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Optimising Gelling Agents, Light Source and After-care to Commercialise Teak Tissue Culture

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Abstract

Mass propagation of Teak (*Tectona grandis* Linn. f.) a commercial species is a priority to increase multiplication rate and meet the growing demand for planting material. The present study has assessed the *in vitro* performance of Teak Tissue Cultures with different gelling agents, different light conditions, and aftercare of *ex vitro* rooted shoots to enhance their survival rate. Multiple shoot formation was induced from excised seedling nodal explants on MS supplemented with BA and Kn and about 5 - 10 shoots were obtained from each explant. Significant variation ($p > 0.05$) was observed in the number of shoots produced by the different clones. Three gelling agents (agar, phytagel and gellan gum), and two light sources (tube light and LED) were tested for enhancing shoot multiplication. No significant difference in *in vitro* growth was observed between clones with different solidifying agents. Teak, however, did not respond favourably to LED lights. Rooting-acclimatization phase was achieved in the nursery with 80 - 95 per cent success. The rooted plants were sprayed with DAP and Humaur to assess the growth performance following transplanting. Significant variations in rooting indicate the existence of physiological variations among the clones. Application of fertilizers promoted an initial boost followed by a steady increase during the rest of the study period. Clones with high multiplication rates under *in vitro* conditions could be selected for commercialization of teak multiplication. Under *ex vitro* conditions, a spray of fertilizers during the initial establishment phase would result in increased vigour of transplantable plants. This would ensure better survival on out planting.

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Introduction

Teak (*Tectona grandis* Linn. f.) as a plantation crop species of the dicot family Verbnaceae, has attracted the attention of both the government and private sectors. It is one of the few valuable hardwood species that is grown increasingly on plantation scale in tropical countries around the globe. With expanding population, and a rising preference for hardwood veneer and wood-based panels, the requirement of good quality teak is on the increase, the result being an imbalance in the demand and supply. Its large-scale cultivation is hampered by various factors, the major one being poor germination of drupes (Masilamani et al. 1997). Higher productivity from the plantations can be achieved by selecting genetically improved stock, which expresses itself during the later stages of growth in plants (Indira and Basha 1999).

Vegetative propagation from shoot-bud and/or root grafting can produce 50000 to 60000 plants from one kg of improved seed (from clonal seed orchards and seed production areas) (Kjaer et al. 1998), but has little practical feasibility commensurate with the demand for planting material (Dharmalingam and Masilamani 1997). Low seed production from the orchards (Kumar 1992) remains a bottleneck for large scale planting. To overcome this, rapid multiplication through micropropagation for mass propagation has been adopted and standardized. Although tissue culture requires intensive capital investment, it has the advantage of saving time and avoiding the problem of unreliable spurious seed supply. The plantlets so obtained are uniform and have improved qualities. The improved genetic material can be introduced faster into the new plantations (Kjaer and Foster 1996). Many research institutions have developed micropropagation protocols for mature explants and seeds (Mascarenhas et al. 1993, Tiwari et al. 2002, Goh and Montenuis 2005, Yasodha et al. 2005). It is believed that different clones require slightly different culture medium for optimum mass multiplication.

Fluorescent tube lights are commonly used in tissue culture laboratories as light sources. They have a wide range of wavelengths (350 - 750 nm) and consume high electricity which ultimately increases the per plant production cost. Light-emitting-diode (LED) light sources are proposed as potential alternatives for growth and development of *in vitro* and greenhouse plants due to their high efficiency and low electricity consumption and lower heat generation (Yeh and Chung 2009). Gupta and Jatothu (2013) and Bello-Bello et al. (2016) reported that LED treatments result in higher frequencies of somatic embryo germination, conversion of a greater number of lateral roots than that of white fluorescent light treatments in *Pinus* species.

High quality seedlings are a pre-requisite to the successful establishment of the plantations particularly on poor soils. Fertilizers play a vital role in boosting the initial growth and development of the seedlings in the nursery. Many

workers have studied the role of fertilizers on seedling growth of teak and found that the requirement varies with the type of the soil used (Totey et al. 1986, Rangaswamy et al. 1990, Kalpana Mishra 1995). In tissue culture raised plants, acclimation facilitates physiological and structural changes to help the plants adapt to new environmental conditions. Therefore, it is necessary to provide sufficient aftercare for plants to adapt to local climatic conditions.

