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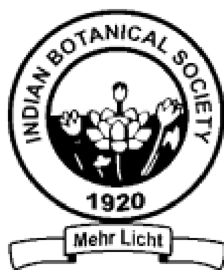

IN VITRO PROPAGATION OF INDIAN TEAK (*TECTONA GRANDIS* L.) FROM LEAF EXPLANTS

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Efficient plant regeneration via direct organogenesis was established using leaf explants of *Tectona grandis* L. were tested for growth and organogenic capacity on MS medium fortified with various concentrations of 6-Benzyl Amino Purine and Kinetin (0.5- 3.0mg/L) either singly or in various combinations of IAA/IBA (0.5-3.0 mg/L). The highest shoot regeneration frequency (100 %) was obtained from leaf explants on MS medium fortified with (1.5 mg/L) IBA/IAA in combination of (2.0mg/L) BAP. At the end of 6 weeks the regenerated shoots were transferred on the same medium (MS + 1.5 mg/L IAA/IBA + 2.0 mg/L BAP) for further proliferation and elongation. The regenerated shoots were rooted with high frequency (60%) in MS medium supplemented with (1.5 mg/L) IBA when compared to other auxin IAA. The *in vitro* raised plantlets were successfully established in green house and transplanted to natural conditions with 70% survival.

Key words: *Tectona grandis In* Vitro regeneration, 6-Benzyl Amino Purine, Kinetin, Leaf explants.

Tectona grandis L., (Verbenaceae) the major tropical hard wood plantation species is a useful indicator for plantation trends in 1980. It constituted 11% of the total area of tropical forests plantation where as in 2010 the corresponding figure was 4%. If the present trend persists, the future outlook for the sustainable supply of quality tropical hardwood is miserable. This is at a time when demand for the raw material, particularly domestic demand, is a forecast to rise. What is required now, to avoid a pending crisis in the sustainable supply of tropical hardwoods is to increase, sustainably, the area under sustainable management in natural forests and supplement this with a significant increase in plantations.

T. grandis is a commercially important, tropical hardwood tree species that has several applications in the lumber industry and is used in agroforestry systems (Galeano *et. al.,* 2015). Teak grows naturally across the Indian subcontinent through Myanmar and Thailand to Laos, and it is common in deciduous forests and well-drained alluvial soils (Pandey and Brown 2000). Central characteristics of its wood include resistance to weathering, strong fiber, and special oil content, which produces premium timber (Feroz *et al.* 2013), making teak one of the world's most valuable hardwood species (Galeano *et al.* 2014). It is cultivated in more than 36 countries with 5.7 million hectares planted worldwide (Santos *et al.* 2014). The propagation of teak via cuttings has been reported (Nautiyal *et al.* 1992) but this method has several limitations and only provides a few propagules from selected individuals. Micropropagation protocols of this species are available (Gupta *et al.* 1980, Devi *et al*. 1994, Tiwari *et al*. 1997).

Clonal propagation technique of propagation has been developed successfully for commercial propagation of selected plus trees of teak (Kaosaard *et al*. 1987, Kaosa-ard and Apavatjarut 1988, 1989). In this technique, shootlets are produced *in vitro* and are then transferred to glasshouse for rooting. The rooted shootlets or plantlets are transplanted for stock production.

Gupta *et al.* (1979) observed multiple shoot formation from excised seedling explants and from 100-year old trees. Small quantities of

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some 50 plants were obtained from tissues excised from mature trees. Palanisamy *et al.* (2009) conducted experiments for establishing clonal plantations of teak with high yielding clones to enhance productivity and selected a total of 41 outstanding trees

For biotechnological improvement programs to successfully introduce new traits into selected trees for enhanced growth and productivity, an efficient regeneration method is necessary. Multiple studies have described shoot regeneration from stem internodes of teak using different plant growth regulators, such as Benzylaminopurine (BAP), Indole-3-acetic acid (IAA), A-naphtalene acetic acid (NAA), and gibberellic acid (GA3) (Tiwari *et al.,* 2002, Shirin *et al.* 2005, Gyves *et al.* 2007). However, no reports have assessed the use of a cytokinin pretreatment in explants to improve shoot regeneration frequency.

The aim of this study was to develop an efficient shoot regeneration protocol for *T. grandis* from leaf explants by testing the effects of different pretreatments, regeneration culture medium, adventitious rooting, and acclimatization.

