The effect of sucrose concentrations and shoot inoculum density on the growth of in vitro shoot of teak (*Tectona grandis* L.)

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Abstract. The teak belongs to the highly prized timber tree. Quality teak trees have been guaranteed by planting clonal planting materials propagated vegetatively, for example, by shoot tip cutting and tissue culture. Tissue culture propagation of teak has been commercially viable even though the efficiency of production can still be improved. This research aimed to study the effect of increasing shoot inoculum density and sucrose concentration requirement for optimum growth of teak shoot in vitro. Inocula of a single nodal shoot in the number of 5 or 9 were inoculated on modified MS basal media with sucrose concentrations treatment of 20, 30, 40, 50, and 60 g l⁻¹. The results showed that the standard sucrose concentration of 30 g l⁻¹ and a higher concentration of 40 g l⁻¹ were optimum for the growth the inocula of the densities tested. Even though the average shoot growth was lower for nine inocula than that of five inocula, the total shoot growth was higher for the former. An increasing number of inocula can be used as a method to increase the efficiency of shoot proliferation stage of teak micropropagation.

1. Introduction

Teak (*Tectona grandis* L.) is a timber tree belonging to the family of *Lamiaceae* grows in the rain forest of India, Myanmar, Laos, and Thailand [1]. The teak plant has high economic values due to its timber quality. Teak timber is strong, durable, and resistant to various insect pests, especially termites [2]. Teak timber is commonly used for building construction, ship buildings, and furniture [3].

Teak has been planted in large areas in an estate system and managed by a government company in Indonesia, as well as in small areas by smallholder farmers [4]. Recently, private individuals or enterprises plant teak as individual investment, corporate investment, or even plantation investment for sale. Teak estates for sale have been advertised by companies in Indonesia and elsewhere, such as in India, Nicaragua Panama. Besides, it had been analyzed economically in Colombia [5]. These new ventures, as well as the conventional ones, will require good quality and homogenous planting materials that can and have been provided by vegetative propagation via shoot tip cuttings or micropropagation via tissue culture of superior mother plants [6].

Reports for tissue culture propagation of teak were abundant and had been published for a period of almost 40 years; however, most of them were still in their early stage and rarely demonstrated their
efficiency for a sufficient period [2-3,7-10]. An efficient protocol with a high rate of propagation is needed for the production of inexpensive teak planting material. The efficiency of tissue culture propagation can be measured by the production of inoculum for subculture per unit vessel, per unit growth medium, or duration of subculture. The production of the inocula as a manifestation of the culture growth is affected by components of culture environment such as light intensity and temperature, as well as the chemical components of the growth medium for given genotypes.

Even though teak tissue culture could be initiated from seedling [10,11], organogenic way from internode [12], most commonly were from exiting meristems contained in nodal explants of elite trees and proliferated by the formation of new meristem axillar resided in leaf axil of the node of multiple shoots or a single shoot [2,3,7,13]. The meristem axillar, together with the carrier tissue, i.e., nodal stem segments, was used as inocula for further shoot proliferation. Therefore, the number of nodal stem segments that can be subcultured is a determinant for efficient propagation and dependent upon the initial number of inocula, and the speed of new meristem formation or shoot proliferation and shoot elongation.

The chemical component of the culture medium consisted of mineral nutrients and organic nutrients. Since tissue culture is a mixotrophic system, the presence of carbon source as an organic nutrient is essential for the growth of plant culture. The most commonly used carbon source was sucrose in the concentration of 20-50 g l\(^{-1}\) [14] even though the most common concentration used was 30 g l\(^{-1}\). Sucrose is not only important as a carbon source but also necessary to maintain the water potential of the medium.

This paper reports the effect of inoculum density and sucrose concentration on the proliferation of teak tissue culture for efficient propagation protocol.

