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# Multiple adventitious shoot formation in Spanish Red Cedar (*Cedrela odorata* L.) cultured *in vitro* using juvenile and mature tissues: an improved micropropagation protocol for a highly valuable tropical tree species

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**Abstract** *Cedrela odorata* L. is a valuable tropical tree widely appreciated for its wood. This species confronts serious problems due to both overexploitation of its natural populations and its susceptibility to the Meliaceae borer *Hypsipyla grandella*, which destroys the apical meristems and produces structural deformations. The rapid introduction of new varieties through clonal forestry has been demonstrated to be the most effective way to improve the production of perennial plantation species. In this work, we report both a protocol for the rejuvenation of elite mature trees of *C. odorata* and the optimization of an *in vitro* culture system to scale up micropropagation. Several media formulations and the use of temporary immersion culture in bioreactors were evaluated. The addition of 20% coconut water to TY17 medium increased the number of adventitious shoots from hypocotyl segments to an average number of 4.68 shoots per explant. To replace coconut water and to define the culture medium, several cytokinins were tested at various concentrations; however, none of them produced the effect of coconut water. Rejuvenation of elite mature individuals was investigated by *ex vitro* grafting of mature tree twigs onto 3-mo-old juvenile trees. Although the grafting had a positive effect on the micropropagation of mature material, the multiplication rate of 1.5 new shoots per explant did not compare to the organogenic capacity of younger materials. Shoot and root elongation as well as acclimatization to *ex vitro* conditions were carried out in a temporary immersion culture of juvenile material using BioMINT® bioreactors. A 3.5-fold increase in shoot elongation and a 4-fold increase in root elongation were achieved compared to material cultured on semisolid media. Furthermore, this culture system allowed for 98% effectiveness in the soil adaptation of the *in vitro*-grown plants. The scaled-up multiplication capacity over a period of 6 mo calculated for the system is above 16,000 plants per mother plant with young materials but is only 125 with mature materials.

**Keywords** Clonal forestry · Grafting · BioMINT® Bioreactor · Rejuvenation · Coconut water

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## Introduction

The Spanish red cedar, also known as cigar box cedar (*Cedrela odorata* Linnaeus [Meliaceae]), is a tropical timber tree highly valued in worldwide markets. *C. odorata* wood is a valuable forest product that can sell for up to five times the value of coniferous hardwoods and that is second in value only to mahogany (*Swietenia macrophylla* King), another member of the Meliaceae family (ITTO 2009). Because of its economic importance, natural populations currently face serious problems associated with overexploitation and environmental degradation (ITTO 2002; Cavers et al. 2004; Lamb et al. 2005). Natural populations of *C.*

*odorata* have suffered large declines due to logging and reduced habitat destruction (Navarro et al. 2004). The International Union for Conservation of Nature (IUCN 2004) lists *C. odorata* as a species that faces a high risk of extinction in the wild in the medium-term future.

Intensive cultivation of *C. odorata* in commercial plantations, which could help alleviate the pressure on natural populations, has been hampered by the borer *Hypsipyla grandella* Zellar (Insecta: Pyralidae). This moth, whose larvae feed on the apical meristem of young trees in the Meliaceae family, causes lateral branching. As a result, the trunk becomes deformed and inadequate for the timber industry (Keay 1996; Briceño–Vergara 1997; Valera 1997; O'Neil et al. 2001). In natural populations, *C. odorata* is genetically diverse, and it is possible to find individuals with a certain degree of resistance to this insect's attack (Newton et al. 1993, 2001; Cavers et al. 2004; Navarro et al. 2004); however, domesticated clonal lines or varieties with this characteristic have been difficult to produce through traditional breeding technologies. As an alternative to classic silviculture techniques, clonal forestry, particularly *in vitro* propagation, has potential to improve the quality and yield of perennial species that have not yet been domesticated (Giri et al. 2004; Robert et al. 2006b). Moreover, the micropropagation of tree species, particularly in *Pinus* and *Populus*, has proven to be a rapid strategy for producing homogeneous clonal planting stock useful for commercial plantations, forest restoration (Merkle et al. 1990; Sutton 2002; Häggman et al. 2006), woody biomass production, and conservation and rejuvenation of elite or rare germplasm (Giri et al. 2004).

Rejuvenation is the *in vitro* manipulation of mature tissues to recreate juvenile phenotypical characteristics such as shoot architecture, rooting, vigor, multiplication rate, and orthotropism (Franclet 1983). Rejuvenation has been reported in *Sequoia sempervirens* (Fouret et al. 1984; Franclet et al. 1987; Franclet and Franclet–Mirvaux 1992), *Sequoiadendron giganteum* (Monteuuis and Bon 1989), *Pseudotsuga menziesii* (Timmis et al. 1992), *Betula papyrifera* (Struve and Lineberger 1988; Brand and Lineberger 1992a, b), *Prunus* sp., *Eucalyptus gunnii*, *Eucalyptus citriodora*, *Pinus pinaster*, and *Salix babylonica* (Franclet et al. 1987). The application of rejuvenation technologies to tissues from mature *C. odorata* plants could be a key factor in the establishment of plantations with elite characteristics. *In vitro* cloning of individuals with proven *H. grandella* resistance will guarantee enough *C. odorata* plant material to not only elucidate the genetic basis of this insect resistance but also isolate and massively propagate *H. grandella*-resistant varieties.

*In vitro* clonal mass propagation presents well-known challenges that are mainly related to production costs, which are due to the intense hand labor required and the losses suffered during acclimatization to the soil (Smith 2000). Reports on other tropical forest species suggest that temporary immersion culture may overcome these challenges by significantly increasing the number and quality of micropropagated plants. Favorable results with this technique have been reported for *Hevea brasiliensis* (Etienne et al. 1997), *Eucalyptus* species (Castro and González 2002), and *Agave* species (Robert et al. 2006b). The bioreactor design is an important element of the successful application of temporary immersion cultures to various stages of the micropropagation process of some species. Successful *in vitro* micropropagation of large plantlets has been reported for agave using the BioMINT® bioreactor (Robert et al. 2006a). Preliminary results by our group on the culturing of foliage species such as *Musa accuminata* and *Capsicum chinensis* confirmed the suitability of this bioreactor system, which was specifically designed to reduce the costs of micropropagation and to increase the quality and acclimatization survival rate of *in vitro*-produced plants. Previous work on the *in vitro* culturing of *C. odorata* include apical shoot culture and followed by encapsulation in alginate (Maruyama et al. 1997a, b) and the micropropagation of trough nodal shoots and hypocotyl fragments derived from seedlings (Maruyama et al. 1989; Cerdas et al. 1998; Valverde et al. 1998; Pérez et al. 2002). However, an optimized system for the high multiplication rates essential for mass clonal propagation of mature elite trees is not yet available.