The objective of this study was to determine the effects of different gelling agents in the media used for growth of the plants under *in vitro* conditions, light quality on *in vitro* shoot proliferation and growth of teak and the effect of fertilizers in supporting acclimation under nursery conditions.

Materials and Methods

Drupes of teak collected from a Clonal Seed Orchard (CSO) comprising of genotypes drawn from different parts of the country were cracked open. The whole seeds were separated and washed with Teepol (0.1%) for five min, followed by distilled water rinsing. The seeds were then surface-sterilized with 0.1 per cent sodium hypochlorite (v/v) for 5 min and 0.1 per cent (w/v) mercuric chloride for 5 min, rinsed three times in sterile distilled water following each treatment. They were, thereafter, germinated singly on water-agar (2%) in test tubes.

When the germinated seedlings attained a height of 3-5 cm, they were dissected into single nodal cultures. Each of these sections was then transferred to medium containing BAP 2.22 µM and Kn (1.16 µM). The pH of medium was adjusted to 5.6 - 5.7 with 0.1 N sodium hydroxide and autoclaved at 120°C for 20 mins.

Three different gelling agents, namely, agar (0.7%), phytagel (0.2%) and Gellan gum (0.25%) were tested to identify the best solidifying agent. Three clones were used for each of experiments. Phytagel is an agar-substitute produced from a bacterial substrate composed of glucuronic acid, rhamnose, and glucose. It produces a high-strength gel, which aids in the detection of microbial contamination. Phytagel provides an economical alternative to agar as a gelling agent. Gellan gum is a water-soluble anionic polysaccharide produced by the bacterium *Sphingomonas elodea*. It produces a highly transparent gel, which allows for a better observation of the root growth compared to conventional agar gel.

Three clones were used for each experiment. The explants were incubated at 23°C in a 16-hour photoperiod provided by white fluorescent tubes and light-emitting diodes (LED). The experiments were laid out in a CRD with ten replications per clone and five cultures per replication. After six weeks of culture, the explants produced shoots. The new shoots were excised and the subculture procedure was repeated every six weeks for a period of six months.

Observations were recorded on per cent cultures responding to shoot formation, average number of shoots per explant, shoot lengths, the number of nodes per explant and harvestable shoots.

Shoots with three pairs of leaves and a height of 4 cm or above were excised and rooted under *ex vitro* conditions. The shoots were treated with IBA at a concentration of 1000 mg/l for 5 min. They were then placed in vermiculite and shifted to covered polytents to maintain high humidity. After 20 - 25 days, the polytents were loosened gradually. After 40 - 45 days the rooted plants were moved to shade house. At the end of the rooting period, the percentage of rooted shoots was determined. Rooting characteristics of these shoots were recorded, including the average number of roots and the length of the longest root. For rooting parameters, 25 plants were maintained per clone.

The requirement of nutrients for development of healthy plants was assessed. DAP and *Humaur* at a concentration of 2 mg/l were given as foliar sprays. Clone SBL was used for the study. DAP is most widely used phosphorus fertilizer, and it is popular because of its relatively high nutrient content and its excellent physical properties. *Humaur* is a bio-organic foliar nutrient, manufactured by Hindustan Antibiotics Ltd., Pimpri, Pune and is reported to contain enzymes, vitamins and organic acid precursors. The plants were sprayed with the chemicals after sunset twice at two months interval. Observations on height, collar diameter and the number of leaves were recorded at monthly intervals for 120 days.

The significance of differences was determined by ANOVA and the significant ($p < 0.05$) differences among mean values were estimated by DMRT. All statistical tests were performed by SPSS version 20.0. The data are presented as means ± Sd, and different letters in the Tables indicate significant differences at $p < 0.05$. Data were transformed into arcsine wherever required to improve the normality of the data distribution. The data presented in percentages were subjected to arcsine transformation before analysis, and then converted back to percentages for convenience of presentation in the Tables and Graphs. The experiments were arranged in a CRD.

