

Plant regeneration in Spanish cedar, *Cedrela odorata* L., using zygotic embryo explants from mature seed and improvement of embryogenic nodule initiation by heat shock

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Abstract Conditions for induction of embryogenic nodules and subsequent somatic embryogenesis in the tropical hardwood *Cedrela odorata* are described. Embryo explants from ungerminated mature seeds were placed on Driver and Kuniyuki Walnut (DKW) medium using 0.8% w/v agar as the gelling agent, plus benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Various phytohormone combinations were tested, from which 5 μ M BA + 50 μ M 2,4-D were chosen as the standard regime based on a maximum frequency of embryogenic nodule occurrence of 20–25% on this medium. Nodules, when excised from the cotyledons and placed on growth-regulator-free medium, produced both plantlets and secondary embryogenic tissue. With extended culture on growth-regulator-free DKW medium, plantlets developed roots and could be transplanted into pots for further growth. The frequency of nodule initiation could be improved by either orienting the cotyledon explants with their lower (abaxial) surface in contact with the medium or imposing a pre-excision period of heat shock. The treatments together were additive. An optimum heat-shock temperature (47°C) and range of exposure times (8–12 h) were defined.

Keywords *Cedrela odorata* · Cedro · Spanish cedar · Hardwood · Heat shock · Somatic embryogenesis

Introduction

Cedrela is a tropical tree genus within the Meliaceae family, subfamily Swietenioideae (Pennington 1981), within which *Cedrela odorata* L. is the most commercially important and widely distributed species of the genus (Cintron 1990). The Swietenioideae subfamily also contains other commercially important genera, such as *Swietenia* spp. (the mahoganies), *Khaya* spp. (African mahoganies), and *Toona ciliata* (Australian red cedar). Common names for *Cedrela* are cedro, Spanish or Mexican cedar, or cigar-box cedar, as its wood is fragrant and resembles the odor of cedar. Cedro is an important fine-timber species used to make cigar boxes, furniture, and paneling (Salazar et al. 2000). It is also used for shade plantings in cocoa and coffee plantations. In addition, it has a number of different nontimber uses as the bark oil contains several sesquiterpenes, which are used in traditional medicine to treat malaria and diabetes (Martins et al. 2003). Also present in the heartwood are limonoids, called gedunins, which are potentially useful as extractives for their antifeedant (Arnason et al. 1987) and antimalarial (MacKinnon et al. 1997; Omar et al. 2003) properties. Leakey and Newton (1994) have called multipurpose, multiple-use trees, like *Cedrela*, “Cinderella” species because of their untapped value.

C. odorata is found throughout Central America, Amazonia, and elsewhere. However, overharvesting for timber has placed the species at risk, with large individuals becoming scarce because of indiscriminate opportunistic cutting, such that the species is now included in the IUCN Red List (Anonymous 2004). Widespread implementation of plantation culture, which would improve timber supply,

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is limited because of risks associated with attack by the mahogany shoot borer, *Hypsipyla grandella* (Zeller), which destroys the terminal shoot bud of young saplings, resulting in a highly branched tree of low commercial value (Newton et al. 1999). However, within-species genetic variation in both morphological types (Navarro et al. 2002) and susceptibility to insect attack (Newton et al. 1999) exist, so selection and breeding followed by upscaled clonal propagation might be used to produce high-value plantation stock.

Despite their high value and the widespread use of Meliaceae species for timber, there are few reports of successful tissue culture, suggesting that Swietenioideae species may be recalcitrant. Shoot organogenesis using meristem culture from nodal segments was first reported in 1988 for *Swietenia macrophylla* (Lee and Rao 1988) and for both *Swietenia mahoganii* and *S. macrophylla* (Venketeswaran et al. 1988). Shortly thereafter, Maruyama et al. (1989) reported organogenesis in *C. odorata*, also using shoot tips as the explant material, and subsequently Nunes et al. (2007) have provided a set of organogenesis protocols for the closely related species, *Cedrela fissilis*. Successful somatic embryogenesis (SE) was initially described in the Swietenioideae, for *S. macrophylla* (Maruyama and Ishii 1999; Maruyama 2006), using *in vitro*-grown shoot tips taken from organogenic cultures. More recently, low-frequency SE has been reported by Muñoz Tuesta (2003) from different parts of aseptically germinated *C. odorata* plantlets and at higher rates by Gonzalez-Rodriguez and Peña-Ramirez (2007), especially from root explants. Recently, SE has also been accomplished using immature zygotic embryos excised from *C. fissilis* (Vila et al. 2009). Both studies used tissues from sterile 2-wk-old germinants. This paper reports on successful SE and plant production using zygotic embryos excised from ungerminated mature *C. odorata* seeds and the stimulation of SE initiation by imposing a period of heat shock during seed imbibition prior to excision.

