Morphological and physiological response of proliferating shoots of teak to temporary immersion and BA treatments.

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Abstract

In vitro shoot proliferation of *Tectona grandis* L. (teak) using temporary immersion system (TIS) and different concentrations of BA is presented. Shoots grown on semi-solid MS medium supplemented with 2% (w/v) sucrose and 4.44 µM of BA were used as starting materials. In the TIS, three BA concentrations (2.22, 4.44 and 6.66 µM) and a cytokinin-free medium (CK-free medium) were assayed. A high average number of shoot was reached, applying 4.44 and 6.66 µM of BA (7.7 and 10.3 shoots/explant), respectively. The high BA concentrations decreased the accumulation of phenolic compounds and the deposition of lignin in the vascular cells of the teak shoots. The morphometric analysis by scanning microscopy revealed that the leaves of the shoots cultured in the TIS in Ck-free medium and with 2.22 and 4.44 µM of BA showed elliptical stomata; however, in the leaves developed with 6.66 µM of BA, the stomata were majorly ring-shaped, raised and wide open. Deformed stomata with broken epidermis of the guard cells, typical of hyperhydric leaves, were also observed. The survival percentages after ex vitro rooting on the IBA (492.1 µM) solution were higher in plantlets from CK-free medium and with 2.22 µM of BA, (96.7 and 91.7%, respectively) than those cultured on semi-solid medium (73%). The shoots from both TIS treatments developed a good root system. Plantlets from 6.66 µM of BA showed the lowest survival percentages (60%). A survival percentage was 100% two weeks after transplanting, and three months after ex vitro transfer, the plants were ready for field plantation. Here we present the first report of the successful propagation of teak by TIS.

Keywords*:* cytokinin, forest plant, hyperhydricity, liquid medium, micropropagation, semi-automation

Abbreviations*: BA* 6-Benzyladenine - *MS* Murashige and Skoog basal medium - *DW* dry weight - *FW* fresh weight - *GAE* Gallic Acid Equivalent - *WP* water percentage - *SEM* scanning electron microscope - *TIS* temporary immersion systems

Introduction

Teak (*Tectona grandis* L.) is one of the world's premier hardwood timbers. It is highly sought after for shipbuilding as well as for interior and exterior luxury furnishings. Teak ranks among the top five tropical hardwood species in terms of plantation area established worldwide. The establishment of new industrial forest plantations requires a lot of time before meeting the high demand for this precious timber. To enhance teak

plantation development different propagation methods have been described, but tissue culture is a commercially feasible method for producing teak plants as uniform as possible on a large scale and in a short time for the plantation industry (Krishnapillay, 2000). In consequence, different propagation protocols on agar-based systems have been described (Monteuuis et al. 1998; Tiwari et al. 2002; Yasodha et al. 2005; Gyves et al. 2007). However, it is well-known that the mass propagation of plants by tissue culture in conventional semi-solid media is labor intensive and costly. Gelling agents contribute significantly to in vitro production costs and limit the automation for commercial mass propagation. Consequently, new studies on in vitro propagation using different culture conditions can contribute to further optimization of the process and to the reduction of production costs (Ziv, 2005).

Using liquid media in micropropagation processes is considered the ideal solution for reducing plantlet production costs and for considering automation (Aitken-Christie et al. 1995). Nevertheless, the advantages of in vitro culture in a liquid medium are often counterbalanced by technical problems such as asphyxia and hyperhydricity, a typically stress-induced change of morphological, anatomical and physiological disorders (see Ziv 1991; Debergh and Read. 1991; Kevers et al. 2004). Different procedures have been developed to avoid these problems (Ziv, 2005). Among them, the use of temporary immersion systems (TIS) to improve in vitro growth and plant quality in different species (see review Etienne and Berthouly, 2002; Quiala et al. 2006; Roels et al. 2006; Aragón et al. 2009, Pérez-Alonso et al., 2009; Yan et al., 2010, Sankar-Thomas and Lieberei, 2011). However, few forestry species, *Eucalyptus sp* (Castro and González, 2002; McAlister et al. 2005), *Pinus radiata* D. Don (Aitken-Christie and Jones, 1987) and *Crescentia cujete* L. (Murch et al. 2004) have been successfully propagated by TIS.

The aim of this report was to determine the optimal concentration of BA for the teak shoot proliferation in the TIS, and to clarify whether the different concentrations of BA during the multiplication stage affect teak plants development under ex vitro conditions. Here we present the first report of the successful propagation of teak in this semi-automatic system.

Materials and Methods

Plant material and growth condition

Apical shoots from plants cultured in greenhouse and cloned from epicormic shoots of 30-years-old teak trees from the Experimental Forest in Villa Clara, Cuba, were cut off. The shoots were surface sterilised with ethanol (70% v/v) for 30 s. After rinsing three times with sterile distilled water, the explants were dipped in a water

