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Influence of 6-Benzyladenine and gelling agent on the reduction of hyperhydricity in *Tectona grandis* L.

Influencia de la 6-Bencilaminopurina y el agente gelificante en la reducción de la hiperhidricidad en *Tectona grandis* L.

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Abstract

The influence of different factors on shoot proliferation and the occurrence of hyperhydricity in teak (*Tectona grandis* L.) have been studied. Four concentrations of BA (2.22, 4.44, 6.66 and 8.88 µM) and a control treatment with 0 BA were examined. Aiming at reducing the costs during commercial propagation by using gelrite in stead of agar, the use of both gelling agent in the proliferation and hyperhydricity was tested. In order to evaluate if hyperhydricity can be reduced by increasing the gelrite concentration in the culture medium, three concentrations (2.0, 2.5 and 3.0 g l^{-1}) were tested in combination with 4.44 µM BA. The proliferation and occurrence of hyperhydricity during 21 successive subcultures were evaluated. The highest proliferation was achieved in the treatments with 6.66 or 8.88 µM BA. They yielded 5.22 and 5.56 shoots/explant, respectively. But also, the highest percent of hyperhydric shoots was achieved in this treatment. Gelrite resulted in a higher proliferation, but also an almost two times higher hyperhydricity as compared to agar-solidified media. Satisfactory reduction in hyperhydricity (18%) was achieved with 3.0 g l^{-1} gelrite. However, the successive subcultures onto proliferation in this treatment favored hyperhydricity compromising shoot quality and it´s competence to proliferate. *In vitro* teak plants were *ex vitro* rooted and then transferred to greenhouse conditions for acclimatization; ten weeks after transfer they were ready for field plantation.

Key words: cytokinin, forestry, micropropagation, morpho-physiological disorder, teak

Abbreviations: *BA*- 6-Benzyladenine, *MS*- Murashige and Skoog basal medium, *IBA*- Indole-3-butyric acid, *ANA*-Naphtalene acetic acid

Resumen

Se estudió la influencia de diferentes factores en la proliferación y la ocurrencia de la hiperhidricidad in teca (*Tectona grandis* L.). Se probaron cuatro concentraciones de BA (2,22; 4,44; 6,66 and 8,88 µM) y un control sin BA. Con el objetivo de reducir los costos durante la propagación comercial se experimentó sustituir el agar por el gelrite, para lo cual se estudió en efecto de ambos gelificantes en la proliferación y la hiperhidricidad de los brotes. Se estudiaron, tres concentraciones de gelrite (2,0; 2,5 and 3,0 g l^{-1}) combinadas con 4,44 µM BA, con el objetivo de evaluar si la hiperhidricidad podía ser reducida incrementando la concentración de gelrite. Se evaluó la proliferación de brotes y la ocurrencia de la hiperhidricidad durante 21 subcultivos. Se logró una alta proliferación de brotes en los tratamientos con 6,66 y 8,88 µM BA (5,22 y 5,56 brotes), pero el porcentaje de brotes hiperhídricos también se incrementó. El gelrite resultó en una alta proliferación de brotes, pero con mayor incidencia de la hiperhidricidad que el medio gelificado con agar. Se obtuvo una reducción satisfactoria de la hiperhidricidad (18%), cuando la concentración de gelrite se incrementó hasta 3,0 g l^{-1}. No obstante, la multiplicación de los brotes en este tratamiento más allá del 11no subcultivo favoreció la hiperhidricidad, lo que afectó la calidad de los brotes y su competencia para la proliferación. Las plantas fueron enraizadas *ex vitro*, transferidas a condiciones de invernadero para su aclimatización y diez semanas después de la transferencia estaban listas para la plantación en campo.

Palabras clave: citoquinina, forestal, micropropagación, desorden morfo-fisiológico, teca.