Materials and Methods

Seed materials. Mature *C. odorata* seed was purchased on two occasions from the Banco de Semillas Forestales, CATIE, Turrialba, Costa Rica (seed lot code BSF072, Las Juntas, Guanacaste province). The seed lot's germination rate was confirmed as >80% using a standard germination test (30 d on a moistened fiber mat at 28°C). For all experiments, seeds were imbibed between two circles of filter paper (to keep them from floating) in distilled water in 250-ml beakers for 16 h (or longer for the lengthiest heat-shock intervals—see under “Experimental treatments” below).

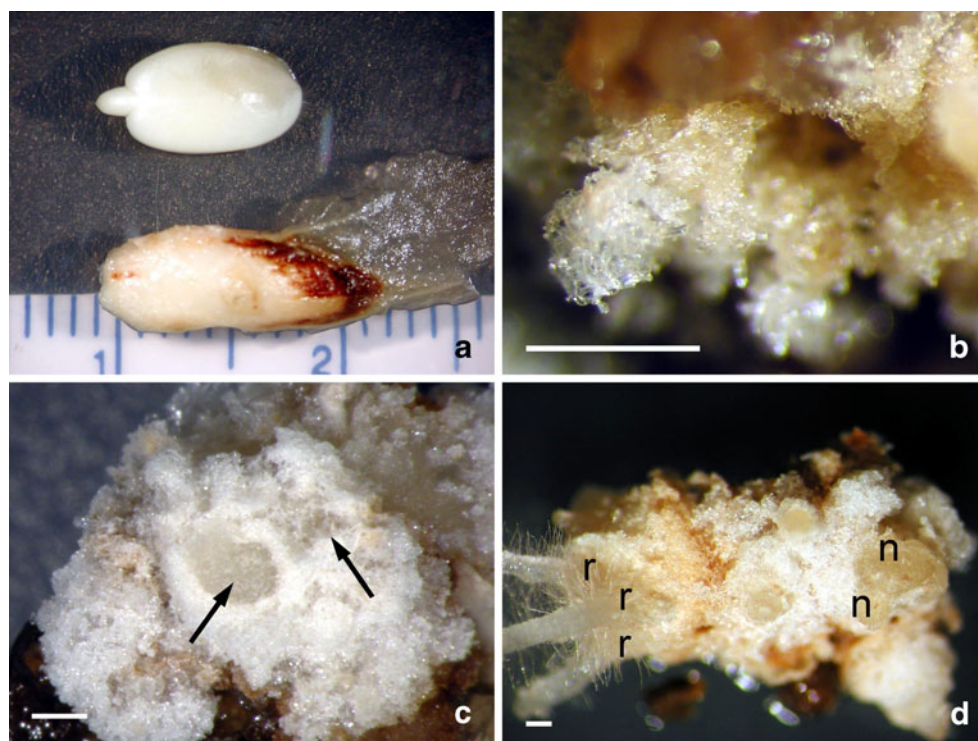
Excision and culture conditions. Imbibed seeds were surface-sterilized by immersion in 0.6% sodium hypochlorite (10% v/v Javex/distilled water) plus 0.5% Tween 20 with stirring for 30 min, then 2 min in 70% ethanol with stirring, followed by three rinses of sterile distilled water of 1–2 min each. The sterile, partially dewinged mature seeds were placed in Petri plates on filter paper moistened with sterile distilled water, and whole zygotic embryos (Fig. 1a) were excised from them with a scalpel and forceps.

