CROP BREEDING AND APPLIED BIOTECHNOLOGY

*ARTICLE*

# Efficient and new method for *Tectona grandis* in vitro regeneration

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Crop Breeding and Applied Biotechnology
17: 124-132, 2017
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332017v17n2a19

**Abstract:** *The aim of this study was to develop a shoot regeneration protocol for Tectona grandis by examining the effects of pretreatments, regeneration culture medium, adventitious rooting, and acclimatization. The best organogenic potential was achieved when hypocotyls and cotyledonary segments from seedlings germinated on a medium containing TDZ, were cultured for four weeks on MS medium supplemented with BAP/$GA_3$. With TDZ pretreatment during seed germination, up to 70% of the cotyledonary segments (0.5 TDZ pretreatment and 1.0 BAP + 0.5 $GA_3$ during regeneration, mg $L^{-1}$) and 60% of the hypocotyl explants (0.1 TDZ pretreatment and 1.0 BAP + 0.5 $GA_3$ during regeneration, mg $L^{-1}$) resulted in shoot regeneration. Finally, 65% of the regenerated shoots rooted, of which 60% were successfully acclimatized ex vitro. Our protocol increases regeneration efficiency for teak and it can serve as a platform for genetic transformation* to improve this important tree species.

**Key words**: *Adventitious rooting, plant growth regulator, plant tissue culture, woody plant.*

## INTRODUCTION

*Tectona grandis* L. f. is a commercially important, tropical hardwood tree species that has several applications in the lumber industry and is used in agroforestry systems (Galeano et al. 2015). Teak grows naturally across the Indian subcontinent through Myanmar and Thailand to Laos, and it is common in deciduous forests and well-drained alluvial soils (Pandey and Brown 2000). Central characteristics of its wood include resistance to weathering, strong fiber, and special oil content, which produces premium timber (Feroz et al. 2013), making teak one of the world's most valuable hardwood species (Galeano et al. 2014). It is cultivated in more than 36 countries with 5.7 million hectares planted worldwide (Santos et al. 2014).

Conventional genetic improvement of forest species for increased yield and pest resistance is time consuming due to long generation time and requires large test sites because of their size (Giri et al. 2004). Furthermore, conventional breeding has had limited success as many trees are outcrossing, producing high degrees of heterozygosity and incompatibility (Poupin and Arce-Johnson 2005). However, biotechnology offers a practical alternative to conventional plant breeding for genetic improvement of tree species.

In vitro plant regeneration relies on the interaction of multiple endogenous

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**Received:** 24 June 2016
**Accepted:** 17 January 2017

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and exogenous factors, including salt composition in the medium, explant/tissue competence, plant growth regulators, and light (Cangahuala-Inocente et al. 2014; Bakhtiar et al. 2016). Cytokinins, auxins, and gibberellins are the three most commonly used plant growth regulators in plant tissue culture (Xie and Hong 2004, Ferreira et al. 2008, Akram and Aftab 2009). An optimal combination of different types and concentrations of plant growth regulators is essential to induce competence in tissue for organogenesis and/or somatic embryogenesis (Wendling et al. 2014). In particular, the relationship between the applied plant growth regulators and the explant's ability to respond to the stimuli provided by the plant growth regulators determines organogenesis (Find et al. 2014). Therefore, successful plant regeneration in tissue culture involves multiple processes, including signal transduction and perception of stimuli from plant growth regulators, redifferentiation of the dedifferentiated tissues, subsequent cell division, and organization to form organs (i.e., root, shoot) (Magyar-Tábori et al. 2010, Almeida et al. 2015). Cytokinins added to the regeneration medium can enhance cell competence for the development of new shoots and therefore increase the number and frequency of regenerating shoots and their subsequent growth rate (Hartmann et al. 2011).

For biotechnological improvement programs to successfully introduce new traits into selected trees for enhanced growth and productivity, an efficient regeneration method is necessary. Multiple studies have described shoot regeneration from stem internodes of teak using different plant growth regulators, such as benzylaminopurine (BAP), indole-3-acetic acid (IAA), α-naphtalene acetic acid (NAA), and gibberellic acid ($GA_3$) (Tiwari et al. 2002, Shirin et al. 2005, Gyves et al. 2007). However, no reports have assessed the use of a cytokinin pretreatment in explants to improve shoot regeneration frequency.

The aim of this study was to develop an efficient shoot regeneration protocol for *T. grandis* by testing the effects of different pretreatments, regeneration culture medium, adventitious rooting, and acclimatization.

## MATERIAL AND METHODS

### Plant material

Open-pollinated seeds from Proteca Biotecnologia Florestal LTDA, Mato Grosso State, Brazil, were obtained manually from fruits. The seeds were sterilized following the protocol proposed by Tambarussi et al. (2015).

### Germination on BAP and TDZ pretreatment

Sterilized seeds were inoculated in a glass flask (2.0×10.0 cm) containing 50 mL of MS basal medium (Murashige and Skoog 1962) supplemented with 30 g $L^{-1}$ sucrose and varying concentrations of BAP (0.0, 0.1, and 0.5 mg $L^{-1}$) or TDZ (0.0, 0.1, and 0.5 mg $L^{-1}$). The culture medium was solidified using 2.3 g $L^{-1}$ Phytagel™ (Sigma Chemical Co®, USA). The pH of all culture medium was adjusted to 5.8 with NaOH (1M) before autoclaving at 121 °C (≈1.0 kgf $cm^{-2}$) for 20 min. Seed germination was carried out under the following light conditions: 25±2 °C with irradiance of 30 µmol $m^{-2}$ $s^{-1}$ and a 16 h photoperiod, using cool white fluorescent bulbs (Philips Eco MASTER®).