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Introduction

Tectona grandis L. (teak) grows naturally throughout southeastern Asia and is one of the most valuable tropical hardwood species on the international market (Gyves *et al.*, 2007). Teak has been introduced into Central America (Panamá) in 1926 from Colombo (Sri Lanka) and in the following 20 years it was naturalized in almost all countries of Central America and the Caribbean (De Camino *et al.*, 1998). Teak is traditionally propagated through seeds and cuttings. However, due to low efficiency of traditional propagation methods to satisfy the high demand of teak, *in vitro* propagation has become an efficient way to produce teak plants as uniform as possible, on a large scale and in a short period of time (Monteuuis *et al.*, 1998; Daquinta *et al.*, 2001; Tiwari *et al.*, 2002; Yasodha *et al.*, 2005; Gyves *et al.*, 2007). However, sometimes the shoot quality is impaired by the occurrence of hyperhydricity (Castro *et al.*, 2002).

Hyperhydricity is induced by the combined action of several physical and/or chemical factors of the culture environment (Gaspar, 1991). Although most plants can adapt to these environmental conditions, some of them become abnormal with a translucent aspect due to chlorophyll deficiency and high water content (Debergh, 1983; Gaspar, 1991). The phenomenon has been considered as a morpho-physiology disorder and many physiological and biochemical changes have been observed (Ziv, 1991; Franck *et al.*, 2004). Some of these factors include the presence in the medium of growth regulators, of large quantities of NH_4^+ and Cl^- ions, the type and concentration of the gelling agent, high relative humidity in the culture vessels (Debergh *et al.*, 1983; Ziv, 1991; Kevers *et al.*, 2004; Hazarika, 2006) and the successive subcultures of the explants in a culture medium with cytokinin (Vieitez *et al.,* 1985).

Cytokinins have been shown to induce hyperhydricity in many species, usually in a concentration- dependent manner and when other conditions in the culture system are not optimized (Ivanova and Van Staden, 2008, Mocaleán *et al.,* 2009).

Gelling agents are not an ''inert'' medium component, and their type and concentration have a significant effect on the performance of tissue cultured plant material (Debergh, 1983; Ziv, 1991; Pereira-Netto *et al.,* 2007), including the occurrence of hyperhydricity (Franck *et al.,* 2004).

Gelrite is a product derived from bacteria (*Pseudomonas elodea*) with consistent quality and high purity. Because substantially smaller quantities produce gels of hardness comparable to agar, gelrite appears to be an economically good gelling substitute for agar (Ivanova and Van Staden, 2010).

The successive subcultures of tissue cultured plant material for a long period of time can lead to a declining of proliferation rate and the occurrence of hyperhydricity (Vieitez *et al.,* 1985; Gómez *et al.,* 2007). Because the efficiency of commercial micropropagation, among others factors, are often determined by the number of plants produced from the initial explant, it is indispensable to know how many subcultures can be carried out during the proliferation stage without compromising the proliferation rate and plant quality.

After a meticulous revision in the literature about the *in vitro* commercial propagation of teak, no one research refers the evaluation of the proliferation of shoot up to the 7th subculture. Otherwise, although several research results have been described to overcome hyperhydricity, in various species, using different strategies such the evaluation of gelling agent type and concentration (Debergh *et al.,* 1981; Franck *et al.,* 2004; Ivanova *et al.,* 2006; Ivanova and Van Staden, 2011), no experience with teak has been reported.

The aim of this study was to determine the optimal concentration of BA to improve teak shoot proliferation (shoots/explants) with low occurrence of hyperhydricity (%). In order to reduce commercial propagation costs, the potential of gelrite and its most proper concentration as a possible replacement of agar was assessed, with special emphasis on achieving high proliferation and low occurrence of hyperhydricity. The study also aims at determining the maximum number of subcultures that can be carried out using the selected combination of BA and gelrite concentration and the development of the micro-propagated plants under *ex vitro* conditions.

Materials and Methods

Plant material and culture conditions

Apical shoots from plants cultured in the greenhouse and cloned from epicormic shoots of 30-year-old teak trees from Cuba were cut off. Shoots were surface-sterilised with ethanol (70% v/v) for 30 s. After rinsing three times with sterile distilled water, 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 full-basal MS (Murashige and Skoog, 1962) medium supplemented with BA (4.44 µM), sucrose (2%; w/v), and solidified with 2.0 g l^{-1} gelrite (Duchefa Biochemie, NL) to induce bud sprouting.