We present here the development and optimization of an *in vitro* culture system for the production of *C. odorata* propagules using explants from both seedlings and mature trees. Using modified TY17 medium (Gonzalez-Rodríguez and Peña-Ramirez 2007) and the BioMINT® temporary immersion system (Robert et al. 2006a), we report high multiplication and *ex vitro* transplantation rates for the efficient large-scale propagation of this species.

## Materials and Methods

*Plant material.* Following recommendations described by Longman (1993), 30-year-old *C. odorata* elite trees were selected from natural populations around Acayucan, Veracruz, Mexico (18°02′40.97″ N; 94°51′14.82″ W). The selection was based on the trees' anatomical features (straight trunks at least 10 m long and 50 cm in diameter) and no signs of *H. grandella* attack (Briceño-Vergara 1997). Ripe fruits and 40-cm long twigs were collected from five of these trees between February and May in both 2005 and 2006. The collected vegetative materials were immediately transferred to the laboratory. Ripe fruits were incubated at 25°C and 10% relative humidity until spontaneous opening occurred, and mature seeds were released. The resulting seeds were

mixed with 1% (*w*/*w*) each of Benomyl® (DuPont; Wilmington, DE), Agrimycin® (Pfizer; New York, NY), and Captan® (Bayer; Leverkusen, Germany) and then kept at room temperature in a black plastic bag for a maximum period of 4 mo.

*Shoot induction from mature tissues.* Two hundred twigs of *C. odorata* mature trees (40 cm in length) were immediately grafted onto juvenile trees (Longman 1993), while 200 other twigs were established directly in soil beds in order to generate axillary buds (Xavier et al. 2003). The grafting process was performed by applying side wedge cuttings on the young trees used as rootstocks and "V" cuttings on the twigs used as scions (Fig. 1*A*). Both the twigs and grafted plants were kept under greenhouse conditions at 35°C and 80% humidity. One week after grafting, young trees were decapitated to induce shoot formation. The apical shoots were eliminated completely by cutting about 10 cm below the apex with removal of all the leaves. The plants were kept under the same greenhouse conditions for three additional weeks. During this period, the responding axillary buds from the rootstocks were excised. After 4 wk, the formation of new shoots was quantified, and the sprouting index was determined. The percentage of sprouting in both the rejuvenated twigs and soil-planted twigs was calculated as follows: 100×(number of responding axillary buds/total axillary buds). The 25-mm-long shoots from these twigs were collected for further use.

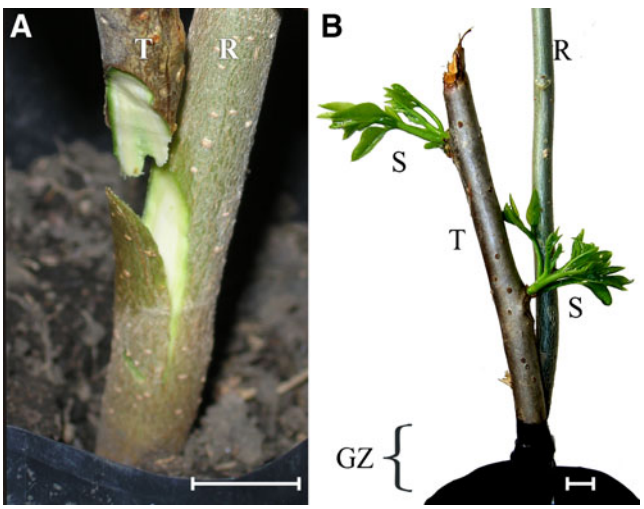


**Figure 1.** *C. odorata* grafting. Young trees (about 3 mo of age) were used as rootstock for the grafting of twigs cut from mature elite red cedar trees. *A*, View of the *C. odorata* grafting process. The cut was performed in a young tree used as the rootstock (*R*) and the scion is a twig (*T*) ready to be placed on the wound. *B*, View of an established twig 4 wk after grafting. The new shoots (*S*) present in the mature twig are noteworthy. *GZ* corresponds to the grafting zone observed in *A*. *Bars* correspond to 1-cm scale.

*Disinfestation and culture establishment.* Shoots excised from twigs and seeds were disinfested by immersing them in a 15% (*v*/*v*) sodium hypochlorite solution (Clorox®; Oakland, CA) supplemented with 200 µl $L^{-1}$ of polyoxyethylene sorbitan monolaurate (Tween 20®) for 20 min, followed by three rinses of 5 min each with sterile, distilled water. The micropropagation ability of axenified shoots from mature twigs was evaluated. The disinfested seeds were germinated aseptically in 2-L, sterile Erlenmeyer flasks (100 seeds/flask) using cotton soaked with distilled water. Seed germination and all of the subsequent culture steps were carried out in a culture room at 28°C with a photoperiod of 16 h light (40 µmol $m^{-2}$ $s^{-1}$; Sylvania T12 Gro–Lux®; Waltham, MA) and 8 h dark. After 2 wk, the emerging seedlings were used as explants for medium optimization experiments. All analytical chemicals, unless stated otherwise, were purchased from PhytoTechnology Laboratories (Lenexa, KS).