### Shoot regeneration by indirect organogenesis

At 20 days after germination on the pretreatment culture medium (BAP or TDZ), seedlings were dissected to isolate the hypocotyls (5±1 mm), cotyledonary intermodal segments (subsequently called "cotyledonary segments") (7±1 mm), and mature cotyledons (15±5 mm) for shoot regeneration. The explants were divided into 16 treatments (Table 1) consisting of either MS basal medium (control, free of plant growth regulators) or supplemented with varying concentrations of BAP, indole-3-butyric acid (IBA), and $GA_3$ (BAP/IBA/$GA_3$; mg $L^{-1}$): ($T_1$) 0.0/0.0/0.0; ($T_2$) 1.0/0.0/0.0; ($T_3$) 3.0/0.0/0.0; ($T_4$) 5.0/0.0/0.0; ($T_5$) 0.0/0.5/0.0; ($T_6$) 1.0/0.5/0.0; ($T_7$) 3.0/0.5/0.0; ($T_8$) 5.0/0.5/0.0; ($T_9$) 0.0/0.5/0.5; ($T_{10}$) 1.0/0.5/0.5; ($T_{11}$) 3.0/0.5/0.5; ($T_{12}$) 5.0/0.5/0.5; ($T_{13}$) 0.0/0.0/0.5; ($T_{14}$) 1.0/0.5/0.5; ($T_{15}$) 3.0/0.0/0.5; ($T_{16}$) 5.0/0.0/0.5. The pH of all culture medium was adjusted to 5.8 with NaOH (1M) before autoclaving at 121 °C (≈1.0 kgf $cm^{-2}$) for 20 min. Explants on the regeneration medium were kept in darkness at 25±2 ºC for one week and then transferred to light conditions (16 h photoperiod and irradiance of 30 µmol $m^{-2}$ $s^{-1}$) for five weeks without subcultures. The medium was replaced for all cultures every three weeks, maintaining consistent treatments. After six weeks, we assessed the percent of adventitious shoot induction (%), number of shoots per explant, and the length (cm) of regenerated shoots.

### In vitro adventitious rooting and acclimatization

Shoots longer than 10 mm were transferred to half-strength MS basal medium supplemented with 0.5 mg $L^{-1}$ IBA, 7.0 g $L^{-1}$ sucrose, and 2.3 g $L^{-1}$ Phytagel™ for in vitro adventitious rooting. After six weeks, complete plantlets measuring approximately 5 cm in height with 2-5 cm of developed roots were washed thoroughly with tap water. They were then transferred to plastic pots (25 cm diameter × 20 cm height) containing soil with vermiculite (1:1, v/v) enriched with ¼ MS salt solution (weekly) and placed in a greenhouse for acclimatization, development, and growth. Once a day, the seedlings were watered manually until all substrate was saturated. After six weeks, survival rate (%) and growth of plantlets (cm) were evaluated.

### DNA extraction, microsatellite amplification and analyses

For 42 seeds, DNA was extracted from 100 mg of leaf tissue per seedling germinated during the study, based on Doyle and Doyle (1990). We used three microsatellite markers specific to teak: CIRAD1TeakH10 (Forward primer: 5´-CGATACCTGCGATGCGAAGC-3´, Reverse primer: 5´-CGATACCTGCGATGCGAAGC-3´, EMBL accession number AJ968933), CIRAD3TeakB02 (Forward primer: 5´-ATGAAGACAAGCCTGGTAGCC-3´, Reverse primer: 5´-GGAAGACTGGGGAATAACACG-3´, EMBL accession number AJ968937), CIRAD4TeakF02 (Forward primer: 5´-CCGGTAAAAAGGTGTGTCA-3´, Reverse primer: 5´-GAGTGGAAGTGCTAATGGA-3´, EMBL accession number AJ968942) (Verhaegen et al. 2005). Microsatellite loci were amplified by PCR for a final volume of 25 µL consisting of buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, and 1.5 mM $MgCl_2$), 20 ng of template DNA, 0.8 mM of each primer, 0.2 mM dNTPs, and 1 U Taq DNA polymerase (Invitrogen). The amplification program for all primers included an initial denaturing step at 96 °C for 3 min, followed by 30 cycles of amplification (94 °C for 30 s, followed by 1 min at 56 °C (specific annealing temperature of each primer pair), and 72 °C for 30 s), and a final elongation step at 72 °C for 7 min. Amplifications were performed with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). The amplification products (5 µL of the total reaction volume) were analyzed based on Tambarussi et al. (2010). Allele scoring was carried out using the 100 bp DNA Ladder (Invitrogen) as the size standard. Analysis of the mating system was based on the mixed-mating model (Ritland and Jain 1981) and the correlated mating model (Ritland 1989), using the program Multilocus MLTR (Ritland 2002). The average coancestry coefficient within progenies was estimated as: $\overline{\theta} = 0.125(1+\hat{F}p)[4\hat{s} + (\hat{t}_m + \hat{s}\hat{t}_m\hat{r}_s)(1+\hat{r}_{p(m)})]$, where $F_p$ (considered as equal to zero) is the parental inbreeding coefficient (Ritland 1989). The frequency of pair wise self-sibs ($P_{SS}$), half-sibs ($P_{HS}$), full-sibs ($P_{FS}$), and self-half-sibs ($P_{SHS}$) within progenies were estimated following Sebbenn (2006).