MATERIALS AND METHODS:

Plants of *Tectona grandis* L. (Verbenaceae) were collected from natural habitat of Mahadevapur Reserved Forest east division in Telangana state and were grown in pots in Botanical Garden of the Institute. Leaf explants were collected from these plants. About 1.0 - 2.0 cm long segments were taken from plants and these were also initially rinsed with 90% ethyl alcohol followed by washing with tap water. Explants was treated with 0.2% solution (v/v) of Tween 20 (Commercial Polyoxyethylene sorbitan monolaurate; S. D. Fine-Chem. Ltd., Mumbai) and kept under running tap water for 30 min. The cuttings were sterilized with 0.1% Mercuric chloride solution (w/v) for three min and then repeatedly washed with sterile distilled water. At the time of inoculation, both ends of leaf explants were trimmed with the help of a scalpel under aseptic conditions.

Culture media and conditions: MS basal medium was supplemented with various plant growth regulators and 3.0% sucrose. The pH of the media was adjusted to 5.8, solidified with 0.8% Difco-bacto agar and autoclaved at 103.4 kPa at 121°C for 15-20 min. A single explant was placed in each culture tube, and incubated at 25 $\pm$ 1°C with a 16 h photoperiod under fluorescent light (40-50 Mol m^{2}S^{-1}).

Shoot proliferation media: MS medium supplemented with 0.5 – 3.0 mg/L of -Benzyl Amino Purine, Kinetin individually, and in combination with (0.5 – 3.0 mg/L) Indole Acetic Acid / Indole Buteric Acid was used for shoot regeneration.

Elongation media: Explants with multiple shoots proliferated on BAP-containing media were transferred to MS medium containing different concentrations of Indole-3-acetic acid (IAA) and Indole Buteric Acid was used for shoot elongation.

Rooting and acclimatization: Shoots obtained only from BAP-containing media were used in rooting experiments, and were transferred to MS medium supplemented with 1.0 mgl^{-1} IAA. After 3 weeks of culture, plantlets recovered were washed with running tap water and agar sticking to the roots removed. Plantlets (a height of 5 - 10 cm) with fully expanded leaves and well-developed roots were transferred to soil and vermiculite (50: 50 v/v) mixture and covered with plastic bags, they were kept in the culture room for 2 weeks before being transferred to soil.

Data analysis: All the experiments were repeated thrice and the standard deviation and standard error were calculated using 20 explants per treatment.

RESULTS AND DISCUSSION:

This investigation was initiated to study the

Table 1: Effect of plant growth regulators on direct shoot proliferation from leaf explants of *Tectona grandis* L on MS medium after 6 weeks of culture.

Plant Growth Regulators mg/L	% of cultures Responding	No. of shoots per explants	Shoot length(cms)
BAP			
0.5	40	1.46 ± 0.12	2.05 ± 0.02
1.0	50	3.32 ± 0.13	3.06 ± 0.24
1.5	70	5.10 ± 0.13	3.09 ± 0.12
2.0	90	8.21 ± 0.13	5.04 ± 0.12
2.5	60	4.00 ± 0.15	4.02 ± 0.15
3.0	52	2.00 ± 0.15	2.02 ± 0.15
BAP+IAA			
2.0+0.5	70	2.05 ± 0.15	2.03 ± 0.05
2.0+ 1.0	75	3.05 ± 0.05	3.20 ± 0.15
2.0+1.5	100	10.08 ± 0.05	4.07 ± 0.14
2.0+2.0	82	8.04 ± 0.15	3.08 ± 0.13
2.0+2.5	64	5.08 ± 0.42	2.03 ± 0.12
2.0+3.0	50	4.04 ± 0.22	1.03 ± 0.12
BAP+IBA			
2.0+0.5	63	2.02 ± 0.14	1.02 ± 0.44
2.0+ 1.0	75	4.02 ± 0.15	2.04 ± 0.05
2.0+1.5	100	5.02 ± 0.15	4.03 ± 0.02
2.0+2.0	64	4.08 ± 0.20	3.06 ± 0.03
2.0+2.5	60	3.02 ± 0.50	2.07 ± 0.12
2.0+3.0	53	2.02 ± 0.50	1.02 ± 0.50

values represent means ±Standard error of 3 replicates per treatment

effect of various cytokinins on shoot proliferation from Leaf explants of *T. grandis*. An initial experiment was conducted to test the effect of various concentrations of different cytokinins BAP and Kn individually and in combination with IAA/IBA on shoot proliferation in Leaf explants (Table 1and 2).

