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Simple Protocol for the Micropropagation of Teak (*Tectona grandis* Linn.) in Semi-Solid and Liquid Media in RITA® Bioreactors and *ex Vitro* Rooting

María Elena Aguilar[1*], Karla Garita[1], Yong Wook Kim[2], Ji-Ah Kim[2], Heung Kyu Moon[2]

[1]Biotechnology Laboratory, Tropical Agricultural Research and Higher Education Center (CATIE), Turrialba, Costa Rica
[2]Division of Forest Biotechnology, National Institute of Forest Science (NIFoS), Suwon, Republic of Korea
Email: *aguilarm@catie.ac.cr

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Abstract

In Latin America the forestry of exotic species such as teak has been increasing in recent decades, due to their advantages in wood quality, rapid growth; and the relative ease of producing clones and their multiplication with respect to native species. Therefore, there is great interest in developing larger-scale propagation strategies that reduce costs and intensive manual labor. Culture in liquid media with temporary immersion and the semi-automation of the system has raised expectations for large-scale micropropagation. We report a protocol for teak, which reuses the primary explants in several culture cycles in semi-solid medium to produce nodal explants for the multiplication phase in temporary immersion bioreactors (RITA®). The control of factors such as cytokinin concentration, explants density, immersion frequencies and culture duration was analyzed. The number of shoots increased with 0.5 mg·l^{-1} of BA (6-Benzyladenine), alone or in combination with 0.5 mg·l^{-1} of Kinetin, with 2 daily immersions of 1 minute each; however, these shoots showed a high degree of hyperhydricity. When 0.05 mg·l^{-1} of BA was used with 1 immersion of 1 minute every 2 days, the hyperhydricity decreased. Although the number of shoots was lower, they showed good length to be used during multiplication and rooting *ex vitro*. Our results suggest that teak micropropagation can be simplified in two phases *in vitro*, the establishment and multiplication; followed by rooting *ex vitro* and acclimatization. This would imply a reduction in production costs, since most of the multiplication would take place in RITA® containers.

Keywords

Micropropagation, Teak, *Tectona grandis*, RITA® Bioreactors, Temporary Immersion, *Ex Vitro* Rooting

1. Introduction

Teak (*Tectona grandis* Linn. F.), is a large and longlived arborescent tree belonging to the Lamiaceae family [1]. This species occurs naturally in India, Myanmar, Laos and Thailand and has become naturalized in Indonesia. It has also been planted throughout tropical Asia, as well as in tropical Africa, Latin America and the Caribbean [2]. Teak is one of the most prized high value timber species due to the exceptional properties of its wood, durability and aesthetic features. Thus, the establishment of industrial plantations has been extended in recent years due also to the total prohibition on harvesting teak from the natural forest [1]. According to Ugalde [2], in tropical America, the silviculture of exotic species has gained recognition and thanks to the unique traits of species like teak, commercial plantations are raising expectations in recent decades. In parallel, there has been an enormous interest in the use of selected clones and vegetative propagation practices for their multiplication. Micropropagation of superior clones through commercial laboratories may be attempted for large scale production of clones in operational planting programs [2]. In addition, it is more and more common for forest companies to request propagation services from laboratories for the multiplication, disease cleansing, and the reinvigoration of mother plants of the selected clones. Micropropagation of teak has often been used due to the difficulty via seminal reproduction because these seeds have hard tegument and low quality and its germination is irregular [3] [4].

In vitro propagation of teak, has been usually restricted to the culture of shoot tips and the multiplication of axillary buds in semi-solid culture medium [3] [5] [6] [7] [8] [9] [10]. In this species, in large-scale *in vitro* production the use of axillary buds allows a high sustainability of the culture and genotypic fidelity compared to de novo procedures; to date, millions of plants have been propagated this way [1]. Few studies have reported on the regeneration of teak plants via organogenesis [11] [12] or somatic embryogenesis [13].

Currently, the mass propagation of *in vitro* plants seeks to reduce production costs, simplifying processes, using liquid culture media, free of gelling agents and taking advantage of the *ex vitro* rooting benefits. It is well known that the mass propagation of plants in semi-solid media is labor intensive and costly. Gelling agents contribute significantly to *in vitro* production costs and limit the possibility of automation for commercial mass propagation [14]. Liquid medium has been recommended as ideal for automated culture and cost reduction during micropropagation [15]. In the same way, *ex vitro* rooting is attractive because it also reduces the cost of production significantly [9]. The disadvantages asso-

ciated with liquid media such as plant asphyxiation and hyperhydricity under these conditions can be avoided by using temporary immersion [16] [17]. This systems have been used to culture somatic embryos of different woody species [18]; some of them from temperate zones such us *Kalopanax septemlobus* [19], *Quercus robur* [20], *Q. suber* [21]; others correspond to woody tropical as *Hevea brasiliensis* [22] [23], *Coffea arabica* [24], *Coffea canephora* [25], *Theobroma cacao* [26], *Elaeis guineensis* [27], and *Bactris gasipaes* [28]. Although there are some reports on the micropropagation of woody plants using the culture of nodal explants in temporary immersion, including *Eucalyptus* [29] [30] [31], *Crescentia cujete* [32], *Malus* [33], *Castanea sativa* [18] [34], *Gmelina arborea* [35], *Psidium guajava* [36] and *Pistacia* [37]; only two of them correspond to *Tectona grandis* [14] [38].

Quiala *et al.*, [14] evaluated the multiplication of teak shoots in the temporary immersion system (TIS) based on two-vessels, described by Escalona *et al.*, [39]. The objective of his research was to determine the optimal concentration of BA (6-Benzyladenine) for shoot proliferation in a TIS and to clarify if different concentrations of BA affect the development of teak plants *ex vitro.* Quiala *et al.* [14] observed an increase in the number of shoots when increasing the concentration of BA (2.22 - 4.44 - 6.66 μM); however, these high levels of BA contributed to the appearance of morpho-physiological disorders in the shoots, indicating the presence of some degree of hyperhydricity. Numerous studies reveal that this phenomenon can be associated with physical and chemical factors in culture *in vitro* [40] [41], as well as high concentrations of BA [42] [43], high relative humidity, mainly in liquid culture media [14] [18] [30] [33] [34] [37], gas accumulation, light, temperature, or other factors [44] [45]. Therefore, the control of culture parameters coupled with the metabolic needs of each biological system will make it possible to make better use of the different *in vitro* multiplication strategies.