### Experimental design and data analysis

The experiments used completely randomized factorial design with five replicates per treatment in both the germination and shoot regeneration phase. Each replicate was composed of a glass flask (1.5 × 10.0 cm) containing five explants. After six weeks with the shoot regeneration medium, collected data were transformed to $[(x+0.5)/100]^{0.5}$, where $x$ is either the frequency of regeneration (%), number of shoots per responsive explant, or shoot length. Parameters were analyzed and subjected to the Shapiro-Wilk test ($P<0.05$) and the Hartley test ($P<0.05$) to check normality and homogeneity of variance between treatments, respectively, before analysis of variance (ANOVA). ANOVA was applied to compare values obtained for the different measured parameters ($P<0.05$ and $P<0.01$). Mean values were compared using the Tukey test ($P<0.05$).

## RESULTS

### BAP and TDZ pretreatment on germination

We assessed the effect of BAP and TDZ application during *T. grandis* seed germination on shoot regeneration efficiency. Seedlings germinated on MS culture medium with no cytokinin pretreatment were used as the control (Figure 1a). The germination percentage on MS culture medium supplemented with 0.1 or 0.5 mg $L^{-1}$ of BAP or TDZ was not affected by the cytokinin pretreatment. However, the seedlings germinated on a cytokinin supplemented medium displayed reduced apical and root tip growth with thick but smaller cotyledons, compared to untreated control seedlings. The phenotype severity varied depending on the type and concentration of cytokinin used on the germination medium. The cytokinin TDZ at 0.5 mg $L^{-1}$ resulted in the most severe abnormal phenotype (Figure 1a).

## Shoot regeneration by indirect organogenesis

Shoot regeneration on a culture medium containing BAP, IBA, or $GA_3$ (at varying concentrations) increased with the use of cytokinin pretreatment during germination. We obtained shoot regeneration in 14 of the treatments (Table 1). No axillary shoot induction was observed from the explants with no cytokinin pretreatment (control, MS culture medium free of plant growth regulators). In addition, no shoot regeneration was achieved on a culture medium without BAP, IBA, or $GA_3$ (Table 1).

The highest percentage of shoot regeneration was recorded for explants from seedlings germinated on pretreatment medium containing 0.1 mg $L^{-1}$ TDZ and 0.5 mg $L^{-1}$ BAP and subsequently cultured on MS culture medium supplemented with 1.0 mg $L^{-1}$ BAP and 0.5 mg $L^{-1}$ $GA_3$ (Tables 1 and 2). Hypocotyls showed a suitable organogenesis process, leaf primordia, and apical meristem development (Figure 1b-c), and a considerable increase in shoot induction (>30% in 13 treatments), compared to the no pretreatment control (Table 1). Specifically, BAP pretreatment with either 0.1 or 0.5 mg $L^{-1}$ showed a 22% increase in shoot induction in all shoot regenerations, compared to explants from seedlings germinated with no pretreatment. In addition, 62.5% of the treatments with different plant growth regulators (Tables 1 and 2) induced shoots (Figure 1b-e), with a maximum average of 2.7 shoots per explant emerging when pretreated with 0.5 mg $L^{-1}$ TDZ. In addition, the greatest number of shoots per responsive explant (i.e., 7 shoots) was achieved from the hypocotyl explants taken from seedlings germinated in the presence of 0.1 or 0.5 mg $L^{-1}$ TDZ (Table 2).