*Optimization of culture medium for organogenic induction of adventitious shoots.* To micropropagate *C. odorata*, ten hypocotyl segments (9 mm long) excised from 2-wk-old seedlings were placed horizontally in baby food jars containing 25 ml of semisolid TY17 medium 1× WPM basal medium (Lloyd and McCown 1981), 0.5× MS vitamins (Murashige and Skoog 1962), supplemented with 0.1% (*w*/*v*) activated charcoal, 2% (*v*/*v*) coconut water (CW), 26.02 µM citric acid, 28.38 µM ascorbic acid, 80 mM sucrose, and 0.25% (*w*/*v*) Gelrite®. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121°C for 15 min (Gonzalez-Rodríguez and Peña-Ramirez 2007). When specified, various concentrations of other components were added: coconut water (5%, 10%, 20%, and 50% *v*/*v*), dicamba (4.5 and 13.5 µM), and other cytokinins, including benzyl adenine (BA), 6-γ,γ dimethyl allyl aminopurine (2iP), or zeatin (Zea) at 0, 4.5, and 13.5 µM. The jars were placed in a culture room with the conditions described earlier. After 3 wk, shoot formation was evaluated as the ratio of newly generated adventitious shoots per explant.

*Successive micropropagation rounds.* To determine the efficiency of the multiplication process, shoots formed *in vitro* were subcultured in TY17 medium, which contained 20% (*v*/*v*) CW and 9 µM gibberellic acid ($GA_3$). After 6 wk in culture, the propagated shoots were counted, measured, and cut into 9 mm internode segments to be used as fresh explants for a new round of adventitious shoot induction under the same culture conditions. This process was repeated three times. A sample of 200 shoots from every multiplication round was elongated and rooted in baby food jars containing 25 ml of half-strength semisolid MS medium, which contained 80 mM sucrose, 9 µM gibber-

ellic acid ($GA_3$) and 0.25% (*w*/*v*) Gelrite®. After 9 wk in the culture room, the plants were established in soil.

*Adventitious shoot induction in roots, leaves, and epicotyls.* To compare the organogenic potential of different tissues, adventitious shoot induction was studied in 9 mm long segments from roots and epicotyls as well as 9 mm leaf disks derived from 2-wk-old seedlings. Under the same conditions described above, BA, 2iP, or Zea was added to the TY17 medium at 0, 4.5, 13.5, and 45 μM with or without 20% (*v*/*v*) CW. After 3 wk in culture, the newly formed adventitious shoots were counted.

*Micropropagation using mature plants.* Axillary shoots collected from soil-established and grafted twigs were used as a source of explants and were cultured as previously described: 9 mm long internode segments were placed in baby food jars containing 25 ml of TY17 medium with 20% (*v*/*v*) CW at a density of 10 explants per container. Plants were incubated as described above. These mature materials were evaluated over three micropropagation rounds and compared with juvenile hypocotyl explants.

*Shoot elongation and rooting using temporary immersion systems.* Around 2,000 adventitious shoots (approximately 20 mm long), which were derived from juvenile material from successive micropropagation rounds, were employed as explants for shoot elongation using temporary immersion bioreactors (BioMINT®) (Robert et al. 2006a, b). To evaluate the effects of sucrose and $GA_3$ on shoot elongation, three concentrations of sucrose (40, 80, and 120 mM) and two concentrations of $GA_3$ (4.5 and 9 μM) were tested. In all cases, 30 individualized adventitious shoots were incubated in bioreactors filled with half-strength MS medium lacking Gelrite® and containing the appropriate amounts of sucrose and $GA_3$. The explants were immersed at a frequency of 1 min every 6 h and incubated at 28°C for a period of 6 wk with a change of fresh medium after 3 wk, as previously described (Robert et al. 2006b). The plantlets were measured under aseptic conditions. After 6 wk, the plantlets were placed again in the BioMINT® bioreactors for root induction in medium containing IBA at a final concentration of 1.48 μM. For comparative purposes, plants were also rooted in semisolid medium containing 0.25% (*w*/*v*) Gelrite®, 80 mM sucrose, and two concentrations of $GA_3$ (4.5 and 9 μM). After 6 wk in culture, the roots were measured and the plants from both conditions were transferred to the soil.

*Acclimatization and soil transfer.* A sample of 50 rooted plants from each treatment was transferred separately to soil in seed germination trays containing wet Germination Mix® (SunGro Horticulture; British Columbia, Canada) and was kept in a greenhouse for 1 mo. The plants were then transplanted to plastic bags containing a wet mixture of 70%/30% (*w*/*w*) peat moss sphagnum (SunGro Horticulture. British Columbia, Canada) and perlite (Perlita de La Laguna S.A. de C.V. Saltillo, México) with an initial pH value of 5.7. Trees were maintained under greenhouse conditions for 3 mo. When the plants reached a minimum size of 50 cm, they were transplanted to the field in an experimental plantation located at the Instituto Tecnológico Superior de Acayucan, Veracruz, México (18°02′39.68″ N; 94°55′23.40″ W). The survival rate of the established plants was calculated 6 mo later as the ratio of surviving plants per total plants transferred to the soil.

*Statistical analysis.* All assays were conducted by employing a minimum of 50 explants for *in vitro* experiments and 50 individuals for *ex vitro* experiments and were repeated three times (except for the soil establishment experiment). The data were analyzed using standard ANOVA procedures. The differences between the means were determined by Fisher's least significant difference (LSD) test with the assistance of Statistica® software package (StatSoft Tulsa OK).

## Results

Micropropagation from elite mature trees was attempted through two separate but complementary approaches: the rejuvenation of elite material by *ex vitro* and *in vitro* culturing of shoots derived from mature material and an optimized hypocotyl micropropagation protocol suitable for maintaining efficient propagation of *C. odorata* vegetative material.

*Rejuvenation of selected materials.* Material collected from mature elite trees was established directly in soil or grafted onto juvenile material as described in the "Materials and Methods" section; 7.5% of the grafted twigs yielded new shoots while over 90% of the twigs established directly on soil yielded shoots (Table 1, the sprouting index). The hypocotyls of these sprouting shoots (Fig. 1) were successfully established *in vitro* in optimized TY17 medium (see the "Adventitious shoot induction" section) and then evaluated for their ability to produce adventitious shoots.