2. Materials and methods

As the source of inocula, shoot cultures of teak mutant ‘MK10’ Research Center of Biology, LIPI was used. The ‘MK10’ is a fast-growing teak mutant that, after 14 years, it has a diameter of 57 cm at chest height. The shoot cultures had been maintained in culture for over a year with a periodic subculture of 5-6 weeks. The cultures were maintained in a stainless steel culture cabinets with a light source of fluorescence lamp at an intensity of 46 µmol.m\(^{-2}\).s\(^{-1}\) for 16 hours daily at a temperature of 25±2°C and humidity of 40%. These cultures were maintained by the inoculation of nodal stem segments maintained in the growth medium. There were five inocula per vessel. The growth medium followed MS growth medium formulation [15] with modification. Nitrogen salts were modified by decreasing NH\(_4\)NO\(_3\) concentration to 1200 mg l\(^{-1}\), increasing KNO\(_3\) concentration to 3033 mg l\(^{-1}\) to give a ratio of NO\(_3\):-NH\(_4\) of 3:1 [16,17], and enriching with 100 mg l\(^{-1}\) myo-inositol, 10 mg l\(^{-1}\) thiamine, 0.1 mg l\(^{-1}\) BA, 30 g l\(^{-1}\) sucrose. The pH of the medium was adjusted to pH 5.7-5.8. The medium was solidified with 8 g l\(^{-1}\) agar. The growth medium as much as 25 ml was contained in a glass vessel of 275 ml volume with 6.6 cm diameter by 9.2 cm height, capped with transparent plastic film, and secured with rubber bands.

An experiment to improve the proliferation rate of this teak mutant was carried out by increasing inoculum density per vessel from 5 to 9 nodal stem segments and by varying the sucrose concentration in the medium to 20, 30, 40, 50 and 60 g l\(^{-1}\). Inocula of one nodal stem segment of first, second, third, or even fourth whenever available from the shoot tip was excised from previous shoot cultures. The inoculum had a stem of 0-1,0-3 cm above and 0,5-0,8 cm below node and half of leaf lamina. There were four replications for each treatment. The initial experiment, according to the treatments, was designated as the initial subculture (S0) and continued to the first (S1) and second subculture (S2). Stem segments from a particular treatment were only subcultured to the same treatment.

Growth variables were observed at 6-7 weeks after culture and included the number of shoot per inocula, number of nodes per shoots, and shoot length. From those variables, the number of nodes per vessel, representing productivity of the treatment combination and internode length, indicating the shoot vigor, were calculated and presented. The number of nodes per vessel was calculated by multiplying the number of shoot per inocula with the number of nodes per shoot with a number of inocula per vessel. The internode length was calculated by dividing shoot length with a number of nodes per shoot. The presence of necrotic apical and the yellowing shoots was also recorded with
digital camera Sony Cyber-shot DSC-WX500. Data were plotted and analyzed with Excel Microsoft Office.

3. Results and discussion

The growth of teak shoot culture from inoculum nodal stem segments was characterized by the emergence of the axillary meristem into a single or two elongating shoots. The shoots grew periodically by forming a pair of new leaves, then internode elongated, and new leaves were formed, and so on. Meanwhile, at the base of the nodal stem segment inocula, callus masses were also formed both submerge in the medium or above medium. Visually, there were significant differences in the growth of the shoot culture under different sucrose concentrations at different inoculum density (Figure 1). However, quantitative observations are depicted as follows.

The number of shoots formed from the nodal stem segment most often was one, as indicated by the average number that was one without error bars. Only very rarely, a maximum of two shoots grew from the nodal stem segments. That mode of shoot growth was consistent and not affected by the concentration of sucrose, inoculum density, or subculture number (Figure 2).

Figure 1. The growth of teak shoot cultures at different inoculum density (5 vs. 9) after seven weeks, after 2nd subculture on the same treatment medium sucrose concentration (20, 30, 40, 50, and 60 g l\(^{-1}\)).

