Importance of Biochemistry in Agriculture & Plant Sciences

Chapter 1

Tissue Culture-Based Micro-Cloning of *Shorea Tumbuggaia* Roxb.--A Globally Endangered Indian Medicinal Tree

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Abstract

The genus Shorea of Dipterocarpaceae family is represented by 196 rainforest tree species. Four species of Shorea i. e, *Shorea assamica*, *S. robusta*, *S. roxburghii* and *S. tumbuggaia* have been documented in India. Of these, *Shorea tumbuggaia* Roxb. commonly known as green dammer tree, is an endemic, globally endangered, semi-evergreen tree, restricted to the Southern Eastern Ghats parts of Chittor, Cuddapah and Nellore districts of Andhra Pradesh and North Arcot and Chengalpattu districts of Tamil Nadu in India. In addition to its timber-yielding potential, the tree is also known for its medicinal properties as an external stimulant and a substitute for Abietis; Resina and Pix Burgundica of European pharmacopoeias. The plant extract is used as a cure for ear-aches in children. The bark is reported as having anti ulcer activity and leaves are used in the treatment of dysentery. The establishment of the seedlings of this tree is very low due to seed dormancy and heavy attack with an unidentified Bruchid beetle insect pests at the pre-or post-dispersal stage of seed resulting in its dwindling population. The present study, therefore, highlights the urgent need for tissue culture interventions in *S. tumbuggaia* propagation for raising quality disease-free propagules for its conservation. Collection tour to South Peninsular region of India (Tirumula Hills, Tirupati, Andhra Pradesh and nearby areas) were conducted for the purpose of collection of plant materials. Stem cuttings and young seedlings of *S. tumbuggaia* were collected and established in the glass-

house at Plant Tissue Culture Laboratory, CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow. Nodal explants excised from fresh sprouts have been cultured *in vitro* for mass propagation.

An *in vitro* plant tissue culture approach provides a feasible alternative for extensive mass manufacturing and conservation of elite and critically endangered medicinal plant species. The presented work describes a proficient *in vitro* seed germination and proliferation procedure for the conservation of an endangered high trade medicinal tree, *Shorea tumbuggaia* Roxb. Given the precarious position of this species, effective conservation is highly indispensable for its continued existence, primarily for ecological reasons and also for forestry purposes as this tree species is a valuable source of timber and oleoresin. In the present study, over 90% *in vitro* seed germination was achieved. Multiple shoot formation was established from seedlings with a maximum of 4.60 ± 0.30 multiple shoots per explant using MS (Murashige and Skoog) medium supplemented with a combination of 6.66μM 6-benzylaminopurine and 0.454 μM thidiazuron. Rooting of multiple shoot cultures of 85% was achieved on half strength MS medium supplemented with 2.24μM indole 3-butyric acid (IBA) with 6.48 ± 0.58 roots per plantlet. Rooted plantlets showed more than 70% acclimatization in potting conditions and were established successfully in a greenhouse, with morphological similarities to mother plants. Clonal fidelity assessment of micropropagated plants with ten inter simple sequence repeat (ISSR) primers reproduced a total of 24 monomorphic bands. The presented work represents the first report of *in vitro* proliferation, conservation, and clonal fidelity assessment of tissue culture generated *S. tumbuggaia* plantlets.

Keywords: Shorea tumbuggaia; Tissue culture; In vitro proliferation; in vitro conservation; clonal fidelity; ISSR

1. Introduction

The genus Shorea of Dipterocarpaceae family is represented by 196 rainforest tree species [13,25]. Of these, *Shorea tumbuggaia* Roxb. commonly known as green dammer, is a globally endangered, semi-evergreen tree, restricted to the Southern Eastern Ghats of Chittor, Cuddapah and Nellore districts of Andhra Pradesh and North Arcot and Chengalpattu districts of Tamil Nadu in India [6]. This resinous tree attains the maximum height of about 20-30m with a maximum width of 150-190 cm. The trunk of the plant is used as flag poles for temples. In addition to its timber-yielding potential, the tree is also known for its medicinal properties as an external stimulant and a substitute for Abietis; Resina and Pix Burgundica of European pharmacopoeias [34]. The plant extract is also used as a cure for ear aches and leaf juice as ear drops in children [27]. Methanolic extract of leaves of this plant has been reported to have antinociceptive and anti-inflammatory activity [14] whereas the bark is a rich source of anti-

ulcer molecules [23]. Leaves are also used in the treatment of dysentery and stem resin is used for making incense sticks. The oleoresin which is exuded from the stem bark of Shorea can be used to cure hydrosis and alexiteric [36]. Besides, antidiabetic and antioxidant activity of *S. tumbuggaia* is also reported in alloxan-induced diabetic rats and revealed that this plant had anti hyperglycemic and hypolipidemic actions [25].