The pH was adjusted to 5.8 before autoclaving. After 48 hours, *in vitro* shoots were transferred to fresh medium to reduce browning. After 30 days the established apical shoots were transferred to the multiplication stage. The micropropagation cycle consisted of a monthly subculture of nodal segments after the removal of the new *in vitro* shoots onto a fresh medium to produce a large number of *in vitro* shoots. The cultures were incubated at 25±2°C with a 16 h light (fluorescent lamps with photon lux light intensity of 40 µmol $m^{-2}s^{-1}$).

Experiment description

Eight *in vitro* shoots were cultured per 25 ml of solid medium, distributed in a 250 ml glass culture container and there were ten replicates (flasks). Three experiments were conducted; each one was repeated three times. For all experiment each treatment comprised 80 explants. After 4 wks of culture, 40 explants were used to evaluate proliferation (number of shoots/explant) and occurrence of hyperhydricity (%) (n=120). The number of shoots per explant (proliferation) was recorded. The newly-formed shoots were classified as normal shoots or hyperhydric shoots, according to their external appearance and the hyperhydricity (%) was calculated. Hyperhydric shoots had thicker, glassy and watery appearance compared to normal shoots.

Effect of BA concentration

In the first experiment, the established apical shoots (figure 1A) were placed onto the multiplication medium. Four concentrations of BA (2.22, 4.44, 6.66 and 8.88 µM) and a control treatment with 0 BA were assessed. The conventional basal MS medium was supplemented with sucrose (2%; w/v), 100 mg l^{-1} myo-inositol and solidified with agar (6.6 g l^{-1}).

Effect of gelling agent type

In the second experiment, two types of gelling agent were compared: agar and gelrite (Duchefa Biochemie, NL) at concentrations of 6.6 and 2.0 g l^{-1} respectively, resulting in the same gel hardness of the media. The gel hardness was determined according to Huang *et al.*, 1995. The basal MS medium was supplemented with sucrose (2%; w/v), 100 mg l^{-1} myo-inositol and 4.44 µM BA.

Effect of gelrite concentration

In the third experiment, three concentrations of gelrite (2.0, 2.5 and 3.0 g l^{-1}) were tested. The basal MS medium was supplemented with sucrose (2%; w/v), 100 mg l^{-1} myo-inositol and 4.44 µM BA.

Effect of the number of subcultures

Teak shoot were multiplied during 21 successive subculture onto basal MS medium supplemented with sucrose (2%; w/v), 100 mg l^{-1} myo-inositol, 4.44 µM BA and solidified with gelrite (3.0 g l^{-1}). Every second subculture, 40 explants were used to evaluate proliferation (number of shoots/explant) and occurrence of hyperhydricity (%).

Ex vitro rooting and acclimatization

For *ex vitro* rooting and acclimatization 40 teak shoots (>2.5 cm long) were harvested at the end of the 11th subculture. Shoots were washed with running tap water and the basal callus was cutting. Shoots were *ex vitro* rooting according to Quiala *et al.* (2012), before being inserted into the substrate. The shoots were then planted in plastic containers (70 hole of 121 cm^3 capacity) and filled with an organic matter (humus and sugarcane mill baggasse): Zeolite (1:1) mixture. Once planted, the shoots were placed in a greenhouse (30 ± 2°C, RH 90%) and maintained under 50% shade with intermittent-mist water sprays to avoid damage due to desiccation. The frequency of survival was recorded after 4 weeks. Ten weeks after transfer the plants were ready for field plantation.

Statistical analysis

The normality of the data was tested using the Kolmogorov-Smirnov test. Prior to statistical analysis, the hyperhydricity (%) data were transformed into arcsine to improve the normality of the data distribution. The significance of differences was determined by analysis of variance (ANOVA), and the significant ($P<0.05$) differences among mean values were estimated by Fisher´s LSD. All statistical tests were performed by SigmaStat software version 3.11. The data are presented as means ± standard error, and different letters in the tables and figures indicate significant differences at $P<0.05$. The data presented in percentages were subjected to arcsine transformation before analysis, and then converted back to percentages for presentation in the tables and graphics. The experiment was arranged in a completely randomized design.