solution containing 2% sodium hypochlorite and 0.2 ml Tween-80 for 10 min, followed by three rinses in sterile distilled water. The explants were then singly placed in test tubes (25 mm × 150 mm) with 10 ml of basal MS (Murashige and Skoog, 1962) medium supplemented with 6-BA (2.22 µM), sucrose (2%; w/v), and solidified with 2.5 g l^{-1} gelrite (Duchefa Biochemie, NL), semi-solid medium, to induce bud sprouting. The medium pH was adjusted to 5.8 before autoclaving. After 48 hours, the shoots were transferred to a fresh medium to reduce browning. After 30 days, the aseptic shoots were cut into single nodes with their respective 2 leaves and placed into a proliferation basal MS (Murashige and Skoog, 1962) medium supplemented with BA (4.44 µM), sucrose (2%; w/v), and solidified with 2.5 g l^{-1} gelrite (Duchefa Biochemie, NL). Eight shoots were cultured per 25 ml of proliferation medium, distributed in a 250 ml glass culture container. The cultures were incubated at 25±2°C and 16-h light (fluorescent lamps with photon lux light intensity of 40 µmol m^{-2} s^{-1}). After the seventh subculture, the explants were inoculated into the TIS. For the semi-solid treatment, 30 explants were cultured during 4 weeks in similar conditions as described above and evaluated at the same time as the TIS. Three replicates were done.

TIS and experimental design

The concept and operation of the TIS used in the experiments were based on the two-vessel system described by Escalona et al. (1999), but using glass vessels with a 2 L capacity (Figure. 1A). Ten apical shoots with two pairs of fully expanded leaves were inoculated per container and 500 ml of proliferation liquid culture medium were used. Glass beads were placed in the bottom of the vessels to support the explants. The system was programmed to transfer the medium and to immerse the explants for 40 seconds every 6 hours.

Three concentrations of BA (2.22, 4.44 and 6.66 µM) and a cytokinin-free medium (CK-free medium) were assayed. In order to test the TIS culture system profitability in relation to the conventional culture methods, a control treatment consisting in shoots cultured on a conventional semi-solid basal MS medium supplemented with BA (4.44 µM) was included. The number of normal shoots (NS), number of hyperhydric shoots (HS), shoot length (cm), stem diameter at the base (mm), number of nodes per shoot, number of leaves per shoot and the rooting percentages were recorded after 4 weeks of culture. Three culture vessels were used in each treatment and the experiment was repeated three times.

Measurement of water content

The liquid culture medium was removed from the vessel in order to determine the content of water. The shoots were collected from the different treatments and then rinsed with distilled water. Immediately after shoot

harvesting, the fresh weight (FW) was recorded, and shoots were drying for 48h at 60 °C and their dry weight determined. The water content (WC) was calculated as:

$$WC(\%) = \frac{(FW - DW)}{FW} x100$$

Measurement of total phenolic content

For the phenol quantification, the teak shoots from the different treatments described above were sampled. Upon collecting, the samples were frozen in liquid nitrogen and stored at –80°C until further use. The samples were freeze-dried and finely ground with a pestle and mortar. Plant extracts were prepared according to Kirca and Arslan (2008) with modifications regarding the amounts of plant materials (Santamaría et al. 2010). The dried plant material (0.075 g) was weighed in a centrifuge tube and 1.5 ml of methanol was added. The suspension was shaken at 1000 rpm at room temperature on an orbital shaker (Thermomixer Compact, Eppendorf) for 1 h. The suspension was centrifuged at 5204 × g for 10 min, and the supernatant was transferred to an 8 ml volumetric flask. The residue was re-extracted with 1.5 ml methanol using the same procedure. After this procedure was repeated three times all extracts were pooled and the volume adjusted to 7.5 ml with methanol. This crude extract was filtered though a filter paper and kept in amber-coloured glass bottles at –18°C until analysis.

The total phenol content was determined according to the Folin–Ciocalteu method (Spanos and Wrolstad 1990). A sample extract (50 µl) was mixed with distilled water (450 µl) and 0.2 N Folin–Ciocalteu reagent (2.5 ml). Two millilitres of saturated sodium carbonate was added, and the resulting mixture was vortexed for 1 min. After incubation at room temperature for 2 h, the absorbance of the solution was measured at 765 nm using a UV/VIS spectrophotometer (Beckman coulter DU1800). The total phenolic content was calculated based on a standard curve of Gallic acid, which was linear within a range of 100–2000 mg l^{-1} (R^2 = 0.9954). The results were presented as the mean of the nine analyses and expressed as milligrams of Gallic acid equivalents per gram dry weight (mg GAE g^{-1} DW).

Histochemistry

The portion of stem between the second and third pair of leaves of the initial explants was excised and 15 µM transversal sections of stem were obtained with a rotation microtome (MOD 1130/Biocut). The micro-chemical test of Wiesner (phloroglucinol-HCl) reaction to staining the transversal section was used to observe the deposition of lignin in the vascular cells. Samples were placed in a 1% phloroglucinol-HCl solution for 10 min and

observed immediately. Bright-field illuminated transversal sections were taken using a microscope Nikon Eclipse E600.

Scanning microscopy

For the scanning electron microscopy (SEM) and morphometric analysis, the second pair of fully expanded leaves from the primary explant culture in TIS and leaves of the seedlings cultured for 30 days in the greenhouse (*in vivo* control) were collected on the same day and hour, and prepared according the procedure described by Robinson et al. (1987) with slight modifications done by Majada et al. 1997. Leaf samples (1 cm^2) were fixed for 16 h in 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 and washed three times with the same buffer. Afterwards, the samples were dehydrated in ethanol series and finally passed to acetone. Samples were dried using the critical-point technique (Blazers CDP030), and then coated with gold in an argon atmosphere for 90 s and 22 mA (Blazers SCD 004 sputter coater). A Jeol scanning electron microscope (JSM 6100) at 15 kV (Universidad de Oviedo, Spain) was used for the observations and photomicrography of the foliar surface.