Excised embryos were placed in Petri dishes at six seeds per plate containing Driver and Kuniyuki Walnut (DKW) basal medium supplemented with 0.1 mg l⁻¹ nicotinic acid and 0.2 mg l⁻¹ thiamine HCl (Driver and Kuniyuki 1984) and solidified with 0.8% w/v agar (Sigma Aldrich Ltd., St. Louis MO). Autoclaving was done for 18 min at 1.5 kg cm⁻² and 115°C. Excised embryos were incubated at 24.5±0.5°C in darkness during the initiation phase, which lasted 20–25 wk, and were transferred to fresh media every 3 wk. During the first three weeks, cultures were checked for contamination daily, and contaminated embryos were removed until no new fungal growth could be detected. For most experiments, 180% of the number of seeds required were excised for each treatment within an experiment, with the goal of achieving 100 of the number of viable explants after accounting for germination and contamination losses. At the end of initiation, promising tissues were removed from the cultures and incubated at 24.5±0.5°C under CW fluorescent light (20±2 μmol s⁻¹m⁻²).

To produce plantlets, the standard treatment was culture of somatic embryos for 4 wk on DKW medium without growth regulators followed by transfer to DKW containing 1 μM indole butyric acid to stimulate root growth. Plantlets with roots 1–3 cm in length were transferred into 400-ml containers (Magenta GA-7, PhytoTechnology Labs, Shawnee Mission, KS) filled with 8/16 mesh (1.2–2.4 mm diam.) attapulgite (microporous clay particles; Agsorb 8/16 LVM, Oil-Dri Corp., Chicago, IL) and 200 ml of hormone-free DKW solution without sucrose.

Experimental treatments: hormone concentrations and ratios. Two experiments were done. The first experiment was intended to determine explant responses within a broad series of phytohormone concentrations. The second used a narrower range of combinations and an increased number of explants per treatment to better define explant responses. Both experiments employed a matrix of combinations of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and the cytokinin benzyladenine (BA). In experiment 1, 36 embryos (six embryos per plate × six plates) per treatment were used, and the concentrations were 0, 2, 4, 10, 20 μM for BA and 2, 10, 20, 40, 100 μM for 2,4-D. In experiment 2, 60 embryos (six embryos per plate × ten plates) per

Figure 1. Early stages of *C. odorata* somatic embryogenesis: (a) partially dewinged seed and initial zygotic explant; (b) clear, loose, filamentous callus, the initial indication of SE; (c) nodules forming within the initial callus; (d) roots and non-embryogenic nodules at approximately 20 wk of culture. All bars 0.1 mm.



treatment were used, and the concentrations were 0, 1, 2, 5, 10 μM for BA and 1, 5, 10, 20, 50 μM for 2,4-D. For these experiments, all embryos were placed on the medium in the same orientation shown in Fig. 1a.

Experimental treatments: heat shock and orientation. Two trials were performed. Half the seeds were exposed to heat-shock treatment during the final part of the imbibition immediately before excision. The heat-shock treatment for these trials consisted of placing the beakers with seeds in a water bath at 42°C for the last 4 h of the imbibition treatment, according to Gonzalez-Rodriguez and Peña-Ramirez (2007). Orientation refers to whether the excised embryo was placed in culture with either the edges of the cotyledons or, alternatively, the abaxial (lower) side of one cotyledon contacting the medium, with the other one removed from the embryo and placed on the medium beside it, also abaxial side down. Seventy embryos (seven embryos per plate \times ten plates) per treatment were used in the first heat-shock/orientation experiment, and 49 embryos (seven embryos per plate \times seven plates) per treatment were used in the second experiment in the heat-shock/orientation trial. In both experiments, the hormone concentrations present in the medium were 5 μM BA + 50 μM 2,4-D.

Explants were examined for the presence of SE nodules after 23 wk of culture in trial 1 and at 8 and 24 wk of culture in trial 2. The trials were assessed both separately and as a merged data set using analysis of variance (ANOVA; SAS version 8.2, SAS Institute Inc., Cary, NC) after square root ($x+1$) transformation.

