RESEARCH

IN VITRO PROPAGATION OF CEDAR (*Cedrela odorata* L.) FROM JUVENILE SHOOTS

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Cedrela odorata L. is one of the most important timber species currently traded in the Caribbean and Central America; however, it has been intensively exploited. *In vitro* techniques and clonal propagation can help to develop new plantations and assist in establishing improvement programs for this species. The aim of this study was to develop a protocol to establish *in vitro* conditions and to micropropagate this species from nodal explants from juvenile cuttings taken from field trees. Disinfection of node explants with 5% propiconazole CE 25 during 3 min resulted in 100% explant disinfection and 60% morphogenic response on those established explants. Shoot development was optimized by cultivating *in vitro* node explants in Murashige and Skoog basal medium supplemented with 2 mg L^{-1} 6-bencilaminopurine and 3 mg L^{-1} naphthaleneacetic acid. This medium resulted in 100% shoot development from the *in vitro* node explants with a 3.93 cm mean height. Rooting was also stimulated 6 wk after individualization of the regenerated plants on the same micropropagation medium with a mean of 3.9 roots per plant. *In vitro* plants did not show morphologic differences when compared to *ex vitro* seeds.

Key words: Organogenesis, *in vitro* propagation, *Cedrela odorata*, *in vitro* rooting, plant growth regulators.

Cedrela odorata L. (cedar, red cedar, Cuban cedar, and Mexican cedar) is a high-value timber species that has been considered vulnerable because of intense exploitation of its natural populations and the lack of intensive propagation systems allowing the establishment of commercial plantations (Cintron, 1990; Patiño, 1997; Plasencia, 1998). In the Americas, the development of hardwood plantations of the Meliaceae family (*C. odorata* and Mahogany) is limited because of the long life cycles of these species, their susceptibility to pest attack, and the lack of farmer interest to plant species with profits in the long term (Ramos, 1998; Pérez, 2006). Moreover, the current propagation of this species is not supporting the fast reproduction in natural forests to sustain its increased demand.

In vitro techniques applied to clonal propagation can help mitigate these drawbacks and provide farmers with high quality planting material (Yanchuk, 2000; Sasson, 2001). The use of modern *in vitro* techniques on woody trees has brought new possibilities not only for rapid tree species multiplication, but also for *ex situ* conservation of important germplasms (Husain *et al*., 2008).

Together with providing multiplication in a limited time and space, *in vitro* propagation of plants (micropropagation) avoids the limitations of the long regeneration cycle of ligneous species and reduces the dependence on sexual reproduction (García-Gonzáles *et al*., 2010). However, *in vitro* culture of woody species is considered very difficult to achieve due to the low regeneration potential of woody tissues and contaminant microorganisms that affect morphogenic efficiency and plant recovery (Husain and Anis, 2009). On the other hand, phenolic compound production from woody tissues also interferes during explant establishment, and phenol production interferes with morphogenetic processes (Husain and Anis, 2009).

Advances in plant micropropagation of *C. odorata* are not still in depth (Pérez *et al*., 2002). Discrete results for *C. odorata* micropropagation using apical buds and nodal segments, isolated from juvenile plants generated from seeds, have already been achieved (Maruyama *et al*., 1989a; 1989b; Orellana, 1997; Pérez *et al*., 2002; Rodríguez *et al*., 2003; Maruyama, 2006; Pérez, 2006). However, it has been found that *C. odorata* is characterized for its recalcitrance to *in vitro* culture due to bacteria and fungi, an oxidative response of *in vitro* tissues after disinfection, and low morphogenic response of explants (Maruyama, 1989b; Pérez *et al*., 2002).

Morphogenic response in *C. odorata* has been benefited by adding cytokinins to the culture medium

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and combining auxins and cytokinins in different concentrations (Maruyama *et al*., 1989a; Pérez *et al*., 2002). Rooting of *C. odorata* under *in vitro* conditions has been achieved by supplementing the basal medium with auxins (Pérez *et al*., 2006). Similarly, it was found that the mineral composition of the basal medium can be a significant factor in the morphogenic response of *in vitro C. odorata* plants (Pérez, 2006) and *Cedrela fisilis*, a species from the same genus (Costa Nunes *et al*., 2002).