The stomatal density (number per mm^2), lengths and widths of the stomata (with guard cells) and pore dimensions, as well as the areas of stomatal pores and of entire stomata, relative to the leaf areas were measured from each photomicrograph by digital image processing software (Image Pro Plus® version 4.5.0.29 for Windows). Stomatal length refers to the distance between the ends of the guard cells, and the width is the distance transversely 'across' them.

Ex-vitro rooting and acclimatization

The teak shoots harvested from the different treatment in TIS and semi-solid culture medium were washed in running tap water, and dipped for two minutes in IBA (492.1 µM) solution before they were inserted, according to the experimental design, into a substrate. The shoots were then planted in 5 cm x 10 cm black polyethylene bag containers filled with organic matter (humus and sugarcane mill baggasse): Zeolite (1:1) mixture. Once set into the substrate, the shoots were placed in a greenhouse (30 ± 2ºC, RH 90%) and then maintained under 50% shade with intermittent-mist water sprays to avoid desiccation damages. The survival percentages were recorded after 30 days and the survivor plants from the different TIS treatments were pooled and transplanted to 10 cm x 15 black polyethylene bag containers. Three-month-old plants were planted in the field and their performance is still being observed.

Statistical analysis

The test of normality (Kolmogornov-Smirnov) was done. The significance was determined by the analysis of variance (ANOVA), and the least significant ($p<0.05$) differences among the mean values were estimated by Fisher's LSD. All the statistical tests were performed with the SigmaStat software. The data were presented as means ± standard error and different letters in tables, and figures indicated significant differences at $p<0.05$. The data presented in percentages were subjected to arcsine transformation before the analysis, and then converted back to percentages for their presentation in the tables.

Results

Effect of BA on shoot proliferation and growth

The TIS improved the in vitro teak shoot proliferation after 4 weeks of culture. In the CK-free medium, a single shoot per explant was produced (Figure 1B). All the shoots grown with 2.22 µM of BA displayed a normal morphology (Figure 1C), an average of 4.1 NS per explant was achieved (Table 1). The result from the TIS culture showed that shoot multiplication were greater in the immersion system, compared to the conventional propagation methods in semi-solid medium. In the TIS an average of 7.7 NS per explant with 4.44 µM of BA was achieved, whereas in the semi-solid medium and average of 3.6 NS per explant was reached with the same BA concentration (4.44 µM) (Table 1). Even though the highest number of NS (10.3 NS per explant) was reached with 6.66 µM of BA (Table 1), this BA concentration was harmful since a high average of HS (4.6 HS per explant) were observed (Figure 1E).

Shoot height and diameter were significantly increased when 2.22 µM of BAP was used (Figure 1C), but both parameters decreased with major concentrations. The number of leaves and nodes per shoot did not differ significantly between treatments (Table 1). Only in the control, a 100% of rooting plants were observed (Table 1). The rooting percentage decreased as the concentration of BA increase, rooting was inhibited with 6.66 µM of BA.

Here, we present the highest number of shoots reported in the literature for teak, up to now, achieving 7.7 and 10.3 NS with 4.44 µM and 6.66 of BA, respectively within 4 weeks of culture in TIS.

HERE TABLE 1 AND FIGURE 1

Measurement of water percentage and total phenolic content

The increment of BA in the culture medium increased the fresh and dry matter accumulation (Figures 2A and 2B), but not the ratio between them (Figure 2C). The shoots developed from all concentrations of BA accumulated, proportionally, more water than the shoots cultured on the CK-free medium (Figure 2D).

HERE FIGURE 2

Significant correlation was observed between total phenol content and BA concentration. A general descending trend in total phenol content in shoots cultured with BA (Figure 3) was observed. The accumulation of phenolics was three, two and one and a half time lower in shoots grown with 6.66, 4.44 and 2.22 µM of BA, respectively than in shoots from the CK-free medium.

HERE FIGURE 3

Histochemistry

The phloroglucinol-HCL staining consistently showed that BA concentration strongly affected the deposition of lignin in the vascular cells. The shoots from the CK-free medium had the most intensively staining lignifications and the highest concentration of BA, the weakest. The deposition of phloroglucinol-positive material in the stem tissue was rather amorphous in shoots cultured with cytokinin compared with the CK-free medium (Figure 4). The vascular system of shoots from the CK-free medium showed a well-organised xylem and homogeneous lignin deposition pattern (Figure 4A). In the shoots grown with 2.22 µM of BA, the organisation of xylem was normal, although some xylem cell walls were not lignified (Figure 4B). Xylem organisation in the shoots grown with 4.44 µM and 6.66 of BA was poor and phloroglucinol-positive material was amorphous, with no identifiable or consistent pattern of deposition and scarce lignifications observed (Figure 4C and 4D)

HERE FIGURE 4

Scanning microscopy

The anatomical examinations revealed that BA affected the development of the stomatal complex. Stomatal density in teak leaves was significantly higher than in the CK-free medium only when 6.66 µM of BA was used (Table 2). The increment in the concentration of BA was accompanied by an increase in the stomatal size and area of the whole stomatal complex.