Experimental treatments: heat-shock temperature and duration. Three thousand six hundred seed embryos were excised and placed in groups of 96 zygotic embryos (six embryos per plate \times 16 plates) per temperature/time treatment combination. Temperatures of 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C were used for the shorter heat-shock times of 1, 2, 4, and 6 h. At longer times of 8, 12, 16, and 24 h, the imbibition temperatures used were only 35°C, 40°C, 45°C, and 50°C. The hormone concentrations were as before: 5 μM BA + 50 μM 2,4-D for all treatments. Cotyledons from each embryo were separated, leaving the hypocotyl attached to one of the two, and plated abaxial side down in pairs. Explant cotyledons were assessed individually for mortality (indicated by no callus growth and bleaching) and for the presence of SE tissues at 23–24 wk. Both viable and older brown (due to phenolics) SE tissues were counted in the assessment. (The latter would ordinarily have been harvested earlier in culture rather than waiting for the full 23 wk.)

Results

Description of somatic embryogenesis. In this study, a consistently occurring early phase of response, within 4–8 wk of culture on DKW initiation medium, was the appearance of clear, loose, and somewhat filamentous callus tissue on both sides of the cotyledons (Fig. 1b, c),

possibly similar to the tissue Venketeswaran et al. (1988) described as “snow-flake” callus in their tissue culture study of mahogany and called “callosa Blanca” by Muñoz Tuesta (2003). Structures of several types emerged from within or beneath this tissue (Fig. 1b), which subsequently developed into either smooth nodules that did not differentiate further, roots (Fig. 1d), or clusters of greenish-white to green-colored nodules.

The green nodules were found to form on the upper cotyledonary surfaces only and occurred in numbers varying from frequently observed small clusters of 10–20 (Fig. 2a) to occasional extensive arrays of 50–100 or more that covered most of the cotyledon explant surface (Fig. 2b). They were smooth, with prominent epidermal cells, and frequently could be sparsely pubescent (Fig. 2c, arrows). Aside from the difference in color, the embryogenic nodules were easily distinguishable from the other type of nodules, which were smooth, brownish-gray in color, and slightly larger (Fig. 2c). Another location where the green nodules occasionally occurred was on either side of the embryo apical meristem (Fig. 2d).

After 20–24 wk of culture, the embryogenic nodules were detached from the underlying cotyledonary tissue for further culture on hormone-free medium. If left on the original explant, nodules turned brown and died, presumably from accumulation of phenolics. After 3–4 mo of monthly subculture, a mixture of plantlets (often malformed), a few roots, and a mucilaginous callus arose from the original nodules (Fig. 3a), which became dark brown

with extended culture. Approximately one third of the nodules produced embryogenic callus. The callus lines, which grew at different rates, were highly nodular and clear to yellowish-white-colored. Depending on the line, numerous immature embryos (Fig. 3b) were generated after a further 3–6 mo of monthly subculture on hormone-free medium. A number of lines of this callus (Fig. 3b, inset) appear to have achieved stable growth on hormone-free DKW medium and were kept in culture for over 2 yr. They continued to generate large numbers of plantlets (Fig. 3c). Although the plantlet morphology is variable (e.g., plantlets with deformed and fused cotyledons are frequently observed), most appear normal, and 5–10% of the plantlets spontaneously develop roots when removed from the callus and subcultured directly onto hormone-free basal medium, after which they can be transferred into nonsterile growth medium (Fig. 3d). Those that have been repotted for further growth in the greenhouse (Fig. 4) are normal-appearing plants that continue to grow rapidly in the nursery.

Optimizing hormone concentrations and ratios. Both hormone concentration experiments were set up as (5) BA × (5) 2,4-D matrix experiments. A wider range of phytohormone concentrations was used in trial 1. Trial 2 concentrations were modified based on the results of trial 1, and a larger number of explants per phytohormone combination (60) were used. Results of both trials are given in Table 1. The optimal BA and 2,4-D concentrations were found to be

Figure 2. Formation of *C. odorata* cotyledonary SE nodules: (a) embryogenic nodules—*e*—in small clusters; (b) a large array of embryogenic nodules covering almost the entire surface of one cotyledon; (c) close-up of embryogenic and nonembryogenic—*n*—nodules: arrows indicate leaf hairs; (d) embryogenic nodules formed at the bud meristem: *c* cotyledon, *s* leaf scar from separated cotyledon. All bars 0.1 mm.