Leaf induced efficient callus and shoots on MS medium containing (1.5 mg/L BAP + 2.0 mg/L) IAA/IBA respectively (Plate1A&1B). Highest number of shoots (10.08 ± 0.05) with 4.07 cm length of shoots and growth response was obtained with IAA and BAP (100%) then Kn and IAA (97%) with (8.04 ± 0.15) shoots/explants (3.08 ± 0.13cm length of shoots). Levels above or below this gradually decreased the frequency of callus induction and shoots formation. After 6 weeks, the actively growing callus were sub cultured on fresh culture medium of the same composition. It enhanced peripheral greening of callus inducing shoot buds. Combination of IAA and Kin showed low response to callus formation compared to IAA and BAP which showed best results from leaf explants culture.

The calli derived from leaf explants were best for regeneration and were sub cultured on MS medium with (1.5 mg/L) BAP + (2.0 mg/L) IAA and (1.5 mg/L) Kn + (2.0 mg/L) IAA. After 6 weeks shoot buds from green callus were regenerated to plantlets.

The combination of (2.0 mg/L) BAP and (3.0 mg/L) IAA is effective and induced minimum percentage of (50%) plantlet formation when compared to (2.0 mg/L) Kin + (3.0 mg/L) IAA (45%) (Table 1& 2). The percentage of culture response in inducing callus and regeneration from callus derived from leaf is low when the concentration of BAP/Kin is increased upto (0.5mg/L). The regenerated micro shoots were sub cultured on the same composition of medium for further shoot proliferation and elongation.

Rooting of Shoots: Micro shoots (3-4 cm) developed from leaf regenerated were excised and cultured on MS medium supplemented with (0.5-2.0mg/L) IAA and (0.5-2.0mg/L)

Table 2: Effect of plant growth regulators on direct shoot proliferation from leaf explants of *Tectona grandis* L on MS medium after 6 weeks of culture.

Plant Growth Regulators mg/L	% of cultures Responding	No. of shoots per explants	Shoot length(cms)
Kin			
0.5	35	1.46 ± 0.12	1.05 ± 0.02
1.0	46	2.02 ± 0.13	3.06 ± 0.24
1.5	65	3.00 ± 0.13	4.09 ± 0.12
2.0	80	5.00 ± 0.13	3.04 ± 0.12
2.5	50	2.00 ± 0.15	2.02 ± 0.15
3.0	52	1.00 ± 0.15	1.02 ± 0.15
Kin+IAA			
2.0+0.5	65	1.05 ± 0.15	1.03 ± 0.05
2.0+ 1.0	70	5.05 ± 0.05	2.20 ± 0.15
2.0+1.5	97	7.08 ± 0.05	4.07 ± 0.14
2.0+2.0	73	8.04 ± 0.15	3.08 ± 0.13
2.0+2.5	60	6.08 ± 0.42	2.03 ± 0.12
2.0+3.0	45	4.04 ± 0.22	1.03 ± 0.12
Kin+IBA			
2.0+0.5	60	1.02 ± 0.14	1.02 ± 0.44
2.0+ 1.0	70	3.02 ± 0.15	2.04 ± 0.05
2.0+1.5	95	4.02 ± 0.15	4.03 ± 0.02
2.0+2.0	60	3.08 ± 0.20	3.06 ± 0.03
2.0+2.5	54	2.02 ± 0.50	2.07 ± 0.12
2.0+3.0	50	1.02 ± 0.50	1.02 ± 0.50

Values represent means ±Standard error of 3 replicates per treatment

Table 3: Rooting ability of regenerated shoots from Leaf explants culture of *Tectona grandis* (L.) cultured on MS medium supplemented with IAA and IBA.

Growth Hormones mg/L		Percentage of Response	Average Number of Shoots (S.E.)
IAA	**IBA**		
0.5	-	43	2.0 ± 0.3
1.0	-	70	3.2 ± 0.3
1.5	-	50	2.3 ± 0.4
2.0	-	45	1.0 ± 0.3
-	0.5	42	1.2 ± 0.5
-	1.0	60	2.0 ± 0.4
-	1.5	50	1.6 ± 0.3
-	2.0	46	1.3 ± 0.3

IBA. Profuse rhizogenesis was observed on (1.0 mg/L) IAA. 1.0 mg/L of IBA alone is most potential in inducing high percentage (70%) of rooting with highest number of roots per shoot (3.2+0.38) when compared to IBA (1.0 mg/l) which induced (54%) of rooting with highest number of roots (2.8+ 0.87) per shoot (Table - 3). Most of the shoots had produced roots within 5 weeks after placing on rooting medium.