The stomata were elliptical-shape, slightly open and sunken in the CK-free medium (Figures 5A and 5B) and with 2.22 µM of BA (Figures 5C and 5D), similar to the leaves from seedlings grown in the greenhouse (Figures 5I and

5J). In the leaves developed with 4.44 µM of BA, the stomata were also elliptical (Figures 5E and 5F) but more open and sited at the same levels as the epidermal cells. In the highest concentration (6.66 µM) of BA, stomata were ring-shaped, raised and wide open (Figures 5G and 5H), and the stomatal area was found to be three times higher than the CK-free medium and two and one and a half times higher than 2.22 and 4.44 µM of BA, respectively. Similarly, the pore area increased proportionally with BA concentration, being 10 times higher with 6.66 µM of BA than the CK-free medium and four and almost two times higher than 2.22 and 4.44 µM of BA, respectively; deformed stomata with broken epidermis of the guard cells were also observed with 6.66 µM of BA (Figures 5K and 5L).

HERE TABLE 2 AND FIGURE 5

Ex-vitro rooting and acclimatization

The simple ANOVA analysis showed that BA concentration affected the ex vitro survival of teak plants during acclimatization. The plants from the CK-free medium and 2.22 µM BA showed higher survival percentages (96.6% and 91.6%, respectively) (Figure 6), and a well-developed root system was observed (Figure 7A and 7B). Although no significant differences of survival percentages were observed between the plantlets grown on the TIS with 4.44 µM of BA (80%) and plantlets from culture in semi-solid medium (73%), a poor-developed root system was observed in the last one (Figure 7E). The plantlets grown in TIS with 6.66 µM of BA achieved the lowest survival percentage (60%) (Figure 6), and a poor-developed root system was observed (Figure 7D).

The survival percentage was 100% two weeks after transplanting (Figure 7F), and three months after the ex vitro transfer, the micropropagated teak plants were ready for field plantation (Figure 7G).

HERE FIGURE 6 AND FIGURE 7

Discussion

According to the literature, BA has been the most commonly cytokinin used in the micropropagation of teak, alone or combined with kinetin or auxin (Gangopadhyay, et al. 2002; Tiwari et al. 2002; Yasodha et al. 2005). Goswami et al. (1999) suggested a protocol for the micropropagation of teak using a basal MS semi-solid medium supplemented with BA and kinetin. In this protocol, an average of 3.7 NS per explant in eight weeks was produced after subculture onto an elongation medium. Tiwari et al. (2002) reported that the placement of the explants in MS medium supplemented with BA (22.2 µM) alone or with IAA (0.57 µM) resulted in the maximum average number of shoots, 5.63 and 5.67 shoot/explant, respectively. These shoots elongated to 3.7 cm within 4 weeks of culture.

Shirin et al. (2005) achieved the highest frequency of shoot proliferation (6.33) on BA (10 μmol·L^{-1}) and NAA (1.0 μmol·L^{-1}) using nodal explants from mature teak shoots. Gyves et al. (2007) reported a high number of shoots for teak. An average number of 4 shoots with 4 nodes were obtained by the authors after 4 weeks of culture.

In the last decade an important number of work has confirmed that temporary immersion stimulates shoot proliferation and growth (see review Etienne and Berthouly, 2002; Quiala et al. 2006; Pérez-Alonso et al. 2009; Sankar-Thomas and Lieberei, 2011; Yan et al. 2011). The high number of shoots obtained in the TIS may be a consequence of the efficient gaseous exchange between the plant tissue and the gas phase inside the vessel. Multiple daily air replacement by pneumatic transfer of the medium prevents the accumulation of gasses like ethylene or CO_2. Additionally, the uptake of nutrients and hormones over the whole explant surface ensures maximum growth (Preil, 2005). Etienne and Berthouly, 2002 suggested that the most important reason for the efficiency of the TIS is that it combines the advantages of both gelled culture (gas exchange) and liquid culture (increased nutrient uptake), which improves the growth of the plantlets.

Shoot proliferation was proportionally enhanced with the concentration of BA as shown in Table 1, but in vitro shoot morphology was strongly affected by BA concentration. In the highest concentration of BA (6.66 μM) several HS were observed. Therefore, this BA concentration was harmful for the teak shoot proliferation in the TIS. Although the mechanism for hyperhydricity remains to be elucidated, it might take place during the axillary bud multiplication stage and has been correlated, among other factors, with the relatively high cytokinin level (Debergh, 1983; Hazarika, 2006). It has been reported that cytokinin induced hyperhydricity in many species, usually in a concentration dependent manner and when other conditions in the culture system are not optimized (Mocaleán et al. 2009; Ivanova and Van Staden, 2011).

Significant correlation was observed between total phenol content and BA concentration. The lowest content of this important secondary metabolite was recorded when 6.66 μM of BA were used. Low phenolics levels have been found in hyperhydric tissues rather than in normal tissues (Perry et al. 1999). Kevers et al. (1984) reported that hyperhydricity was associated with several biochemical deviations, among others the phenolic metabolism. In teak, phenolics have been associated to a defence mechanism against dehydration and pathogen attack. The in vitro teak leaves are covered with particular globose trichomes, appearing to be glandular ones contained phenol droplets within them (Bandyopadhyay et al. 2004).

The histochemical study revealed that the deposition of lignin in the vascular cells of the teak shoots decreased as the concentration of BA increased, being strongly affected in shoots cultured with 6.66 μM of BA.

