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IMPROVED MICROPROPAGATION METHODS FOR TEAK

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YASODHA, R., SUMATHI, R. & GURUMURTHI , K., 2005. Improved micropropagation methods for teak. To meet the project demand for teak seedlings in India, a micropropagation technique was developed using seeds of clonal seed orchards (CSOs). A comprehensive method for good shoot multiplication, cost- effective rooting and application to a wide range of genotypes was developed. Seeds collected from different clones and cultures were established in MS (Murashige & Skoog) medium supplemented with 22.2 µM Benzyl adenine (BA) and 11.62 µM Kinetin (Kn). Rapid shoot proliferation was conducted in alternate growth hormone concentrations of 2.22 µM BA and 1.16 µM K for two subcultures followed by 4.40 µM BA and 2.32 µM Kn for one subculture in MS basal medium. Optimum rooting of 80-100% rooting was achieved using healthy shoots of 4.0 cm and above, treated with 1000 ppm Indole-3-butyric acid (IBA) solution. Compared with conventional seedlings, where one hectare of CSO produces planting material for just 17 ha, micropropagation could increase planting stock by several folds.

Key words: **micropropagation - teak - phytagel - *ex vitro* rooting**

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Introduction

Tectona grandis is a valuable timber species grown on a large-scale by State forest departments, private companies and farmers in India. The annual planting target of teak in India is about 1.2 million hectares. Planting stock production is by seed, although there are many disadvantages, including poor fruit production, empty fruits and a low germination rate (Joshi & Kelker 1971, Gupta & Kumar 1976). Reliable seed sources are also limited. Since 1960, eight hundred hectares of clonal seed orchards have been established in India. One hectare of fully stocked CSO produces approximately 40 kilograms of fruit (Kumar 1992). Maximum planting stock production is 10 million seedlings, which works out to 10-12% of the total planting. Entomophily, non-synchronous flowering, self-incompatibility and a short pollination period compound the problem of seed yield (Hedegart 1973, 1976). Under natural conditions, fruit development varies from 0.4 to 5.1% and self-incompatibility is reported to be 96 to 100 %. Various vegetative propagation methods like grafting, budding and rooting of cuttings are being practiced for teak multiplication (Rawat & Kedharnath 1968, Nautiyal *et al.* 1991, 1992, Nautiyal & Rawat 1994, Goh & Monteuuis 1997). However, the quantity of production is limited because of the poor rooting of cuttings collected directly from mature trees without any pretreatment for rejuvenation, limited availability of rootstocks and intensive maintenance of stock plants. Further, higher rooting percent is achieved with specific type of shoot cuttings only (Monteuuis 1995). Micropropagation as a tool for clonal propagation of teak to overcome these problems has been advocated (Monteuuis *et al.* 1998, Devi *et al.* 1994, Mascarenhas *et al.* 1987, 1993, Apavatjrut *et al.* 1988). Although commercial level production strategies are worked out for the mass micropropagation of teak (Monteuuis *et al.* 1998), techniques have not been available. In the present study, we have developed a comprehensive method to amplify the genetically-improved seeds obtained from clonal seed orchard, which can be used on a commercial scale.

Materials and methods

Plant material

Fruits collected from clonal seed orchard (CSO) of Maharashtra were used in this study. Endocarps of the fruits were broken and seeds were removed for aseptic germination. Surface sterilisation of the seeds was done with aqueous solutions of 0.1% sodium hypochlorite for 5 minutes and 0.1% mercuric chloride for 5 minutes, followed by three rinses with sterile double distilled water. The surface-sterilised seeds were germinated individually on the medium containing agar (0.7%) and sucrose (3%) devoid of growth regulators.

Culture establishment

In optimisation experiments for culture establishment and shoot multiplication, individual identity of *in-vitro*-grown seedlings was not maintained. Nodal segments of 1-cm length were excised from 60-75 days old seedlings (6-8 cm in height) and placed on the following culture media.

a. MS 1- Murashige & Skoog's medium (1962) (MS) supplemented with 0.67 µM Benzyladenine (BA), 0.70 µM Kinetin (K), and 0.4% agar (Mascarenhas *et al.* 1993).
b. MS 2- MS medium supplemented with 22.2 µM BA, 11.62 µM Kinetin and 0.7% agar (modified from Devi *et al.* 1994). After 5 days of inoculation, the explants were transferred to MS + 2.22 µM BA + 1.16 µM K + 0.7% agar.
c. SH- Schenk & Hilderbrandt (1972) Medium containing SH macronutrients, MS micronutrients and organic additives, 1.48µM Indole-3-butyric acid (IBA), 4.40µM BA, 3% sucrose and 0.7% agar (Apavatjrut *et al.* 1988)

All the media ingredients used in this study were obtained from Hi-Media laboratories, India, except for the growth regulators and phytagel, which were obtained from Sigma Chemical Co., St. Louis, Mo.