We have established a protocol for teak multiplication, combining the culture of semi-solid and liquid media in Temporary immersion bioreactors (RITA®) during the proliferation phase. The reuse of the primary nodal explants [46] in several multiplication cycles allowed the optimization of the initial explant. This work focused on developing a simplified protocol for teak multiplication that allows the efficient production of *in vitro* shoots and rooted plants of good quality in the greenhouse.

2. Material and Methods

2.1. *In Vitro* Establishment

This work was done using plants from rooted cuttings adult trees of selected clones from the Hojancha, Canton Agricultural Center in the province of Guanacaste, Costa Rica. For the introduction of explants *in vitro*, terminal shoots in active growth were harvested from mother plants located in the nursery. Previously, the shoots were immersed for 30 minutes into a solution that contains 3

$g \cdot l^{-1}$ of Manzate, Benlate and Agrimicin, followed by three rinses on sterile distilled water. Next, the shoots were subjected to a double disinfection in calcium hypochlorite $Ca(OCl)_2$ at 10% and at 8% for 20 and 15 minutes respectively, followed by three rinses with sterile distilled water. Finally, the apical explants were placed in culture vials (28 mm × 98 mm) containing 10 ml of MS initial culture medium [47] supplemented with 0.2 $mg \cdot l^{-1}$ of Kinetin and 0.1 $mg \cdot l^{-1}$ of Indol Acetic Acid (IAA) and 30 $g \cdot l^{-1}$ of sucrose. Agar 7 $g \cdot l^{-1}$ was used as gelling agent (SIGMA) and the pH is adjusted to 5.7.

2.2. Shoots Multiplication

2.2.1. Multiplication in Semi-Solid Medium

During the multiplication phase in semi-solid medium, nodal explants from a node were used, derived from shoots of the initial culture phase. Based on published results [3] [5] [6] [7] [8] [10], the effect of BA 0.5 - 1.0 and 1.5 $mg \cdot l^{-1}$ in combination with Kinetin 0.5 $mg \cdot l^{-1}$ was evaluated to determine the number and length of shoots produced per explant during 45 days of culture. The experiment consisted of three replicates of 25 culture vessels containing 20 ml of semi-solid medium and three explants each. In order to make better use of the primary nodal explant, it was reused several times to produce several cultures of shoots, according to the methodology used by Lardet *et al.* [46] in *Hevea brasiliensis*. The base of the primary nodal explant was transferred to fresh medium (subculture of the primary nodal explant) to produce a new generation of shoots, taking advantage of the axillary buds at the base. In addition, shoots from the primary nodal explant were used as secondary nodal explants to develop a new cycle of multiplication in semi-solid medium (**Figure 1**). Then, primary nodal explant was cultured for three cycles of 30 days each in vessels containing three explants in 20 ml of MS semi-solid multiplication medium supplemented with 0.05 $mg \cdot l^{-1}$ of BA. This assay was replicated using a 0.5-liter vessel with 100 ml of semi-solid multiplication medium in order to maximize the space of the container and the production of shoots. The number of explants per recipient was evaluated for R3 = 3 explants, R4 = 4 explants and R5 = 5 explants during three cultures lasting 30-days.

2.2.2. Multiplication in Liquid Medium in RITA® Bioreactors

Multiplication in temporary immersion bioreactors RITA® (CIRAD, France) was based on the evaluation of BA concentration, with or without Kinetin, and the frequency of immersion. The shoots used in RITA® come from the multiplication phase in semi-solid medium (**Figure 1**). The number of explant (30 - 60) per RITA®, and duration of the culture cycle (30 - 45 days) were initially defined. In this experiment the MS medium was supplemented with 0.5 $mg \cdot l^{-1}$ of BA and Kinetin. For all experiments, four RITA® containers were used per treatment containing 200 ml of liquid MS medium; once the initial density and culture time were defined, 30 explants and 30 days of culture were used. The RITA® containers were installed in an automated ventilation system, connected to an air