Hypolignification is characteristic of the hyperhydric tissues (see review Hazarika, 2006). The microchemical testing on transverse sections of the stems of the hyperhydric plantlets of *Simmondsia chinensis* (Link), revealing that there was scarce lignification and that xylem organisation was poor (Apóstolo and LLorente, 2000). We suggest that in some way BA affects the metabolism of phenolics, majorly lignins or their precursors, and the lack of this important heteropolymer turns the vascular cell wall weak, less hydrophobic, and permeable to water, causing hyperhydricity. So, those teak plants with poor-lignified cell walls and a low accumulation of phenolics must be more susceptible to dehydration and pathogen attack during acclimatization.

In general, the stomatal sizes appeared to be proportionally related to the BA concentration. The in vitro leaves from the CK-free medium and those cultured with 2.22 µM of BA showed heterogeneity in the stomata size and shape, which is attributable to the presence of stomata in the different development stages, typical of the rooting phase as reported for *Nicotiana tabacum* (Tichá et al. 1999) and *Eucalyptus grandis* × *Eucalyptus urophylla* (Louro et al. 1999). This shape is typical of the stomata of in vivo plants and has been observed in different species during in vitro propagation (Pospíšilová et al. 1999; Tichá et al. 1999). In the highest concentration of BA (6.66 µM) abnormal stomata, ring-shaped, raised and wide open, typical of hyperhydric leaves, were observed. Martre et al. (2001) reported that a high cytokinin concentration in the medium could induce an abnormal development of the stomatal complex. The scanning microscopy study of the in vitro hyperhydric plantlets of *S. chinensis* (Link) showed that leaves had very thin cuticles and malformed stomata at the epidermal level (Apóstolo and LLorente, 2000). In the hyperhydric leaves of carnation Majada et al. (2001) stated that, although the stomata had ellipsoidal guard cells sited at the same level as the epidermal cells, some stomata were raised above the leaf surface, supported on the epidermal cells.

Rooting is one of the most difficult steps in the micropropagation of woody species, and forest rooting is usually performed ex vitro because of the low frequency of in vitro rooting. However, in vitro rooting is often preferred because the plants perform much better in terms of plant quality because of the advantage of already possessing roots during the acclimatization phase (De Klerk 2002). The teak plantlets from the CK-free medium and cultured with 2.22 µM of BA that showed lower water and greater phenol contents in the biomass, a normal morphology of the whole stomatal complex and a well-organised xylem and a homogeneous lignin deposition pattern were more suitable for acclimatization. Such plants developed roots spontaneously in the TIS. In this sense, TIS-improved teak micropropagation enhanced the in vitro hardening of plants and acclimatization. The TIS have improved in vitro plant quality and made acclimatization safer for different species (Majada et al., Rodríguez et al. 2008;

Aragón et al. 2010; Yang and Yeh, 2008; Sankar-Thomas et al. 2008, Yan et al. 2010). The teak plants from 4.44 µM of BA which showed some morpho-physiological disorders during the multiplication stage displayed good development and survived (80%) during acclimatization, suggesting that plantlets were able to revert to a normal morpho-physiological state after the transfer to ex vitro conditions. However, the teak plantlets from 6.66 µM of BA which presented several morpho-physiological disorders survived at low percentages during acclimatization. We speculated that such plants had a high rate of water loss from the large number of stomata which probably did not learn till then how to close at the sense of desiccation outside the cultural environment and maybe they failed to close at the right time during transplantation. Otherwise, these plants with low phenolic content and lack of lignin developed a deficient water transportation system. As a result, they had an inefficient management of water and were consequently more susceptible to dehydration and they did not survive during acclimatization. In accordance with Bandyopadhyay et al. 2004, water is the most important component of plants and inefficient management of it may lead to poor rate of hardening and transplantation. On the other hand, we showed in our experiments that the in vitro conditions prior to acclimatization were important for the ex-vitro growth of the micropropagated teak plants, which is consistent with other authors' results with different species (Kadleček et al. 2001, Hazarika, 2006, Mocaleán et al. 2009). The definition of the proper or inadequate anatomical and physiological characteristics of the micropropagated plants allow for the selection of those plants which are able to survive during the acclimatization stage (Hazarika, 2006)

In conclusion, the use of TIS-improved teak micropropagation enhances both shoot proliferation and growth. The in vitro teak plants from the CK-free medium and cultured with 2.22 µM of BA, which showed a lower water and greater phenol contents in the biomass, a normal morphology of the whole stomatal complex and a well-organised xylem and homogeneous lignin deposition pattern were more suitable for acclimatization. We found that the concentration of 4.44 µM of BA was more suitable for the teak shoot proliferation in TIS, since the average of NS was higher than culture in semi-solid medium. Furthermore, the survival percentages did not differ between both cultural systems during acclimatization. In the future, different strategies such as the addition of osmotic agent like polyethylene glycol, as well as CO_2 supply in the vessel and the application of forced ventilation may play an important role to improve teak plants quality without compromising the high average number of shoots achieved in this BA treatment. The teak plantlets from the highest concentration of BA (6.66 µM) presented numerous anatomical defects and physiological disorders and they majorly did not survive the critical acclimatization stage. The results in the current studies provide, for the first time, information on the rapid and successful propagation of teak by TIS.