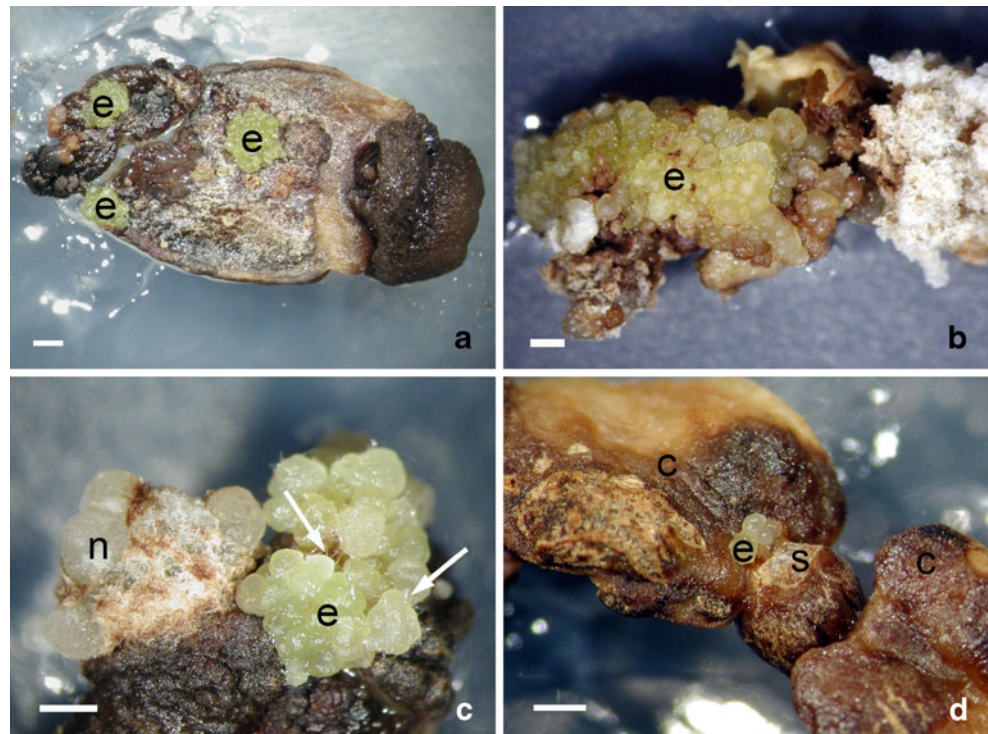
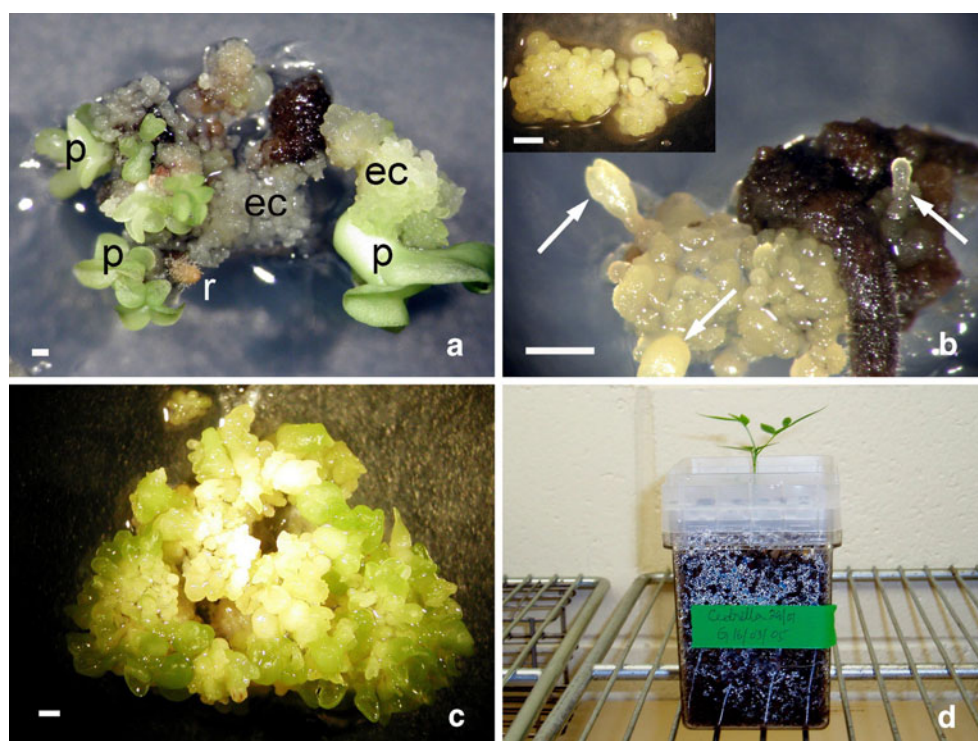