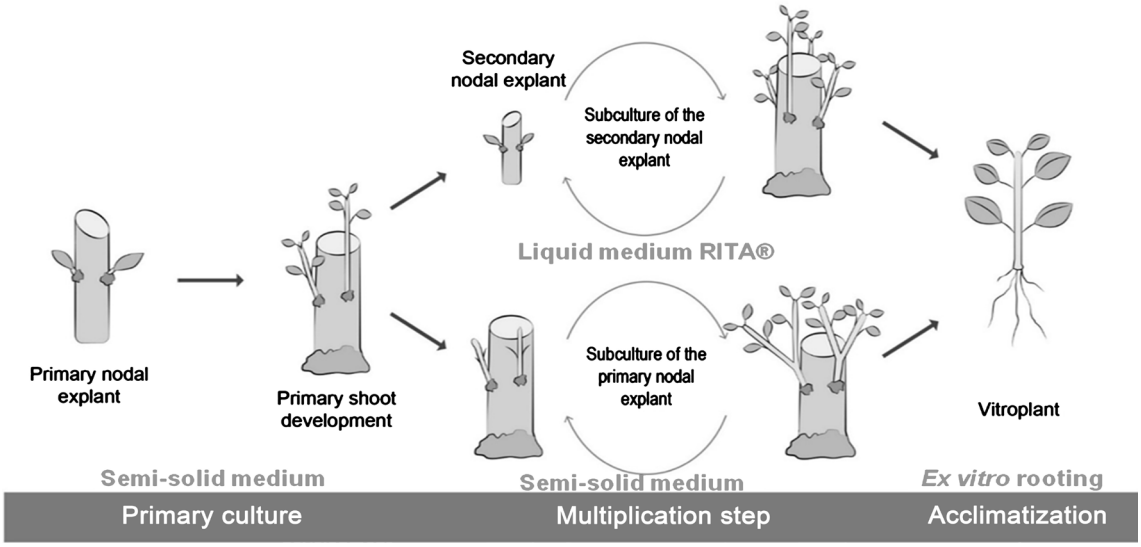

Figure 1. Multiplication of teak including reuse of the primary explant and the subculture of primary and secondary nodal explants in semi-solid and liquid medium in temporary immersion (RITA®). Aguilar, M.E., Ortiz, J.L., Kim, Y.W., Kim, J.A. and Moon, H.K. (2017) Contributions of somatic embryogenesis and other *in vitro* propagation techniques to the genetic improvement of tropical woody species: *Coffee arabica*, *Tectona grandis* and *Gmelina arborea*. In: Sung Park Y and Trontin JF (eds) Somatic Embryogenesis and Other Vegetative Propagation Technologies. Fourth International Conference of the IUFRO Unit 2.09.02, La Plata, Buenos Aires, Argentina pp 59-78.

compressor and a timer to regulate immersion frequency and duration. Next, the effects of BA (0.5 mg·l^{-1}) and Kinetin (0.5 mg·l^{-1}) were evaluated in combination and individually, and in a medium free of these regulators. In this experiment 2 immersions per day of 1 minute each were used at 12 hour intervals.

In order to improve the teak multiplication in RITA® containers, two treatments were also evaluated, which differed in their concentrations of cytokinins, 0.5 BA and 0.5 Kinetin mg·l^{-1} described earlier and compared with 0.05 mg·l^{-1} of BA. The effect of 1 and 2 daily immersions of 1 minute each was also compared. The evaluation was done individually for two culture cycles. Finally, the effect of immersion frequency on the number and quality of the shoots produced with 0.05 mg·l^{-1} of BA was evaluated. For this, 1 and 2 daily immersions were compared with 1 and 2 immersions applied every 2 days. In both cases, the immersion time was 1 minute.

2.3. *Ex Vitro* Rooting and Acclimatization

Shoots approximately 2 cm in length were cut from the highest quality shoots produced in semi-solid culture medium and different experiments in RITA®; and were taken to the greenhouse and planted in trays containing peat moss (Germination Mix, Canadian Sphagnum 65%, horticultural perlite, horticultural vermiculite) as a substrate. Three trays were used with 50 shoots each per treatment. As a rooting agent, the commercial hormone MAGIC ROOT (EVER GREEN) powder was applied on the basis of the shoots containing 0.3% IBA (w/w) as active ingredient. During acclimatization, the trays were placed in the

greenhouse inside plastic tunnels with a relative humidity close to 100%. An sprinkler irrigation regimen of 15 seconds, 3 or 4 times per day was used, depending on weather conditions. Two weeks after initiating the transfer of the plants to the nursery, the first fertilization was implemented with slow release Osmocote® (19-6-10) (1 g·Plant^{-1}). Additionally, fertilization was performed with complete granulated N-P-K formula (1 g·Plant^{-1}) at 30 days, and one supplement of chelated foliar fertilizer (Bayfolan® Forte) in a concentration of 5 ml l^{-1} that was applied every 8 days. In all cases the final evaluation was made after 60 days of acclimatization.

2.4. General Culture Conditions

All media were enriched with 30 g·l^{-1} of sucrose and the pH was adjusted to 5.7. Cultures were kept in a photoperiod of 12 hours light and 12 hours dark (provided by white light LED lamps) at a temperature of 27˚C ± 2˚C.

2.5. Statistical Analyses

Data were treated by analysis of variance (ANOVA), which was completed using Infostat Statistical Software [48]. The Fisher test was also used for comparing means (α = 0.05). The variables evaluated were: percentage of budbreak (%), mean No. of shoots per explant, mean length of shoot (cm), percentage of hyperhidric shoots and shoots in rosette in some cases (%) and percentage of survival during rooting-acclimatization (%).

3. Results

3.1. *In Vitro* Establishment

The establishment of apical teak explants *in vitro* was slow, between 45 and 60 days of culture in the initial medium was necessary to obtain shoots appropriate for multiplication; about 40% of the apices were lost due to oxidation or contamination.

3.2. Shoots Multiplication

3.2.1. Multiplication in Semi-Solid Medium

In the multiplication phase, the MS semi-solid medium supplemented with 0.5 - 1.0 and 1.5 mg·l^{-1} of BA and 0.5 mg·l^{-1} of Kinetin allowed the development of numerous shoots, but with callus formation at the base of these. In addition, in some cases the formation of hyperhidric shoots was observed, being higher with the increase in BA concentration. However, the combination of 0.5 mg·l^{-1} of BA with 0.5 mg·l^{-1} of Kinetin produced the highest number of shoots, with an average length of 3.4 cm and the least callus formation at the base (**Table 1**).

The reduction of the cytokinin source led us to use 0.05 mg·l^{-1} BA during the multiplication phase, obtaining a lower number of shoots but of better quality than those previously obtained. **Figure 2** shows the number and average length of shoots produced during three culture cycles from the primary nodal explant

Table 1. The effect of BA and Kinetin on the number, length and callus formation at the base of teak shoots in a semi-solid culture medium.