Shoot multiplication

In the first experiment, the effect of MS medium containing different concentrations of BA (1.11, 2.22 and 4.44 µM) and Kinetin (0.58, 1.16, 2.32 and 4.6 µM) were tested to standardise the concentrations of the growth regulators for optimal growth. For each treatment, 10 nodal segments of *in-vitro*-developed shoots were used. All the nodal segments excluding the apical bud were used for the experiment. In the second experiment, MS medium with agar (0.7 and 0.8%), phytagel (0.2, 0.25 and 0.3%) and combinations of agar and phytagel (0.35 and 0.1%; 0.4 and 0.125% respectively) were tested to identify a suitable solidifying agent. Twenty culture flasks were used for each treatment, with a single cluster per flask having two to three shoots per cluster.

Multiplication of improved genotypes

Quality seeds obtained from the following CSO clones were germinated *in vitro* and fast growing seedlings were selected and used for large-scale multiplication.

Name of the clone	**Origin of the clone**	**Source**
MHAL A9	Maharastra	Maharashtra CSO
MHAL A3	Maharastra	Maharashtra CSO
TNT 10	Tamil Nadu	Maharashtra CSO
SBL 01	Andhra Pradesh	Maharashtra CSO

Individual seeds were treated as genotypes and germinated as described and maintained separately throughout the experiment. Fifty-five days after germination, seedlings were cut into single nodal segments and placed on culture initiation medium (MS 2) for five days then transferred to the shoot multiplication medium. Shoot multiplication was carried out in 2-step culture method as optimised in the previous experiments.

Subculture Method

For continuous shoot production, the nodal segments of the *in-vitro*-formed shoots were used. The response diminished after 5-6 subcultures. The shoots were therefore subcultured by two different methods.

1. Node planting (single nodes derived from *in vitro* grown shoots),
2. Horizontal placement (placing the stem segments with at least 3 nodes horizontally on the culture medium after removing the leaves and apical bud)

Data on number of shoots produced and number of shoots suitable for rooting were recorded.

Rooting

Ex vitro rooting experiments were performed with the micro cuttings of various lengths (2-3, 3-4, 4-5 cm) obtained from the four genotypes. Twenty micro cuttings were used for each treatment. Bases of the cut ends were dipped in 1000 ppm IBA solution for 5 minutes and inserted directly into net pots (64 cc) containing vermiculite presoaked in water. Prior to auxin treatment, the lamina was clipped leaving the top pair of expanded leaves. The net pots were placed in polytents under shade, closed tightly on all sides to maintain high humidity (approximately 95%). After 20-25 days, the polytents were opened gradually. Data on rooting were recorded after 35 days of treatment.

Culture conditions

Cultures were maintained under 12-h photoperiod with the light intensity of 40 µmol $m^{-2}s^{-1}$ at 25 ± 2 ^{0}C. The media were supplemented with 20% sucrose, adjusted to the pH of 5.7 ± 0.1 prior to the addition of gelling agent and autoclaved at 121 ^{0}C and 108 kPa for 20 minutes.

Stomatal studies

Stomatal studies were conducted using the *in-vitro*-grown normal shoots, vitrified shoots, acclimatized plants (leaves formed in culture) and seedlings. Three leaves from three different shoots/plants were used. Lower epidermal peelings from each leaf were taken. Stomatal index (SI) was determined after examining 10 fields of view per epidermal strip. SI was calculated using the following formula.

$$SI = \frac{\text{No. of stomata}}{\text{No. of stomata + No. epidermal cells)}} \times 100$$

Diameter and length of the stoma and stomatal pore were determined after examining 100 fully opened stomata. Guard cell behaviour of the stomata was studied by incubating the leaves in dark for 10-15 minutes. The significance of differences among the treatments was established by one-way analysis of variance. Percentage data was subjected to arcsine transformation prior to analysis.