Acclimatization of the plantlets: After *in vitro* rooting the regenerated plantlets were taken out and were washed carefully to remove agar and then transferred to pots containing sterile vermiculite. Each pot was enclosed in a polyethylene bag after watering and maintained in a plant growth chamber at 25+1°C under 16-h illumination with fluorescent lamps. Bags were progressively opened weekly. The percentage of survival was found to be 70% and the plants were morphologically identical to the acclimatization. Now plantlets were transferred to large pots filled with garden soil and farmyard manure (1:1) in the open parental

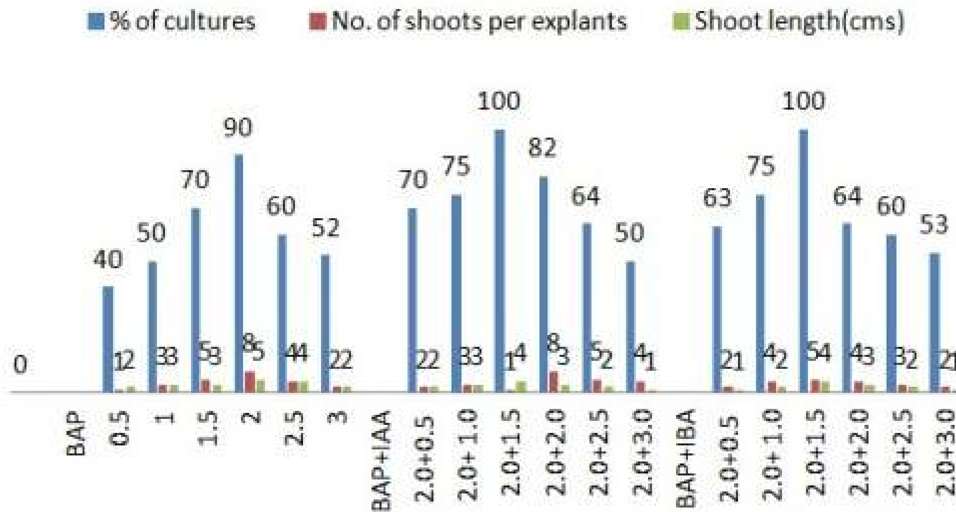

Figure 1: Effect of plant growth regulators on direct shoot proliferation from leaf explants of *Tectona grandis* L on MS medium after 6 weeks of culture

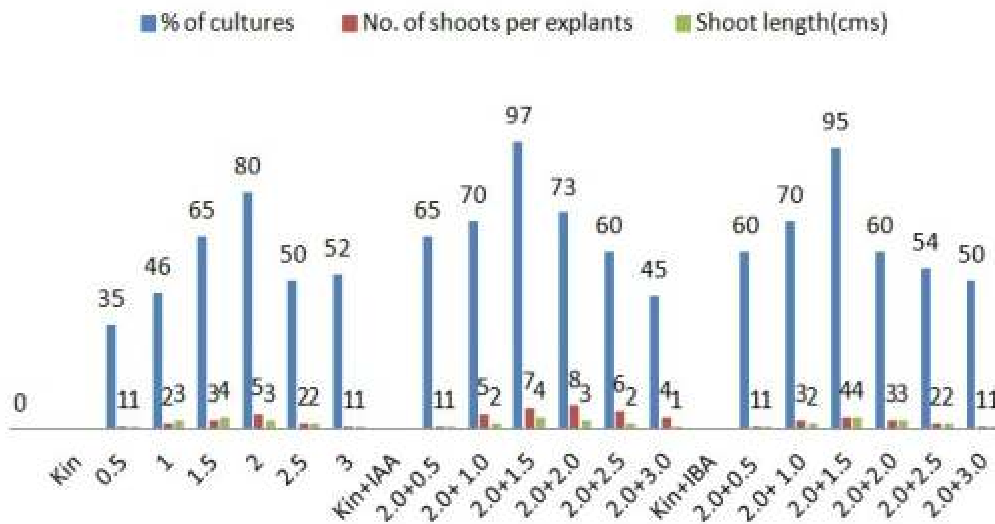

Figure 1: Effect of plant growth regulators on direct shoot proliferation from leaf explants of *Tectona grandis* L on MS medium after 6 weeks of culture

plants. In all experiments a minimum of three plates were cultured. Each single treatment consisted of five to ten explants per plate. Data recorded at four weeks included the number of shoots per explants, length of shoots and rooting. Data were statistically analyzed using one way analysis of variance.