Results and Discussion

Seeds germinated within two weeks of inoculation. The seedlings showing a height of above 4 cm were selected for further multiplication. Each plant was cut into 3 - 4 nodal segments and placed in MS supplemented with BAP and Kn (Yasodha et al. 2005). Significant differences ($p < 0.05$) were observed between the sources. Germination per cent varied from 25.8 to 87 (Fig. 1) with a mean of 61.32 and Sd of 17.65. Variations in germination of teak, with less than 25 per cent germination, has been reported by Prasad and Jalil (1986) and Indira and Basha (1999). Mathew and Vasudeva (2003) reported low germination and a high

CV (47.54) between seedlots of a CSO of teak. It is suggested that the age of the ortets could influence germination. In the present study, though differences were observed between the clones, the average germination and CV of the seedlots was high (28.78).

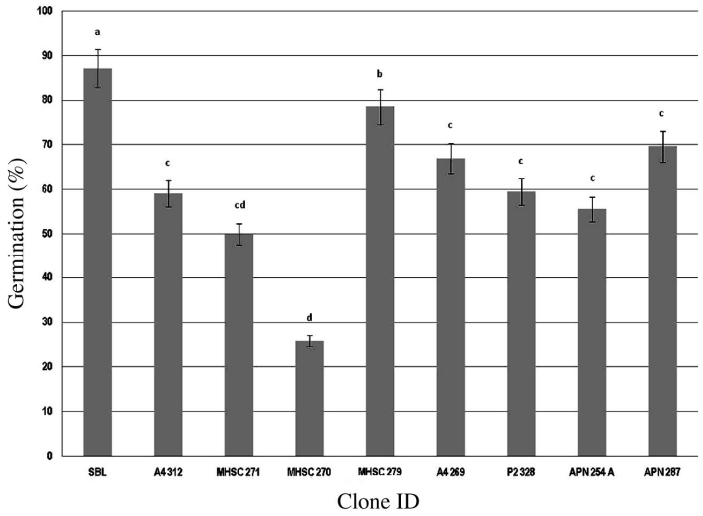

Fig 1. Differential response in germination of teak from different sources under *in vitro* conditions.

Table 1. *In vitro* response of clones of *Tectona grandis* after 6 months.

Sl. No.	Clones	No. of shoots per explant	Rootable shoots per explant	Total rootable shoots
1.	SBL	10.04 ± 2.62^{ab}	7.83 ± 3.12^{a}	36.13^{a}
2.	A_4 312	8.92 ± 3.29^{ab}	7.15 ± 2.15^{ab}	18.74^{ab}
3.	MHSC 271	6.98 ± 1.40^{abc}	5.44 ± 0.69^{b}	25.77^{a}
4.	MHSC 270	3.80 ± 2.59^{c}	2.20 ± 1.10^{c}	23.44^{a}
5.	MHSC 279	10.70 ± 4.74^{a}	8.15 ± 5.85^{a}	21.24^{a}
6.	A_4 269	7.64 ± 2.83^{abc}	5.52 ± 4.16^{ab}	27.63^{a}
7.	P_2 328	7.46 ± 1.87^{abc}	4.44 ± 1.57^{ab}	25.40^{a}
8.	APN 254A	7.43 ± 0.75^{bc}	7.21 ± 0.25^{ab}	7.21^{b}
9.	APN 287	5.47 ± 0.82^{ab}	4.32 ± 0.59^{ab}	9.50^{b}

A two- to three-folds multiplication of shoots was observed from the nodal segments (Table 1). The *in vitro* shoots were sub-cultured in fresh medium of the same composition. They yielded 4 - 5 shoots each within 3 - 4 weeks. The mean number of shoots produced per subculture was 4.6 and they varied from clone to

clone. After 180 days, clone MHSC-279 showed maximum shoot proliferation (10.7 shoots per explant) followed by SBL (10.04). Repeated subcultures favoured axillary bud development and formation of multiple shoot cultures, a prerequisite for commercial production. Significant variation ($p < 0.05$) was observed in the number of shoots produced *in vitro* by the different clones. Slight modifications in the medium by increasing or decreasing the cytokinin concentrations, resulted in loss of morphogenetic potential or an increase in hyper-hydricity of the explants (data not shown here).