Results

Effect of BA concentration

Shoot proliferation was observed with all tested BA concentrations (table 1). On media with 2.22 µM BA, an average of 2.84 shoots per explant was produced, also the lowest percent of hyperhydricity was achieved (table 1). Compared to the control, the higher proliferation of shoot was obtained when explants were grown on a medium containing either 6.66 or 8.88 µM BA, yielding 5.22 and 5.56 shoots per explant, respectively. However, the occurrence of hyperhydricity was also higher (25 and 35%, respectively).

The best results on shoot proliferation were achieved on media with 4.44 µM BA since an average of 4.88 shoots per explant was produced (table 1), similar to the treatments with 6.66 µM BA, but with lower occurrence of hyperhydricity (15%) (table 1).

Effect of gelling agent type

In this experiment the potential of gelrite as a possible replacement of agar was assessed with special emphasis on achieving high proliferation and low occurrence of hyperhydricity. As such commercial propagation costs could be reduced.

Significant differences associated with the used gelling agent were observed after 4 weeks of culture. Shoot proliferation was significantly higher with gelrite (4.84 shoots per explant) than with agar (3.70 shoots per explant). However, hyperhydricity was almost two times higher (34%) with gelrite as compared to agar-solidified media (16%; table 2).

Effect of gelrite concentration

The increase of gelrite concentration from 2.0 to 2.5 g l^{-1} or 3.0 g L^{-1} did not affect the multiplication; significant reduction was no detected (table 3). Increasing the gelrite concentration reduced hyperhydricity. However, a significant reduction (18.1%) was achieved only at 3.0 g l^{-1} gelrite (table 3).

Effect of the number of subcultures

Shoot proliferation increased from the 1st to the 5th subculture (3.1 to 6.0 shoots per explant). Proliferation remained similar from the 6th to 11th subculture, ranging between 6.2 and 5.9 shoots per explant (figure 2A), while no significant increment of hyperhydricity were recorded from the 1st to the 11th subculture (figure 2B), ranging between 16.1 to 18.6%. However; more than 11 successive subcultures onto proliferation media with 4.44 µM BA and 3.0 g l^{-1}gelrite resulted in low proliferation (figure 2B) and high hyperhydricity from the 12th to the 21st subculture. A ranging between 27.3 to 53.1% of the new shoots was hyperhydric. Furthermore, while healthy shoots with dark green color were majorly observed from the 1st to 11th subculture (figure 2B), from the 15th to the 21st subculture (figure 2B), whole explants, including all new shoots, with translucent and glassy appearance and a pale green–brown color (figure 1C).

Table 1. Effect of BA concentration on proliferation (number of shoots per explant) and hyperhydricity (%) of *T. grandis* shoots

[BA] (µM)	Proliferation (shoots/explant)	Hyperhydricity (%)
0.00	1.05±0.62 d	---
2.22	2.34±0.79 c	1 d
4.44	4.88±0.43 b	15 c
6.66	5.22±0.71 ab	25 b
8.88	5.56±0.52 a	35 a

Shoot explants were cultured on MS-based medium gelled with agar (6.6 g l^{-1}). Data were collected after 4 weeks culture. Values represent the means (40 shoots per treatment, repeated three times, n=120) and EE=Standard Error. Data presented in percentages were subjected to arcsine transformation before analysis. Distinct letters in the same column indicate significant differences according to F-LSD test at $P < 0.05$.

Ex vitro rooting and acclimatization

In vitro shoots from the 11th subculture produced new leaves within 15 days. Shoots surviving one month after planting (figure 1 D). Treating shoots with an IBA (492.1 µM) solution for 2 min resulted in 75% rooting. Ten week after transfer plants were ready for field plantation (figure 1E).