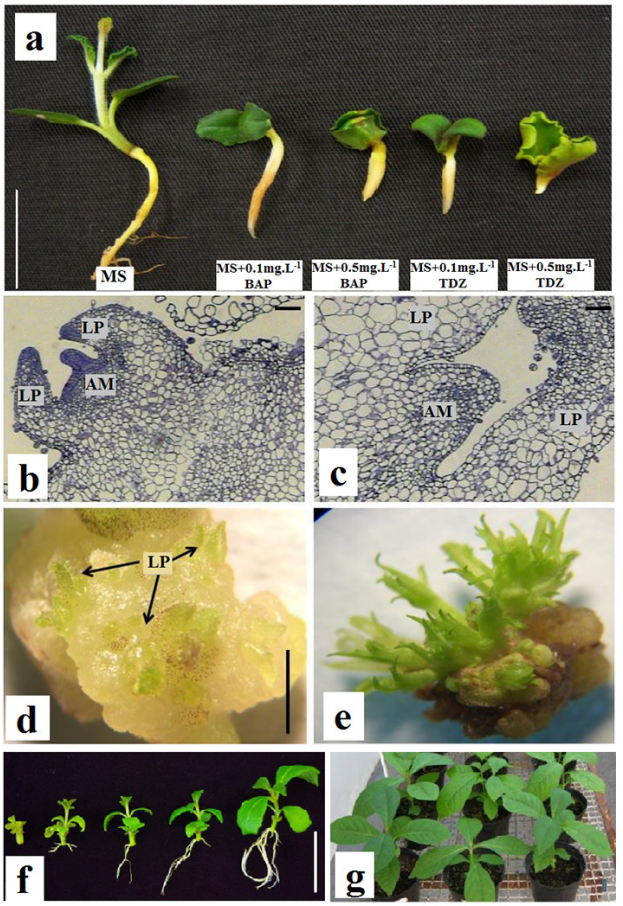


***Figure 1.*** Organogenesis of *Tectona grandis*. (a) Effects of BAP and TDZ at 20 days after germination (Bar = 1 cm); (b-c) Indirect organogenesis in adventitious buds from hypocotyls by histological analysis (LP = leaf primordia, AM = apical meristem, Bar = 50 µm); (d-e) Regeneration after 28 days (Bar = 5 mm); (f) In vitro root sequential development, left to right: 5, 22, 30, 42, and 70 days; (g) plants in the greenhouse at 72 days after acclimatization.

The addition of 3.0 or 5.0 mg $L^{-1}$ BAP and 0.5 mg $L^{-1}$ $GA_3$ in the regeneration culture medium promoted shoot growth with at least one shoot reaching a length greater than 1.3 cm at six weeks (Table 3). The explants treated with 0.1 or 0.5 mg $L^{-1}$ TDZ during germination showed greater subsequent shoot growth, at 1.7±0.6 cm and 1.5±0.5 cm, respectively, after 80 days, compared with 1.0 cm for the control (Table 3). The largest shoot growth (2.7 cm, 80 days after regeneration) was observed in the shoots regenerated from cotyledons pretreated with 0.5 mg $L^{-1}$ BAP. In general, explants from the seedlings germinated in the presence of 0.1 and 0.5 mg.$L^{-1}$ TDZ showed significant increases in shoot induction and subsequent shoot growth, compared to BAP treatment at the same concentrations (Figure 1a, Table 1). Remarkably, the highest regeneration frequencies were achieved from cotyledonary segments (70% of regeneration) and hypocotyls (60% of regeneration) when the seedlings germinated in the presence of 0.5 and 0.1 mg $L^{-1}$ TDZ, respectively, and the explants subsequently cultured on MS medium supplemented with 1.0 mg $L^{-1}$ BAP and 0.5 mg $L^{-1}$ $GA_3$ (Tables 1 and 2).

## Microsatellites analysis

In order to evaluate the genetic variability of the seeds used to obtain organogenesis from different pretreated explants, we analyzed the mating system and estimate relationship parameters and coancestry. Microsatellite analyses of our seed pool showed that seedlings were composed mainly of half-sibs (81%), followed by self-half-sibs (16%), full-

sibs (3%) and self-sibs (1%) (Table 4). The average coancestry coefficient within progenies (Θ) was 0.136. Due to the detected selfing and correlated mating in progenies, the average coancestry coefficient within progenies (Θ) was higher than expected for half-sib populations (Θ = 0.125). This difference can influence the organogenetic potential of collected seedlings.

***Table 1.*** Percentage of *Tectona grandis* regeneration based on the effects of cytokinins used in germination (MS - control, BAP, or TDZ, mg $L^{-1}$) and regeneration medium with different plant growth regulators (BAP, IBA, and $GA_3$, mg $L^{-1}$) after 80 days of in vitro culture