Along with the abovementioned results, fundamental studies have been developed in very young plants, which limit their application in breeding programs based on the selection and multiplication of promising clones. Pérez (2006) found that the morphogenic response of *in vitro* tissues from 10-yr-old plants was variable; moreover, morphogenic response of the established tissues could depend on the juvenility of the donor plant tissues. Therefore, this study was conducted to establish a protocol for *in vitro* establishment and micropropagation of *C. odorata* from juvenile shoots taken from field trees.

MATERIALS AND METHODS

Plant material

Plant material was obtained from adult *C. odorata* plants previously located at the Seed Farm (21°20' N, 77°52' W) of the Experimental Agroforestry Station in the eastern Cuban province of Camagüey. The trees were 10 to 12 yr old with a mean height of 12 m.

Establishment of *C. odorata* from buds with shoot development

Cuttings were prepared from juvenile seasonal shoots to induce shoot development. They were placed in a sterile water solution containing 5 mg L^{-1} (22.2 μM) of 6-bencilaminopurine and maintained during 3 wk at 25 ± 1 °C under a 12:12 h photoperiod. Light intensity was regulated to 50 μmol m^{-2} s^{-1} in the growing chamber.

Evaluation of different bud disinfection methods

To introduce and establish *in vitro C. odorata*, juvenile shoots were selected from previously induced cuttings. Shoots up to 5 cm long were selected and extracted from the cuttings and prepared as 1.0- to 1.5-cm long nodals. For nodal surface disinfection of nodal segments, the following conditions were evaluated: 1) Sodium hypochlorite solution at 1 and 5% during 10, 20, and 30 min; and 2) propiconazole CE 25 solution ((2*RS*,4*RS*;2*RS*,4*SR*)-1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1*H*-1,2,4-triazole, Tilt, Syngenta Monthey AG, Monthey, Switzerland) at 1 and 5% during 3, 5, and 10 min.

The percentages of disinfected explants, shoot survival, shoot development from buds, and shoot rooting were evaluated.

For all the experiments, plant material was washed with detergent water for 15 min, and then washed three times with distilled sterile water. Tissues were dried on absorbent paper for 10 min and then established in the shoot development medium. Each treatment was replicated three times with 10 nodal segments per replicate. The homogeneity of the variance was determined through the Bartlett Test ($\alpha = 0.05$). Percentage data were transformed according to the expression $y = \sqrt{x}$. For the processing and analysis of the results an ANOVA and the Tukey HSD multiple range test ($p = 0.05$) were carried out in order to compare treatment means. All the statistical analyses were carried out with the Statgraphics Plus 5.0 software (StatPoint, Warrenton, Virginia, USA).

Disinfected nodal explants were established in MS (Murashige and Skoog, 1962) basal medium supplemented with 2 mg L^{-1} (8.88 μM) 6-bencilaminopurine (BAP), 30 g L^{-1} sucrose, and pH was adjusted between 5.6 and 5.7 according to the recommendation for this species (Pérez *et al*., 2002). Initial explants were maintained during 12 wk under a 12:12 h photoperiod and 100 μmol m^{-2} s^{-1} light intensity. Room temperature was maintained at 25 ± 1 ºC.

Effect of different culture media on shoot induction and rooting of *in vitro C. odorata* plantlets

For the multiplication step, the effect of indoleacetic acid (IAA) and naphthaleneacetic acid (NAA) at three concentrations on node and leaf formation, plant height, and rooting was evaluated. These parameters are decisive in establishing a micropropagation method when the multiplication rate is based on pre-existing buds from nodal segments.

Nodal segments were taken from 12-wk-old shoots induced in modified MS basal medium as already described. For shoot induction and efficient plant elongation, this medium was supplemented with the following auxin treatments: 1) IAA 1.0 mg L^{-1} (5.70 μM); 2) IAA 2.0 mg L^{-1} (11.42 μM); 3) IAA 3.0 mg L^{-1} (17.12 μM); 4) NAA 1.0 mg L^{-1} (5.37 μM); 5) NAA 2.0 mg L^{-1} (10.7 μM); and 6) NAA 3.0 mg L^{-1} (16.07 μM).