Discussion

Effect of BA concentration on multiplication and hyperhydricity

In general, increasing the concentration of BA from 2.22 to 8.88 µM increased shoot proliferation, but the frequency of hyperhydricity also increased. According to the literature, BA is the most commonly used cytokinin in micropropagation of teak, alone or combined with kinetin or auxin (Monteuuis *et al.,* 1998; Gangopadhyay *et al.,* 2002; Tiwari *et al.,* 2002; Yasodha *et al.,* 2005; Gyves *et al.,* 2007; Akram and Aftab 2009). Hyperhydricity during tissue culture of teak has already been reported by Castro *et al.* (2002). They refer to the best results on shoot proliferation (2.5 shoots/explant) with 2.22 µM BA, with low occurrence of hyperhydricity (5%). Similar to our results, the authors point out that a higher concentration of BA results in higher proliferation but also in higher hyperhydricity. Goswami *et al.* (1999) suggested using a basal MS solid medium supplemented with BA and kinetin for teak shoot multiplication, achieving a mean of 3.7 normal shoots per explant in 8 weeks after subculture; but the number of hyperhydric shoots was not reported. Tiwari *et al.* (2002) showed that placement of the explants in MS medium supplemented with BA (22.2 µM) and a subsequent elongation step with 22.2 µM BAP + 0.25 µM IAA resulted in the maximum number of shoots after 8 weeks of culture. Although the BA concentration was high, the authors do not refer to the occurrence of hyperhydricity. Gyves *et al.* (2007) reported a high number of shoots for teak (4 shoots/explant) after 4 weeks

Table 2. Effect of gelling agent type on proliferation (number of shoots per explant) and hyperhydricity (%) of *T. grandis* shoots

Gelling agent	Proliferation (shoots/explant)	Hyperhydricity (%)
Agar	3.70± 0.81b	16 b
Gelrite	4.84± 1.01a	34 a

Shoot explants were cultured on MS-basal supplement with 4.44 µM BA. Data was collected after 4 weeks culture. Values represent the means (40 shoots per treatment, repeated three times, n=120) and EE= Standard Error. Data presented in percentages were subjected to arcsine transformation before analysis. Distinct letters in the same column indicate significant differences according to F-LSD test at $P < 0.05$.

Table 3. Effect of gelrite concentration on proliferation (number of shoots per explant) and hyperhydricity (%) of *T. grandis* shoots

Gelrite (g l-1)	Proliferation (shoots/ explant)	Hyperhydricity (%)
2.0	4.71± 0.21a	29 a
2.5	4.66± 0,36a	25 a
3.0	4.61± 0.41a	18 b

Shoot explants were cultured on MS-basal supplement with 4.44 µM BA. Data was collected after 4 weeks culture. Values represent the means (40 shoots per treatment, repeated three times, n=120) and EE= Standard Error. Data presented in percentages were subjected to arcsine transformation before analysis. Distinct letters in the same column indicate significant differences according to F-LSD test at P < 0.05

of culture. They took steps to overcome hyperhydricity problems by modifying the MS medium (reducing the ammonium quantity) and by adding pectin (from grape must) to reduce the water content in the medium and by adding auxin and giberelin. According to general observations, these changes probably contributed to the improvement of the culture quality reducing visual hyperhydricity symptoms. However, although these techniques solve the hyperhydricity problems, the addition of pectin and a complex mix of three regulators of growth certainly increase the cost of commercial teak propagation.

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 in the culture medium (Debergh 1983; Hazarika 2006). It has been reported that cytokinin induces hyperhydricity in many species, usually in a concentration-dependent manner and when other conditions in the culture system are not optimized (Ivanova *et al.,* 2006; Moncaleán *et al.,* 2009).

Effect of gelling agent type on hyperhydricity and multiplication

Agar was found to better control hyperhydricity than gelrite. The low frequency of hyperhydricity on a medium with agar might be due to a sulphated galactan in agar (Nairn *et al.,* 1995), being able to control hyperhydricity. However, agar is expensive (Huang *et al.,* 1995). From an economical point of view, during commercial propagation the gelling agent represents almost 90% of one unit (1 l) of culture medium (Pérez *et al.,* 2000).