The number of rootable shoots was highest in MHSC 279, while it was the least in MHSC 270, which also recorded the lowest shoot multiplication (3.8). The variation in response to shoot multiplication under similar environmental conditions could be attributed to the genotypic differences existing between them. Clone SBL produced maximum harvestable shoots (36.13) while clones of APN produced the least (7.21 and 9.5) per explant at the end of six months.

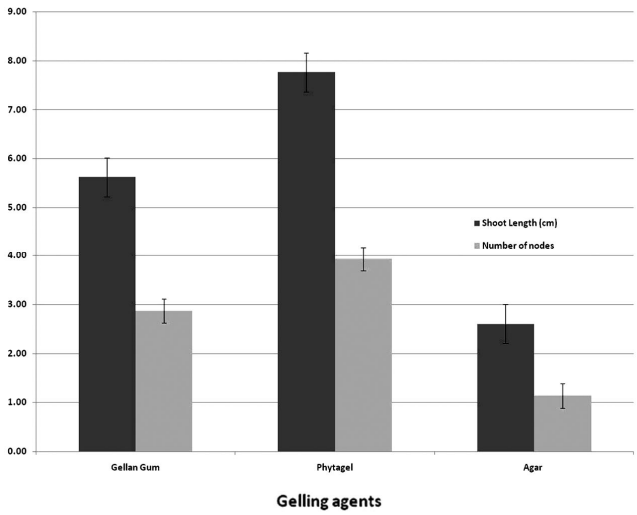

Fig 2. Effect of different gelling agents on shoot multiplication in teak.

Workers have advocated the synergistic effect of cytokinin and auxin in teak (Shirin et al. 2005). Surender and Narender (2009) reported the use of MS fortified with cytokinin and auxin for multiple shoot induction. Using BA, adenine sulphate and IAA, Rout et al. (2008) have reported the induction of multiple shoots in *Acasia* micropropagation. Efficient shoot multiplication in teak from apical nodes and nodal segments requires the presence of BA (Junior et al. 2009), with IAA (Tiwari et al. 2002) or TDZ (Kozgar and Shahzad 2012). In the present study, good shoot multiplication was achieved with minimal concentrations of 2.22 μM BA and 1.12 μM Kn.

Singh and Mishra (2016) reported an optimum gelling strength of 5.8 g/l agar for good growth in teak. Yasodha et al. (2005), however, stated that the number of shoots, growth of shoots and morphology was greatly affected by the strength of the gelling agent. In the present study, significant differences ($p < 0.05$) were observed in growth with respect to the gelling agent used in the study.

The preference of gelling agents was in the order phytagel > gellan gum > agar. The length of the shoots averaged 8 cm with 4 nodes. However, there was no significant difference between clones in different solidifying agents.

Light quality strongly influences plant development. This refers to the color or wavelength reaching the plant's surface (Johkan et al. 2010). Utilization of light emitting diodes (LEDs) for plant growth in controlled environment has emerged as an attractive low-cost alternative technology (Yeh and Chung 2009). The application of traditional fluorescent and LED lighting in relation to growth of the microshoots of teak was tested. Plants grew normally and without symptoms of disorder indicating that the culture conditions were appropriate. But, teak did not respond favourably to LED lights (Fig. 3). All three clones showed a similar trend in response to application of LED lights.

