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In Vitro Morphogenesis of *Toona ciliata* and *Swietenia* Hybrid

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ABSTRACT

The *Meliaceae* are of great importance in construction and furniture-making. *Toona ciliata* is a member of this family originally from the Himalayan Region; in Cuba it is known as Himalayan Cedar. The *Swietenia* natural hybrid results from a cross between *Swietenia mahogany* x *Swietenia macrophylla*. Natural regeneration occurs in these species by seed and grafting but such propagation is limited, while there exist no reports on their *in vitro* culture. The objective of this paper was to promote callus formation and plant regeneration in theses two species. Two to three year-old mature *T. ciliata* and 40 year-old *Swietenia* hybrid trees were used. The rachis of *Toona* and the inflorescences of *Swietenia* were taken from young branches from these plants, disinfected in a 0.25% (w/v) mercuric chloride solution for 10 min followed by three rinses in autoclaved distilled-water. They were then established on Murashige and Skoog culture medium supplemented with 0-1 mg/L thidiazuron. Nodular callus, induced in 22-44% of all explants in *T. ciliata* and in 40-70% of explants in *Swietenia* were maintained on TDZ indefinitely and demonstrated strong, regenerative morphogenic characteristics. Shoots sprouted from six-month-old callus in the dark and *T. ciliata* plants could regenerate in the light. In *Swietenia* hybrid we obtained somatic embryo-like structures in callus derived from inflorescences. This is the first successful report of callus induction and plant regeneration from mature explants in these two important forestry species.

Keywords: callus, Himalayan Cedar, mahogany, Meliacea, plant growth regulators, plant regeneration
Abbreviations: IBA, indolebutyric acid; **TDZ**, thidiazuron or N-1,2,3-thiadiazol-5-yl-N-phenylurea

INTRODUCTION

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

Toona ciliata and *Swietenia* hybrid belong to the *Meliaceae* family and are useful native trees in the Himalayan region and Caribbean islands, respectively. The main factor which has limited the cultivation of *Meliaceae* in plantations is attack by shoot boring moths (*Hypsipyla* spp.), which are widespread throughout the tropics. *Hypsipyla grandella* is found throughout Central and South America (except Chile). Himalayan Cedar is known to be resistant to damage caused by *H. grandella*. Species within the *Meliaceae* have traditionally been propagated by seeds in nurseries, similar to Spanish Cedar and Mahogany. However, high-quality clonal propagation is more desirable.

Swietenia is generally considered to comprise three species *Swietenia mahogany* (L) Jacq, *S. macrophylla* King and *S. humilis* Zucc. The tree species are poorly defined biologically, partly because they hybridize freely (Mayhew and Newton 1998). For example, naturally occurring hybrids of *S. mahogany* x *S. macrophylla* occur in Cuba, Puerto Rico and other Caribbean islands. Similar populations are probable in other countries where plantations of two progenitor species exist.

Only a few Meliacious plant tissue culture studies have been carried out, including *Cedrela montana* (Carrizoza and Serrano 1997), *Cedrela odorata* and *Swietenia macrophylla* (Valverde *et al*. 1998), *Azadirachta indica* (Soneji *et al*. 2001), *Melia azedarach* (Handro and Floh 2001; Sharry and Teixeira da Silva 2006) and *Cedrela fissilis* (da Costa *et al*. 2002). 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 induce callus and if possible, somatic embryogenesis, and to subsequently promote plant regeneration in *T. ciliata* (Himalayan Cedar) and *Swietenia* hybrid. Achieving this goal through the optimization of *in vitro* conditions would ensure the clonal propagation of these important forestry species.