Gelrite promotes hyperhydricity in teak and similar results have been found with various species (Franck *et al.,* 2004; Ivanova and Van Staden, 2010). The hypothesis about the effect of gelrite on hyperhydricity seems to be related to its physical structure as supported by Ivanova *et al.,* (2006). They found that higher levels of endogenous cytokinins were detected in shoots of *A. polyphylla* grown on gelrite media than those grown on agar-gelled media.

Shoot formation was higher on a medium gelled with gelrite as compared to agar. Similar results were reported for *Malus domestica* (Pasqualetto *et al.,* 1988), *Allium cepa* (Jakse *et al.,* 1996) and *Scrophularia yoshimurae* (Tsay *et al*., 2006). However, in agreement with Ivanova and Van Staden, (2010), we assumed that this effect of the gelling agent on the proliferation rate appears to be species-specific because more shoots were produced on media with agar of *A. polyphylla*.

Effect of gelrite concentration on hyperhydricity and multiplication

Increasing gelrite concentration reduced hyperhydricity. Different authors refer to a similar positive effect of controlling hyperhydricity by increasing gelrite concentration in woody species such as *Picea abies* (Bornman and Vogelmann, 1984) and *Malus spp*. (Pasqualetto *et al.,* 1988). However, in contradiction to our results, they refer to a negative effect on shoot proliferation. In our results, a negative side effect of the gelrite concentration was not observed, maybe because the amount of gelrite added from one treatment to another was low. Similar results were also reported for *A. polyphylla* (Ivanova and Van Staden, 2010). These authors report that increasing the gelrite concentration, and so decreasing the water availability in the media, reduced significantly the occurrence of hyperhydricity. However, in contradiction to their results, we did not achieve a negative side effect of this approach for shoot regeneration since no statistical differences were detected at $p<0.05$ level.

Effect of the number of subculture on multiplication and hyperhydricity

The successive subcultures onto proliferation media with 4.44 µM BA and 3.0 g l^{-1}gelrite of more than the 11th one resulted in low proliferation and high hyperhydricity. Similar results were reported for *Castanea sativa* Mill (Vieitez *et al.,* 1985). These authors pointed to a negative effect of growth regulators on hyperhydricity and low proliferation rate upon an increasing number of subcultures. A poor shoot multiplication was found with *Chimonanthus praecox* where the amount of viable tissue persistently decreased with each subculture *(*Kozomara *et al.,* 2008). The quantity of *Eucalyptus globulus* shoots decreased from the 3rd to the 6th subculture when shoots were successively subcultured every 50 days on a proliferation medium with 4.44 µM BA and 0.05 µM naphthalene acetic acid (ANA) (Gómez *et al.,* 2007).

Apparently, *in vitro* teak shoots are displaying various degrees of hyperhydricity. Some of them do not show macro-morphological perceptible changes, but the