Teak is a large deciduous tree, with a tall straight stem. It stands out the most valuable of all known timbers for its extraordinary durability. Once seasoned, teak timber does not split, crack, shrink or alter its shape. It is not very hard and heavy but can give a beautiful appearance after being polished. With such preferences it has been over exploited for many centuries. Thus, teak plantation must be established and propagated rapidly to replenish the natural stand of those used areas. In order to meet those urgent demands, both extensive and intensive cultivation of teak is being undertaken. A major problem of teak plantation is that the seeds in general have very low germination rates (30-60%) due to difficulties to remove seed dormancy.

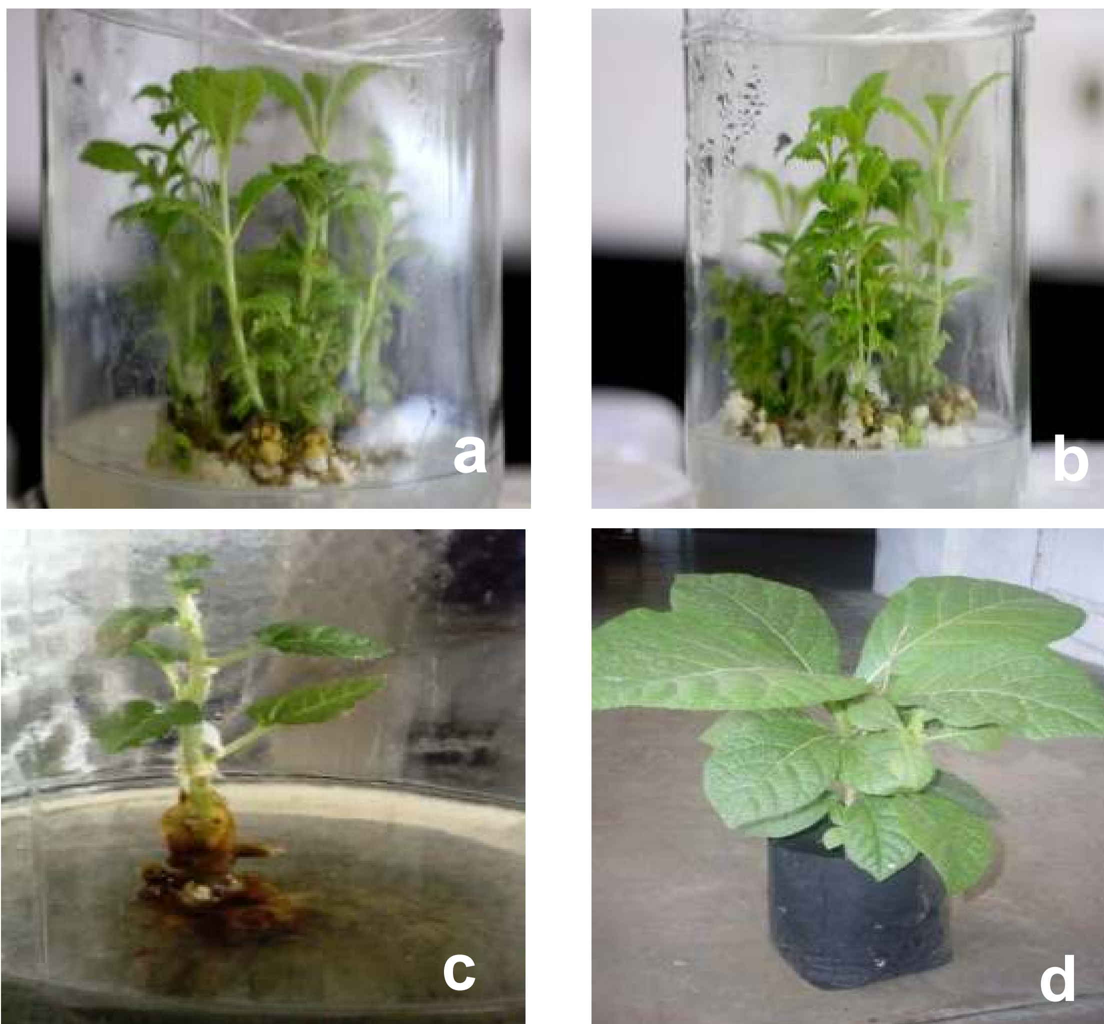

Plate-1(A-B): *In Vitro* Plantlet regeneration from Leaf explants culture in *T. grandis*. **A.** *In Vitro* Plantlets on MS+ (2.0mg/LBAP+1.5mg/LIAA) **B.** Multiple shoots on MS+ (2.0 mg/LKin+2.0mg/L IAA) **C.** *In Vitro* rooting of micro shoots on MS+IAA (1.0mg/L) after six weeks **D.** Hardening of plantlet

In this paper, *in vitro* propagation, using Leaf explants culture has been found a reliable method of *in In Vitro* propagation of teak. Leaf explants were induced to produce multiple shoots, followed by root induction under *in vitro*.

Baghel *et al.* 2008 assessed shoot induction from hypocotyls, cotyledonary axis, and cotyledons without pretreatment for *Tectona grandis*, They observed mean shoot induction frequencies ranging from 14 to 63% from explants placed in MS culture medium supplemented with BAP, NAA, and Kinetin (at varying concentrations), with the best plant regeneration produced by cotyledonary axis. In our study, we found similar shoot regeneration frequencies, ranging from 50 to 70%, which varies widely depending on explant type, pre-treatment, and regeneration culture medium with combinations of plant growth regulators. Ranasinghe and Berlyn (1996) propagated teak successfully from stem segments and leaf stalks taken from seedlings or trees using a modified MS medium. Root formation occured within 2 months after planting the explants on the same medium with 3.5ppm NAA and 0.25ppm BAP. Multiple shoot formation was induced from excised seedling nodal explants on MS medium fortified with IBA (0.3mg1) and BA (1mg1). Approximately 2-5 shoots