| BAP[1] | IBA[1] | $GA_3$[1] | -- MS (control)[2] -- | | | --- 0.1 BAP[2] --- | | | --- 0.5 BAP[2] --- | | | --- 0.1 TDZ[2] ---- | | | --- 0.5 TDZ[2] --- | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | $0.0^{B}$ |
| 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 15.0 | 0.0 | 0.0 | 15.0 | 15.0 | 0.0 | 5.0 | 20.0 | 0.0 | 20.0 | $6.0^{AB}$ |
| 3.0 | 0.0 | 0.0 | 0.0 | 0.0 | 30.0 | 0.0 | 0.0 | 20.0 | 10.0 | 0.0 | 5.0 | 20.0 | 0.0 | 0.0 | 15.0 | 5.0 | 15.0 | $8.0^{AB}$ |
| 5.0 | 0.0 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 20.0 | 0.0 | 0.0 | 0.0 | 0.0 | 10.0 | 5.0 | 0.0 | 20.0 | 15.0 | $5.3^{AB}$ |
| 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | $0.0^{B}$ |
| 1.0 | 0.5 | 0.0 | 0.0 | 0.0 | 20.0 | 15.0 | 0.0 | 0.0 | 15.0 | 0.0 | 30.0 | 45.0 | 0.0 | 40.0 | 30.0 | 10.0 | 0.0 | $13.7^{AB}$ |
| 3.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 20.0 | 0.0 | 30.0 | 25.0 | 0.0 | 30.0 | 25.0 | 0.0 | 15.0 | 25.0 | 0.0 | 30.0 | $13.3^{AB}$ |
| 5.0 | 0.5 | 0.0 | 0.0 | 0.0 | 20.0 | 0.0 | 0.0 | 30.0 | 0.0 | 0.0 | 20.0 | 5.0 | 0.0 | 15.0 | 0.0 | 10.0 | 10.0 | $7.3^{AB}$ |
| 0.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 5.0 | 10.0 | 0.0 | 0.0 | 40.0 | 0.0 | 40.0 | $7.0^{AB}$ |
| 1.0 | 0.5 | 0.5 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 25.0 | 10.0 | 10.0 | 25.0 | 55.0 | 30.0 | 25.0 | 55.0 | 0.0 | 40.0 | $19.0^{A}$ |
| 3.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 15.0 | 0.0 | 20.0 | 40.0 | 0.0 | 15.0 | $6.7^{AB}$ |
| 5.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 | 25.0 | 20.0 | 0.0 | 20.0 | 20.0 | 0.0 | 20.0 | 20.0 | 0.0 | 5.0 | $9.3^{AB}$ |
| 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 10.0 | 0.0 | 0.0 | 20.0 | 0.0 | 25.0 | 10.0 | 10.0 | 0.0 | 20.0 | 0.0 | 10.0 | $7.0^{AB}$ |
| 1.0 | 0.0 | 0.5 | 0.0 | 0.0 | 5.0 | 0.0 | 0.0 | 15.0 | 0.0 | 10.0 | 20.0 | 15.0 | 0.0 | 60.0 | 70.0 | 15.0 | 55.0 | $17.7^{A}$ |
| 3.0 | 0.0 | 0.5 | 0.0 | 0.0 | 5.0 | 15.0 | 0.0 | 25.0 | 25.0 | 0.0 | 0.0 | 20.0 | 30.0 | 15.0 | 15.0 | 0.0 | 40.0 | $12.6^{AB}$ |
| 5.0 | 0.0 | 0.5 | 0.0 | 0.0 | 5.0 | 10.0 | 0.0 | 20.0 | 0.0 | 0.0 | 20.0 | 15.0 | 0.0 | 30.0 | 40.0 | 0.0 | 35.0 | $11.7^{AB}$ |
| Average | | | $0.0^{e}$ | $0.0^{e}$ | $6.5^{cd}$ | $5.0^{cd}$ | $0.0^{e}$ | $14.7^{ab}$ | $8.4^{bcd}$ | $1.3^{de}$ | $13.4^{ab}$ | $16.9^{ab}$ | $5.0^{cd}$ | $15.6^{ab}$ | $24.4^{a}$ | $3.8^{cd}$ | $20.6^{ab}$ | 9.0 |

BAP = benzylaminopurine; IBA = indole-3-butyric acid; $GA_3$ = gibberellic acid; MS = basal medium (Murashige and Skoog 1962); TDZ = thidiazuron; Cs = cotyledonary segment; Co = cotyledon; Hy = hypocotyls. [1] Regeneration medium; [2] Germination medium.
For Averages, means followed by the same capital letter (column), or the same lowercase letter (row) do not differ significantly by Tukey test (P<0.05).

***Table 2.*** Number of *Tectona grandis* shoots based on the effects of cytokinins used in germination (MS - control, BAP, or TDZ, mg $L^{-1}$) and regeneration medium with different plant growth regulators (BAP, IBA, and $GA_3$, mg $L^{-1}$) after 80 days of in vitro culture

| BAP[1] | IBA[1] | $GA_3$[1] | -- MS (control)[2] -- | | | --- 0.1 BAP[2] --- | | | --- 0.5 BAP[2] --- | | | --- 0.1 TDZ[2] --- | | | ---0.5 TDZ[2] --- | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | $0.0^{B}$ |
| 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.3 | 0.0 | 0.0 | 1.7 | 3.5 | 0.0 | 1.0 | 2.7 | 0.0 | 2.3 | $0.9^{AB}$ |
| 3.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.7 | 0.0 | 0.0 | 4.3 | 2.0 | 0.0 | 3.5 | 2.3 | 0.0 | 0.0 | 2.0 | 2.0 | 2.0 | $1.6^{AB}$ |
| 5.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 0.0 | 2.3 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 | 4.0 | 0.0 | 2.7 | 2.0 | $1.0^{AB}$ |
| 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | $0.0^{B}$ |
| 1.0 | 0.5 | 0.0 | 0.0 | 0.0 | 2.3 | 1.0 | 0.0 | 0.0 | 5.5 | 0.0 | 3.0 | 2.5 | 0.0 | 4.0 | 1.6 | 1.0 | 0.0 | $1.4^{AB}$ |
| 3.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 2.5 | 0.0 | 3.7 | 2.2 | 0.0 | 4.0 | 1.5 | 0.0 | 4.3 | 2.0 | 0.0 | 3.0 | $1.6^{AB}$ |
| 5.0 | 0.5 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 | 0.0 | 4.5 | 0.0 | 0.0 | 3.7 | 1.0 | 0.0 | 2.7 | 0.0 | 1.0 | 1.0 | $1.3^{AB}$ |
| 0.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.5 | 0.0 | 0.0 | 3.0 | 1.0 | 0.0 | 0.0 | 4.0 | 0.0 | 5.0 | $1.1^{AB}$ |
| 1.0 | 0.5 | 0.5 | 0.0 | 0.0 | 3.5 | 0.0 | 0.0 | 2.5 | 1.0 | 1.0 | 2.3 | 3.2 | 2.5 | 4.8 | 2.4 | 0.0 | 5.0 | $1.9^{AB}$ |
| 3.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 0.0 | 2.5 | 0.0 | 3.7 | 2.7 | 0.0 | 1.0 | $0.8^{AB}$ |
| 5.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 5.5 | 2.0 | 0.0 | 3.3 | 1.3 | 0.0 | 3.7 | 2.0 | 0.0 | 1.0 | $1.3^{AB}$ |
| 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 3.0 | 0.0 | 5.0 | 0.0 | 1.5 | 0.0 | 1.3 | 0.0 | 1.0 | $0.9^{AB}$ |
| 1.0 | 0.0 | 0.5 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 6.9 | 0.0 | 3.0 | 5.0 | 1.3 | 0.0 | 7.0 | 3.2 | 4.0 | 7.0 | $2.6^{A}$ |
| 3.0 | 0.0 | 0.5 | 0.0 | 0.0 | 1.0 | 1.5 | 0.0 | 4.5 | 1.7 | 0.0 | 0.0 | 3.3 | 3.0 | 3.0 | 1.0 | 0.0 | 5.0 | $1.6^{AB}$ |
| 5.0 | 0.0 | 0.5 | 0.0 | 0.0 | 1.0 | 1.0 | 0.0 | 5.3 | 0.0 | 0.0 | 3.5 | 2.0 | 0.0 | 4.3 | 2.0 | 0.0 | 5.5 | $1.5^{AB}$ |
| Average | | | $0.0^{c}$ | $0.0^{c}$ | $1.3^{abc}$ | $0.5^{c}$ | $0.0^{c}$ | $2.8^{a}$ | $1.2^{abc}$ | $0.3^{c}$ | $2.3^{ab}$ | $1.2^{abc}$ | $0.6^{c}$ | $2.6^{a}$ | $1.7^{abc}$ | $0.7^{bc}$ | $2.6^{a}$ | 1.2 |