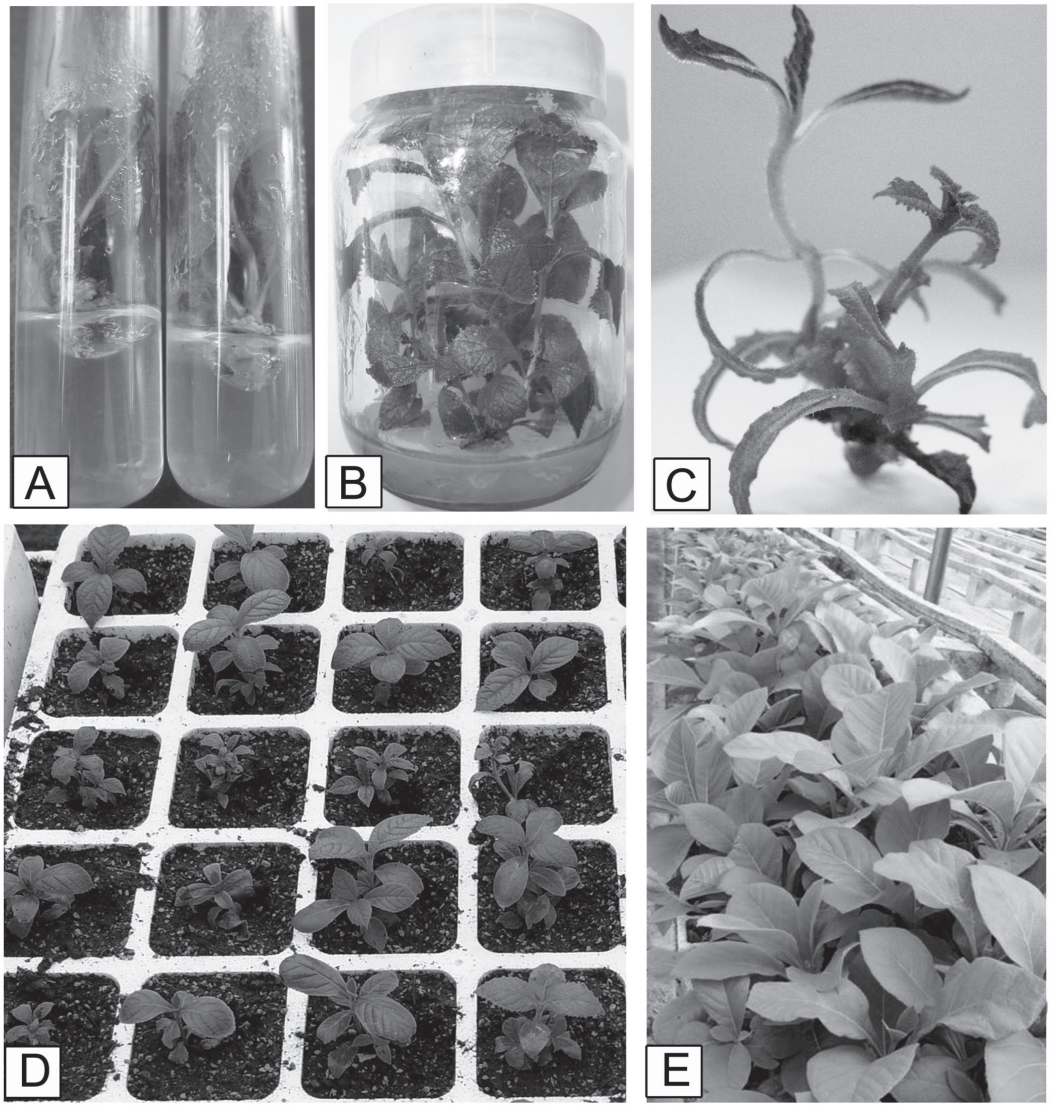

Figure 1. Micropropagation of 30-year old teak (*T. grandis*). **A** Apical shoots *in vitro* established in basal MS medium supplemented with BA (4.44 µM). **B** Glass culture container with *in vitro* teak shoot from the 3st subculture in basal MS medium supplemented with BA (4.44 µM), and solidified with 3.0 g l^{-1} gelrite. **C** *In vitro* teak shoot showing hyperhydricity symptoms, observed from the 15th to the 21st subculture (note that whole explants, including all new shoot, display translucent and glassy appearance and a pale green–brown color). *Ex vitro* rooting and acclimatization of teak plantlets cultured during 11th subculture in solid medium. **D** 4-week old plants after transfer to *ex vitro* conditions. **E** 10-week old plants after transfer to *ex vitro* conditions ready for field plantation.

subculture of this type of shoot for a long time to new vitrifying conditions (culture medium with BA and gelrite) may lead to over-accumulate severe morpho-physiological disorder, reducing proliferative competition and quality. According to Kevers *et al*. (2004), the process of hyperhydricity is generally considered as reversible. The authors point out that the hyperhydric state (of newly formed shoots) can also be maintained through several subcultures without too much change, but subculturing hyperhydric shoots in vitrifying conditions may lead to severe damage, including death of the whole shoot as such, through apparent necrosis of all primary meristems.