*In vitro propagation of rejuvenated materials.* From both soil-established and grafted twigs, well-formed shoots approximately 25 mm long (Fig. 1*B*) were excised and then cultured in optimized semisolid TY17 medium in

**Table 1.** Organogenic responses in mature and juvenile *C. odorata*

| Explant source | Sprouting index (percentage) | Shooting index[x] |
|---|---|---|
| Mature twigs in soil | 95±2.1 | 0.6±0.27a |
| Mature twigs grafted | 7.5±1.0 | 1.5±0.47b |
| Juvenile (Hypocotyl) | NA | 4.7±0.12d |

The number of shoots formed from axillary buds on mature twigs was quantified and is reported as the sprouting index. Adventitious shoots were used as the explant source for *in vitro* micropropagation; here, hypocotyl explants were used as an adventitious shoot induction reference. After 4 wk of culture in TY17 medium supplemented with 20% coconut water and 13.5 μM dicamba, adventitious shoots were quantified and are reported as the shooting index

*NA* not assayed

[x] Data in this column represent an average ± mean standard error of four subsequent rounds of micropropagation. *Letters* indicate significant differences by LSD tests at $P \leq 0.05$, $n=20$

order to evaluate the ability of the rejuvenated material to proliferate *in vitro*. The micropropagation results, as depicted in Table 1, indicate that an average of only 0.6 and 1.5 new shoots per explant were produced by the soil-established and grafted twigs, respectively. Successive subcultures (four rounds) did not alter these results. Experiments aimed at increasing the biomass are currently being performed.

*Effect of coconut water and dicamba on adventitious shoot induction.* To micropropagate *C. odorata*, our group previously developed TY17 medium (Gonzalez-Rodríguez and Peña-Ramirez 2007). With this formulation, we have been able to induce both proembyogenic calli and adventitious shoot formation on *C. odorata* hypocotyl segments harvested from *in vitro* germinated seedlings. This report focuses on the organogenic induction of adventitious shoots for micropropagation.

To induce optimal adventitious shoot induction, three different concentrations of dicamba (0, 4.52, and 13.57 μM) and five different concentrations of CW (0%, 5%, 10%, 20%, and 50%) were used to supplement TY17 medium in a 3×5 matrix arrangement. In all treatments, adventitious shoots developed from the 9-mm hypocotyl explants after 3 wk in culture. Medium containing 13.5 μM dicamba and 20% (*v*/*v*) CW induced the highest number of slightly vitrified, deep-green, and well-defined adventitious shoots (Fig. 2*A*). Based on our previous experience, material showing this phenotype is the best explant for micropropagation. The number of shoots formed increased with the CW concentration (Fig. 3). Supplementation with 5% (*v*/*v*) CW in TY17 medium increased the shooting index only slightly from media lacking CW, but higher levels of CW yielded averages of 2.94, 3.78, and 3.8 shoots per explant when 10%, 20%, and 50% CW, respectively, was added. Dicamba further increased the number of shoots in the presence of 20% or 50% CW, but not at 10% CW. The highest number of shoots induced per explant was 4.6 at 13.5 μM dicamba and 20% CW. As previously reported (Gonzalez-Rodríguez and Peña-Ramirez 2007), some proembryogenic-like callus formation was also observed in all treatments (Fig. 2*B*).

*Effects of cytokinins and coconut water on adventitious shoot induction.* To establish a defined medium for adventitious shoot induction, CW was replaced with various cytokinins (BA, 2iP, or Zea) at four different concentrations (0, 4.5, 13.5, and 45 μM). Table 2 shows that none of the tested cytokinins was as effective as CW at the concentrations used, although BA and Zea had a positive effect at 13.5 μM. In all cases, a slight reduction was observed at the highest concentration (45 μM), indicative of a supra-optimal level. The concentration of 13.5 μM BA was the most effective treatment in the absence of CW, generating an average of 2.68 new shoots per explant. In the presence of 20% CW, none of the cytokinins increased the number of shoots formed; however, at 45 μM BA, the same supraoptimal reduction was observed (Table 2).

*Effects of coconut water and cytokinins on adventitious shoot induction from different explant sources.* To determine the organogenic potential of other tissues, the above experiment was repeated on root, leaf, and epicotyl explants (Table 2). The response of all of these tissues was inferior to that of the hypocotyls irrespectively of the presence or absence of CW. The roots were the most second most responsive to CW, whereas the leaf tissues were the least responsive. When epicotyl segments were used as explants, the addition of any of the three cytokinins without CW had a slightly positive effect on the formation of shoots. Zeatin appeared to be the most effective cytokinin when CW was not present in the medium, particularly on the roots, inducing an average of 1.4 adventitious shoots per explant. All shoots were able to sustain successive micropropagation rounds with the same efficiency as those from other sources (data not shown).

*Effects of dicamba, coconut water, and BA on adventitious shoot induction.* Finally, we investigated the effects of adding dicamba at three different concentrations (0, 4.5, and 13.5 μM) to TY17 medium supplemented with 13.5 μM BA with or without 20% (*v*/*v*) CW. Figure 4 shows that addition of 4.5 μM dicamba did not produce a significant increase in *C. odorata* shooting frequency, but a two-fold increase was observed at 13.5 μM. Although this increase was significant, all of the treatments that contained CW surpassed this value (Fig. 4). The best shooting