RESULTS AND DISCUSSION

The potential benefits of the use of clonal planting stock in reforestation programs have long been recognized. However, to achieve the maximum possible genetic gain for teak improvement, both sexual reproduction and vegetative multiplication must be followed (Kaosa-ard *et al.*1998). It can be accomplished through micropropagation using CSO seed grown aseptically.

Culture establishment

Incubation of 40 days of culture in MS1 and SH medium provided good shoots (Figure 1) beyond which there was vitrification (MS 1) or excessive callus formation at the base (SH) (Table 1). The protocol of Devi *et al.* (1994) (MS medium with 22.2 µM BA and 4.6 µM K) produced malformed and vitrified shoots when the explants were maintained on the same medium for more than 15 days. However, quality shoot production was obtained when the nodes were transferred to MS medium with 2.22 µM BA and

1.16 µM K after 5 days of culturing in MS2 medium (Table 1). Tiwari *et al* (2002) also recommended the use of high concentration of BA (22.2 µM BA) during culture establishment of teak.

Table 1. Effect of media composition on culture establishment of teak

Media Code	Media composition	Mean number of shoots ± S.E	Mean shoot height (cm) ± S.E	Culture response (%)	Culture morphology*
MS 1	MS +0.67 µM BA +0.70 µM K + 0.4% Agar (Mascarenhas *et al.* 1993)	1.5± 0.4	4.2 ± 0. 7	62	Partially vitrified shoots
MS 2	MS + 22.2 µM BA + 11.62 µM K + 0.7% agar** (Modified from Devi *et al.* 1994)	2.8 ± 1.1	2.3 ± 0.4	55	Normal shoots
SH	SH macronutrients + MS micronutrients + 3% sucrose + 1.48 µM IBA + 4.40 µM BA + 0.7% agar (Apavatjrut *et al*.1988)	1.2 ± 0.5	4.5 ± 0.9	60	Callus formation at the base of the explant

* Recorded after 3 subcultures

** After 5 days the explants were transferred to MS + 2.22 µM BA + 1.16 µM K + 0.7% Agar

Figure 1 Nodal segment showing bud initiation

Table 2. Effect of cytokinins on shoot multiplication of teak (MS + 0.7% Agar)

Cytokinins	**Mean no. of shoots ± S.E**	**Mean shoot height in cm ± S.E**
1.11 µM BA 0.58 µM K	0.6 ± 0.4	2.2 ± 0.9
2.22 µM BA 1.16 µM K	1.8 ± 0.8	5.6 ± 2.2
4.40 µM BA 1.16 µM K	1.8 ± 0.6	5.6 ± 1.9
4.40 µM BA 2.32 µM K	2.5 ± 1.0	4.2 ± 1.2
4.40 µM BA 4.60 µM K	1.5 ± 0.9	2.9 ± 0.8

Shoot multiplication

After about four to five subcultures on the MS medium with 2.22 µM BA and 1.16 µM K the shoot growth and multiplication rate declined. Optimisation was achieved by transferring the shoots to various concentrations of BA and K with 0.7% agar (Table 2). High frequency shoot proliferation (Table 2) with good shoot production was achieved in the medium containing 4.40 µM BA and 2.32 µM K, and the mean height of shoots was 4.2 cm (Figure 2). Continuous subculturing (4-5 subcultures each with 40

days interval) on the same medium resulted in vitrification, which was overcome by varying the concentrations of agar or phytagel alone, or combinations were used with 4.40 µM BA and 2.32 µM K (Table 3). Although phytagel improved shoot multiplication, some cultures still showed vitrification in 0.2% concentration. Increasing the concentration of phytagel to 0.25% produced normal cultures (90%) with more shoots, (average of 3.5), compared to agar medium. However, maintenance of cultures over four months in MS medium with 4.40 µM BA, 2.32 µM K and 0.25% phytagel again led to vitrification. Therefore, a 2-step culture method was developed with two culture cycles in 2.22 µM BA, 1.16 µM K and 0.25% phytagel followed by one cycle in 4.40 µM BA, 2.32 µM K and 0.25% phytagel.