BAP = benzylaminopurine; IBA = indole-3-butyric acid; $GA_3$ = gibberellic acid; MS = basal medium (Murashige and Skoog 1962); TDZ = thidiazuron; Cs = cotyledonary segment; Co = cotyledon; Hy = hypocotyls. [1] Regeneration medium; [2] Germination medium.
For Averages, means followed by the same capital letter (column), or the same lowercase letter (row) do not differ significantly by Tukey test (P<0.05).

***Table 3.*** Shoot height (cm) of *Tectona grandis* based on the effects of cytokinins used in germination (MS - control, BAP, or TDZ, mg $L^{-1}$) and regeneration medium with different plant growth regulators (BAP, IBA, and $GA_3$, mg $L^{-1}$) after 80 days of in vitro culture

| BAP[1] | IBA[1] | $GA_3$[1] | -- MS (control)[2] -- | | | --- 0.1 BAP[2] --- | | | --- 0.5 BAP[2] --- | | | --- 0.1 TDZ[2] --- | | | --- 0.5 TDZ[2] --- | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | Cs | Co | Hy | |
| 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | $0.0^{B}$ |
| 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.2 | 0.0 | 0.0 | 1.7 | 1.8 | 0.0 | 2.3 | 1.3 | 0.0 | 1.0 | $0.6^{AB}$ |
| 3.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.1 | 0.0 | 0.0 | 1.0 | 1.5 | 0.0 | 1.0 | 1.3 | 0.0 | 0.0 | 1.0 | 2.0 | 1.0 | $0.7^{AB}$ |
| 5.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.2 | 1.8 | 0.0 | 1.7 | 1.0 | $0.6^{AB}$ |
| 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | $0.0^{B}$ |
| 1.0 | 0.5 | 0.0 | 0.0 | 0.0 | 1.0 | 1.3 | 0.0 | 0.0 | 2.0 | 0.0 | 1.0 | 1.4 | 0.0 | 1.4 | 1.0 | 2.0 | 0.0 | $0.7^{AB}$ |
| 3.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 1.0 | 1.3 | 0.0 | 1.7 | 1.3 | 0.0 | 1.0 | 1.3 | 0.0 | 1.3 | $0.7^{AB}$ |
| 5.0 | 0.5 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 | 1.0 | 1.0 | 0.0 | 1.0 | 0.0 | 2.0 | 0.4 | $0.5^{AB}$ |
| 0.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 1.0 | 1.0 | 0.0 | 0.0 | 2.7 | 0.0 | 1.3 | $0.5^{AB}$ |
| 1.0 | 0.5 | 0.5 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 1.0 | 1.0 | 1.5 | 1.3 | 1.2 | 2.4 | 1.2 | 1.4 | 0.0 | 1.3 | $0.9^{AB}$ |
| 3.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 2.0 | 0.0 | 1.4 | 1.0 | 0.0 | 1.3 | $0.4^{AB}$ |
| 5.0 | 0.5 | 0.5 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 1.2 | 1.3 | 0.0 | 1.0 | 1.7 | 0.0 | 1.7 | 1.5 | 0.0 | 1.0 | $0.7^{AB}$ |
| 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 0.0 | 1.0 | 0.0 | 1.0 | 1.0 | 2.5 | 0.0 | 1.0 | 0.0 | 1.0 | $0.6^{AB}$ |
| 1.0 | 0.0 | 0.5 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 1.0 | 0.0 | 4.0 | 1.0 | 1.3 | 0.0 | 1.3 | 1.0 | 3.0 | 1.0 | $1.0^{A}$ |
| 3.0 | 0.0 | 0.5 | 0.0 | 0.0 | 1.0 | 1.0 | 0.0 | 1.0 | 1.0 | 0.0 | 0.0 | 1.3 | 2.7 | 1.0 | 1.0 | 0.0 | 1.0 | $0.7^{AB}$ |
| 5.0 | 0.0 | 0.5 | 0.0 | 0.0 | 1.0 | 1.0 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.0 | 0.0 | 1.1 | 0.8 | 0.0 | 1.8 | $0.6^{AB}$ |
| Average | | | $0.0^{b}$ | $0.0^{b}$ | $0.5^{ab}$ | $0.4^{ab}$ | $0.0^{b}$ | $0.8^{ab}$ | $0.6^{ab}$ | $0.3^{ab}$ | $0.8^{ab}$ | $1.1^{a}$ | $0.6^{ab}$ | $1.0^{a}$ | $0.9^{a}$ | $0.7^{ab}$ | $0.9^{a}$ | 0.6 |