Figure 3. Structures arising from excised SE nodule culture: (a) plantlets—*p*—and embryogenic callus on the original excised nodule (dark brown); (b) *inset*: typical SE callus in long-term culture and close-up of embryos (arrows) arising from embryogenic callus; (c) prolific plantlet formation on SE callus cultured on hormone-free medium; (d) potted *Cedrela* of three different ages (approximately 12, 18, and 24 m) grown from germinated plantlets. All bars 0.1 mm.



within the 5–10 and 20–50 μM ranges, respectively. Based on these results, 5 μM BA + 50 μM 2,4-D were chosen for use in all subsequent experiments.

Effect of heat shock and orientation. During the course of initiation, *C. odorata* explants warp and twist as the cotyledons open out, and rapid callus growth deforms

them. Anecdotal observations during the hormone matrix experiments (where all explants were initially placed flat on the medium with the cotyledons still appressed) suggested that the opening and random twisting of the explant cotyledons to either edge-on or flat orientation on the medium surface could influence the rate of embryogenic nodule production. Results from a small preliminary experiment (not shown), where heat shock was used as a pretreatment, also suggested that heat shock during imbibition can promote embryogenic nodule initiation.

The two initial trials in which either edge-on or abaxial side-down cotyledon orientations were tested with or without a heat-shock pretreatment indicate that either placing the lower cotyledon surface or applying a heat-shock treatment prior to excision increases the frequency of embryogenic nodule formation (Fig. 4). Applied together, both practices appear to be additive. The experiments were performed on two different seed lots with similar results, although the first experiment used seed that had been in storage for several yr and whose viability was diminishing. A *t* test (not shown) performed on trials 1 and 2 data showed that they were not significantly different. Therefore, a two-way ANOVA (using a statistics package under SigmaPlot 11.1) was done on the combined data sets and is summarized in Table 2. The data from the two combined trials show that the flat orientation significantly improves initiation ($P=0.003$) as does heat shock compared with the standard regime ($P=0.002$), with a smaller but nonetheless significant interaction between the two ($P=0.048$).

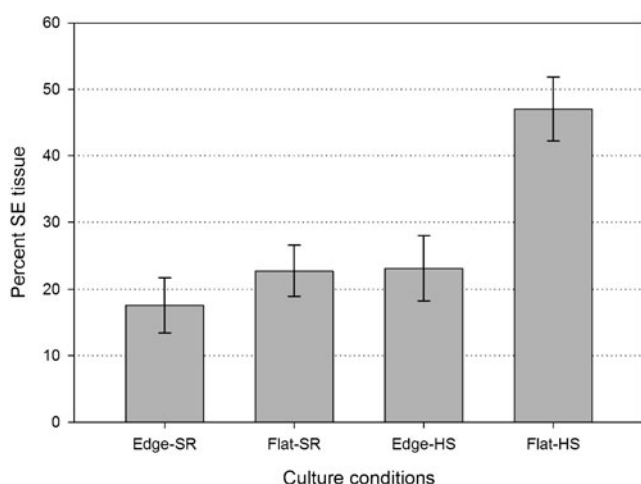


Figure 4. *C. odorata* explants with SE nodules observed (combined from two separate trials) expressed as a percentage of the total live explants. Treatments: *SR* standard regime (no heat shock), *HS* heat-shock pretreatment, *edge* cotyledons placed in contact with the medium edge-on, *flat* abaxial (lower) cotyledon surface in contact with the medium (bars: standard error of the mean).