BA	Kinetin	No. of shoots per explant	Shoot length (cm)	Callusing (%)
($mg \cdot l^{-1}$)				
0.5	0.5	3.5^{a}	3.4^{a}	28.6
1.0	0.5	2.7^{b}	3.6^{a}	30.0
1.5	0.5	3.0^{ab}	2.8^{a}	47.0

*Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

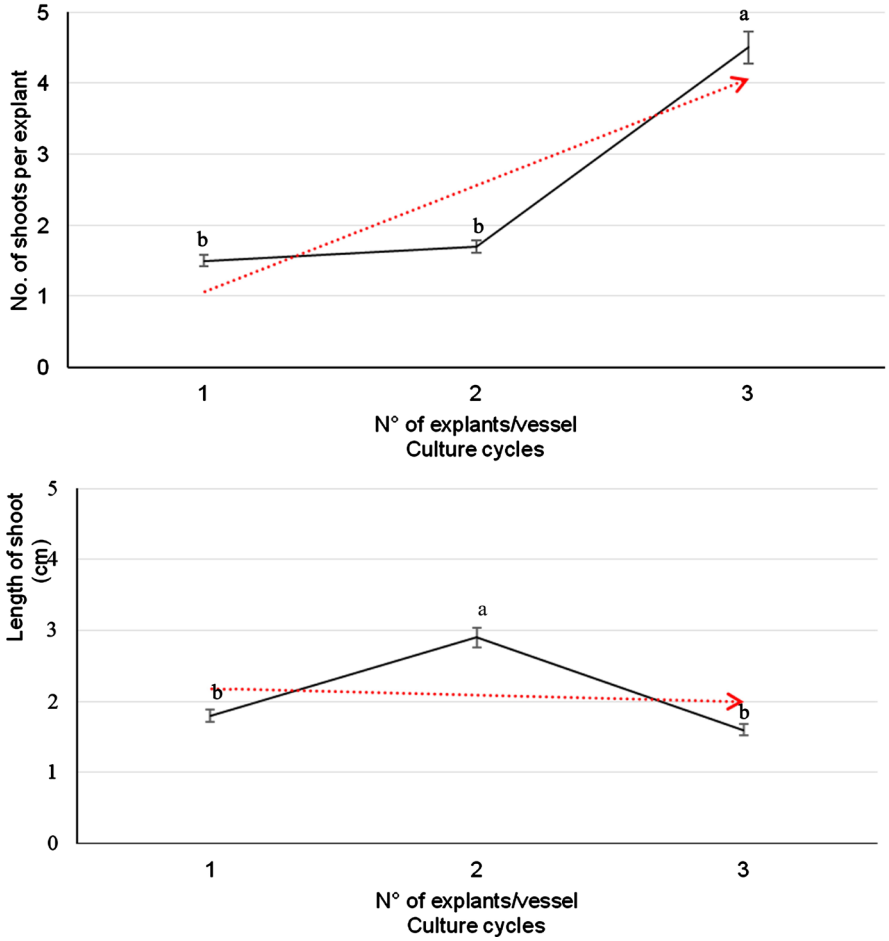

Figure 2. Number and average length of shoots produced during three culture cycles (1 - 2 - 3) from the primary nodal explant cultured in semi-solid medium. The dotted red line represents the linear trend. Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

cultured in vessels containing 20 ml of semi-solid medium with 0.05 $mg \cdot l^{-1}$ of BA. The number of shoots increased with each culture cycle, being significant in the third cycle where they tripled (4.5) with respect to the first cycle (1.5). However, shoot length was lower in the third culture cycle as the number of shoots produced increased. When a larger vessel containing 100 ml of medium was used, the production of shoots per explant showed exponential growth in each

culture cycle (Figure 3). However, the highest number of shoots was observed in R4 in the three cultures, being highly significant in the third culture (8.1), with a p value < 0.0001; the R3 (5.1) and R5 (4.9) treatments did not show significant differences. A homogeneous shoot length was observed in all treatments, except for the third subculture where R4 with 4 explants and R5 with 5 explants showed the highest numbers of shoots, but of shorter length, respectively (3.5 - 2.7 cm). The best condition in terms of shoot production yield and shoot quality is the R4 condition with the highest number of shoots larger than 3 cm. Some R5 shoots showed growth in rosettes and scorched leaves.

3.2.2. Multiplication in Liquid Medium in RITA® Bioreactors

Table 2 (Experiments A and B) shows the effect of two culture densities of 30 and 60 nodal explants per RITA®, in a medium supplemented with 0.5 mg·l^{-1} of BA and Kinetin. The average number of shoots per explant increased only slightly with a density of 30 explants per RITA®, showing significant differences in the first 45-day culture experiment. Average shoot length was favored by the culture time, being the best at 45 days of culture with 30 or 60 initial explants (Experiment B). In this experiment, a density of 30 explants per RITA® during 30 days of culture resulted in a total of 90 better quality shoots per container,

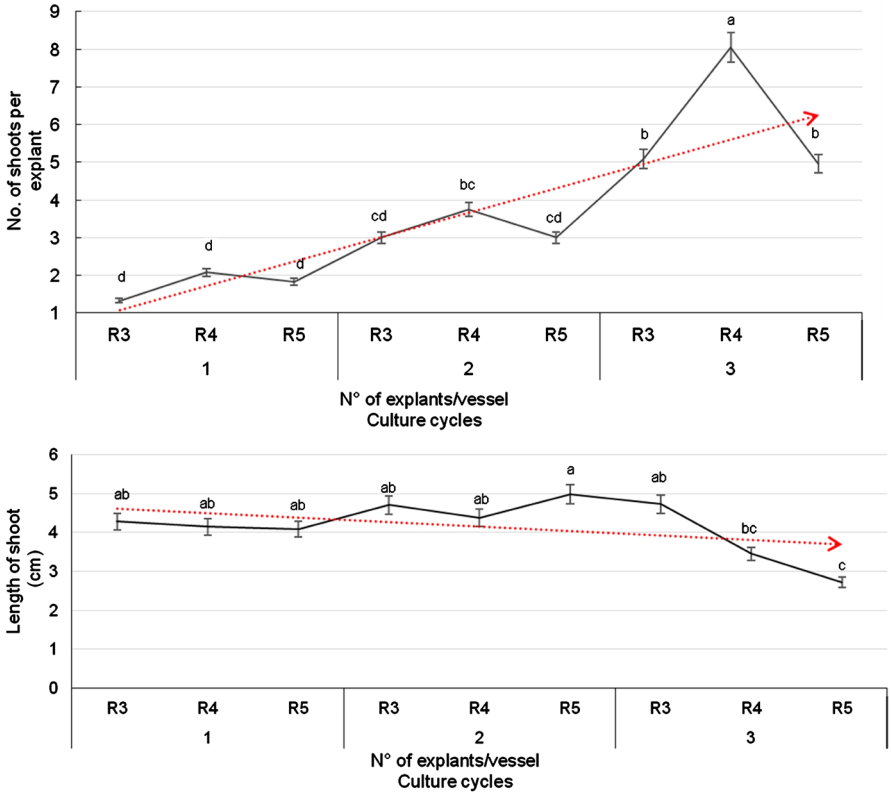

Figure 3. Number and average length of shoots produced during three subcultures (1 - 2 - 3) from the primary nodal explant cultured in semi-solid medium. R3, R4 and R5 correspond to the number of primary nodal explants for each recipient. The dotted red line represents the linear trend. Means with the same letter are not significantly different (LSD Fisher; p > 0.05).

