiplication of elite materials. Somatic embryogenesis (SE) is one of the most promising strategies for tree propagation due to the possibility of producing artificial seeds, the ability to store and rapidly mobilize germplasm and the opportunity for genetic manipulation. We report here the induction of indirect SE in *C. odorata* from calli derived from immature zygotic embryos after 12 weeks of culture. Macroscopic, histological, and scanning electron microscopic analyses of the calli revealed the presence of embryogenic cell clusters that formed cotyledonary embryos with clear bipolar structures and no vascular connections with the mother tissue. Different media preparations containing combinations of diverse auxins and cytokinins are known to have different effects on the type and frequency of embryogenic structures. Embryo conversion was achieved using an MS-based medium [Murashige T, Skoog F (1962) Physiol Plant 15:473–497, 1962] supplemented with abscisic acid, and transfer to soil was successful at a rate of 75%. The method described here provides a basis for optimizing the clonal propagation and genetic manipulation of this valuable species.

Keywords Clonal forestry · Dicamba · Indirect somatic embryogenesis

Abbreviations
ABA   Abscisic acid
GA_3 Gibberellic acid
IBA   Indolebutyric acid
IZE   Immature zygotic embryo
PGR   Plant growth regulator
SE   Somatic embryogenesis

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Introduction

Spanish red cedar (*Cedrela odorata* Linnaeus) is a tropical timber species with a natural distribution from southern Mexico to northern Argentina (Pennington and Styles 1975). Its hard aromatic wood is highly appreciated worldwide, being extensively used by the fine-furniture and construction industries (Lamb 1968). The demand for this forest resource, which fetches high prices on the world market, is second only to mahogany (*Swietenia macrophylla* King) (ITTO 2010). This demand and uncontrolled overextraction from natural populations during the last

200 years (Cavers et al. 2004) has led to the International Union for the Conservation of Nature (IUCN 2004) designating *C. odorata* a species facing a high risk of extinction in the wild within the medium-term future. In addition, the selective logging of individuals with elite properties may have resulted in some of the desirable genetic characteristics being lost in the natural population (Cornelius and Watt 2003; de la Torre et al. 2008), possibly also explaining why most of the materials available for restoration or commercial plantations are highly susceptible to pest attack.

The great susceptibility of available materials to the Meliaceae borer *Hypsipyla grandella* Zeller (Lepidoptera: Pyralidae) has frustrated virtually all efforts to establish large-scale commercial plantations. Cornelius and Watt (2003) looked for natural mechanisms of resistance and suggested the existence of *C. odorata* individuals with a degree of tolerance against *H. grandella* attack, raising the possibility that tolerant individuals could be cloned.

Genetic improvement of forest species using traditional approaches is a very slow process due to their long life-span, the size of the trees, recalcitrant seeds, genetic diversity, and sexual incompatibility (Park 2002). Clonal forestry has proven to be the best option for achieving genetic gains in a short period of time. In timber species, somatic embryogenesis (SE) offers unique advantages, such as higher rates of propagule production per single induction event (Pullman and Johnson 2002; Yildirim et al. 2006), cryopreservation of embryogenic calluses (Park 2002), rejuvenation (von Aderkas and Bonga 2000; Ravindra and Nataraja 2007), the ability to produce artificial seeds (Aquea et al. 2008) and, remarkably, a low rate of production of chimeric plants derived from genetic transformation experiments (Bajaj 1995; Álvarez and Ordás 2007). Among tropical wood species, SE has been reported for other Meliacea members, such as *Melia azedarach* (Vila et al. 2007), *Azadirachta indica* (Shrikhande et al.

1993; Rout 2005), *Swietenia macrophylla* (Maruyama and Ishii 1999), and *Cedrela fissilis* (Vila et al. 2009).

We have previously reported on a protocol for *C. odorata* micropropagation in which hypocotyl segments were used as starting material and TY17 medium containing the plant growth regulator (PGR) dicamba was used as the induction culture medium (Gonzalez-Rodríguez and Peña-Ramírez 2007; Peña-Ramírez et al. 2010). Using this protocol, we observed the induction of both adventitious shoot formation and the development of some proembryogenic calli. With the aim of generating a more efficient propagation method, we continued exploring SE induction and report here on a protocol for the induction of SE and plant regeneration in *C. odorata*.