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Table 1. The effect of BA on shoot proliferation of teak after 4 weeks of culture in the TIS and semi-solid system.

[BA] (µM)	Number of NS per explant	Number of HS per explant	Shoot length (cm)	Stem diameter (mm)	Number of leaves per shoot	Number of nodes per shoot	Rooting (%)
SS	3,62±0.89^{c}	0.6±0.35^{c}	6.10±0.65^{c}	1.40±0.021^{b}	10.40 ±0.46^{a}	5.50±0.52^{a}	0
CK-free	1.30±0.23^{d}	---	6.75±0.17^{c}	1.25±0.017^{bc}	11.00±0.43^{a}	5.80±0.23^{a}	100
2.22	4.10±0.15^{c}	---	8.80±0.17^{a}	1.84±0.050^{a}	11.80±0.91^{a}	6.10±0.73^{a}	50
4.44	7.70±0.49^{b}	2.6±0.21^{b}	7.25±0.38^{bc}	1.49±0.023^{b}	11.20±0.84^{a}	6.00±0.31^{a}	20
6.66	10.3±0.39^{a}	4.2±0.31^{a}	5.80±0.25^{d}	1.20±0.021^{c}	10.80±0.58^{a}	5.40±0.65^{a}	0

TIS, Immersion frequency every 6 h, immersion time 40 seconds. *SS,* Shoots cultured on semi-solid medium. *NS,* Normal shoots. *HS,* Hyperhydric shoots. *CK-free*, CK-free medium.

*Values represent the means ± standard error (10 shoots per TIS, 30 shoots per treatment, repeated three times, n=90) and different letters in the same columns indicate significant differences as determined by F-LSD test at $P < 0.05$.

Table 2. Morphometric analyses of the teak shoot leaf surfaces developed with different concentrations of BA after 4 weeks of culture in TIS.

[BA] (µM)	Leaf area* (cm^2)	Stomatal density** (mm^2)	Stomatal length*** (µm)	Stomatal width*** (µm)	Stomatal area*** ($µm^2$)	Pore length*** (µm)	Pore width*** (µm)	Pore area*** ($µm^2$)
CK-free	2.34±0.04^{b}	608±6.36^{b}	13.47±0.56^{d}	10.88±1.72^{cd}	134.4±4.74^{c}	6.61±0.41^{d}	1.46±0.12^{c}	8.12±0.50^{d}
2.22	2.94±0.08^{a}	600±7.01^{b}	18.25±0.20^{c}	14.04±1.33^{c}	213.70±5.06^{bc}	9.67±0.32^{c}	3.56±0.17^{bc}	21.63±1.01^{c}
4.44	2.57±0.08^{b}	618±6.92^{ab}	22.18±0.46^{b}	18.03±1.00^{b}	290.82±3.87^{b}	11.91±0.37^{b}	5.62±0.25^{b}	49.66±1.90^{b}
6.66	2.31±0.06^{b}	647±5.44^{a}	26.94±0.61^{a}	26.14±3.81^{a}	486.83±4.43^{a}	15.46±0.51^{a}	14.0±0.46^{a}	91.01±2.80^{a}

TIS, Immersion frequency every 6 h, immersion time 40 seconds. *CK-free,* CK-free medium.

Values represent the means±standard error (*n=20, **n=25, ***n=25). Different letters in the same columns indicate significant differences as determined by F-LSD test at $P < 0.05$.

Figure 1. Shoot developed in TIS with different concentrations of BA after 4 weeks of culture. (A) Double-vessel system with teak shoots. (B) Shoot from the CK-free medium. (C) 2.22 µM of BA. (D) 4.44 µM of BA. (E) 6.66 µM of BA. (Immersion frequency every 6 h, immersion time 40 seconds)