Conventionally, *S. tumbuggaia* is a self-pollinating plant and propagated by seeds. The extensive exploitation of this plant, habitat degradation, and other abiotic interferences including low seedling germination rate, incapability of seedlings to contend with neighbor vegetative populations, high rates of seed and flower abortion, seed predation by insect pests at the pre- or post-dispersal stage, less availability of viable seeds, and non-annual ephemeral flowering, has brought *S. tumbuggaia* to the IUCN list of endangered medicinal tree plant species [9]. For the ecosystem restoration and reforestation, efficient propagation methods are required to generate the plant material in natural habitats where populations are most depleted. Therefore, immediate attention towards maintained cultivation and conservation are required to guarantee the unremitting supply of good quality plant material [3,11,24,29]. Plant tissue culture-based *in vitro* proliferation strategies have been used for the propagation of a large number of threatened plants in *ex-situ* conservation programs [30,31]. *In vitro* plant tissue culture-based micropropagation provides several significant advantages over conventional vegetative propagation. It is used to reduce the high cost of rapid production and establishment of good quality plant material resulting from limited stock plant material supply and inefficient traditional vegetative propagation [19]. Members of Dipterocarpaceae are generally considered non-amenable to *in vitro* tissue culture. To date, there has been only one report of tissue culture of Shorea genus with limited success reported [35].

In the present study, optimized procedures were developed for efficient *in vitro* culturing for seed germination, proliferation, and rooting followed by greenhouse acclimatization of *S. tumbuggaia* using shoot tips derived from aseptically grown seedlings. This appears to be the first report on the procedure for *in vitro* proliferation and conservation of *S. tumbuggaia*. This practice can be employed for the purpose of conservation using large-scale propagation for forestation and ecosystem restoration programs. The concern for the genetic fidelity of the progeny developed from *in vitro* conserved grown seedlings is highly required to check the relatedness of progeny with mother plants, so the clonal fidelity of *in vitro* raised plantlets was also evaluated for the first time using ISSR markers.

2. Materials and Methods

2.1. Collection of Plant Material and *In Vitro* Seed Germination

Healthy and young hanging matured fruits (with dry wings and hard endocarp; **Figure:1a, b**) were collected in the fruiting season (April and May) from the scattered population of

S. tumbuggaia, from different locations of Tirumala Hills, Tirupati, Andhra Pradesh, India (Latitudes 13° 40′59.7° N, Longitude 79° 20′ 49.9°E and altitude 853 M above sea level). For in vitro seed germination, the hard and stony endocarp of mature fruits was first removed (**Figure:1c**). Seeds were washed thoroughly with 5% (v/v) Tween®20 solution (Sigma-Aldrich®, St Louis, MO) and 70% (v/v) Savlon®(Jhonson and Jhonson, New Delhi, India) for 10 min followed by a 5 min treatment of antifungal Bavistin ® (5% (w/v); BASF

India Ltd., Mumbai, India) for pre-sterilization, followed by rinsing 4 to 5 times with autoclaved distilled water to remove any residue from earlier treatments. Final sterilization was done with 0.1% (w/v) $HgCl_2$ solution (Sigma-Aldrich®) for 3 to 4 min, followed by rinsing 4 to 5 times with sterile double distilled water under aseptic conditions. After sterilization, seeds were blot dried and cultured in culture tubes (50 mL; Borosil, New Delhi, India) containing seed germination medium composed of half strength MS (Murashige and Skoog medium [20], HiMedia ®, Mumbai, India) fortified with 3% (w/v)sucrose (HiMedia®) and 0.8% (w/v) agar (plant tissue culture grade, HiMedia®). The pH of all medium combinations was adjusted to 5.8 with 0.1 N HCl (HiMedia®) or 0.1NNaOH (HiMedia®) before autoclaving at 121°C (103.4 kPa) for 15 min. Ascorbic acid at 100 mgL−1 (Sigma-Aldrich®) and 0.02% (w/v) activated charcoal (HiMedia®) were also added to the germination medium to prevent the leaching of phenolics from the germinated seeds. The percentage of seed germination was recorded 4 wk after the seeds were placed on the seed germination medium. Fifty seeds were used for the germination treatment and the experiment was performed three times.