Table 1. Effect of 2,4-D and BA interactions on embryogenic nodule formation in *Cedrela*

BA concentration (μM)	Hormone trial		2,4-D concentration (μM)							
	No. 1	No. 2	1	5	10	10	20	20	40	100
0		0	0.0	0.0	5.9	2.4		2.1	0.0	
	1		0.0	0.0	2.3		14.8	14.8	11.5	
2		2	0.0	0.0	8.3	5.7	20.6	12.2	23.3	12.5
	4		0.0	0.0	13.3		5.9		15.4	12.0
	5		0.0	0.0		7.5		17.8	25.9	
10		10	4.3	0.0	12.0		24.1		24.2	12.5
	20		9.4		4.0		23.1		17.6	16.1

Italicized values in the table are BA/2,4-D combinations at which the highest frequency of nodule formation was observed. Results are expressed as the percentage of embryo explants where embryogenic nodules were observed (explants per phytohormone combination: $n=36$ in experiment no.1; $n=60$ in experiment no. 2)

The best combination of imbibition times and temperatures could be determined graphically by generating a 2-D contour plot (using SigmaPlot 11.1), shown in Fig. 5, based on the presence of embryogenic nodules for each time/temperature combination as a percentage of the surviving live explants. The maxima (embryogenic nodule initiation values $\geq 40\%$) actually defined by the data points fell within the temperatures range of $45\text{--}50^\circ\text{C}$ for a period of 8–12 h, with the interpolation suggesting that a temperature value midway between the two temperatures of $47\text{--}48^\circ\text{C}$ for approximately 9–10 h may be optimal. Temperatures above 55°C for 8 h or less were lethal, as was 50°C or more at 12 h or longer. Although a higher frequency of nodule formation was observed, an increase in the number of nodules (or larger clusters) per explant was not.

Table 2. Results of a two-way ANOVA on the effects of heat shock and orientation on induction of embryogenic tissue in *Cedrela*

Factor	Combined trial 1 and 2				
	df	SS	MS	F	P
Orientation (O)	1	3,474.561	3,474.561	9.770	0.003
Heat shock (H)	1	3,657.734	3,657.734	10.285	0.002
H \times O	1	1,450.230	1,450.230	4.078	0.048
Residual	62	22,049.498	355.637		
Total	65	30,710.920	472.476		

Analysis was performed on the combined data set of trials 1 and 2 (initial no. of explants in each treatment: $n=70$ in trial 1; $n=49$ in trial 2). Results are significant at $P<0.05$

Discussion

This report indicates that zygotic embryos from mature stored *C. odorata* seed may be successfully cultured using

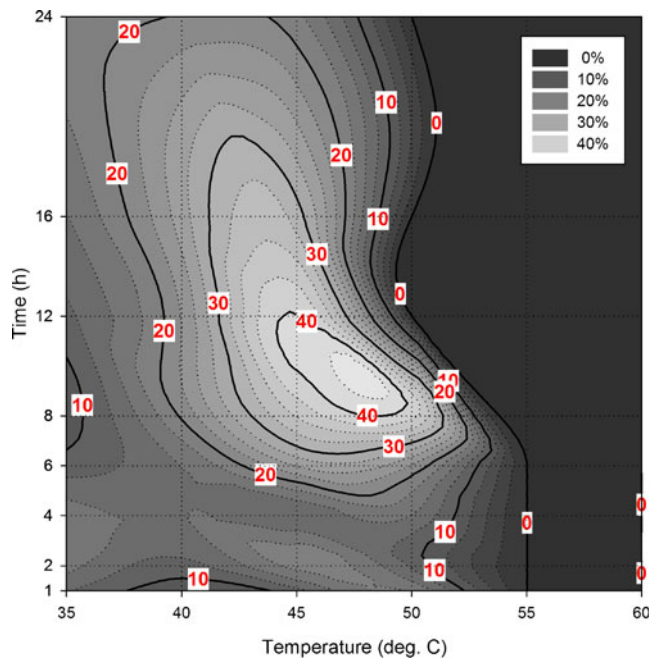


Figure 5. Contour plot of the percentage of SE tissue initiation observed at different heat-shock imbibition times between 1 and 24 h and temperatures from 35°C to 60°C . Isolines defined by solid and dashed lines, and percentages (numbers within boxes) delimit the combinations of incubation times and temperatures at which a similar frequency of embryogenic nodule formation was observed. Maximum response was within the range of $47\text{--}49^\circ\text{C}$ for 9–10 h. Temperatures within the area bounded by 0% (darkest colored region) were lethal, and no nodules were produced.

