Morfogénesis *in vitro* de *Toona ciliata* a partir de raquis de hojas jóvenes con tidiazuron

In vitro morphogenesis of *Toona ciliata* from young leaf rachis using thidiazuron

Marcos Daquinta[*], *Yarianne Lezcano*[*], *Mariela Cid*[*], *Danilo Pina*[*], *Romelio Rodríguez*[*]

ABSTRACT

The Meliacea are of great importance in construction and furniture-making. *Toona ciliata* is a Meliacea originally from the Himalayan region; it is known as Himalayan Cedar in Cuba. Natural regeneration occurs in this specie by seed diffusion and grafting; such propagation is limited. The object of this paper was to promote callus formation and plant regeneration in *Toona ciliata* from *in vitro* propagation. Two to three year old mature trees (*Toona ciliata*) were used. Rachis were taken from young branches from these plants. They were disinfected in 0.25% (w/v) mercuric chloride solution for 10 min followed by three rinses in autoclaved distilled-water. They were then established in MS supplemented with 0-1 mg/L thidiazuron culture medium. Nodular calluses were obtained having good morphogenic characteristics. Shoots sprouted from six-month-old calluses in the dark and plant regeneration was done in the light. AIB shoots were rooted in MS medium supplement with 1 mg/L IBA.

Key words: callus, Himalayan Cedar, Meliacea, growth regulators, plant regeneration.

Abbreviations: IBA- indolebutyric acid, thidiazuron-N-1,2,3-thiadiazol-5-yl-N-phenylurea.

RESUMEN

Las Meliáceas son de gran importancia en la construcción y fabricación de muebles, entre otras aplicaciones. *Toona ciliata* es una Meliácea originaria de la región del Himalaya; en Cuba se conoce como cedro del Himalaya. La regeneración natural de esta especie es por semillas e injertos. Los procesos de propagación en la producción de esta especie son limitados. El objetivo de este trabajo fue lograr la formación de callos y la regeneración de plantas en *Toona ciliata* para la propagación in vitro. Se usaron árboles de dos a tres años de *Toona ciliata*. A partir de estas plantas se utilizaron raquis de hojas jóvenes las cuales se desinfestaron con bicloruro de mercurio al 0.25% (w/v) durante 10 minutos, se lavaron con agua destilada estéril y se establecieron en el medio de cultivo MS suplementado con 0-1 mg/L de Tidiazuron. Se obtuvieron callos nodulares con buenas características morfogénicas. Se lograron brotes en callos de seis meses de edad en la oscuridad y la regeneración de plantas en la luz. El enraizamiento de los brotes se obtuvo en el medio de cultivo MS con 1 mg/L AIB.

Palabras clave: callo, cedro del Himalaya, Meliácea, reguladores del crecimiento, regeneración de plantas.

INTRODUCTION

Toona ciliate, belongs to the *Meliaceae* family, is a useful native tree in the Himalayan region.

The main factor which has limited the cultivation of *Meliaceae* in plantations is attack by shoot boring moths (*Hypsipyla spp)*, which are widespread throughout tropics. *Hypsipyla grandella* is found

* Agricultural engineers at the Cell and Tissue Culture Laboratory, Bioplant Centre, Universidad Ciego de Ávila, carretera a Morón km. 9. CP 69450. Cuba.

Recibido: Marzo 16 de 2004 Aceptado: Noviembre 22 de 2005

throughout Central and South America (except Chile). Himalayan Cedar is known to be resistant to damage caused by *Hypsipyla grandella*. The specie has been traditionally propagated by seeds in nurseries, similar to Spanish cedar and Mahogany.

However, high-quality clone propagation is more desirable.

Meliacious plants are amongst the most commercially important tropical timber species, dominating international trade in those areas where they are native.

Only a few Meliacious plant tissue culture studies have been carried out. It is worth mentioning those regarding *Cedrela montana* (Carrizoza and Serrano, 1997), *Cedrela fissilis* (Da Costa et ál., 2002), *Melia azedarach* (Handro and Floh, 2001), *Azadirachta indica* (Soneji et ál., 2001) and *Cedrela odorata* and *Swietenia macrophylla* (Valverde et ál., 1998). There is a lack of reference material concerning *Toona ciliata* shoot regeneration from somatic tissue; only seed regeneration has been documented.

The aim of this work was to promote callus formation and plant regeneration in *Toona ciliata* (Himalayan Cedar) in order to establish clonal propagate this on of in vitro conditions.