Figure 2 Multiple shoot production

Plant regeneration via shoot proliferation is aimed at the production of large number of normal regenerated plants. In the present study, the number of shoots produced, growth of shoots and culture morphology were greatly affected by the concentration of cytokinins and gelling agent's strength. Ziv (1991) reported that vitrification is associated with *in vitro* conditions favorable for optimised growth and proliferation. The decline in the response of cultured nodes observed in this study may be due to the

changes in physiological factors under culture conditions. Cachita (1991) reported that explants of same origin and nature, or of the same type behave differently in the culture media because of their varying physiological and metabolic properties. The 2-step culture method maintained proliferation and normal shoot production for almost two and half years.

Table 3. Effect of gelling agent on shoot multiplication of teak (MS + 4.40 μM BA + 2.32 μM K)

Gelling agent		Mean number of shoots ± S.E	Mean shoot height in cm ± S.E	Cultures vitrified (%)	Culture morphology
Agar					
	0.70%	1.8 ± 0.9	5.0 ± 1.2	40	Normal shoots
	0.80%	1.2 ± 0.6	3.2 ± 1.0	0	Shoots with very short inter nodes
Phytagel					
	0.20%	3.9 ± 1.8	4.0 ± 1.5	30	Normal shoots
	0.25%	3.5 ± 2.2	4.2±1.2	10	Normal shoots
	0.30%	1.5 ± 0.8	2.2 ± 0.9	10	Part of the shoots dried
Agar + Phytagel					
0.35% + 0.1%		2.8 ± 0.5	3.1 ± 0.9	55	Normal shoots
0.4% + 0.125%		3.2 ± 0.7	2.8 ± 1.2	40	Normal shoots

Shoot Production of improved genotypes

To increase micropropagation efficiency, the stem segments were subcultured horizontally or as single nodes using 2-step culture method. The mean number of shoots produced from horizontally placed stem ranged from 6.0 (MHAL A9) to 9.3 (TNT 10) (Table 4). Nodal segments of TNT 10 produced 1.8 shoots, while SBL 01 and MHAL A3 produced 2.5 shoots. The number of shoots suitable for rooting (> 4 cm length) was 0% in the case of MHAL A3 and 38% in SBL 01. Nodal cultures significantly influenced the production of shoots with length of >4 cm ($P< 0.005$), the percent of shoots with > 4 cm varied from 25 (MHAL A3) to 55 (SBL 01). The shoots produced from horizontally-placed stem segments were healthy.

However, most of the micropropagation protocols of teak use nodal segments for multiplication (Apavatjrut *et al.* 1988, Mascarenhas *et al.* 1993, Tiwari *et al*. 2002). The cultures, which show poor shoot elongation, may be subcultured as clusters to increase the production of rootable shoots. In this method 3-4 rootable shoots were produced per culture flask. Multiple shoots produced from the nodes or horizontal stem were cultured as clusters to increase the number of shoots suitable for rooting. The number of shoots collected for rooting increased up to 62 % (SBL 01) when the shoot clusters were maintained on the medium for one culture cycle (Table 5).

Table 4. Subculturing method and multiple shoot production of teak

Clone	Subculture method	Mean no. of shoots ± S.E	Percent shoots > 4 cm
TNT 10	Horizontal shoot	9.3 ± 1.0	27
	Node	1.8 ± 1.2	40
SBL 01	Horizontal shoot	6.2 ± 0.9	38
	Node	2.5 ± 1.8	55
MHAL A9	Horizontal shoot	6.0 ± 0.6	8
	Node	2.2 ± 1.0	35
MHAL A3	Horizontal shoot	6.6 ± 1.3	0
	Node	2.5 ± 2.1	25

Table 5. Data on shoot cluster culture of teak

Clone	Mean no. of shoots ± S.E	Percent shoots > 4 cm
TNT 10	4.5 ± 0.5	45
SBL 01	4.5 ± 0.5	62
MHAL A9	5.3 ± 0.6	42
MHAL A3	5.6 ± 0.5	45

Ex vitro rooting

There was no significant difference ($P< 0.05$) within and between the genotypes for the root characters like number of roots and root length except for longest roots. Shoots of 4-5 cm length produced 80 – 100 percent rooting (Table 6). Root systems were well branched with normal root hair development (Figure 3). The length of the longest root was reached up to 15.0 cm. After 40-45 days, the rooted plants were

moved to shade house (Figure 4). The survival rate of the *ex vitro* rooted plants was 85- 90% in net pots. When the hardened plants produced 2-3 pairs of new leaves, they were transferred to polybags (20 cm x 7.5 cm) with potting mixture containing sand, soil and composted coir pith (1:1:3 v/v) (Figure 5). However, Tiwari *et al*. (2002) reported the *ex vitro* rooting of *in vitro* raised micro shoots with 77.9% survival of the plantlets.