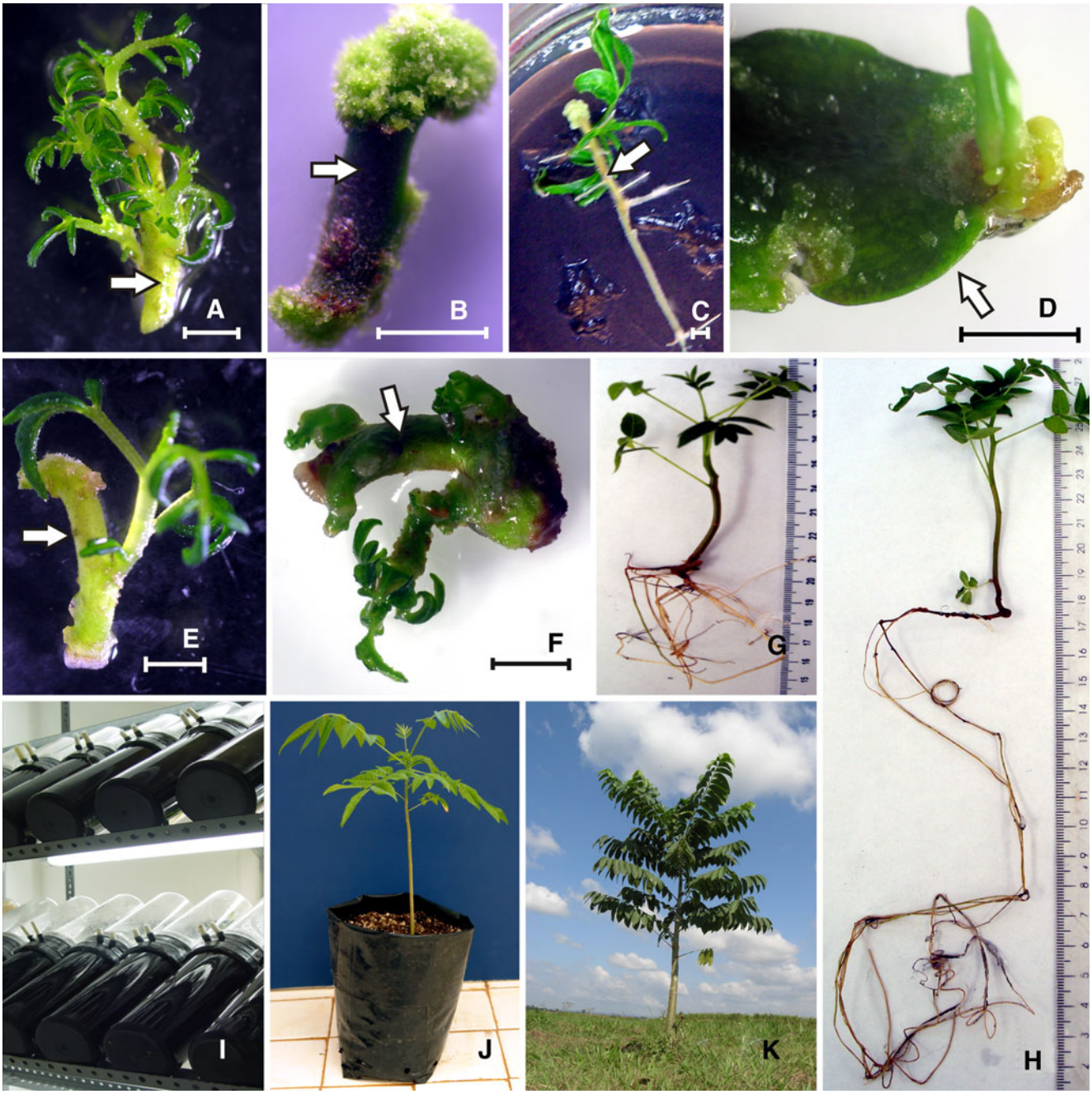


**Figure 2.** *In vitro* regeneration of *C. odorata*. *A*, Typical adventitious shoot induction from hypocotyl fragments. *B*, Proembryogenic-like calli formed on an explant. Adventitious shoot formation from roots (*C*), leaves (*D*), or epicotyls (*E*). *F*, Adventitious shoot formation on stem fragments from vegetative mature material. *G*, *H*, Whole architecture of a typical propagule rooted in semisolid medium (*G*) or in a temporary immersion culture system (*H*). *I*, BioMINT® bioreactors used for temporary immersion culture. *J*, Micropropagated tree transferred to soil. *K*, Micropropagated tree established in a field. *Bars* from (*A*) to (*F*) correspond to 3 mm. *G*, *H*, scaled in centimeters. *Red arrows* from (*A*) to (*F*) point to mother tissue.

frequency was obtained with the treatment that contained 20% CW, 13.5 μM BA, and 13.5 μM dicamba. The addition of BA, however, did not substantially increase the shoot frequency value when compared with the treatment that just contained 20% CW and 13.5 μM dicamba.

*Successive micropropagation rounds*. To calculate both how many plants can potentially be produced using this optimized medium and its protocol's reproducibility in subsequent micropropagation rounds, shoots derived from the experiment in Fig. 3 were employed for three extra

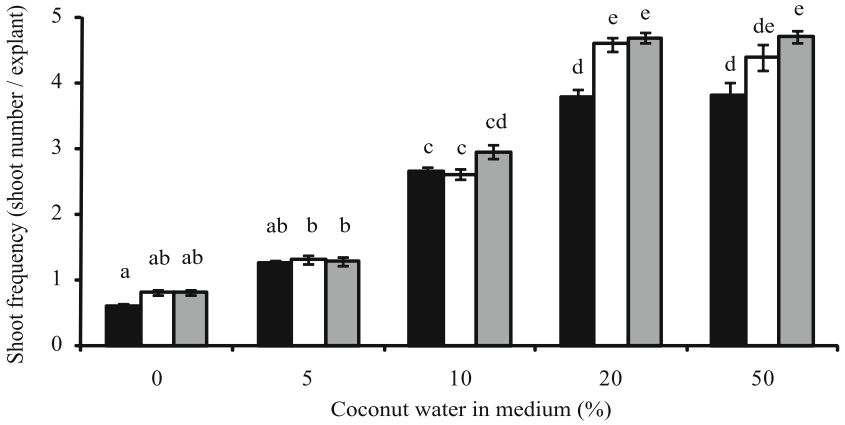


**Figure 3.** Effects of coconut water and dicamba on adventitious shoot regeneration. Hypocotyl explants 9 mm long were excised from 2-wk-old seedlings and incubated in TY17 medium containing increasing concentrations of coconut water with dicamba at 0 μM (*black bars*), 4.52 μM (*white bars*), and 13.57 μM (*gray bars*). The mean standard errors are shown *above the bars* and the *letters* indicate significant differences by LSD tests at $P \leq 0.05$, $n = 50$.

rounds of induction. The 9-mm-long internode stem segments were placed on semisolid TY17 medium containing 20% CW, 13.5 μM BA, and 13.5 μM dicamba. As shown in Table 3, the multiplication rate remained constant from one micropropagation round to the next with an average of 18 new explants obtained from each cultured hypocotyl segment. On average, 4.68 new shoots each yielded 3.8 stem segments of adequate size for further induction.