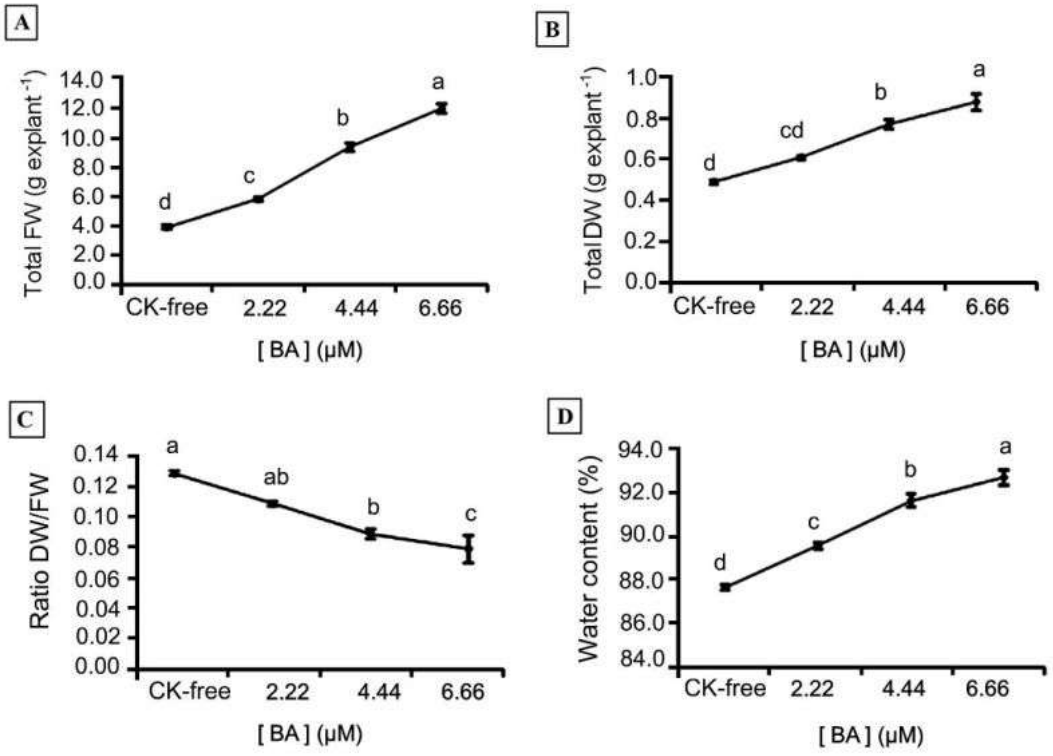

Figure 2. Effects of BA in the biomass growth of the teak shoots developed in TIS after 4 weeks of culture. (A) Total FW. (B) Total DW. (C) Ratio DW vs. FW. (D) Water percent. *Each value is the mean for 90 shoots ± standard error of the mean.

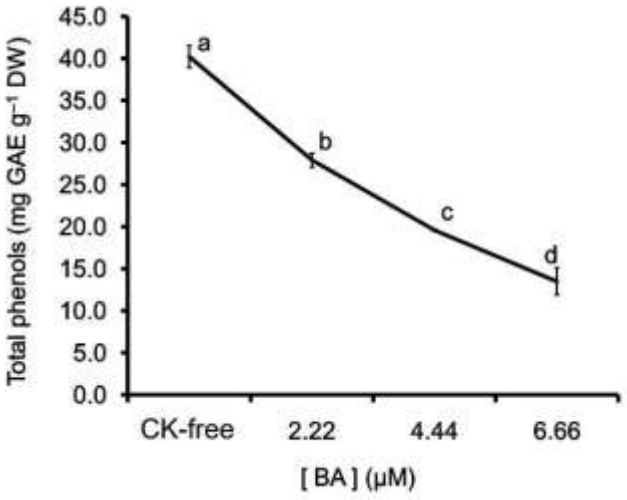

Figure 3. Total phenol content in the teak shoots developed in TIS with different concentrations of BA after 4 weeks of culture. *Total phenols are calculated as mg of GAE per g DW, and values represent the means ± standard error (n =9). Different letters indicate significant differences as determined by F-LSD test at $P < 0.05$

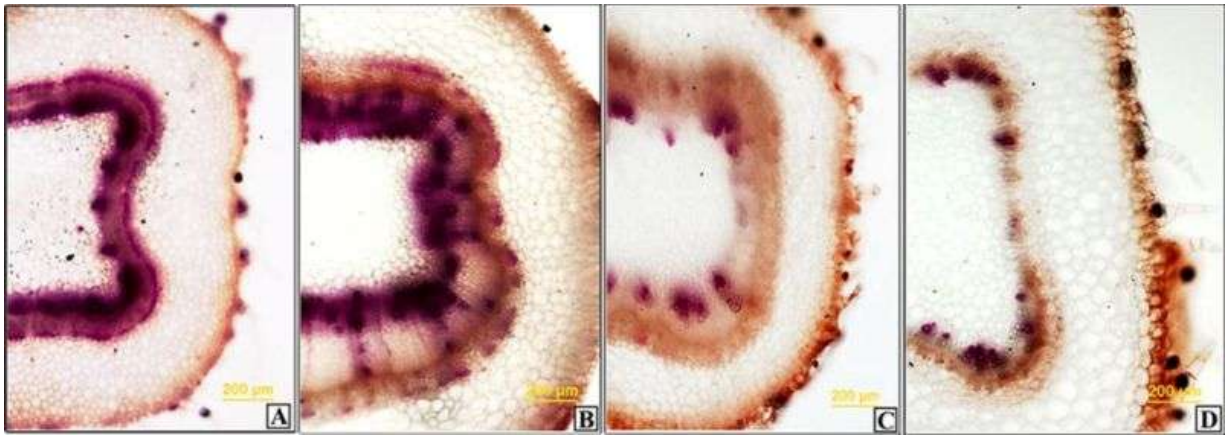

Figure 4. Light microscopy comparison of the lignin accumulation in the transverse section (phloroglucinol-HCl stained) in the teak shoots developed in TIS with different concentrations of BA. (A) CK-free medium. (B) 2.22 µM of BA. (C) 4.44 µM of BA. (D) 6.66 µM of BA (bars= 200 µm).