BAP = benzylaminopurine; IBA = indole-3-butyric acid; $GA_3$ = gibberellic acid; MS = basal medium (Murashige and Skoog 1962); TDZ = thidiazuron; Cs = cotyledonary segment; Co = cotyledon; Hy = hypocotyls. [1] Regeneration medium; [2] Germination medium.
For Averages, means followed by the same capital letter (column), or the same lowercase letter (row) do not differ significantly by Tukey test (P<0.05).

### In vitro adventitious rooting of regenerated shoots and acclimatization

Shoots greater than 1 cm were excised from the explants and placed on a rooting medium to induce adventitious roots. Most roots began appearing two weeks after induction, with full root formation after six weeks (Figure 1f). We observed that 65% of the shoots produced roots on the rooting medium (½MS + 0.5 mg $L^{-1}$ IBA + 7 g $L^{-1}$ sucrose). After transferring plantlets to plastic pots and placing them in a greenhouse, 60% of plantlets showed well-developed leaves and roots survived and grew suitably after transfer (Figure 1g). The entire procedure, from tissue culture initiation to plant establishment in a greenhouse, took about four months.

## DISCUSSION

### Indirect organogenesis

Baghel et al. (2008), assessing shoot induction from hypocotyls, cotyledonary axis, and cotyledons without pretreatment for *Tectona grandis*, observed mean shoot induction frequencies ranging from 14 to 63% from explants placed in MS culture medium supplemented with BAP, NAA, and Kinetin (at varying concentrations), with the best plant regeneration produced by cotyledonary axis. In our study, we found similar shoot regeneration frequencies, ranging from 5 to 70%, which varies widely depending on explant type, pretreatment, and regeneration culture medium with combinations of plant growth regulators. Unlike Baghel et al. (2008), we did not observe regeneration from cotyledons or cotyledonary segments in the absence of pretreatment, suggesting that the pretreatment with cytokinin had a significant impact on shoot regeneration.

Teak is preferentially allogamous (Kertadikara and Prat 1995) and seeds from open-pollinated populations were used in this study. Even with 81% half-sibs (Table 4), the coancestry coefficient was higher within progenies than expected for half-sib populations. Subsequently, genetic differences are expected between each seed given that teak presents high levels of heterozygosis (Fofana et al. 2009). In addition, teak presents different levels of relatedness within progenies (Kertadikara and Prat 1995), affecting the variation in tissue regeneration between treatments (e.g., additives, dominant and epistatic effects) (Kielly and Bowley 1997). If the mating system involves mixtures of outcrossing, selfing, and correlated mating, the open-pollinated progenies will contain mixtures of half-sibs, self-sibs, full-sibs, and self-half-

sibs (Ritland 1989). Thus, relatedness within and among progenies will be higher than expected in half-sib progenies and it will influence genetic and physiological traits. Shoot induction by cytokinin pretreatment (especially TDZ) was positive (Table 1) and genetic variability did not seem to be an important factor in the prevention of organogenesis.

***Table 4.*** Mating system and relationship parameters for the pool of 42 *Tectona grandis* seeds

| Parameter | Seed pool (±SD) |
|---|---|
| Multilocus outcrossing rate: $t_m$ | 0.914 (±0.104) |
| Single-locus outcrossing rate: $t_s$ | 0.906 (±0.069) |
| Mating among relatives: $t_m - t_s$ | 0.008 (±0.047) |
| Multilocus paternity correlation: $r_{p(m)}$ | 0.032 (±0.034) |
| Percent of pairwise self-sibs: $P_{SS}$ (%) | 1.0 |
| Percent of pairwise half-sibs: $P_{HS}$(%) | 81.0 |
| Percent of pairwise full-sibs: $P_{FS}$(%) | 3.0 |
| Percent of pairwise self-half-sibs: $P_{SHS}$(%) | 16.0 |
| Coancestry: Θ | 0.136 |

SD = standard deviation.

## Cytokinin pretreatment relevance

The observed effect of cytokinin pretreatment appears to be robust across the varying genetic backgrounds of the explants. The use of synthetic cytokinins (BAP or TDZ) is advantageous not only because they promote shoot induction, but also because they are immune to degradation by cytokinin oxidase, thereby increasing the effectiveness of the pretreatment (Werner et al. 2001). Reports on *Curcuma longa* and *Curculigo orchioides* show that explants obtained from seedlings germinated in the presence of TDZ are more responsive in shoot regeneration than explants with no treatment (Prathanturarug et al. 2005, Thomas 2007). This observation suggests that the ability of the explants to regenerate could be stimulated by pretreatment. In all treatments assessed herein, the percentage of regeneration increased with pretreatment, but for cotyledons regeneration from the explants required pretreatment with TDZ or BAP.