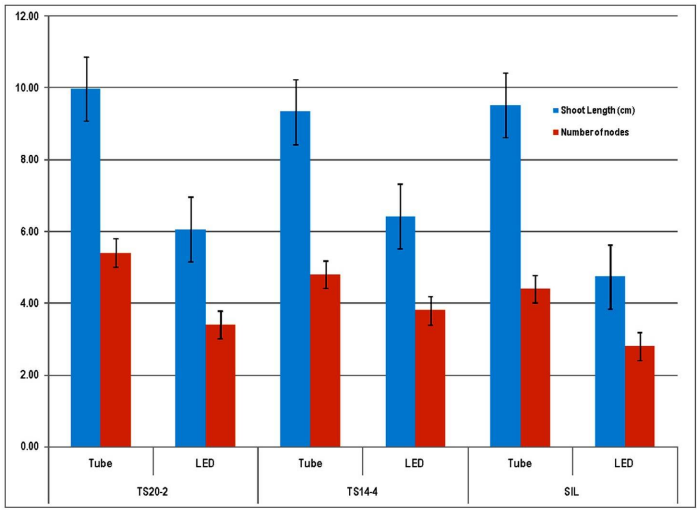

Fig. 3. Effect of different light sources on shoot multiplication in three clones of teak.

Astolfi et al. (2012) reported that the plant response to light quality differs with species. Cherry and oak showed no significant difference in biomass accumulation between LED and fluorescent grown seedlings while beech, lettuce (Kim et al. 2004) and grapes (Poudel et al. 2008) showed improved growth. Red and blue lights have the greatest impact on plant growth (Shukla et al. 2017) because they are the major energy sources for photosynthetic CO_2 assimilation in plants (Lin et al. 2013). Replacing white LEDs with red or blue lights may

enhance plant growth in teak. Furthermore, the effects of varying light spectra and their influence on the rate of *in vitro* plant growth needs to be addressed.

Ex vitro rooting responses did not vary significantly between different clones (data not shown). The mean rooting efficiency was 61% with an Sd 3.45 and CV of 5.6%. Maximum roots developed in clone P2 328 (4.00) while clone MHSC 270 showed a minimum (1.65). The differential response to rooting reveals that the clones vary in relation to their physiological functions. The plantlets were then transferred to the shade house.

Table 2. *Ex vitro* growth response of clones of *Tectona grandis*.

Sl. No.	Clones	Root length (cm)	Shoot length (cm)	Number of leaves
1.	SBL	15.8 ± 0.8^{ab}	7.8 ± 1.0^{e}	7.0 ± 1.1^{a}
2.	A_4 312	14.3 ± 0.3^{ab}	8.6 ± 0.9^{de}	4.1 ± 1.4^{bcd}
3.	MHSC 271	16.0 ± 0.7^{a}	8.0 ± 0.8^{de}	4.0 ± 0.8^{bcd}
4.	MHSC 270	14.1 ± 1.4^{b}	10.4 ± 1.7^{abc}	3.8 ± 0.7^{bcd}
5.	MHSC 279	14.6 ± 0.8^{ab}	12.1 ± 1.9^{a}	4.0 ± 0.2^{bcd}
6.	A_4 269	9.4 ± 1.6^{d}	10.6 ± 1.1^{ab}	4.6 ± 0.2^{bc}
7.	P_2 328	11.1 ± 1.9^{c}	8.6 ± 1.2^{de}	5.0 ± 1.0^{b}
8.	APN 254A	5.6 ± 1.7^{e}	9.7 ± 1.2^{bcd}	3.3 ± 1.6^{cd}
9.	APN 287	6.5 ± 1.4^{e}	8.8 ± 0.9^{cde}	3.1 ± 0.5^{d}

The growth of plants varied with respect to clones. Root length was the highest for MHSC 271 (16 cm) while the least was recorded in APN clones (5.6 and 6.5 cm). Shoot length was the highest in MHSC 279 (12.1 cm) and least in SBL (7.8 cm). On the contrary, SBL recorded maximum number of leaves (7) while APN 287 recorded the lowest. Mohan and Aishwarya (2012), reported the maximum shoot length of 6.1 cm and the maximum root length of 6.2 cm at the end of 90 days on the response of tissue cultured teak to PSB (Phosphate solubilising bacteria).

Nursery techniques have a substantial influence on seedling performance after out-planting (Li et al. 2011, 2012 Takoutsing et al. 2012). Results of the effects of fertilizer treatments on tissue cultured teak is shown in Fig 4. During the four-month-period, the treatments did not show significant difference for height. The collar diameter and number of leaves showed significant variations ($p < 0.05$).