MATERIALS AND METHODS

Young leaves from *Toona ciliate* (2-3 years old) branches were selected; pinnate leaves were cut from branches for collecting plant material in the least destructive way possible. These explants were then washed thoroughly under running tap-water.

The young leaves were surface-sterilized by immersing them in 0.25% (w/v) mercuric chloride solution for 10 min followed by three rinses in sterile distilled-water. The leaves were carefully removed under sterile conditions and discarded. Rachis were sliced into 5 to 10 mm sections and inoculated into MS medium (Murashige and Skoog, 1962), supplemented with thidiazuron (0, 0.10, 0.25, 0.50 and 1.0 mg/L).

Medium pH was kept at 5,6 to 5,8 by using sodium hydroxide prior to adding agar. The medium was sterilized in an autoclave at 1.2 kg/cm^2 and 121 °C for 15 minutes. Explants were cultured in a growth chamber at 25± 2 °C in the dark.

Percentage data were converted by angular transformation for statistical analysis: arc sin $\sqrt{x}$. Variance was analysed by completely random design and Duncan test used for comparing means at 5% level.

RESULTS AND DISCUSSION

Rachis explants formed calluses in all media within 4 weeks, except those cultured in MS which did not contain growth regulators. Table 1 shows that the percentage of callus formation in *Toona cilita* rachis segments was particularly effective with addition of 0.25 mg/L thidiazuron.

Table 1. Percentage of callus formation in *Toona ciliate* young leaf rachis segments with different levels of thidiazuron (n=50). Differences letters indicate significant differences at p<0.05 (Duncan).

Thiadiazuron (mg/L)	Callus in young leaf rachis segments (%)
0.00	0 c
0.10	37.5 a
0.25	44.5 a
0.50	22.2 b
1.00	22.2 b

The best response was achieved with smaller growth regulator levels in *Toona ciliata* young leaf rachis segments; higher callus formation was achieved in *Toona* rachis with 0.10 and 0.25 mg/L TDZ. The highest callus formation percentages (44.5%) were found in medium supplemented with 0.25 mg/L TDZ. Callus multiplication from rachis was higher in *Toona* (Himalayan Cedar) than *Khaya nyasica* and other Meliacious species (unpublished data); this was possible because the explants were taken from young plants.

Barrueto et ál., (1997, 1999) achieved the highest percentages of callus formation and plant regeneration from *Miconia sp* and *Eucalyptus grandis x*

E. urophylla leaves and plantlet nodes with thidiazuron.

Callus were produced from rachis taken from the youngest 3-4 leaves detached from young branches in TDZ-containing induction medium (figure 1-A). Morphogenic response could have been attributed to the juvenility of the petiolar (rachis) cells rather than of distal segments. Most calluses proliferated and formed shoots during subculture in the same callus-inducing medium (MS supplemented with 0.25 mg/L TDZ). The callus was six months old when regeneration was initiated. Shoot production in the callus kept in the dark was higher, as that kept in the light was 0% (figure 1-B). However, plant regeneration was achieved in the light (figure 1-C) in culture medium which did not contain plant growth-regulators.

Environmentally controlled laboratory conditions played a more critical role for growth in terms of successfully applying the micropropagation protocol developed by Venkateswarlu (1999) than changing medium hormones. Darkness was a fundamental factor in inducting shoots in this work.

Soneji et ál. (2001) have reported that rachis explants isolated from shoots grown *in vitro* in MS medium gave rise to callus and shoot regeneration in callus cultures. This was achieved on transfer to hormone-free MS medium.

Petiolar cotyledon segments were more morphogenic to shoot-bud differentiation than distal cotyledon segments. TDZ was highly effective in inducing shoot-buds, but arrested shoot growth in *Albizia chinensis* (Sinha et ál., 2000).

Very young petiole (rachis) explants exhibited higher regeneration potential in *Toona ciliate* compared to leaf explants (unpublished data); regeneration efficiency was found to be highly dependent on callus