Materials and methods

Plant materials

Following the recommendations described by Longman (1993), elite 15-year-old *C. odorata* trees were selected from commercial plantations around Acayucan, Veracruz, Mexico (18°02′40.97″N; 94°51′14.82″W). Unripe fruits (9 weeks post-anthesis) were collected from the chosen trees between January and March 2007 and dissected to isolate the immature zygotic embryos (IZEs) (Fig. 1). The embryos were kept immersed in 0.1% (v/v) Plant Preservative Mixture (PPM; PhytoTechnology Laboratories, Lenexa, KS) for 24 h. All analytical chemicals, unless stated otherwise, were purchased from PhytoTechnology.

Establishment in vitro and induction of SE

One thousand IZEs were cut into pieces and placed in petri dishes (10 explants per dish), each containing 25 ml of full-strength MS (Murashige and Skoog 1962) medium
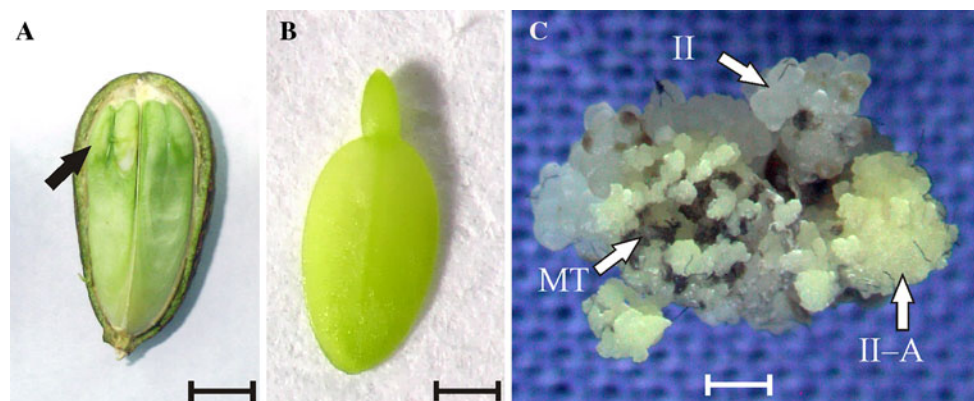
Fig. 1 Immature zygotic embryo isolation and callus induction. **a** Open unripe *Cedrela odorata* fruit (*arrow* immature seeds), **b** immature zygotic embryo, **c** transition from calli type II to type II-A (*MT* mother tissue). *Scale bars*: (**a**) 10 mm, (**b**) 2 mm, (**c**) 1 mm

supplemented with 13.57 μM dicamba, 80 mM sucrose, and 0.25% (w/v) GelRite (induction medium). The pH was adjusted to 5.7 prior to autoclaving. The dishes were incubated at 28°C under a 16/8-h (light/dark) photoperiod with light supplied at an intensity of 40 μmol m^{-2} s^{-1} (photon flux; Sylvania T12 Gro-Lux; Osram Sylvania, Waltham, MA). After 9 weeks, the cultures were analyzed, and the emerging undifferentiated callogenic structures were classified according to their appearance, color, and texture. Only Type II-A calli were subcultured to fresh medium every 3 weeks for 12 weeks under dark culture conditions or a 16/8-h (light/dark) photoperiod. Emerging type II-A calli were examined for differentiated embryo-like bipolar structures as described in the following sections.

Analysis of embryogenic structures

Histological analysis. Representative II-A calli were gradually dehydrated and embedded in hydroxyethyl-methacrylate according to the manufacturer's instructions (Historesin; Leica, Wetzlar, Germany). Microtome sections (thickness 0.03–0.05 mm) were stained with 0.05% tolui-dine blue and observed and photographed under an Olympus BX42 microscope equipped with a CCD camera.

Scanning electron microscopy. Representative II-A calli were dehydrated and dried with supercritical CO_2 (Balzers CPD 020 Critical Point Dryer; Bal-Tec, Schalksmuhle, Germany) and further dissected as required. The samples were mounted on metallic stubs with carbon conductive