*Elongation, rooting, and soil establishment.* Rooting and elongation of *C. odorata* shoots were evaluated using BioMINT® temporary immersion culture bioreactors (Robert et al. 2006a) and standard semisolid culture. The effects of gibberellic acid ($GA_3$) at three concentrations (0, 4.5, and 9 μM) and sucrose at three concentrations (40, 80, and 120 μM) were tested in half-strength MS medium. After 6 wk in culture, the shoot length was measured. As

**Table 2.** Effect of several cytokinins and coconut water on shoot induction in several explants

| Explant | PGR added (μM) | Without coconut water | | | 20% coconut water | | |
|---|---|---|---|---|---|---|---|
| | | BA | 2iP | ZEA | BA | 2iP | ZEA |
| Hypocotyl | 0.0 | 0.7±0.2ab | 0.7±0.2ab | 0.7±0.2ab | 4.5±0.2ef | 4.6±0.2ef | 4.6±0.3ef |
| | 4.5 | 0.9±0.2ab | 0.4±0.2a | 1.3±0.2ab | 4.6±0.2ef | 4.4±0.3ef | 4.5±0.3ef |
| | 13.0 | 2.6±0.2bcde | 1.1±0.2ab | 1.8±0.2abc | 4.6±0.3ef | 4.7±0.1f | 4.7±0.1f |
| | 45.0 | 2.3±0.1abcd | 0.8±0.1ab | 1.7±0.1abc | 3.7±0.2cdef | 4.1±0.3def | 4.1±0.4def |
| Root | 0.0 | 0.2±0.1a | 0.2±0.1a | 0.2±0.1a | 2.2±0.0f | 2.2±0.2f | 2.2±0.2f |
| | 4.5 | 0.3±0.1a | 0.4±0.2a | 0.6±0.2ab | 2.2±0.2f | 2.0±0.3ef | 1.8±0.3def |
| | 13.0 | 0.7±0.1abc | 0.5±0.2ab | 1.2±0.2bcd | 2.3±0.2f | 2.1±0.2ef | 2.2±0.2f |
| | 45.0 | 0.6±0.2ab | 0.5±0.2ab | 1.4±0.2cde | 1.7±0.2def | 1.6±0.2def | 2.3±0.2f |
| Leaf | 0.0 | 0.1±0.1a | 0.1±0.1a | 0.1±0.1a | 0.2±0.1a | 0.3±0.1ab | 0.3±0.1ab |
| | 4.5 | 0.3±0.1ab | 0.2±0.1a | 0.4±0.2ab | 0.9±0.2ab | 0.7±0.2ab | 0.9±0.2ab |
| | 13.0 | 0.6±0.2ab | 0.3±0.1ab | 0.9±0.2ab | 0.9±0.2ab | 0.9±0.1ab | 1.3±0.1b |
| | 45.0 | 0.8±0.1ab | 0.5±0.2ab | 0.7±0.2ab | 0.6±0.2ab | 0.6±0.2ab | 0.7±0.1ab |
| Epicotyl | 0.0 | 0.3±0.1a | 0.3±0.1a | 0.4±0.2a | 1.1±0.1abc | 1.1±0.1abc | 1.1±0.1abc |
| | 4.5 | 1.0±0.2abc | 0.4±0.2ab | 1.4±0.2abc | 1.7±0.2c | 1.6±0.2bc | 1.7±0.1c |
| | 13.0 | 1.3±0.1abc | 0.7±0.1abc | 1.6±0.2bc | 1.5±0.2abc | 1.1±0.2abc | 1.9±0.1c |
| | 45.0 | 1.3±0.1abc | 0.7±0.1abc | 1.6±0.2bc | 1.3±0.2abc | 0.9±0.2abc | 1.7±0.2c |

Explants (9 mm long) were excised from 2-wk-old seedlings. They were placed on different shoot induction media containing the cytokinins BA, 2iP, or ZEA at several concentrations. The shoots were quantified 6 wk later. Average values ± mean standard errors are shown. The *letters* next to values correspond to significant differences by LSD tests at $P \leq 0.05$, $n = 50$. Mean comparison analysis were performed independently per explant type.

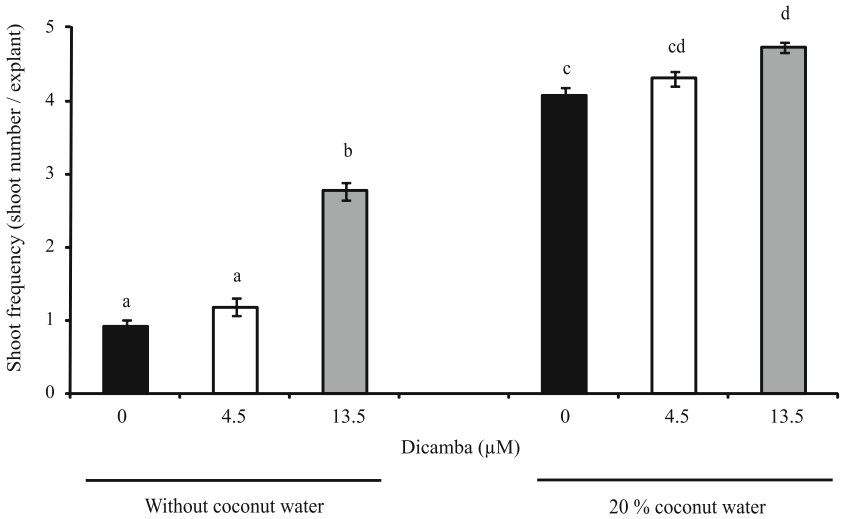


**Figure 4.** Effect of combinations of dicamba, benzyl adenine, and coconut water on shoot induction from hypocotyls. Hypocotyl explants (9 mm long) were excised from 2-wk-old seedlings. The hypocotyls were placed on different shoot induction media containing 13.5 μM benzyl aminopurine with or without coconut water and dicamba at several concentrations (0 μM, *black bars*; 4.5 μM, *white bars*; and 13.5 μM, *gray bars*). The shoots were quantified 6 wk later. The mean standard errors are shown *above the bars* and the *letters* indicate significant differences by LSD tests at $P \leq 0.05$, $n=50$.

shown in Fig. 5*A*, in the absence of $GA_3$, the level of sucrose did not have any significant effect on shoot elongation; however, $GA_3$ did increase shoot elongation at all sucrose concentrations (white and gray bars). The 120-μM sucrose concentration was supraoptimal in all cases. A synergistic effect between 80 mM sucrose and 9 μM $GA_3$ (gray bar) produced the longest shoots (11 cm), which represent a 350% increase when compared to the elongation reached in semisolid culture (3 cm) under the same culture conditions.