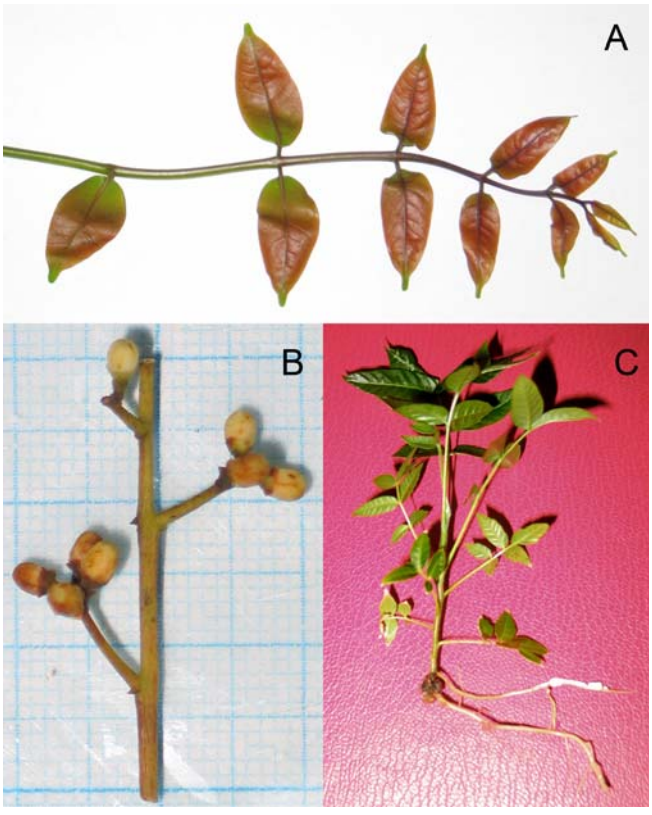

Fig. 1 (**A, C**) *Toona ciliata* leaves; (**B**) *Swietenia* hybrid inflorescence.

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Short Communication

MATERIALS AND METHODS

Young leaves from *Toona ciliata* (2-3 years old) branches and inflorescences (5 cm of terminal section) of *Swietenia* hybrid were selected from a naturally-growing tree in a forestry enterprise at Ciego de Ávila, Cuba. Pinnate leaves were cut from branches in the least destructive way possible. These explants were then washed thoroughly under running tap-water for 15 minutes.

The young leaves of *T. ciliata* (**Figs. 1A, 1C**) were surface-sterilized by immersing them in a 0.25% (w/v) mercuric chloride solution for 10 min and the 40 year-old *Swietenia* inflorescences (**Fig. 1B**) for 5 min followed by three rinses in sterile distilled water. The leaves were carefully removed under sterile conditions and discarded. The rachis were sliced into 5-10 mm transverse sections yielding explants 5-10 mm in size, and flowers were sliced into longitudinal sections and inoculated onto MS medium (Murashige and Skoog 1962), supplemented with thidiazuron (Duchefa, The Netherlands) at 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 (bacteriology A BIOCEN, Cuba) at 6 g/L. 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 √x. Variance was analyzed by a completely random design and Duncan's test of significance was applied in the comparison of means at the 5% level.

RESULTS AND DISCUSSION

Toona ciliate

Rachis explants from *T. cilita* formed callus on media containing all concentrations of TDZ within four weeks. Callus did not form on growth regulator-free MS medium. **Table 1** shows the percentage of callus formation in rachis segments was most effective when 0.25 mg/L TDZ was added, even though a significantly similar high level of callus could be induced at 0.10 mg/L.

Table 1 Percentage of callus formation in *Toona ciliate* young leaf rachis segments with different concentrations of thidiazuron (n = 50). Differences letters within a column indicate significant differences at $P < 0.05$ (Duncan's test).

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

Callus induction from the rachis of *T. cilita* was higher than in *Khaya nyasica* and other Meliacious species (unpublished data); this was possible because the explants were taken from young plants.

Barrueto *et al*. (1997, 1999) achieved a high percentage of callus formation and plant regeneration in *Miconia* sp. and *Eucalyptus grandis* x *E. urophylla* leaves and plantlet nodes when TDZ was used.

Callus was produced from rachises taken from the youngest 3-4 leaves detached from young branches after culture for 6 weeks on TDZ-containing induction medium (**Fig. 2A**). The positive, morphogenic response could be attributed to the juvenility of the petiolar (rachis) cells rather than that of those in distal segments. Most calluses proliferated and formed shoots during subculture when maintained on the same callus-inducing medium (MS supplemented with 0.25 mg/L TDZ) in the dark. The callus was six months old when regeneration began. Shoot production from callus kept in the dark was higher than that kept in the light (0%; **Fig. 2B**). However, plant regeneration was achieved in the light (**Fig. 2C**) on culture medium which did not contain plant TDZ.

Venkateswarlu (1999), who developed a micropropagation protocol for neem, claimed that environmentally controlled laboratory conditions (growth room conditions undefined in that study) played a critical role in determining growth than changing medium hormones. Darkness was a fundamental factor in the induction of shoots in our study.

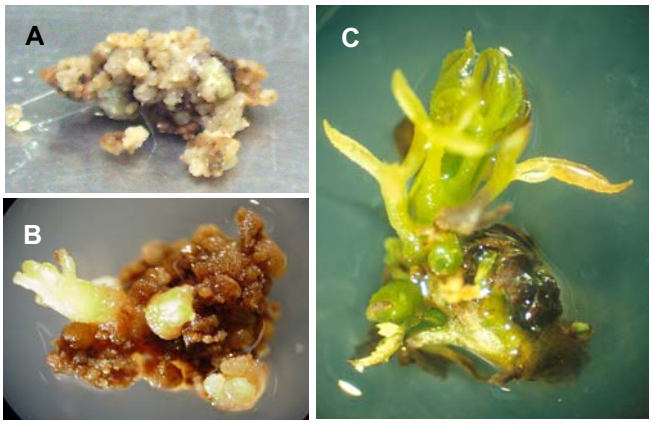

Fig. 2 ***Toona ciliata*** **plantlet production.** (**A**) Nodular callus obtained from young branch rachis. (**B**) Shoots derived from callus. (**C**) Well-developed shoot with secondary buds developing at the base.