Fig. 2 *C. odorata* somatic embryo development. **a** Globular somatic embryos in type II-A calli continuously formed concomitantly with embryo development, **b** an more detailed view from of **a** where heart, torpedo, and cotyledonary embryos can be observed developing in the same cluster, **c** a differentiated bipolar cotyledonary embryo after 9 weeks of culture, **d** a germinated embryo consisting of true leaves, primary and secondary roots, and a well-elongated stem >10 mm, **e** a plantlet established in soil developing under greenhouse conditions. *Scale bars*: (**a**) 200 μm, (**b**) 2 mm, (**c**) 1.5 mm, (**d**) 10 mm, (**e**) 20 mm
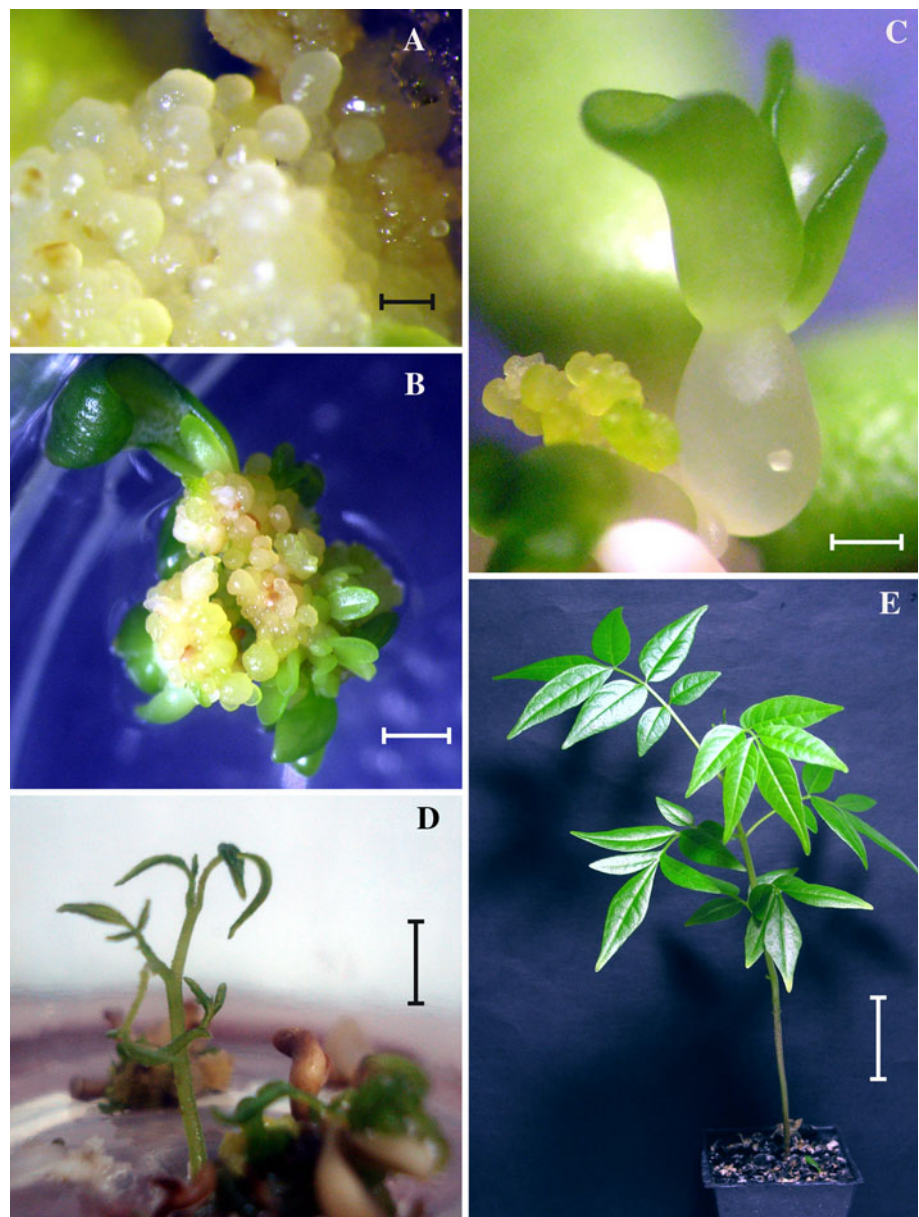
adhesive tape, sputter coated with colloidal gold, and observed at 10–20 kV using a Zeiss DSM 940A scanning electron microscope (Zeiss, Oberkochen, Germany).

Embryo germination and plantlet differentiation

One hundred and forty masses of embryogenic cotyledonary structures were divided into seven groups. Three groups were cultured in petri dishes containing 25 ml of semisolid MS medium supplemented with gibberellic acid (GA$_3$) at 0.5, 5 or 15 µM, respectively, and three groups were cultured in medium containing 0.5, 5 and 15 µM abscisic acid (ABA), respectively. The last group was cultivated in PGR-free medium as a control. After 9 weeks, the percentage of germinated embryos was calculated. Plantlets derived from the germinated embryos were transferred individually to Magenta boxes containing 40 ml of full-strength WPM basal-salt medium supplemented with 1× MS vitamins, 40 mM sucrose, and 0.13% (w/v) Gelrite. Plants were maintained in an environmental chamber under a 16/8-h (light/dark) photoperiod for 2 months before being transferred to soil.