The number of nodes from the shoot cultures determines the rate of proliferation since it is used as the inoculum. The number of nodes between cultures with low inoculum density and high inoculum density did not follow a certain trend, but it tended to be dependent on specific sucrose concentration. High-density inoculum tended to produce a significantly higher number of nodes (Figure 3). Upon subculture to first and second, high inoculum density significantly produced more nodes than low density at sucrose concentration of 20-40 g l\(^{-1}\). At a higher concentration of 50-60 g l\(^{-1}\), the difference was not significant. The higher density tended to produce a lower number of average of eight nodes as compared to average of 16 nodes at a lower density at the third subculture. Increasing sucrose concentration tended to decrease the number of nodes. This trend was more noticeable when the inoculum density was higher. A significant reduction of node number from inoculum with high density occurred at 50-60 g l\(^{-1}\) after three passages (Figure 3c).
Figure 2. The effect of sucrose concentration on the average number of shoots per inocula of shoot culture of teak ‘MK10’ seven weeks after culture at: a) initial experiment (S0), b) first subculture (S1), c) second subculture (S2).

Figure 3. The effect of sucrose concentration on the total number of nodes per vessel of shoot culture of teak ‘MK10’ seven weeks after culture at: a) initial experiment (S0), b) first subculture (S1), c) second subculture (S2).
Figure 4. The effect of sucrose concentration on the average shoot length of shoot culture of teak 'MK10' seven weeks after culture at: a) initial experiment (S0), b) first subculture (S1), c) second subculture (S2).

The treatment of increasing sucrose concentration tended to decrease the shoot length of the shoot culture to very minimal at a concentration of 60 g l$^{-1}$ (Figure 4). In the subsequent experiment, the first subculture resulted in different shoot length between density at the lowest sucrose concentration of 20 g l$^{-1}$. At the same time, no differences were observed at higher sucrose concentration (Figure 4b). The tendency of decreasing shoot length with sucrose concentration became more pronounced at the 2nd subculture (Figure 4c). Comparison between inoculum density treatments indicated that their effects on shoot length generally were not significant, even though a significant decrease in shoot length was observed after three subcultures at lower sucrose concentration of 20-30 g l$^{-1}$.

Figure 5. The effect of sucrose concentration on the average internode length of shoot culture of teak ‘MK10’ seven weeks after culture at: a) initial experiment (S0), b) first subculture (S1), c) second subculture (S2).
Internode length is essential in preparing inoculum for the proliferation stage. Too short (rosette) shoots would produce a small number of inocula, while the most suitable length was about 1 cm. Inoculum density did not significantly affect the internode length. However, the results indicated that increasing sucrose concentration tended to decrease the internode length. There were no significant differences in internode length at sucrose of 20–40 g l\(^{-1}\). However, a significant decrease of internode length occurred at a higher concentration of 50–60 g l\(^{-1}\) (Figure 5). The high error bar at sucrose concentration of 60 g l\(^{-1}\), for nine inocula, at the 2\(^{nd}\) subculture indicated that some shoots failed to grow, or they grew with rosette shoots. Internode length of 0.7 to 1.2 cm occurred at the treatment of sucrose 20–40 g l\(^{-1}\), which seemed optimal since the inoculum length was generally about that size, where 0.5-0.7 cm of the segment was planted submerged in the growth medium, while 0.2-0.3 cm segment above the node was exposed to the air part of the medium.

The proliferation system for teak micropropagation was developed based on one step elongation of one axillary shoot from inoculum nodal stem segments, in which phyllotaxis had two axillary meristems. After the new shoots elongated, the shoots were cut into one-nodal-stem segments, and each nodal stem segment was used as an inoculum. Then, it was inoculated again in the fresh growth medium for further proliferation. This common practice of 1-step proliferation is more efficient than the 2-step proliferation consisting of 3-4 weeks induction of callus phase at the base of stem segment followed by 3-4 weeks of shoot elongation that employs two kinds of growth media [18,19]. The developed protocol contained a low concentration of plant growth regulator BA of 0.1 mg l\(^{-1}\) and allowed one axillary shoot to grow and form 2-4 more nodes with expanded leaves and axillary meristems in the leaf axils. The nodes with axillary meristems then continuously serve as the nodal stem segment inocula for further proliferation. With this protocol, a consistent multiplication rate of 3-4 fold per 4-6 weeks could be achieved. This method of propagation via shoot elongation by employing a low growth regulator resulted in a 3-4 multiplication rate on 7-8 weeks period, which in line with a previous report [6]. This protocol is more preferable than the multiple shoots induction in teak tissue culture since the latter involves the use of higher concentration or a complex mixture of growth regulators [13] or a potent growth regulator such as thidiazuron [10,11], which requires subsequent culture in a growth regulator-free medium to induce shoot elongation [11] and prolong propagation processes.