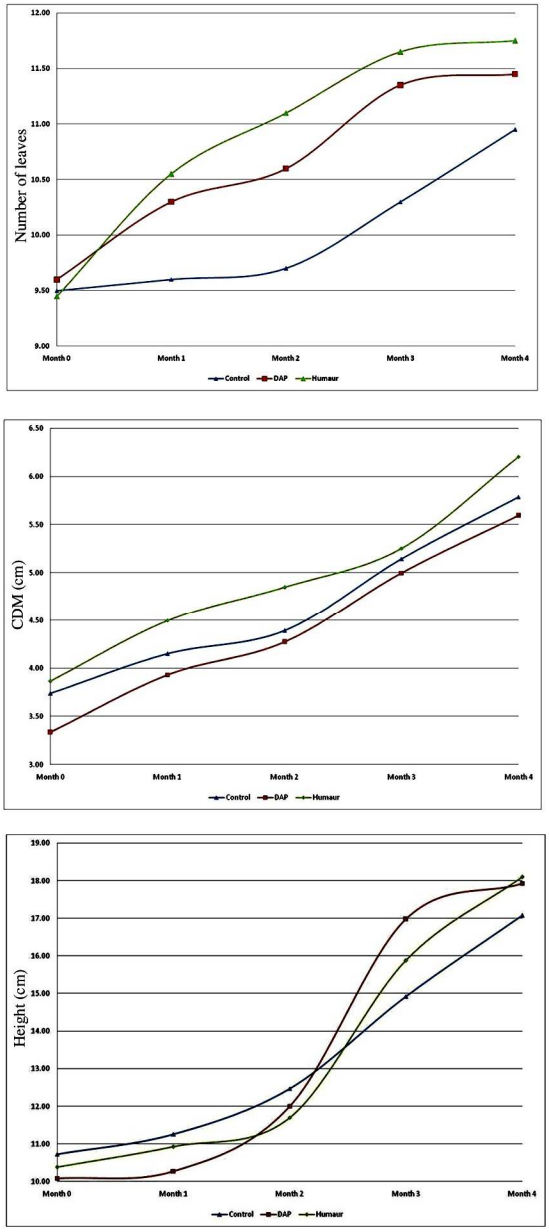

Fig. 4. Effect of fertiliser applications on growth performance of teak in terms of (a) height, (b) CDM and (c) number of leaves.

Collar diameter was the highest in *Humaur* treated plants (6.21 cm) over other treatments. Superior performance could be due to the absorption of nutrients by the rootlets thereby increasing growth. Similar was the case with the

number of leaves put forth by the plants. This increase could be attributed to the increased availability of nutrients for plant growth. Application of fertilizers promoted an initial boost (at the end of one month) followed by a steady increase in the following months.

Sturdiness quotient (SQ) refers to the ratio of the height of the seedling to the root collar diameter and expresses the vigour and robustness of the seedling. No significant differences were found in mean sturdiness quotient at $p < 0.05$. The mean sturdiness of the three treatments revealed lower sturdiness ratio values. A small quotient indicates sturdy plants with a greater chance of survival, especially on windy or dry sites. Bayala et al. (2009) have reported that seedlings with quality indicators out of the acceptable ranges (> 6) are likely not to perform well once they leave the nursery. Such seedlings appear thin, long and etiolated. In the present study, the TC raised plants of teak were observed to be sturdy, suggesting good survival rates when outplanted.

Teak growers continue to face difficulties in obtaining sufficient planting materials, particularly those with improved genetic quality due to bottlenecks like low seed production in orchards, poor germination and high variability in planting stock. An easy and efficient procedure for mass producing teak clones is micropropagation. It is also a safe way for ensuring true-to-type material. Commercialisation of the technology would involve effective use of resources with minimal investments so that cost of per plant production is reduced substantially. The study has involved modifying medium requirements, light sources and post-care of the *in vitro* grown plants to produce quality plantable teak material.

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