Plantlet acclimatization

Elongated plants with stems approximately 100 mm long were planted into germination trays containing a wet mixture of peat moss (sphagnum; SunGro Horticulture, Abbotsford, B.C., Canada) and perlite (Perlita de La Laguna S.A. de C.V., Saltillo, México) in a 70/30 (w/w) proportion with an initial pH of 5.7. Trees were maintained under greenhouse conditions for 5 months, and when the plants reached a minimum size of 50 cm, they were transplanted to an experimental plantation located at the Instituto Tecnológico Superior de Acayucan, Veracruz, Mexico (18°02′39.68″N; 94°55′23.40″W). The survival rate of the established plants was calculated 6 months later as the ratio of surviving plants to total plants transferred to the field.

Results and discussion

Callus induction

Based on a previously reported proembryogenic-like callus induction protocol (Gonzalez-Rodríguez and Peña-Ramírez 2007), SE induction experiments were carried out using IZEs from unripe fruits starting material (Fig. 1a, b). In previous studies, IZEs have also been the main source of explants for the establishment of other Meliacea SE protocols (Shrikhande et al. 1993; Rout 2005; Vila et al.

Fig. 3 Ultrastructural analysis of somatic embryos. **a** A scanning electron microscopy (SEM) image of isodiametric cell clusters showing active mitotic transitions (*arrows*). **b** An more detailed view of **a** where multiple meristematic zones with active continuous cell division can be observed. **c** A typical globular embryo where the formation of a differentiated structure from a suspensor cell aggregate can be observed. At this point, globular embryos are starting to differentiate, as indicated by the presence of a protodermic cell layer formation (*p*) over the surface of the embryo. **d** The emergence of a heart-to-torpedo-shaped embryo where signs of tissue arrangement across the embryo structure can be observed (*arrow*). The embryo can also be seen to be surrounded by undifferentiated cell masses formed of isodiametric cells with defined nuclei, dense cytoplasm, and mitotic transitions typical of embryogenic calli. **e** Slice of an embryo with clearly developed cotyledonary structures, a protodermic cell layer, and complete independence from the mother tissue. **f** SEM observations of heart-shaped embryos developing from embryogenic calli. Symmetric non-fasciated structures are consistently observed. **g** SEM observation of a whole type II-A calli embryogenic cell aggregate. The *image* shows the unsynchronized development of *C. odorata* somatic embryos at different developmental stages, from cell clusters to cotyledonary embryos (*right to left*). **h** Histological preparation of an apical shoot from a germinated embryo showing well-differentiated conductive tissue (*arrow*) as well as dermic layers. *Scale bars*: **a**, **b** 100 µm, **e**, **h** 200 µm

2007). After 9 weeks of culture in induction medium, almost all explants had responded by forming calli. These undifferentiated structures formed two different types of calli that varied in color, texture, appearance, and friability (data not shown). They were denoted type I and type II and appeared at a 3:1 proportion, respectively. Type II calli were the most friable and continued to grow (Fig. 1c), while type I calli failed to propagate on successive subcultures, eventually losing their phenotype; some masses of type II calli produced organogenic structures, but most became necrotic and died.