Following a similar experimental design, rooting was achieved by supplementing TY17 medium with 1.48 μM IBA (Fig. 5*B*). When cultured by temporary immersion, the elongation of roots followed a pattern almost identical to the one observed for shoot elongation. The plants rooted in BioMINT® bioreactors produced roots three times longer and biomass (dry weight; data not shown) ten times higher than plants rooted in semisolid medium. However, significant structural differences were observed: longer primary roots and few lateral hairy roots were present in plants cultured in temporary immersion (Fig. 2*G* and *H*). Rooted plantlets were acclimatized in plastic bags (Fig. 2*J*) and transferred to the field (Fig. 2*K*) as described in "Materials and Methods." Three months later, a survival rate of 98% was calculated (Table 1).

## Discussion

Efficient protocols for *C. odorata* clonal propagation, whether from organogenesis or somatic embryogenesis, are essential for the genetic improvement, germplasm conservation, and mass propagation of this species. This work was aimed at establishing a system to massively propagate elite trees of *C. odorata* collected from a tropical forest in the southeast of Mexico near Acayucan, Veracruz. The main objectives were as follows: (a) development of a protocol for the rejuvenation of mature elite trees and (b) optimization of the culture conditions for mass micropropagation.

Rejuvenation of mature vegetative tissues is indispensable for high-yield propagation of selected *C. odorata* elite

**Table 3.** Shooting index, *in vitro* propagation, and soil establishment of *C. odorata*

| Micropropagation round | Initial explants | Shooting index | New explant/shoot | Yield | Soil establishment (%) |
|---|---|---|---|---|---|
| 1 | 192 | 4.62±0.27 | 3.94±0.08 | 18.23±0.35 | 98.33±1.19 |
| 2 | 1,000 | 4.56±0.80 | 3.82±0.40 | 17.43±1.20 | 98.33±0.26 |
| 3 | 1,000 | 4.66±0.70 | 3.86±0.30 | 18.01±1.00 | 98.14±0.26 |

*C. odorata* stem fragments were cultured for 4 wk in TY17 medium. The resulting adventitious shoots were excised to produce 9 mm stem explants, which were used to generate a second micropropagation round. The yield was calculated as the number of new explants obtained per single mother tissue. Average values ± mean standard error are shown. No significant differences between means were obtained by LSD tests at $P \leq 0.05$, $n=50$

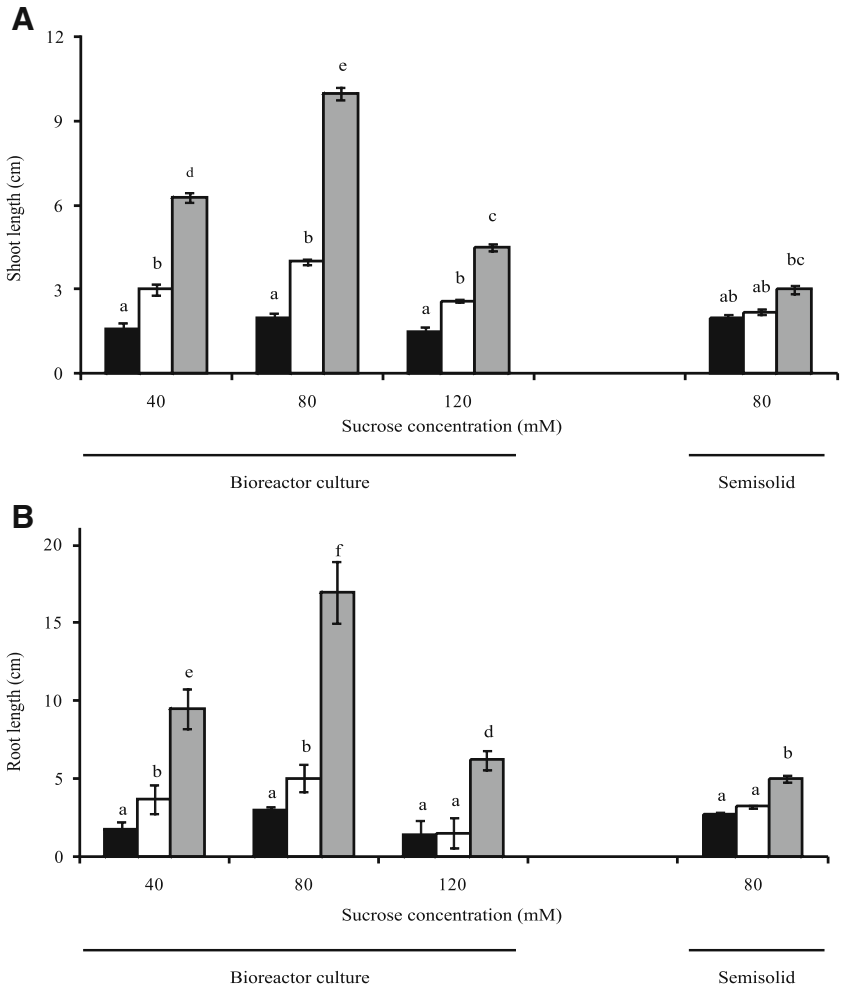


**Figure 5.** Combined effect of $GA_3$ and sucrose on shoot (*A*) or root (*B*) elongation employing BioMINT® bioreactors or semisolid medium culture. Nonrooted shoots (approximately 1 cm long) were incubated either in bioreactors at an experimental density of 25 explants per container or in baby food jars containing MS medium. Different concentrations of sucrose (40, 80, or 120 mM) and gibberellic acid (0 μM (*black bars*), 3 μM (*white bars*), and 9 μM (*gray bars*)) were tested. Six weeks later, shoot and root lengths were measured. The mean standard errors are shown *above the bars* and the *letters* indicate significant differences by LSD tests at $P \leq 0.05$, $n=50$.

materials. Our results indicate that more effort needs to be made towards this objective. At this point, our results do not indicate the extent to which the induction of axillary shoots from twigs established in soil or grafted results in rejuvenation of the tissues, as the emerging shoots performed poorly in micropropagation when cultured under our best conditions.