From the result, the costs during commercial propagation of teak could be reduced by using gelrite in stead of agar, but the advantage is limited since the successive subculture more than 11th subculture affects the shoots quality and its competition to proliferate. Because the often cultures transferred to freshly media with agar and lower cytokinin concentrations successfully recover from hyperhydricity, alternating the subculture of teak shoots into agar-gelled medium + 4.44 µM

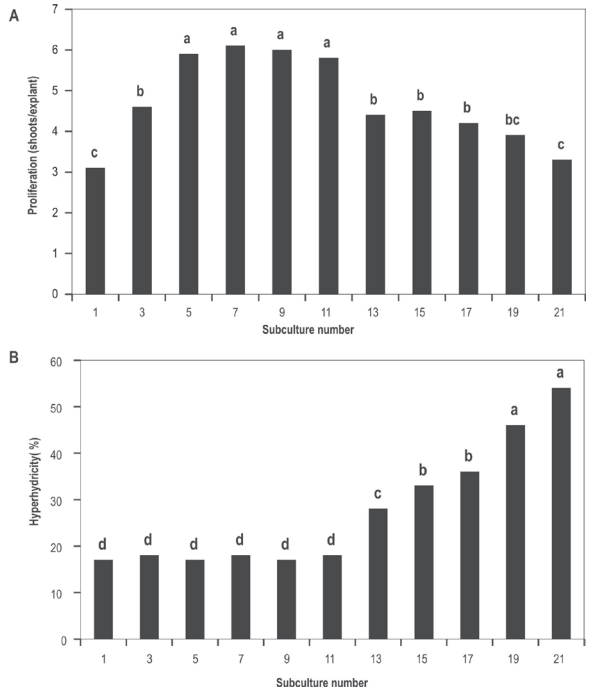

Figure 2. A) Proliferation (shoot/explant). **B)** Hyperhydricity (%) of *T. grandis* shoots formed from the 1st to the 21st subculture. Data in each subculture were collected after 4 weeks culture. Values represent the means (40 shoots per each subculture). Data presented in percentages were subjected to arcsine transformation before analysis. Distinct letters in the same column indicate significant differences according to F-LSD test at $P < 0.05$.

BA and gelrite-gelled medium + 2.22 µM BA, could be a solution for achieve high shoot proliferation controlling hyperhydricity upon the 11th subculture passage.

Ex vitro rooting and acclimatization

A high survival rate was observed with *ex vitro* rooted teak shoots. Rooting is one of the most difficult steps in micropropagation of woody species, and forest rooting is usually performed *ex vitro* because of the low frequency of *in vitro* rooting. *In vitro* rooting is often preferred because plants perform much better in terms of plant quality as they have the advantage of already possessing roots during the acclimatization phase (De Klerk, 2002). However, *ex vitro* rooting of teak is attractive because of the simultaneous rooting and hardening of plants. It also cuts the cost of production significantly (Tiwari *et al.,* 2002).

In this study, we achieved a frequency of survivor of 75%, but in an earlier study, we found that it is possible to enhance the quality of teak plants obtained from solid culture, by using a temporary immersion system with 0 or with 2.22 µM BA in the last subculture of the multiplication stage, achieving a high frequency of survivor (96.7 and 91.7%, respectively) during acclimatization (Quiala *et al.,* 2012).

In conclusion, the present study shows that even though BA is indispensable for the *in vitro* propagation of teak, high concentrations of this cytokinin result in more occurrence of hyperhydricity. Otherwise, the type of gelling agent is critical for controlling hyperhydricity during *in vitro* propagation of teak. When agar was substituted with gelrite, a high proliferation was achieved, but hyperhydricity also increased. By increasing gelrite concentration to 3.0 g l^{-1} was an effective strategy for reduce hyperhydricity without affecting shoot proliferation, but maintaining the explants in this conditions (4.44 µM BA + gelrite) during successive subcultures enhance hyperhydricity.

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