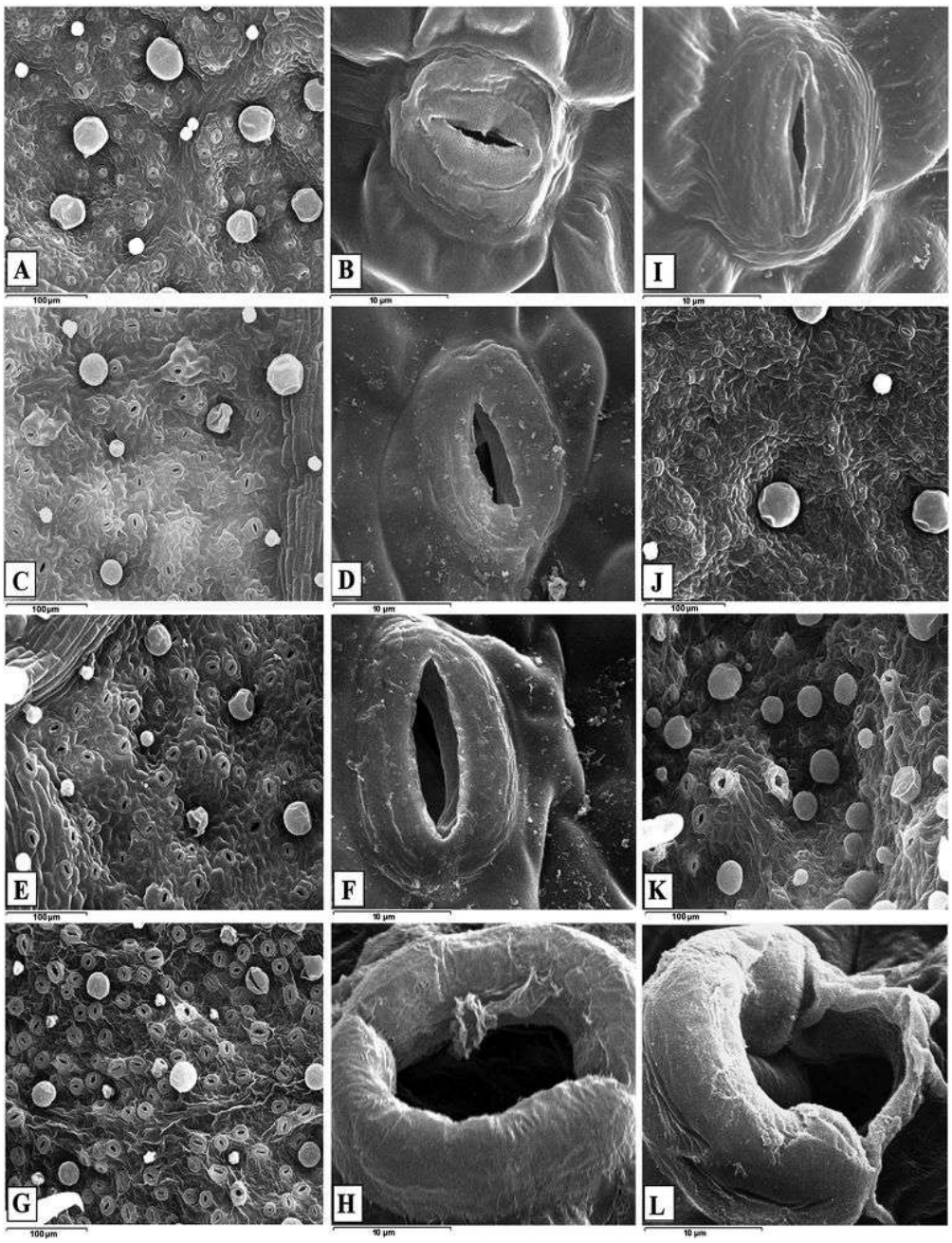

Figure 5. Scanning electron micrographs of the abaxial leaf surfaces of the teak shoots cultured in TIS with different concentrations of BA. (A, B) CK-free medium. (C, D) 2.22 µM of BA. (E, F) 4.44 µM of BA. (G, H) 6.66 µM of BA. (I, J) Seedling. (K, L) In vitro hyperhydric leaves. (A, B, C, E, G, I, K, bars= 100 µm). (B, D, F, H, J, bars= 10 µm). (L, bars= 20 µm).

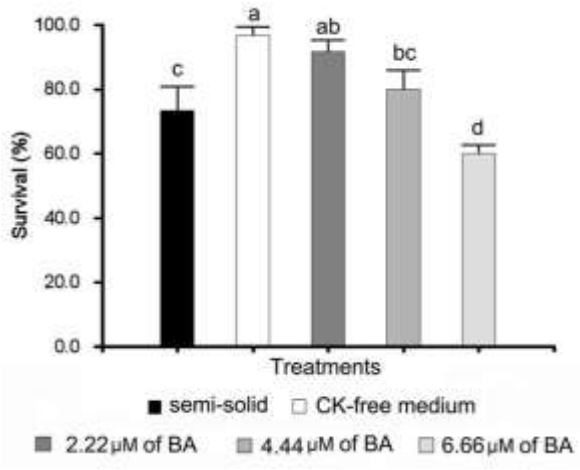

Figure 6. Survival percentages of teak plantlets during acclimatization. Data were taken after four weeks in ex vitro conditions. *Values represent the means (20 replicates per treatment; repeated three times) and different letters on the bars indicate significant differences as determined by F-LSD test at $P < 0.05$.

Figure 7. Ex vitro rooting and acclimatization of teak plantlets (four weeks after transfer) cultured in TIS with different concentrations of BA and semi-solid system, (A) Cytokinin-free medium, (B) 2.22 µM of BA, (C) 4.44 µM of BA, (D) 6.66 µM of BA, (E) Semi-solid system, (F) Six-week-old plants after transfer to ex vitro condition, (D) Three-months-old plants after transfer to ex vitro condition ready for field plantation.