Explants were prepared as uninodal or binodal segments depending on size, approximately 5 mm long. These segments were cultured in their respective treatments at a 12:12 h photoperiod, 100 μmol m^{-2} s^{-1} light intensity, and 25 ± 1 ºC. Sucrose concentration in the basal medium was increased in this step to 50 g L^{-1}. Thirty nodal explants were evaluated per treatment, which were subcultured with a 4-wk frequency until the experiment's final evaluation. To induce root formation, developed shoots were separated from the original nodal segment and cultured in their respective culture medium without eliminating leaves and no explant manipulation.

Evaluating the number of nodal segments, leaf emission, and shoot height was carried out after an 8-wk culture of the nodal segments in each culture media. Root formation was evaluated after 6 wk from the time plants were individualized. Plant height was determined with graph paper; explants were placed on the paper, the initial

start of plant shoot development was established as the basal side, and the apical bud of the shoot as the superior point. Results were processed and analyzed by ANOVA and the Tukey HSD multiple range test ($p = 0.05$) in order to compare treatment means. All the statistical analyses were carried out with the Statgraphics Plus 5.0 software (StatPoint, Warrenton, Virginia, USA).

RESULTS AND DISCUSSION

Effectiveness of disinfection methods

Disinfection of *C. odorata* buds behaved in a complex way (Table 1), and coincided with previously documented results (García *et al*., 2004; Pérez *et al*., 2006). The 30-min disinfection with sodium hypochlorite at 1 and 5%, although effective for disinfection, produced tissue oxidation and death of all disinfected nodal segments. Washing the explants with sodium hypochlorite at 1% for 20 min yielded the best results with bud survival percentages significantly higher than the other treatments. Nevertheless, the percentage of bud disinfection diminished significantly and neither root formation nor shoot development of the nodal segments showed significant differences with the other sodium hypochlorite treatments.

Phytotoxicity symptoms, as a result of sodium hypochlorite treatment, were also observed as tissue depigmentation with progressive discoloration that was not recovered even in light conditions.

Eliminating the microorganism with Propiconazole CE 25 increased explant survival, shooting and rooting percentages as compared to the sodium hypochlorite treatments (Table 1). Shoot induction and development from buds occurred in 60% of the explants that survived the disinfection step; rooting was also observed in 40% of the explants. In both cases, results were statistically higher than the other treatments.

Treatments with high concentrations of propiconazole did not produce death as a result of tissue oxidation. Bud death was observed after depigmentation and a total or sectorized chlorosis of the tissue. The main losses caused by contamination occurred during the first week, and fungi and bacteria were the main contaminant agents that were apparently of environmental origin or present on the tissue surface. In subsequent propagation cycles, the presence of endogen contaminants in the *in vitro* plant material was not observed.

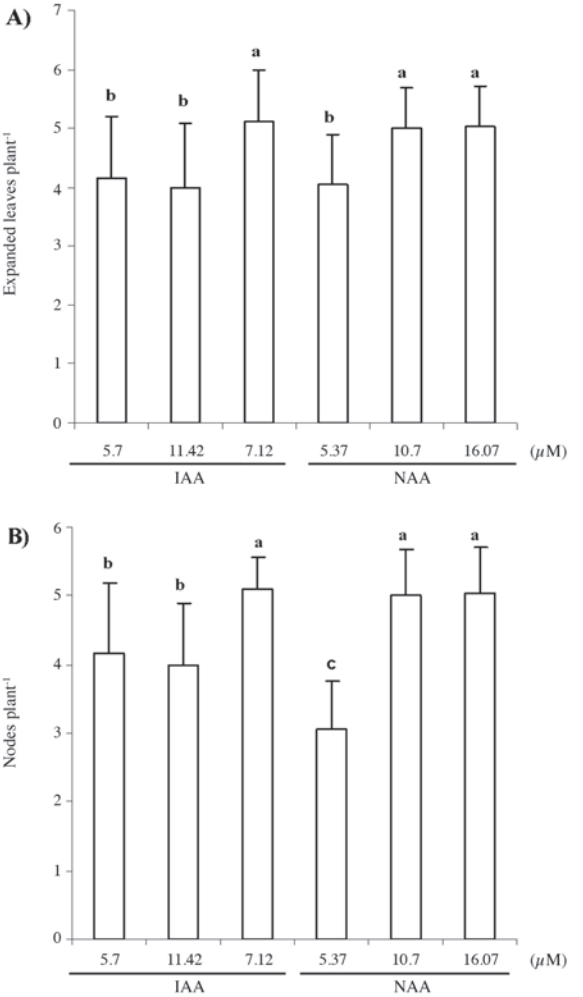
Table 1. Effectiveness of different disinfectants at different concentrations and exposure time on the disinfection and *in vitro* establishment of *Cedrela odorata* vegetative buds. Data were taken 16 wk after node establishment under *in vitro* conditions.