Soneji *et al*. (2001) reported that rachis explants isolated from neem shoots grown *in vitro* on MS medium supplemented with 9 µM BAP and 5 µM 2iP gave rise to callus and shoot regeneration in callus cultures. Following a pulse period, shoot induction was achieved following transfer to plant growth regulator-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 al*. 2000). In contrast, Sharry and Teixeira da Silva (2006) noted that the type and concentration of PGR played a vital role in the success of neem somatic embryogenesis and subsequent plant regeneration, noting that 1-naphthlaeneacietic acid and 6-benzyladenine, coupled to gibberellic acid were more important than light conditions.

Very young petiole (rachis) explants exhibited a higher regeneration potential in *T. ciliata* than leaf explants (unpublished data); regeneration efficiency was found to be highly dependent on callus type, i.e. effective in nodular callus. Nodular callus cultivated on MS medium supplemented with 0.25 mg/L TDZ was able to produce adventitious shoots 45 days after transfer. In conclusion, the presence of TDZ in the medium was capable of inducing indirect shoot regeneration via callus. Following this essential TDZ pulse, shoots could be induced directly without an intermediate callus phase on TDZ-free MS medium in the light.

Previous reports also demonstrate 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 shoots were subsequently excised and cultured on semisolid (6 g/L agar) MS medium supplemented with indolebutyric acid (IBA) at 1 mg/L to induce rooting and thus complete plantlet formation. Neither visually obvious physiological abnormalities nor hyperhydricity were observed.

In future studies, histological examination of shoot origin will be carried out to confirm the mode of regeneration. The present work however, indicates the feasibility of rapid shoot production in Himalayan Cedar. Further refinement of the *in vitro* techniques developed in this study (and establishment of a reproducible protocol) will facilitate the production of clonal plantlets in their thousands.

Swietenia hybrid

Nodular callus was obtained from immature inflorescences of *Swietenia* hybrid, exhibiting a strong morphogenic capacity (**Fig. 3**). It took eight weeks from the beginning of

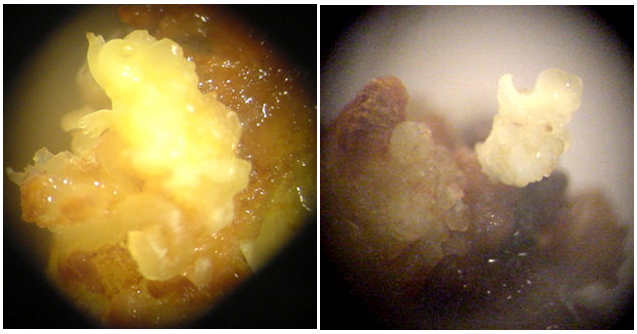

Fig. 3 Callus with somatic-embryogenic-like structures obtained from inflorescences of *Swietenia* on MS culture medium with 0.25 mg/L TDZ.

culture to form somatic embryo-like structures in these callus cultures. Somatic embryogenesis or similar organogenesis from mature tissues of this plant have never been reported.

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 al*. 1998). A relatively high dose of TDZ was used by Vila *et al*. (2003) in *Melia azedarach* (also a member of the *Meliaceae*) for obtaining somatic embryogenesis from immature zygotic embryos.

This study reports a clear and simple method for the rapid and consistent regeneration of shoots obtained from *Toona ciliata* rachis explants on TDZ-supplemented (0.25 mg/L) MS medium in complete darkness. Parallel methodologies employing TDZ have also been shown to be applicable to explants derived from other Meliacious plants. Following shoot initiation, harvested shoots can be further regenerated on MS medium containing 1 mg/L IBA.

The same medium under the same growth conditions was also shown to promote morphogenesis in *Swietenia* hybrid. This is the first report (to our knowledge) of shoot regeneration from Himalayan Cedar young leaf rachis and embryogenic-like callus induction from *Swietenia* hybrid inflorescences.

Our results show that shoots obtained *in vitro* have great potential as an alternative for propagating and conserving these species highly valued for their wood. By providing a suitable regeneration medium, our results open up opportunities for molecular biologists to examine physiological aspects of these plants in an *in vitro* environment, and provide a basis for genetic transformation studies. More research is currently being undertaken to develop effective protocols for regenerating shoots into plants.

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