with 22% hyperhydricity. Even when the culture time was increased to 45 days with a density of 30 explants, it was observed that the hyperhydricity of the shoots was lower (42% and 35%) than when 60 explants were used per RITA® with culture times of 30 and 45 days. The increase in the number of shoots per RITA® (156 - 168) produced at an initial density of 60 explants resulted in 75% (Experiment A) and 86% (Experiment B) hyperhydric shoots when the culture time was 45 days.

Table 3 shows that the concentration of 0.5 mg·l^{-1} of BA alone or in combination with Kinetin, produced a significant number of shoots; however, a high percentage of them were hyperhydric; 72% and 44.6%, in each case, respectively (Figure 4(a), Figure 4(b)). In the medium with 0.5 mg·l^{-1} of Kinetin, only 45% of the explants responded by budbreak, and 45% of the shoots produced showed hyperhydricity. In the absence of regulators, 22% of the shoots were hyperhydric. The height of the shoots was similar between treatments; however, there were significant differences between the two treatments with BA. When using 0.5 mg·l^{-1} of BA, the highest number of shoots was produced (3.3) but they were

Table 2. Effect of the number of initial explants and the duration of the culture cycle in RITA® on the number, length and percent hyperhydricity of teak shoots using 2 daily immersions of 1 minute each.

No. explants per RITA®	Culture time (Days)	No. of shoots per explant	No. of shoots per RITA®[a]	Shoot length (cm)	Hyperhidric shoots (%)
Experiment A					
30	45	3.5[a]	105.0	2.9[a]	42.0
60	45	2.6[b]	156.0	2.7[a]	75.0
Experiment B					
30	30	3.0[a]	90.0	3.2[b]	22.0
30	45	2.9[a]	87.0	3.6[ab]	35.0
60	30	2.8[a]	168.0	3.3[b]	62.0
60	45	2.8[a]	168.0	3.7[a]	86.0

[a]Estimated value based on the average number of shoots per explants; *Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

Table 3. Effect of the concentration of BA and Kinetin alone or in combination on the number and quality of teak shoots produced in RITA®, under 2 daily immersions of 1 minute each.

BA (mg·l^{-1})	Kinetin (mg·l^{-1})	Budbreak (%)	No. of shoots per explant	Shoot length (cm)	Hyperhidric shoots (%)
0.0	0.0	83.3	1.2[c]	2.4[ab]	22.0
0.5	0.0	100.0	3.3[a]	2.2[b]	72.5
0.0	0.5	45.0	1.3[c]	2.4[ab]	45.0
0.5	0.5	87.7	2.0[b]	2.7[a]	44.6

*Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

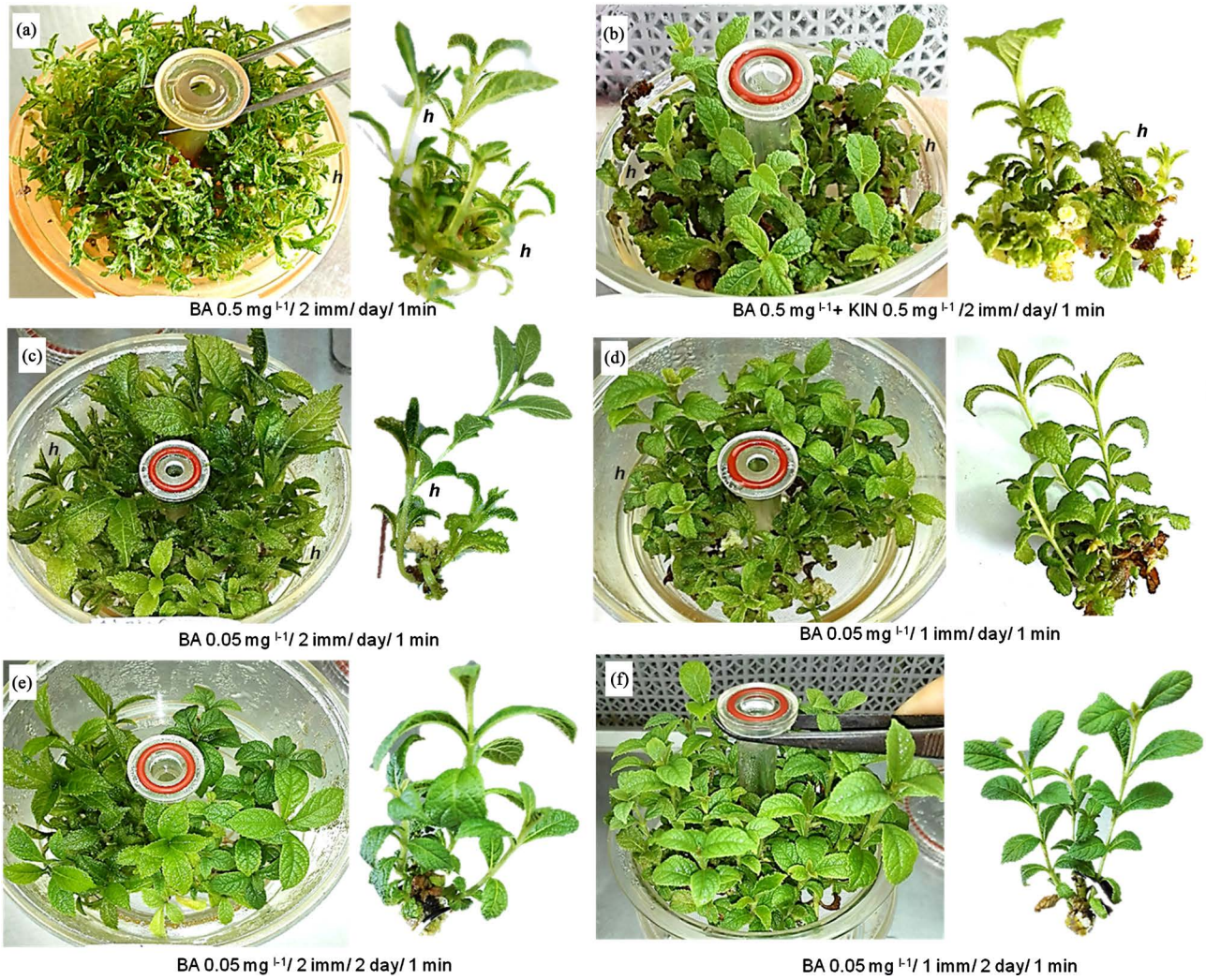

Figure 4. Teak shoots grown in RITA® after 30 days of culture. Shoots from 2 daily immersions (a) 0.5 mg·L^{-1} BA; (b) BA and Kinetin (0.5 mg·L^{-1}); (c) 0.05 mg·L^{-1} BA; (d) 0.05 mg·L^{-1} BA and 1 immersion per day. Shoots from immersions every 2 days; (e) 0.05 mg·L^{-1} BA and 2 immersions; (f) 0.05 mg·L^{-1} BA and 1 immersion; (h) Hyperhydric shoots.

shorter in length (2.2 cm).

Table 4 shows the results of two culture cycles in RITA®, comparing the effect of the BA and Kinetin mixture at a concentration of 0.5 mg·l^{-1} each, and with BA alone at a concentration of 0.05 mg·l^{-1}, under the effect of 1 and 2 daily immersions of one minute each. Very similar responses were observed in the two culture cycles; however, significant differences were found between treatments in each cycle, indicating that the highest concentration of BA and Kinetin (0.5 mg·l^{-1}), in both immersion frequencies, negatively affected the quality of the shoots, as shown in Table 3. The largest number of shoots were achieved in these treatments, but the highest percentages of hyperhydricity and rosseting were observed in these shoots (Figure 4(b)); each one of these variables being greater with 2 daily immersions of 1 minute each. The formation of callus at the base of the shoots was also superior in these treatments. The treatments with 0.05 mg·l^{-1} BA in the two immersion frequencies studied showed lower numbers

of shoots; however, these had the best quality and greater length, which allowed continuation of the multiplication cycles; these shoots were less hyperhydric and show minimal or no rosetting and callus formation at the base (Figure 4(c), Figure 4(d)).

Table 5 presents data on the evaluation of four immersion frequencies and a concentration of BA (0.05 $mg \cdot l^{-1}$). Significant differences were observed between the immersion frequencies and the variables evaluated. The highest number of shoots per explant was achieved when one (3.3) and two immersions (3.2) per day were used; however, the highest shoot hyperhydricity (26% and 29%) and an asynchrony in the development as was also observed in the previous experiment. With immersions every two days, the number of shoots was lower (2.3 and 1.7), but the quality of those shoots was significantly superior, since only 7% were hyperhydric, and the size of the shoots was superior (3.7) or equal (2.2) to the other treatments (Figure 4(e), Figure 4(f)). The treatment of 1 immersion every 2 days together with the addition of 0.05 $mg \cdot l^{-1}$ of BA was the best treatment to

Table 4. Effect of the concentration of BA and Kinetin and the frequency of immersion on the number and quality of teak shoots produced in RITA® in two culture cycles.

BA	Kinetin	N° immersions per day (1 min)	Budbreak (%)	No. of shoots per explant	Shoot length (cm)	Hyperhidric shoots (%)	Rosetting shoots (%)
($mg \cdot l^{-1}$)							
First cycle							
0.5	0.5	1	93.0	1.5^{b}	1.5^{c}	41.0	13.0
0.5	0.5	2	98.0	2.1^{a}	1.4^{c}	77.0	33.0
0.05	0.0	1	93.0	1.3^{b}	1.8^{b}	17.0	5.0
0.05	0.0	2	98.0	1.3^{b}	3.1^{a}	13.0	0.0
Second cycle							
0.5	0.5	1	100.0	1.9^{a}	1.8^{b}	47.0	17.0
0.5	0.5	2	100.0	2.0^{a}	2.1^{b}	46.0	37.0
0.05	0.0	1	100.0	1.3^{b}	3.9^{a}	11.0	4.0
0.05	0.0	2	100.0	1.2^{b}	3.8^{a}	8.0	2.0

*Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

Table 5. Effect of immersion frequency on the number, length and quality of teak shoots produced in RITA® containers.

BA ($mg \cdot l^{-1}$)	N° immersions (1 min)	Budbreak (%)	No. of shoots per explant	Shoot length (cm)	Hyperhidric shoots (%)
0.05	1 every day	100.0	3.3^{a}	2.2^{b}	29.0
	2 every day	96.0	3.2^{a}	3.4^{a}	26.0
	1 every 2 days	100.0	2.3^{b}	3.7^{a}	7.0
	2 every 2 days	100.0	1.7^{c}	2.2^{b}	7.0

*Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

this date for the production of better quality teak shoots using the temporal immersion culture in RITA® containers.

3.3. *Ex Vitro* Rooting and Acclimatization

Table 6 presents the number of shoots for each immersion treatment taken to acclimatization and the percentage of survival after 30 days in the greenhouse. In the case of shoots from an immersion frequency once a day a lot of shoots was lost due to hyperhydricity; therefore, only 48 shoots were acclimatized with a survival of 37.5%. The best survival (51.6%) occurred in the shoots from the 1 immersion every 2 days. The treatment of 2 immersions every 2 days had less shoots (1.7) and they were shorter (2.2 cm), with 38.5% survival rate during rooting and acclimatization.