Treatment	Mean of disinfected nodal segments	Survival	Rooting	Shoot development
		%		
A	7.00 ± 1.00b	13.09 ± 12.54b	8.33 ± 14.43a	13.09 ± 12.54ab
B	10.00 ± 0.0c	0a	0a	0a
C	10.00 ± 0.0c	0a	0a	0a
D	10.00 ± 0.0c	0a	0a	0a
E	5.33 ± 0.58a	62.22 ± 3.85c	37.78 ± 3.85b	37.78 ± 3.85c
F	10.00 ± 0.0c	60 ± 0.0c	40 ± 10.00b	50 ± 0.0c
G	10.00 ± 0.0c	60 ± 10.00c	30 ± 10.00b	33.33 ± 11.55bc
H	10.00 ± 0.0c	46.67 ± 5.77c	26.67 ± 11.55b	30.00 ± 10.00bc

Treatments A to D: Sodium hypochlorite 1% (A, 20 min) (C, 30 min); 5% (B, 20 min) (D, 30 min). Treatments E to H: propiconazole CE 25 1% (E, 3 min) (G, 5 min); 5% (F, 3 min) (H, 5 min). Different letters mean that there are differences among treatments. ANOVA, Tukey HDS Test ($p < 0.05$). n = 3.

Morphogenic response of *C. odorata* nodes under different PGR treatments

Effect of PGR on leaf and stem development. Morphogenic response and plant development showed a direct relationship in the culture mediums with the best results (Figure 1). Treatments with 17.12 μM IAA, 10.7 μM, and 16.07 μM NAA induced the best leaf formation and stem development with significant differences with other treatments (Figure 1A). In these media, it



Data were taken 8 wk after node establishment. Different letters mean that there are significant differences among treatments. Tukey Test HSD ($p > 0.05$) n=30.

Figure 1. Effect of interaction of different concentrations of indole-3-acetic acid (IAA) or naphtaleneacetic acid (NAA) with 6-bencilaminopurine (BAP) (8.88 μM) on the morphogenic response of *Cedrela odorata* juvenile nodal segments under *in vitro* conditions. (A) Leaf emission. (B) Nodal segment emission.

was observed that the leaf lamina of the *in vitro* plants developed better, and it is probable that this effect is related to a better photosynthetic efficiency that has influenced plant development and higher rooting. In the case of the 17.12 μM IAA treatment, a similar number of leaves was observed with regards to the treatments with the best results. Likewise, the number of nodal segments was benefited more in the treatments supplemented with 17.12 μM IAA and NAA at 10.7 and 16.07 μM, and differed significantly from the other treatments (Figure 1B). The greater number of nodal segments can benefit a higher multiplication rate and greater efficiency of the *in vitro* propagation protocol. In woody species, propagation from nodal segments can be limited by the low nodal formation and scarce separation of these, which complicates its manipulation. For *Melia azedarach* L., a good response has been obtained in nodal segments cultured in MS medium supplemented with IAA (0.06 µM) and BAP (4.44 µM), as well as with IAA and kinetin (4.65 µM) (Thakur *et al*., 1998).

Nevertheless, morphogenic response in *C. odorata* has been obtained with relatively low cytokinin levels (Pérez *et al*., 2002; Pérez, 2006). In the case of somatic embryogenesis, embryo germination was achieved with lower concentrations of both growth regulators (Muñoz Tuesta, 2003). For *S. macrophylla,* it was found that 0.5 mg L^{-1} (2.46 µM) of indole-3-butyric acid (IBA) did not have a significant influence on shoot production and stem development compared with cytokinin 2-isopentenyladenine (2-iP) at different concentrations. It was also found that there was no significant effect of the interactions between these two regulators on the morphogenic response of this Meliaceae species (Flores, 2001).