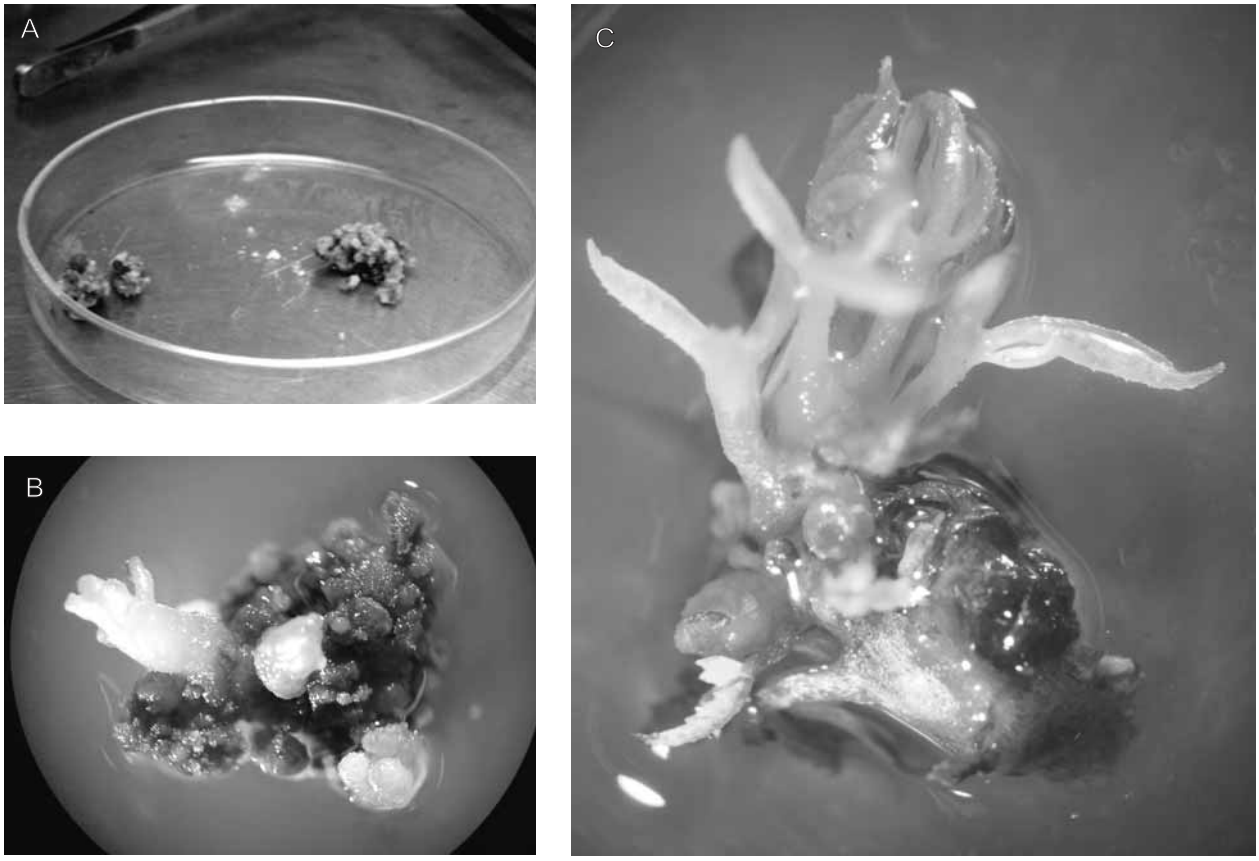

Figure 1. *Toona ciliata* plantlet production. (A) Nodular callus obtained from young branch rachis. (B) Shoots derived from callus. (C) Well-developed shoot with another bud at the base.

type. Calluses cultivated in MS medium supplemented with 0.25 mg/L TDZ were able to produce adventitious shoots 45 days after transfer. The presence of TDZ in the medium was capable of inducing regeneration.

This observation also corroborated previous reports on the effective role of TDZ in promoting shoot formation in many woody trees, having an inhibitory effect on growth and elongation at higher concentrations (Huetteman and Preece, 1993).

The use of TDZ induces a diverse array of cultural responses ranging from callus induction to somatic embryo formation. Several physiological and biochemical events are likely to be influenced by TDZ in cells but these may or may not be directly related to inducing morphogenic responses (Murthy et ál., 1998). A relatively high dose of TDZ was used by Vila et ál. (2003) in *Melia azedarach* (which also belongs to the Meliacea) for obtaining somatic embryogenesis from immature zygotic embryos.

The shoots were excised and cultured in MS medium supplemented with indolebutyric acid (IBA) to induce rooting and thus complete plantlet formation. The plantlets were obtained by transferring the shoots to MS semisolid medium supplemented with 1 mg/L IBA in the light.

However, histological examination of shoot origin must be carried out to confirm the mode of regeneration. The present work indicates the feasibility of rapid shoot production. Further refinement of the *in vitro* techniques developed in this study (and establishing a protocol) will facilitate plantlets being produced in their thousands.