Table 7 compares survival after 60 days of *ex vitro* rooting and acclimatization of shoots from semi-solid and liquid culture media at the same immersion frequencies previously evaluated. The best survival to acclimatization (90%) occurred with shoots in semi-solid media (Figure 5(a)), but this was not significantly different from the survival of shoots using one (86%) or two (85%) immersions every 2 days (Figure 5(b)). Shoots produced under a regimen of 1 or 2 immersions per day were the most vulnerable during acclimatization (Figure 5(c));

Table 6. Survival to *ex vitro* rooting and acclimatization of teak shoots cultured at different frequencies of immersion in RITA® containers.

Treatment	No. of acclimated shoots	No. of surviving plants	Survival (%)
1 immersion every day (1 min)	48	18^{b}	37.5
2 immersions every day (1 min)	128	34^{ab}	26.6
1 immersion every 2 days (1 min)	128	66^{a}	51.6
2 immersions every 2 days (1 min)	65	25^{b}	38.5

*Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

Table 7. Survival to *ex vitro* rooting and acclimatization of teak shoots cultured in semi-solid and liquid medium at different immersion frequencies in RITA® containers. 150 shoots per treatment were used.

Treatment Form of Culture	Survival (%)	Shoot length (cm)	No. of nodes	No. of main roots	Root system length (cm)
Semi-solid medium	90.0	28.2^{a}	9.0^{a}	2.3^{cd}	5.9^{c}
Liquid medium (RITA®)					
1 immersion every day (1 min)	66.0	28.2^{a}	8.6^{ab}	2.4^{bc}	8.4^{ab}
2 immersions every day (1 min)	20.5	28.4^{a}	9.0^{a}	1.8^{d}	6.7^{bc}
1 immersion every 2 days (1 min)	86.0	20.4^{b}	7.5^{b}	2.9^{ab}	9.1^{a}
2 immersions every 2 days/(1 min)	85.0	12.8^{c}	6.5^{b}	3.1^{a}	8.8^{a}

*Means with the same letter are not significantly different (LSD Fisher; $p > 0.05$).

Figure 5. *Ex vitro* rooting and acclimatization. (a) Shoots from semi-solid media. Shoots from RITA®; (b) 1 immersion every 2 days; (c) 2 immersions per day; (d) Plants from semi-solid medium; (e) Plants from 1 immersions every 2 days (RITA®); (f) Plants after 60 days.

as was observed in the previous experiment. For example, shoots with 2 daily immersions showed only 20.5% survival to acclimatization; most of these shoots suffered dehydration during the planting process in a tray (**Figure 5(c)**). After 60 days in the greenhouse, the plants from the semi-solid medium and those with daily immersions showed greater length and number of nodes, but their roots were shorter (**Figure 5(d)**). Meanwhile, plants subjected to a regime of 1 or 2 immersions every 2 days had shorter lengths and fewer nodes (**Figure 5(e)**). However, these plants developed good adventitious root systems, with 3 main roots on average and numerous secondary roots (**Figure 5(e)**), and survival equal to that of plants from semi-solid media in the greenhouse (**Figure 5(f)**).

4. Discussion

In the micropropagation of teak, the establishment phase *in vitro* is one of the most delicate, first, because it is the most vulnerable to contamination of the explants; second, because the *in vitro* response can be limited when adult material

is introduced. Goh and Monteuuis [1] found that when introducing apices and nodal explants of teak trees of different ages, only 70% and 30% respectively were reactive and free of contaminants. In our case, the reuse of the primary explant in several multiplication cycles on semi-solid medium allowed the plant production in a simple way, avoiding contamination from new introductions of explants. In addition, a stock of *in vitro* mother plants to produce shoots is ensured. The best shoots were produced in the larger culture vessels with 4 explants. Possibly, the larger size of the containers improved culture atmosphere and consequently the development of shoots. In *Hevea brasiliensis*, the primary explants were commonly reused in several multiplication cycles for the clonal multiplication of elite genotypes [46].

In micropropagation, strategies have been directed towards increasing multiplication rates, with a reduction in labor-intensive and production costs [16] [49]. The use of cytokinins, mainly BA, liquid culture media and the automation of the culture system are among the proposed solutions. The tissues in liquid culture media are in greater contact with the nutrients, resulting in faster growth [16]. However, culture conditions that promote rapid growth often result in structurally and physiologically abnormal plants [50]. One of the major obstacles is hyperhydricity [49]. This is a physiological disorder induced by physical and chemical factors [40] [41], such as high relative humidity, high levels of growth regulators, the accumulation of gases inside containers, and the intensity of light, among others [44] [45]; that generate stress conditions during *in vitro* culture [40]. BA is the most commonly used cytokinin in the micropropagation industry due to its effectiveness and affordability [42]. However, studies carried out using semi-solid media [41] [43], and liquid media in TIS [14] [18] [30] [33] [34] [37] determined that high level of cytokinin acts as an inducer of hyperhydricity in many species. Moncalean *et al.* [43] observed that the period of exposure to BA had a stronger influence than its concentration on the quality of *Actinidia deliciosa* plants, affecting both the relative water content and the retention capacity. In semi-solid media at high BA concentrations (1.0 - 1.5 $mg{\cdot}l^{-1}$) in combination with Kinetin (0.5 $mg{\cdot}l^{-1}$), we observed hyperhydricity in teak shoots and a strong callus formation in the base. Calluses were also reported by other authors in teak shoots when high BA concentrations were used alone or in combination with other regulators [3] [8] [10] [51]. A negative impact of this callus on the length of teak shoots was observed by Mendoza de Gyves *et al.* [3]. However, these authors do not mention the formation of hyperhydric shoots.