The morphogenic response exhibited by *C. odorata* plants in this study could be related to three fundamental factors: 1) influence of the genotype; 2) explants originated from plants maintained in basal MS medium without any growth regulators, the regulator interactions introduced to the medium may have affected the endogen balance of growth regulators, and consequently, the response to the evaluated morphogenic processes; and 3) the juvenile state of the starting shoots (Figure 2) can determine oxidant compound emission that affects the morphogenic response. It has been documented that the relationship between cytokinin and auxin endogen concentrations play a more important role in differentiation and elongation than the net concentrations of these hormones present in the culture medium (Centeno *et al*., 2003), and it is therefore probable that this relationship is optimum in the treatments with the best results.

Plantlet elongation and rooting under different PGR treatments. The influence of the different culture media on elongation showed a greater difference in all treatments. As shown in Figure 3, the mean height in the treatment supplemented with 3 mg L^{-1} NAA was significantly greater than in the other treatments and had a mean height of 3.93 cm. It has been observed in *C. odorata* that BAP induces tissue differentiation more effectively than stem elongation when applied individually in the culture medium (Pérez, 2006). In the treatments with a low auxin level in the basal medium (5.70 µM IAA 1.0 mg L^{-1}; 11.42 µM IAA 2.0 mg L^{-1}; 17.12 µM IAA 3.0 mg L^{-1}, and 5.37 µM NAA 1.0 mg L^{-1}) short internodal segments were generated.

Increasing NAA concentrations improved shoot height while IAA treatments had no significant effect, probably due to a more efficient synergism between NAA with

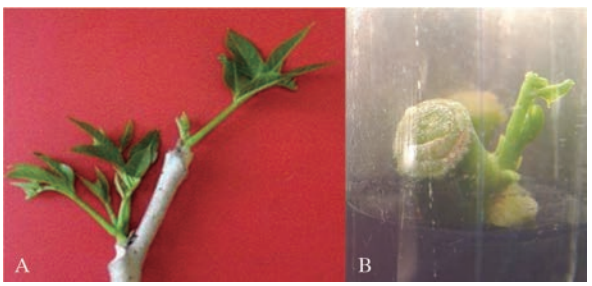
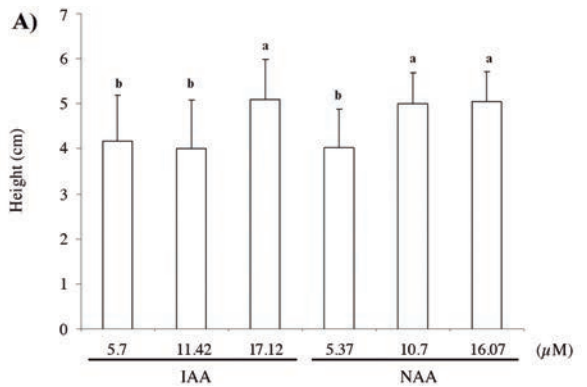
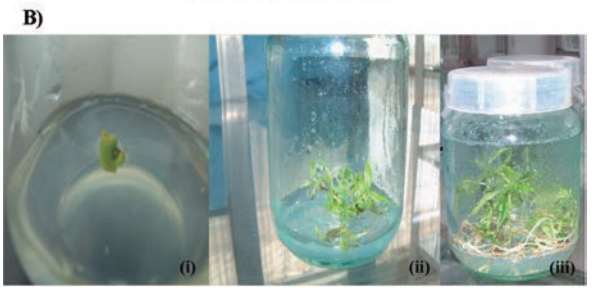


Figure 2. Explant source (A) for *in vitro* disinfection and establishment of *Cedrela odorata* juvenile shoots and morphogenic development of established explants 10 d after being planted (B).



Different letters mean that there are significant differences among treatments. Tukey Test HSD (p > 0.05) n = 30.



In vitro-established nodes 10 d after culture; (ii) morphogenic development from nodal segments 30 d after culture; (iii) morphogenic development 45 d after culture.