The TDZ pretreatment in this study resulted in an increase of 43% in the number of explants that survived for all culture medium, with shoots produced from all surviving explants (Table 2) and the occurrence of leaf primordia (Figures 1D and 1E). Cotyledonary segments and hypocotyls pretreated with 0.5 and 0.1 mg $L^{-1}$ TDZ, respectively, showed the most favorable regeneration values (70% and 60%, respectively) (Table 1). When BAP was used as pretreatment for two strawberry cultivars (*Fragaria* × *ananassa*), it increased shoot regeneration by up to 20% on a medium containing BAP at 0.5 mg $L^{-1}$, IBA at 0.5 mg $L^{-1}$, and $GA_3$ at 0.2 mg $L^{-1}$ (Sorvari et al. 1993). In addition, pretreating explants with BAP at 1.8 mg $L^{-1}$ positively influenced the subsequent success of tissue culture in *Pinus pinaster*, in which the BAP pretreatment promoted the morphogenesis of leaf explants (David et al. 1982). For mulberry (*Morus alba* L.), adventitious bud formation was also observed after BAP pretreatment (Oka and Ohyama 1981). The role of cytokinins in shoot induction has been described for *T. grandis*, including 0.5 and 1.5 mg $L^{-1}$ BAP (Gyves et al. 2007, Tiwari et al. 2002), 1.0 mg $L^{-1}$ kinetin (Devi et al. 1994), and 0.5 mg $L^{-1}$ TDZ (Kozgar and Shahzad 2012). However, our report describes for the first time the effect of cytokinin pretreatment on *Tectona grandis* regeneration.

The addition of adequate levels of TDZ cytokinin is known to increase shoot numbers in other trees, such as *Pterocarpus marsupium* (Husain et al. 2007), *Fraxinus americana* (Palla and Pijut 2011), *Prunus salicina* (Canli and Tian 2009), *Abies fraseir* (Kim et al. 2009), *Melia azedarach* (Vila et al. 2007), *Quercus rubra* (Vengadesan and Pijut 2009), *Pongamia pinnata* (Sujatha and Hazra 2007), *Acrocomia aculeate* (Moura et al. 2009), and *Cinnamomum pauciflorum* (Kong et al. 2009). Finally, cytokinins play a central role during the cell cycle and influence numerous developmental processes, including the formation of shoot meristems, demonstrating their function beyond maintaining the cell cycle (Werner et al. 2001).

## Adventitious rooting and acclimatization

Shoots excised from explants and placed on rooting medium (½MS + 0.5 mg $L^{-1}$ IBA + 7 g $L^{-1}$ sucrose) induced adventitious roots (65%) two weeks after induction, with full root formation after six weeks (Figure 1f). For the rooting medium, we used a protocol similar to Baghel et al. (2008) (MS + 1.0 mg $L^{-1}$ IBA + 7.0 g $L^{-1}$ sucrose), but with a lower IBA concentration. The use of IBA at low concentrations is recommended since it does not interfere with shoot growth and root elongation (Gyves et al. 2007). Similarly, Shirin et al. (2005) obtained 66% of adventitious rooting when using MS supplemented with 2.6 mg $L^{-1}$ NAA. After transferring to a greenhouse, 60% of the teak plantlets in this study grew suitably. Teak plantlet growth under greenhouse conditions has not been a problem, with normal survival rates of more than 60% (Tiwari et al. 2002, Shirin et al. 2005, Gyves et al. 2007, Akram and Aftab 2009). The whole process from tissue culture to greenhouse plant establishment was successful for teak, with each step being essential to obtain complete plants from indirect regeneration. Thus, the time span for complete regeneration was relatively short for a tree species.

Herein, we describe an efficient indirect regeneration protocol through organogenesis from *Tectona grandis* seedling explants. Cytokinin pretreatment increased organogenic competence of teak explants collected from seeds. In particular, TDZ pretreatment was effective in inducing shoot regeneration. Despite the fact that the plant material used in this study came from open-pollinated seeds with varied genetic makeup, shoot regeneration and subsequent adventitious rooting of the regenerated plants were successful. The best organogenic potential was achieved when hypocotyls and cotyledonary segments from seedlings were germinated on a medium containing TDZ and cultured for four weeks on MS medium supplemented with BAP and $GA_3$. Finally, the protocol described herein can significantly increase regeneration efficiency for teak. This regeneration protocol can serve as a platform for genetic transformation to improve this important tropical tree species with significant economic value.

## ACKNOWLEDGMENTS

Evandro V. Tambarussi and Marcelo Rogalski were supported by “Fundação de Amparo à Pesquisa do Estado de São Paulo” (FAPESP - Fellowship Process number 07/05795-0 and 08/00046-2, respectively). Esteban Galeano was supported by “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES - Fellowship Process 58271008) and “Fundação de Amparo à Pesquisa do Estado de São Paulo” (FAPESP- Fellowship Process 13/06299-8). The authors would like to thank “Proteca Biotecnologia Florestal” Company for kindly providing teak seeds.

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