This work has reported methods which have been developed for rapidly and consistently regenerating shoots obtained from *Toona* rachis explants; these have been shown to be applicable to explants derived from other Meliacious plants. A protocol for *Toona ciliata* rachis explant propagation of regenerated plantlets obtained via adventitious shoot formation from callus cultures should thus include incubation in TDZ-supplemented MS medium (0.25 mg/L) in complete darkness for promoting shoot initiation and thereafter shoots should be further regenerated in MS medium containing 1 mg/L IBA.

The protocol described in this publication for inducing shoots from *Toona ciliata* rachis in a short time and with minimum subculturing is original, simple and reproducible. This study has also revealed the usefulness of TDZ in *Toona* clone multiplication. This is the first report (to our knowledge) concerning shoot regeneration from Himalayan Cedar young leaf rachis. Results obtained in our experiments have shown that the shoots (or somatic embryos) have great potential as an alternative for propagating and conserving this species. More research is needed for developing an effective method for regenerating shoots into plants.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Mariano Toribio from Instituto Madrileño de Investigaciones Agrarias y Alimentarias (España) for his help with the photography and Mr. Conroy Cassan Huggins (Saint Vincent and the Grenadines) for his critical reading of the manuscript.

The document has been completely revised for publication in this journal by Jason Garry (IBUN).

BIBLIOGRAPHY

Barrueto, L. P.; Gomes, A. C. M.; Da Costa, S. B. R.; Teixeira, J. B. 1997. Micropropagation of *Miconia sp*, a woody melastomaceae from Brazil, using thidiazuron as plant growth regulator. *Revista Brasileira de Fisiología Vegetal*. 9(1): 21-25.

Barrueto, L. P., Machado, A. C. M. G.; Carvalheira, S. B. R. C.; Brasileiro, A. C. M. 1999. Plant regeneration from seedling explants of *Eucalyptus grandis x E. urophylla*. *Plant Cell, Tissue and Organ Culture*. 56: 17-23.

Carrizoza, M. S. y Serrano, C. 1997. Propagación de *Cedrela montana* por cultivo *in vitro*. Memorias del IV Congreso, tomo II, Pontificia Universidad Javeriana, Colombia, pp. 255-260.

Da Costa, E.; Volkmer de Castillo, C.; Netto, F.; Viana, A. M. 2002. *In vitro* culture of *Cedrela fissilis* Vellozo (Meliaceae). *Plant Cell, Tissue and Organ Culture*. 70: 259-268.

Handro, W.; Floh, E. I. S. 2001. Neo-formation of flower buds and other morphogenetic responses in tissue cultures of *Melia azedarach. Plant Cell, Tissue and Organ Culture*. 64: 73-76.

Huetteman, C. A.; Preece, J. E. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture*. 33: 105-119.

Murashige, T.; Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* (15): 473-497

Murthy, B. N. S.; Murch, S. J.; Saxena, P. K. 1998. Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. *In Vitro Cell. Dev. Biol. Plant*. 34: 267-275.

Pierik, R. L. M. 1990. Rejuvenation and micropropagation. In Nijkamp, H. J. J., Pllasbander, L. H. W.; Aartrijik, J. Vaan (eds). *Progress in Plant Cellular and Molecular Biology*. Kluwer Acad. Pub., pp. 91-101.

Sinha, R. K.; Majundar, K.; Sinha, S. 2000. *In vitro* differentiation and plant regeneration of *Albizia chinensis* (Osb) Merr. *In Vitro Cell. Dev. Biol. Plant*. 36: 370-373.

Soneji, J. R.; Rao, P. S., Marte, M. 2001. Enhanced regeneration of multiple shoots and plantlets in young cotyledons of Neem, *Azadirachta indica* A. *Juss. Physiol. Mol. Biol. Plants*. 7(2): 175-180.

Valverde, L.; Dufour, M.; Villalobos, V. M. 1998. *In vitro* organogenesis in *Albizia guachapele, Cedrela odorata* and *Swietenia macrophylla. Revista Biológica Tropical* 46(2): 225-228.

Venkateswarlu, B. 1999. Micropropagation of plus Neem selected for Azadirachtin content and evaluation of field-transferred plants. In International Tree Biotechnology Meeting, India, pp. 46-49.

Vila, S. A.; González, H. Rey; Mroginski, L. 2003. Somatic embryogenesis and plant regeneration from immature zygotic embryos of *Melia azedarach* (Meliacea). *In Vitro Cell. Dev. Biol. Plant* 39(5): 283-287.