were obtained within 10-15 days from each explant in a growth chamber at 25°C with a light source of 100 lux for 16 h/day. For the rooting medium, we used a protocol similar to Baghel *et al.* (2008) (MS + 1.0 mg /LIBA + 8.0 g/Lsucrose), but with a lower IBA concentration. The use of IBA at low concentrations is recommended since it does not interfere with shoot growth and root elongation (Gyves *et al.* 2007). Similarly, Shirin *et al.*(2005) obtained 70% of adventitious rooting when using MS supplemented with 1.0 mg/ L IAA. After transferring to a greenhouse, 60% of the teak plantlets in this study grew suitably. Teak plantlet growth under greenhouse conditions has not been a problem, with normal survival rates of more than 60% (Tiwari *et al.* 2002, Shirin *et al.* 2005, Gyves *et al.* 2007, Akram and Aftab 2009). The whole process from tissue culture to greenhouse plant establishment was successful for teak, with each step being essential to obtain complete plants from indirect regeneration. Thus, the time span for complete regeneration was relatively short for a tree species.

Development of large-scale plant production system will be possible only when efficient rooting and hardening procedures are available (Bonga, 1992). Traditionally, micro propagated shoots subject to an *in vitro* rooting for the plant production. However, this method often produces non-functional roots and callus at the base resulting in poor vascular connection between shoot and root (Nemeth, 1986; Gaspar and Coumans, 1987). Alternatively, an *ex vitro* rooting procedure have been applied in several woody perennials for the large-scale plant production (Moncousin, 1991). Many investigations described different methods of culturing tissues of one of the most important woody species *Tectona grandis* L. Teak tissues grow well on MS medium containing 1.0ppm glycine (Narasimhan *et. al.,* 1970).

Herein, we describe an efficient direct regeneration protocol through organogenesis from *Tectona grandis* seedling explants. Cytokinin pretreatment increased organogenic competence of teak explants collected from seeds. In particular, BAP pre-treatment was effective in inducing shoot regeneration. Despite the fact that the plant material used in this study came from open-pollinated seeds with varied genetic makeup, shoot regeneration and subsequent adventitious rooting of the regenerated plants were successful. The best organogenic potential was achieved when Leaf segments from seedlings were germinated on a medium containing BAP and IAA and cultured for six weeks on MS medium supplemented with BAP and IAA/IBA. Finally, the protocol described herein can significantly increase regeneration efficiency for teak. This regeneration protocol can serve as a platform for genetic transformation to improve this important tropical tree species with significant economic value.

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