Development of somatic embryos

The characteristics of type II cultures suggested a proembryogenic callus so they were maintained under one of two photoperiod conditions: the 16/8-h (light/dark) photoperiod and total dark. After 12 weeks of culture in MS induction medium, type II calli were analyzed by microscopic observation, which revealed that 58% of the calli had formed aggregates of embryos at all developmental stages that resembled the globular, heart, torpedo, and cotyledonary structures found in developing somatic embryos of dicotyledonous species (Litz and Jarret 1991; Finer 1994). This new embryogenic tissue, termed type II-A (Fig. 1c), differs from type II by the complete absence of mother tissue, a more intense yellow color, emerging unsynchronized globular to cotyledonary structures, higher vigor, and an even greater friability (Fig. 2a). The induction rate of type II-A calli was around 20% based on the results of three independent induction experiments, demonstrating the reliability of this method.
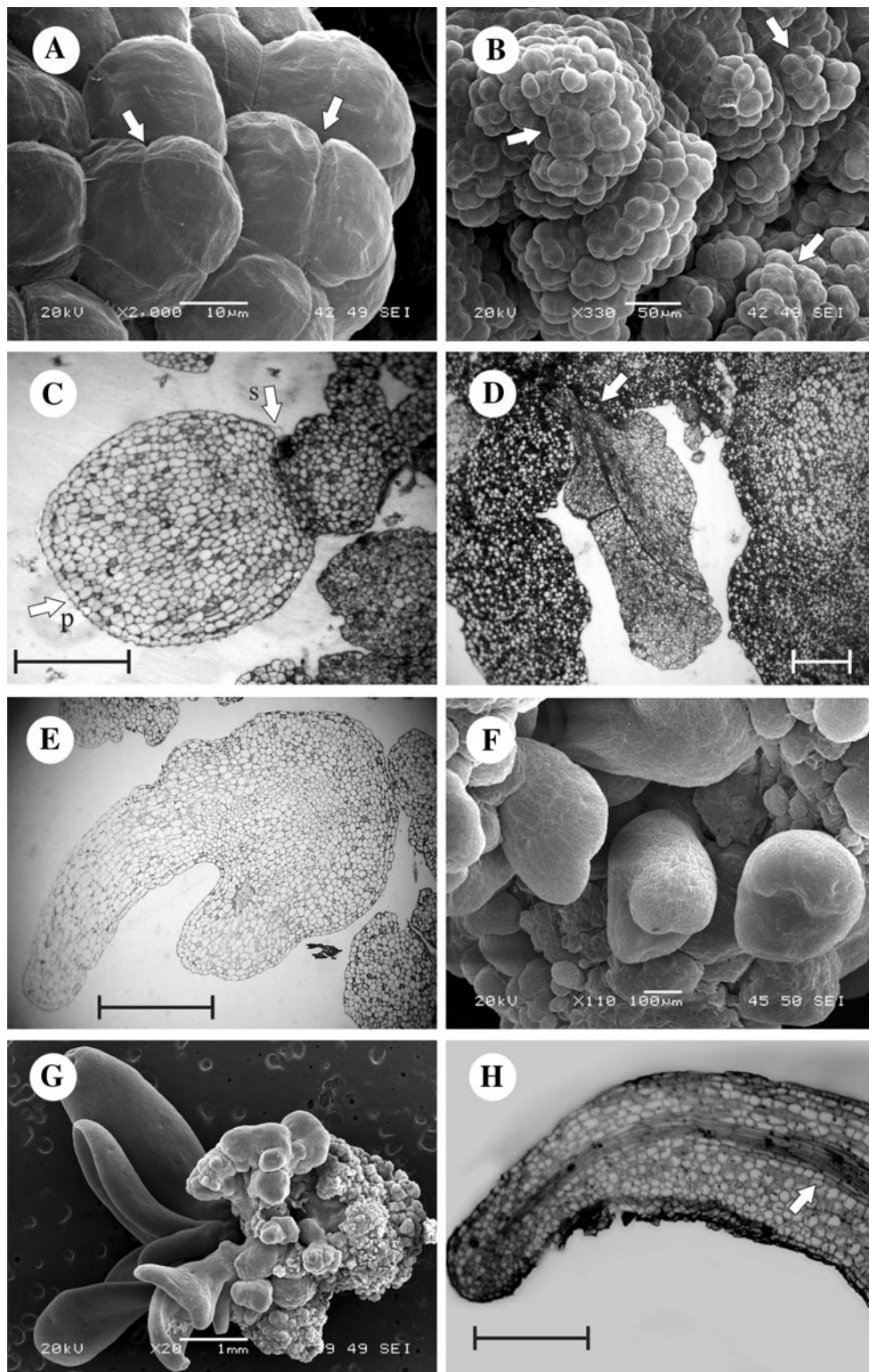
Development of somatic embryos

Somatic embryo development was achieved in a single step in the presence of dicamba, which has been reported to enhance the induction of SE in other tropical timber species, such as *Eucalyptus grandis* (Titon et al. 2007) and *E. globulus* (Pinto et al. 2002); however, dicamba is the least used PGR in SE protocols (Jiménez 2005).