Figure 3. (A) Influence of indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA) at different concentrations, and 6-bencilaminopurine (8.88 μM) interaction on *in vitro Cedrela odorata* plant height. (B) Explant size of a subcultured nodal segment.

cytokinin and/or *C. odorata* endogen hormonal contents. It is possible that the interaction between BAP and IAA benefits cellular elongation at higher IAA levels than those evaluated in these experiments whereas the interaction between BAP and NAA can benefit differentiation at low NAA levels and cellular elongation at levels above 2 mg L^{-1} (10.7 µM) when combined with BAP. On the other hand, this behavior can be associated with a better nutrient absorption because of the number of roots generated in the treatments and higher auxin concentrations. It was found in *C. fissilis* that the interaction between NAA (2.5 µM) and BAP (5 µM) increased plant elongation; however, a higher concentration of NAA reduced plant development when combined with BAP and when BAP was eliminated from the basal medium (Costa Nunes, 2002). The auxin:cytokinin ratio, displaced towards auxins, may have limited explant organogenic capacity (Centeno *et al*., 2003), thus benefiting apical dominance and internodal elongation.

For *C. odorata* nodal segments cultured *in vitro* from juvenile plants, 1 cm long shoots were obtained in basal MS medium supplemented with 2.2 µM BAP although no significant differences were found with the other treatments with 2-iP and kinetin (Pérez *et al*., 2002). The best response for *Melia azedarach* L. was obtained for growth and nodal formation at low IAA levels while height was favored when IAA levels increased (Thakur *et al*., 1998). In *S. macrophylla,* the best nodal emission was obtained with BAP at 4 mg L^{-1} (17.76 µM), and adding NAA or IAA to the medium at different concentrations benefited root and plant height development (Astorga *et al*., 1996). Furthermore, de Schottz *et al*. (2007) found that high concentrations of BAP (20 µM) and 2-iP (2 µM) caused a significant reduction in plant development and internodal length, which affected the multiplication rate.

Treatments supplemented with 3 mg L^{-1} NAA and 3 mg L^{-1} IAA induced the best root formation; NAA treatments induced more efficiently than IAA when it was added to the basal media. There was a direct relationship between rooting and concentration increase of NAA and IAA (Figure 4).

Regenerated roots in the treatment supplemented with 2 mg L^{-1} NAA were more vigorous and pigmented along the side with a good formation of secondary short roots. Roots induced under 3 mg L^{-1} NAA showed partial tissue oxidation, which were probably due to the phytotoxic effect of some of the components of the basal culture medium. It was observed in *C. odorata* that the culture medium's mineral and sucrose composition had a significant effect on root formation and development (Pérez *et al*., 2006).

Previous studies demonstrated that 1 mg L^{-1} (5.37 µM) NAA benefited root emission in nodal segments introduced *in vitro* from seedlings previously germinated under greenhouse conditions (Pérez *et al*., 2006); however, no results were obtained from buds introduced from adult

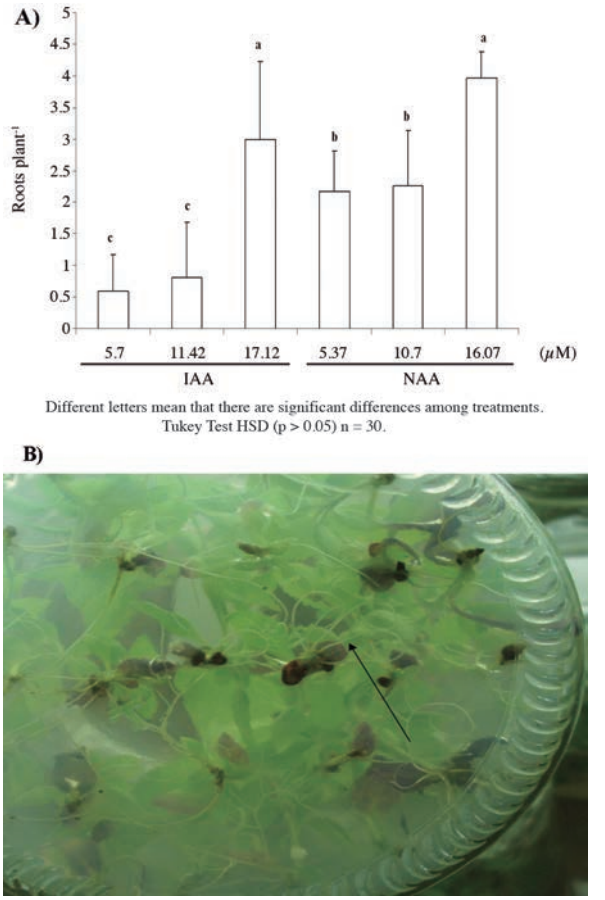


Figure 4. (A) Influence of indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA) at different concentrations, and bencilaminopurine (8.88 µM) interaction on plant rooting of *Cedrela odorata in vitro* plants. (B) Root emission (black arrow) from micropropagated *C. odorata* plants 30 d after being planted in the rooting medium.