Table 6. Effect of shoot height on *ex vitro* rooting of micropropagated shoots of teak

Clone/Shoot height (cm)	No. of roots	Mean root length	Length of longest root	Increment in shoot height	Shoots rooted (%)
TNT 10					
2-3	2.5a	5.2a	6.6dcb	0.8a	65
3-4	3.2a	6.1a	12.5ba	0.8a	100
4-5	3.8a	8.0a	14.8d	1.2a	100
SBL 01					
2-3	3.2a	3.0a	3.3d	0.7a	35
3-4	2.4a	5.1a	6.7dcb	0.4a	50
4-5	3.2a	3.1a	5.0dc	0.7a	80
MHAL A9					
2-3	4.2a	4.0a	6.2dcb	0.4a	40
3-4	3.0a	8.5a	12.3ba	0.4a	40
4-5	2.8a	10.0a	15.0a	0.8a	80
MHAL A3					
2-3	3.0a	8.4a	11.9cba	0.4a	35
3-4	2.8a	9.0a	10.9cba	0.6a	40
4-5	2.0a	8.4a	14.3a	0.6a	90

Means within a column followed by the same letter are not significantly different at the 5% level (Student Newman Keul's test)

Figure 3 Root development in microcuttings

Figure 4 *Ex vitro* rooted microcuttings hardened in the shade house

Figure 5 Hardened plantlets ready for field planting

Stomatal studies

Stomatal nature and their behavior in tissue culture plants are useful in devising the strategy at various stages of acclimatization. *In-vitro*-developed leaves lacked cuticles but had unicellular and uniseriate trichomes scattered all over the leaf surface. Normal seedlings have branched multicellular trichomes covering the entire leaf surface. Stomatal structure of *in vitro* grown leaves differed markedly from that of normal seedlings. Stomata of leaves in culture were larger in size and circular in shape with a larger stomatal aperture (Table 7). These stomata did not close in the dark, whereas the normal stomata in the seedlings responded. When tissue-culture-raised plants were hardened, the lamina expanded and a thin layer of cuticle was formed and the stomata became functional. The phenology and stomata of new leaves formed was very similar to normal leaves. There was no significant difference in stomatal index between in *vitro* leaves, acclimatized leaves and seedling leaves (Table 7).

Table 7. Stomatal size and stomatal index (SI) of micropropagated and seedling leaves of Teak

Sample	Stomata (μm)		Pore (μm)		Stomatal Index
	Width	Length	Width	Length	
***In vitro* leaves**	19.0a	19.4a	5.3a	9.3ba	26.3ba
Acclimatized leaves	14.2b	15.9b	2.8b	7.2b	26.8ba
Seedlings	13.8b	16.2b	2.1b	7.6b	27.2ba

Means within a column followed by the same letter are not significantly different at the 5% level (Student Newman Keul's test)

Commercial propagation strategy suggested for teak

Commercial micropropagation is already practiced world-wide for many ornamentals, horticultural crops and forestry species. Thorpe *et al.* (1991) suggested the importance of micropropagation for the commercial production of forest trees, as it is an integral part in any tree improvement program. Results reported here constitute a promising step towards large-scale *in vitro* propagation of a species in which conventional propagation has been very difficult. Thus, the present study used the seeds obtained from the clonal seed orchards. The protocol was also tested in a commercial tissue culture laboratory and about 5000 plants have been produced in a period of one year with 10 starter cultures. Compared to seed

propagation of teak where one ha. of CSO produces planting stock for only 17.0 ha., micropropagation can increase the planting stock by 500 times.

At present Indian tissue culture units are mainly involved in propagation of crops and ornamentals for export markets. However, in recent years, production for the domestic market has increased from 10% to 40% (Kumar 1994). Also availability of labor at much cheaper rates encourages tissue culture propagation in India. Most of commercial propagation laboratories are situated in Maharashtra, Karnataka, Andhra Pradesh, Tamil Nadu and Kerala, where teak is grown extensively (Govil & Gupta 1997). The capacity of these units could be utilized for multiplication of teak, to enhance the availability of improved planting stock.

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