We quantified the percentage of embryogenic structure formation under the photoperiod and dark culture conditions, respectively. Data from the two conditions revealed no significant difference between the treatments, both of which yielded about 44% globular, 18% heart, 12% torpedo, 17% early cotyledonary, and 9% late cotyledonary structures. Under the dark condition, the late cotyledonary structures remained uncolored (data not shown), while under the photoperiod condition, they presented a deep-green color (Fig. 2b). This phenotype may be an indication of plastid differentiation oriented toward adaption to autotrophic metabolism, similar to the photodifferentiation that takes place in zygotic embryos. Homologous embryo photodifferentiation developmental patterns have been described in *Pinus taeda* (Pullman et al. 2003).

Structural analysis of embryogenic type II-A calli

Stereoscopic analysis of embryogenic type II-A calli after 3 weeks of induction showed highly proliferating clusters composed of globular structures (Fig. 2a) that covered most of the callus and were surrounded by a few embryos at more advanced developmental stages (Fig. 2b). Globular structures were constantly formed, suggesting the presence of a repetitive SE process (Fig. 2c). Examination of type II-A calli by SEM and optical microscopy revealed the presence of embryogenic clusters composed of cells ranging from 30 to 40 μm in length with isodiametric structures (Fig. 3a, b) that were, apparently, undergoing cell division. Histological sections of these clusters showed cells with dense cytoplasm and prominent nuclei (Fig. 3c), and these characteristics remained constant over at least 12 subculture cycles (48 weeks). These cellular masses showed different degrees of organization with multiple meristematic zones (Fig. 3b), including clusters with protodermic formation surfaces, cell proliferation, and the formation of globular structures without vascular connections between the emerging embryo and the original tissue (Fig. 3c). Monitoring of individual structures showed that the transition from globular to late cotyledonary stage was very rapid, taking approximately 15 days, and was immediately followed by the formation of a bipolar meristematic zone. SEM observations showed differentiation from globular to bipolar heart and cotyledonary structures (2 weeks from undifferentiated cluster to late cotyledonary), with a low frequency of fasciated structures (<0.1%; Fig. 3d–f). Type II-A calli showed a high degree of unsynchronized but well-structured somatic embryo formation in their different phases in a single cluster (Fig. 3g). Histological studies of late cotyledonary structures showed well-organized vascularization and apical shoot formation (Fig. 3h). The growth of type II-A calli measured as fresh weight gain and cell packed volume were determined in a time-course

experiment over 12 weeks. A fourfold increase was observed for both parameters after four subcultures, indicating active proliferation. After 1 year of continuous culture, type II-A calli maintained the same embryogenic competence, which may suggest the presence of repetitive indirect SE events (Namasivayam 2007).

Embryo conversion and plantlet acclimatization

To induce embryo development beyond the late cotyledonary phase, GA₃ and ABA were tested. No embryo conversion was observed in the control or with any of the concentrations of GA₃ tested, but 3.8 μM ABA induced up to 12% conversion. Embryo conversion began with the appearance of a bipolar structure (Fig. 2c), ulterior formation of shoots with true leaves, stem and root elongation, and secondary root formation (Fig. 2d). ABA has been reported to induce embryo conversion (Litz and Jarret 1991); however, in our experiments, the efficiency was quite low. To increase embryo conversion, other authors have used ABA at higher concentrations (up to 98 μM (Vales et al. 2007) and evaluated its effects in combination with osmolarity modulators, such as polyethylene glycol (Yildirim et al. 2006) or mannitol (Uddin et al. 1990; Pullman et al. 2003) and different carbon sources (Blanc et al. 1999; Vila et al. 2007). Further studies using higher ABA concentrations combined with cytokinins, other carbon sources, alternate gelling agents, nitrogen, amino acids, and osmotic modulators in the medium may help to increase *C. odorata* somatic embryo conversion rate. A total of 19 out of 20 plants from the experiments were successfully established in soil, indicating the correct development of functional root and photosynthetic systems; in all cases, a normal development pattern similar to plantlets derived from seeds was observed (Fig. 2e). However, only 15 plants survived the transfer to field, resulting in a total transfer efficiency of 75%.

The development of an efficient somatic embryogenesis and regeneration protocol in *C. odorata* is needed for gene transfer. Traits such as insect resistance, abiotic stress tolerance, growth speed, and biomass gain are key elements to the genetic improvement of this species that potentially could lead to the establishment of cost-effective commercial plantations for wood production. Such plantations would alleviate the pressure of logging on natural populations, which is the ultimate goal for the conservation and proliferation of the remaining germplasm diversity.

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