plants. It was also found that root induction from node segments derived from *in vitro*-germinated seeds was inhibited when BAP was added to the basal medium at 2.2 and 6.5 µM (Pérez *et al*., 2002). For *C. fissilis*, a tree from the same genus, the best rooting was obtained when shoot explants were cultured in basal medium supplemented with 1.25 µM IBA after 35 d of culture (Costa Nunes *et al*., 2002).

It has been documented for other woody species that the combination of BAP and kinetin from 0.1 up to 2 mg L^{-1} combined with low levels of IBA (0.1 mg L^{-1}, 0.5 µM) benefited shooting, but rooting was stimulated only when the IBA concentration was higher than 1.5 mg L^{-1} (4.9 µM) (Chalupa, 2003).

Root formation from adult plant tissues in the Meliaceae family has been difficult to achieve. Success with *in vitro* mahogany trees has been limited (Astorga *et al*., 1996), and for *C. odorata* rooting, it has only been successful with seedling-derived tissues or *in vitro*-germinated plants (Pérez *et al*., 2002; 2006). Organogenic formation of *S. macrophylla* roots was benefited by 5.0 mg L^{-1} (26.85

μM) NAA treatments for 5 d and a subsequent subculture in 2.0 mg L^{-1} NAA (10.7 μM) for 4 wk (Lopes *et al*., 2001) while treatments with IBA did not produce any interesting results.

CONCLUSIONS

Disinfection of *C. odorata* nodal segments using Propiconazole CE 25 allowed a high percentage of surface explant disinfection, explant survival, and an efficient morphogenic response from juvenile shoots. Adding 8.88 μM BAP and 16.1 μM NAA to the basal medium induced a good morphogenic response in nodal segments during the micropropagation step. Plant rooting was successful under NAA treatments and might allow the *ex vitro* culture of the micropropagated *C. odorata* plantlets. These results allowed developing a rapid protocol for *in vitro* establishment and micropropagation of *C. odorata* from juvenile shoots isolated from field trees.

Propagación *in vitro* de cedro (*Cedrela odorata* L.) a partir de brotes vegetativos juveniles. El cedro (*Cedrela odorata* L.) es una de las especies más importantes para el sector agroforestal caribeño y centroamericano pero se encuentra bajo una presión intensa de explotación debido a su alta demanda. Las técnicas de cultivo *in vitro* y propagación clonal pueden contribuir a atenuar este inconveniente y dotar a los productores con plantas de alta calidad genética y de ciclos biológicos y productivos más reducidos. El objetivo de este trabajo fue desarrollar un protocolo de establecimiento *in vitro* y micropropagar la especie a partir de segmentos nodales de estacas juveniles obtenidas de plantas de campo. El estudio de ocho métodos de desinfección dejó establecido que lavados de los segmentos nodales con propiconazol CE 25 al 5% durante 3 min posibilita un 100% de desinfección de los segmentos nodales y un 60% de brotación de las yemas. La adición de 2 mg L^{-1} de 6-bencilaminopurina y 3 mg L^{-1} de ácido naftalenacético al medio basal Murashige y Skoog propició una brotación del 100% de los segmentos nodales *in vitro* y una altura promedio de 3,93 cm a las 8 semanas. La misma concentración de reguladores del crecimiento propició la formación de 3,9 raíces por planta a las 6 semanas de individualizada. Las plantas adaptadas *ex vitro* no presentaron diferencias morfológicas con las plantas germinadas a partir de semillas.

Palabras clave: organogénesis, propagación *in vitro*, *Cedrela odorata*, enraizamiento *in vitro*, reguladores del crecimiento.

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