Since the number of nodal inoculum determines the number of nodes produced, thus, it is important to evaluate the number of inoculum for proliferation. Our results indicated that inoculating nine stem segments did not decrease the quality of shoots, but significantly increased the number of nodes produced compared to that of the standard of inoculation of five stem segment per vessel containing 25 ml growth medium at sucrose concentration of 30-40 g l\(^{-1}\). Increasing inoculum density has been shown in bananas to decrease the proliferation rate, the size and the weight of the individual shoot, but increase the total number of shoots produced [20]. Increasing the inoculum density of shootlets of lemongrass in a temporary immersion system also increased the fresh weight of the shoots per vessel [21]. It seems that the limiting factor for the proliferation of teak shoot cultures still needs to be explored to develop the most efficient propagation protocol.

Standard sucrose concentration of 30 g l\(^{-1}\) with a deviation of 10 g l\(^{-1}\) was still satisfactory for the growth of teak shoot cultures, regardless of the inoculum density and its continual growth. Standard sucrose concentration of 30 g l\(^{-1}\), such as in MS formulation, had been commonly employed for teak shoot cultures [7,13] and found to be optimal [22]. It had also been reported to be optimal for efficient regeneration of several other trees such as Acacia arabica [23], Calliandra tweedii [24], dan Elaeocarpus robustus Roxb [25]. However, some studies reported that sucrose concentration of 40 g l\(^{-1}\) was optimum to increase the shoot induction and shoot number of black plum [26]. Meanwhile, the effect of increasing sucrose concentration from 15 mM to 44-58 mM to increase the number of axillary shoots was observed in two out of three blueberry cultivars tested [27]. High sucrose concentration was needed for the initial growth of Almond in vitro [28]. Shoot growth responses in vitro for some trees species, such as walnut [29], were reported to respond parabolically to sucrose concentration.

Higher sucrose concentrations of 50-60 g l\(^{-1}\) were inhibitory to teak shoot growth in vitro. Increasing sucrose concentration from 30 g/l to 60 g/l decreased the relative growth rate of leaf
number and root number of sago palm plantlet [30]. Carbohydrate such as sucrose functions in tissue culture medium as an energy and carbon source, metabolites, and osmoticum [31]. Sucrose concentrations, which are higher than the optimal concentration, are often inhibitory to chlorophyll synthesis [32] and decrease the greening of protocorm of Cymbidium [33]. Increasing sucrose concentration would decrease the osmotic potentials of the medium [31]. Furthermore, increasing sucrose concentrations beyond 4-5% exerted osmotic stress and resulted in progressive inhibition of cell growth [34]. Increasing sucrose concentration in the medium from 3 to 6% decreased water potentials in leaf and probably exerted osmotic stress and resulted in the slow growth of plantlets of Alocasia amazonica [35].

Many tree species were provided with lower concentrations or no sucrose in the tissue culture medium. It was meant as a strategy to force the shoots to change from heterotrophic to autotrophic growth stage [36]. The transition to the autotrophic growth stage is important for successful acclimatization and subsequent production of planting materials. However, for teak, the transition from in vitro to ex vitro growth condition has not been a problem. Teak shoots from tissue culture acclimatized well and gave rise to plants in vivo with a high rate [18].

4. Conclusion

Many tree species were provided with lower concentrations or no sucrose in the tissue culture medium. It was meant as a strategy to force the shoots to change from heterotrophic to autotrophic growth stage. The transition to the autotrophic growth stage is important for successful acclimatization and subsequent production of planting materials. However, for teak, the transition from in vitro to ex vitro growth condition has not been a problem. Teak shoots from tissue culture acclimatized well and gave rise to plants in vivo with a high rate.

Acknowledgments

This research was funded from Non-Tax State Revenue Jati Platinum LIPI, Research Center for Biology, Indonesian Institute of Sciences. All authors contributed equally to this work.

5. References

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