DKW in combination with BA and 2,4-D to induce embryogenic nodules at a high frequency (20–25%) on explant cotyledons and subsequently, using the basal medium, to both develop plantlets from the excised nodules and maintain stable SE tissue in culture. The DKW medium was originally developed for walnut shoot organogenesis (Driver and Kuniyuki 1984) and has since been used for SE culture in a number of *Juglans* cultivars (Tulecke et al. 1995), as well as for organogenesis in other nut trees (Yu and Reed 1993; Kumar and Sharma 2005). However, DKW is employed infrequently in other woody species.

Application of different abiotic stresses during the initial phases of culture has been shown to stimulate early events in SE development (Helleboid et al. 2000; von Aderkas and Bonga 2000). Heat shock is easily imposed and has been demonstrated to stimulate SE in tissues and species as diverse as microspores of *Brassica*, tobacco, wheat (Touraev et al. 1997), cork oak (Bueno et al. 1997), ginseng shoots (Asaka et al. 1993), and chicory leaves (Helleboid et al. 2000). It is evident that early heat shock applied to *C. odorata* can stimulate cotyledonary embryogenic nodule production. However, because a mixed, open-pollinated seed lot was used, it cannot be determined from these trials whether the treatment stimulated recalcitrant zygotic embryos that would not otherwise have developed nodules or whether the heat shock was equally promotive across all the explants. The additive interaction with orientation suggests that hormones and nutrients in the medium may not translocate well over several millimeters of distance in edge-oriented cotyledons, and more extensive abaxial contact is required for efficient induction. Any effect that heat shock may have on SE maturation and plantlet formation currently remains unknown.

The production of embryogenic cultures, plantlets, and somatic seedlings from mature zygotic embryos of *C. odorata* is reported here for the first time, although SE has also been reported recently using immature seed explants from the closely related *C. fissilis* (Vila et al. 2009). Using organogenesis to propagate *C. odorata* from shoot tips was demonstrated by Maruyama et al. in 1989, whereas the current study describes a simple protocol to generate embryogenic nodules and SE callus and plantlets using embryos excised from mature seed, so SE also may be a potentially viable route for clonal propagation and ultimately useful in propagating progeny from controlled crosses. Unlike Gonzalez-Rodriguez and Peña-Ramirez (2007), we did not observe the loss of embryogenic potential from *C. odorata* seed stored for over 5 yr, although the percentage of germination did decrease slightly.

Using a mixed seed lot, clusters of nodules (Fig. 2a–d) could be excised from 30% to 50% of the embryo cotyledons from the best heat-shock treatments. Although many of the nodules turned brown and subsequently died

during extended subculture on growth-regulator-free medium, viable callus (Fig. 3a, b) could be separated and subcultured from 20% to 40% of them, suggesting an SE callus yield of approximately 10–15% from the initial embryo explant population. Calculating the yield of plantlets from the SE callus is difficult because most of the individual growing callus lines could continuously produce tens to hundreds of plantlets (Fig. 3c) on extended subculturing, with spontaneous root formation occurring in under 10% of them. However, as has been noted elsewhere (Merkle et al. 2003), the ability to initiate SE and produce plantlets is only the first of several challenges that must be met. A number of the cultural steps in *C. odorata* tissue culture remain to be optimized: embryogenic nodule survival, SE maturation, germination, and plantlet development in order to produce the large numbers of acceptable-quality somatic seedlings needed for a commercial or industrial program; this process has already begun elsewhere (Gonzalez-Rodriguez and Peña-Ramirez 2007; Pena-Ramirez et al. 2008).

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