Twig grafting diminished the sprouting ability of axillary buds by nearly 90%. Several authors have previously shown that axillary bud induction in grafted material can be affected by a transmissible signal that possibly alters the gibberellin/cytokinin balance (Mapelli and Kinet 1992; Beveridge 2000). In *C. odorata*, grafting reduced the number of lateral shoots but allowed for the regeneration and multiplication of stem segments via *in vitro* micropropagation (Table 3). A large set of experiments to rejuvenate grafted scions with growth regulators is currently being undertaken in our laboratory. To optimize the culture medium for *C. odorata* micropropagation, we modified the previously described TY17 medium (Gonzalez-Rodríguez and Peña-Ramirez 2007) by supplementing it with various concentrations of CW. As shown in Figs. 2*A* and 3, CW had a significant positive effect on the rate of adventitious shoot induction. At the optimal concentration of 20%, CW produced the highest shoot induction frequency reported so far for this species (up to 4.68 shoots per explant). This finding is consistent with other reports revealing a positive effect of CW on tissue proliferation of juvenile and mature explants of woody species (Vengadesan et al. 2000; Rahargo and Litz 2002; Mechada et al. 2003; Moon et al. 2008). This result on tissue proliferation resembles a cytokinin-like effect, which might be due to the cytokinins present in CW (Ge et al. 2005; 2006).

To produce a defined culture medium, we tried to substitute the CW with various cytokinins. As shown in Table 2, cytokinins did induce adventitious shoot formation; however, none of the cytokinins produced similar multiplication rates as those obtained with 20% CW. These observations are common in tissue culture and can be explained by the presence of a very complex cocktail of cytokinins and other compounds in CW, which makes it irreproducible (Ge et al. 2004, 2005, 2006). In addition, organogenesis is a complex process in which components such as oligopectins and arabinogalactans play key roles as cell wall-priming molecules (Fernando et al. 2007; Popielarska-Konieczna et al. 2008). Since such biomolecules could affect the formation of the extracellular matrix, it is plausible that CW components may supply or trigger the biosynthesis these molecules, thereby affecting the number and quality of organogenic primordia clusters in *C. odorata* explants.

Dicamba, a growth regulator similar to auxins in its callogenic effect, is another component of the optimized TY17 medium. Dicamba had a slight but significant effect on adventitious shoot induction in the presence of CW (Table 2; Fig. 4). In general, the addition of either dicamba or BA only produced a weak increase in the number of shoots per explant, making the use of CW irreplaceable for the optimized TY17 medium. Further efforts to obtain a defined medium are in process. We are currently exploring higher auxin/cytokinin ratios and different plant growth regulators, including anti-auxins and abscisic acid. In addition, CW induced the development of adventitious shoot formation on the epicotyls, roots, and leaves of *C. odorata* seedlings (Table 2). The ability to use these tissues would increase the available starting material from a unique selected mother plant, even though the efficiency is much lower than when hypocotyls are used as explants.

Subsequent micropropagation rounds of *C. odorata* juvenile and mature explants showed that this method is efficient and reproducible (Table 3), which are essential properties for sustaining any mass propagation program. Our data indicate that after the first micropropagation round, a single explant can produce between four and five new shoots, each of which can be segmented into three to four new explants for a second micropropagation round. This method results in an average multiplication efficiency of 18 new explants per stem segment at each round. Considering that the resulting shoots need to go through an elongation stage, the whole process takes 9 wk per cycle. Therefore, over a period of 6 mo, a single mother plant from which three hypocotyl segments are cut could yield a minimum of 50 ($3 \times 4.6 \times 3.8 = 52.4$) stem segments in the first round of culture, 900 ($50 \times 4.6 \times 3.8 = 916$) in the second, and around 16,000 in the third. This multiplication rate is the most efficient method for the micropropagation of *C. odorata* reported thus far and is at least twice as efficient as that reported for *C. fissilis* (Costa-Nunes et al. 2002).

When compared to micropropagated juvenile material derived from seedlings, the mature explants were significantly less responsive to shoot induction (Table 1). Notably, for shoots derived from grafted twigs, the micropropagation rate was at least two times higher than the values obtained from soil-planted twigs (Table 3). This observation is consistent with other reports on rejuvenation of mature trees; for example, an up to 10-fold increase of lateral branching was reported on mature explants grafted onto juvenile trees of *S. sempervirens* (Huang et al. 1992, 2003). The balance of cytokinins has been proposed as the main factor responsible for rejuvenation in grafted material (Valdés et al. 2002, 2003a, b), and the level of juvenility of the receptive material may also be critical (Franclet et al. 1987). Additional studies, such as successive grafting and subculturing in temporary immersion bioreactors, are being carried out to induce juvenility in *C. odorata* mature trees.

Shoot and root elongation of *C. odorata* propagules was achieved more efficiently in temporary immersion culture using BioMINT® bioreactors, as both tissues grew much better than in semisolid culture medium (Fig. 5). Furthermore, the *ex vitro* survival rate of plants grown in BioMINT® bioreactors was near 98%, indicating that the plantlets were pre-acclimatized for transfer to *ex vitro* conditions. Similar results have been obtained in other species (Etienne and Berthouly 2002; Robert et al. 2006b). These results clearly show that the BioMINT® system is an efficient preadaptation method.

The *C. odorata* micropropagation system reported here is thus far the most efficient propagation method reported for this species in terms of numbers of regenerated plants per explant and successful acclimatization and establishment. However, the lack of a defined medium is a disadvantage that might pose standardization problems. Our laboratory is committed to the development of new alternatives for efficient *C. odorata* clonal propagation through either organogenesis or somatic embryogenesis.

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