The quality of the teak shoots was also affected during multiplication in RITA® containers. Different degrees of hyperhydricity were related not only to the BA concentration, but also to explant density, culture time, and immersion frequency. Studies with eucalyptus [30], hybrid chestnut [18], and bamboo [52] showed that the density of culture in TIS can generate negative or positive responses in the morpho-physiological development of the plants. In teak, the lower density (30) of initial explants by RITA® during 30 days of culture did not

significantly affect the multiplication rate; but hyperhydricity was reduced and, consequently, the quality of the shoots was improved. Comparatively, a low density of explants by RITA® negatively affected the multiplication rate in eucalyptus [30]; while in the hybrid chestnut increased hyperhydricity [18]. Otherwise, in bamboo a high density of explants caused morphological variations in the leaves, possibly due to physiological and anatomical disorders induced by the increased water content, and the reduction in total chlorophyll content [52].

In other woody species such as apple [33], eucalyptus [30], chestnut [34] and pistachio [37], control of hyperhydricity in TIS was performed by reducing the concentration of cytokinin and adjusting the immersion frequency [18]. In teak, the morphology of the shoots in TIS was very affected by the BA concentration; the shoots produced with 2.2 μM of BA had a normal morphology, but at concentrations of 4.44 and 6.66 μM of BA the hyperhydricity increased [14]. In our study, the number of teak shoots in RITA® increased when 0.5 $mg{\cdot}l^{-1}$ BA was used alone or in combination with Kinetin, and 2 daily immersions of 1 minute; however, these shoots were very hyperhydric. Quiala *et al.* [14], using immersions of 40 seconds every 6 hours, found a significant correlation between low total phenol contents and hypolignification of the shoots at 6.66 μM of BA. Kevers *et al.* [53] and Hazarika [50] suggest that BA affects the metabolism of phenols, particularly lignins or their precursors; and in its absence the vascular cell wall is weak, less hydrophobic and permeable to water, causing hyperhydricity. The excessive accumulation of water in the tissues, indicated by a high fresh weight, is also a symptom of hyperhydricity [14] [18] [52]. The hyperhydricity of the teak in RITA® decreased considerably (7.0%) by reducing the BA to 0.05 $mg{\cdot}l^{-1}$ and the immersion frequency to 1 immersion of 1 minute every two days. Although the number of shoots was low (2.3); there was better use of the shoots since the length was longer (3.7 cm), compared with the treatments where 1 or 2 immersions were used daily. A lower hyperhydricity was also obtained during the germination of somatic embryos of *Coffee arabica* in RITA®, when the number of daily immersions was reduced [38] [54]. Very often, high multiplication rates coincide with high rates of hyperhydricity [14] [42] [49]. According to Etienne and Berthouly [16] the immersion time, *i.e.* duration or frequency, is the most decisive parameter for system efficiency, in addition to optimization of nutrient medium volume, especially during shoot proliferation. Similarly, Ahmadian *et al.* [49] suggest that immersion frequency is a very important parameter to control nutrients and hyperhydricity. They consider that the interval between immersions plays a definitive role in growth and multiplication; once the contact of the explants with the nutrient medium occurs during each immersion. In teak, we seek a balance between the number and length of shoots to continue multiplication cycles in order to produce good quality shoots for the *ex vitro* rooting and acclimation in the greenhouse.

Rooting is one of the most difficult phases during the micropropagation of woody species; in trees, it is usually done *ex vitro* due to the low response of *in*

vitro rooting [14]. Although numerous researchers have successfully practiced the *in vitro* rooting of teak shoots in semi-solid medium [3] [12] [51] [55]; others have opted for rooting *ex vitro* [8] [9] [14] [38]. *Ex vitro* rooting is attractive to micropropagators because of simultaneous rooting and hardening of plants and additionally reduces the number of steps required in micropropagation [9]. It is a cost-effective technique that can save labor, time and energy in the plant propagation system [56]. Goh and Monteuuis [1] mention 90% success during the rooting-acclimatization of teak shoots without rooting substances. The use of physiologically rejuvenated starting material guarantees *ex vitro* rooting, under favorable environmental conditions, even in the absence of rooting hormones. There is a direct impact between the quality of the shoots produced and the success during *ex vitro* rooting and acclimatization. In our case, the best survival during this phase occurred in the shoots coming from semi-solid medium and RITA® with 1 immersion every 2 days, which corresponds to the treatment that originated the best shoots during the multiplication. Similarly, the temporary immersion culture of *Crescentia cujete* [32], and eucalyptus [30] in RITA®; and of teak in twin containers [14], favored rooting and survival during acclimatization. *Eucalyptus sp.* [29] [31] and *Hevea brasiliensis* [23], are successful examples that show the potential of temporary immersion bioreactors in the production of clones in large-scale bio-factories. However, more studies should be carried out to confirm the application of this technique on a commercial scale, considering the control of contamination and rooting *ex vitro* [4].

In conclusion, we propose a simplified formula for the micropropagation of superior teak materials that involves three phases: 1) Apex culture in semi-solid medium for *in vitro* establishment; 2) Multiplication by reusing the primary explant in semi-solid medium and the subculture of primary and secondary nodal explants in liquid medium in temporary immersion (RITA®); 3) *Ex vitro* rooting-acclimatization in the greenhouse. It reduces the continual introduction of apices with better use of the initial explants, more reactive and contaminant-free. The primary explants are reused in several cycles of multiplication in semi-solid media and liquid in temporary immersion. The multiplication rate in RITA® with 30 initial explants doubles 8 times with respect to the semi-solid medium in vessels with 4 initial explants. At the end of the process, to significantly increase the number of shoots for rooting *ex vitro*, the micropropagation protocol is simplified in a single semi-solid medium for establishment; and a second medium, semi-solid or liquid for multiplication. While in the greenhouse, rooting occurs with the application of a commercial hormone. Therefore, by eliminating the gelling agent during the multiplication cycles in RITA®, and during rooting *ex vitro*, production costs will be significantly reduced.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

RITA	Temporary immersion bioreactor
MS	Murashige and Skoog
6-BA	6-Benzyladenine
IAA	Indol-3-acetic acid
IBA	Indol-3-butyric acid
TIS	Temporary immersion system