2.2. Multiple Shoot Induction and Proliferation

The apex of *in vitro* germinated seedlings was removed using a sterilized blade. Shoot tips (1.0 –1.5 cm) were separated from 4-wk-old seedlings and initially cultured for 2 wk on half strength MS medium fortified with 3% (w/v) sucrose (Hi Media ®) and 0.8%(w/v) agar (plant tissue culture grade, Hi Media®), and then shifted to full strength MS medium fortified with 3% (w/v) sucrose (Hi Media®) and 0.8% (w/v) agar (plant tissue culture grade, Hi Media®) having different concentrations (2.27–13.6μM) of 6-Benzylaminopurine (BAP) (Sigma-Aldrich ®),2.32–13.92μM Kinetin (KN) (Sigma-Aldrich®) and 2.27–13.6μM Thidiazuron (TDZ) (Sigma-Aldrich®), either individually or in double combinations to obtain multiple shoot cultures. Morphogenic responses like shoot multiplication response, number of shoots per explant, length of shoots, rooting response, number of roots per explant, and length of roots were measured during the course of study. The number of explants responding and the number of shoot buds induced per responsive explants were recorded every 2 d for the initial 2 wk and then at weekly intervals. Induction of roots in the multiple shoot cultures was optimized by culturing well established healthy multiple shoots on half and full strength MS medium, alone or supplemented with 0.224–4.48μM indole 3-butyric acid (IBA) (Sigma-Aldrich®), 0.285–5.70μMindole 3-acetic acid (IAA) (Sigma-Aldrich®), or 0.268–5.36μM

1-Naphthalene acetic acid (NAA) (Sigma-Aldrich®). All cultures were maintained at 26 ± 2°C under a 16 light photoperiod using cool white fluorescent tubes (Philips, Gurgram, India) of 48 µmol $m^{-2}s^{-1}$ intensity.

2.3. Acclimatization and Establishment of Plantlets in Soil

In vitro rooted plantlets were carefully removed from the culture vessel and gently rinsed with sterile distilled water to remove adhered agar. Rooted plantlets were submerged in Bavistin® (5%, w/v) solution for 5 min and transplanted in pots containing an autoclaved mixture of 1:1:2 proportion of garden soil, vermin compost (Cocogarden ®, Dindigul, India), and vermiculite (Keltech Energies Ltd., Bangaluru, India). The potted plantlets were maintained in the greenhouse for acclimatization and were covered with a translucent polythene bag to retain humidity. Plants were supplemented with half strength liquid MS medium lacking sucrose biweekly. The polythene bag was gradually opened and fully removed after 4 wk of acclimatization and then plants were transferred to earthen pots with garden soil and allowed to grow under greenhouse conditions for further development.

2.4. DNA Isolation and Genetic Fidelity Analysis by ISSR

The genomic DNA from the leaves of one mother plant and nine randomly selected *in vitro* grown plants derived from different seeds collected from this same mother plant was isolated using the CTAB method [15]. DNA quality and yield were analyzed and measured by 0.8% (w/v) agarose gel (Sigma-Aldrich®) electrophoresis and Nanodrop™ (ND1000, Thermo Fisher Scientific ®, Waltham, MA) methodology according to manufacturer's protocol. For ISSR analysis, 10 universal UBC primers (University of British Columbia, Vancouver, Canada) were randomly selected and tested. The PCR amplification was carried out using a reaction mixture of 25µl volume containing 2.5µl of polymerase chain reaction buffer (10X), 1.0µl of MgCl2(25 mM),1.0µ l of dNTPs(100mM),0.2µl of DNA Taq polymerase from DNA Taq polymerase Kit (Sigma-Aldrich ®), and 1.0µl primer, 2.0µl of template DNA (40 ng), and 17.3µl of milli-Q water. PCR amplification was programmed for initial DNA denaturation at 95°C for 5.0 min (one cycle), followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 10 min (one cycle). PCR amplifications using ISSR primers were performed at least three times and only the reproducible PCR products were scored. Amplified products were resolved on 1.5% (w/v) agarose gel (Sigma-Aldrich®).The gels were stained with Nancy 520 (Sigma-Aldrich®) and documented using (Nighthawk™, pdi Inc., New York). 1 kb DNA ladder (1 kb RTU, BR Biochem, New Delhi, India) was used as the molecular standard for the identification appropriate bands amplified with ISSR markers. The amplified bands were visualized under UV light and imaged using a Gel Documentation System (XR-Quantity One®, BioRad® Laboratories, Hercules, CA).

2.5. Experimental Design and Statistical Analysis

The *in vitro* proliferation experiments were performed in a completely randomized design (CRD), performed three times with a minimum of 50 seeds and 20 healthy shoots for each treatment. Observations were made after 7 d interval for shoot multiplication and rooting experiments. Data obtained from seed germination, shoot multiplication and rooting experiments are represented as a mean ± standard error (SE). Statistical differences between results for plant growth regulators used for multiplication and rooting were evaluated by two-way analysis of variance (ANOVA) and post hoc Tukey's HSD test using SPSS V. 17.0, and the values at $p<0.05$ were considered statistically significant. For the genetic stability and clonal fidelity analysis study, clearly visible, and reproducible bands within the size range of 250 to 1500 bp were scored. The genetic association of mother plants with *in vitro* raised plants was assessed by calculating the Jaccard similarity coefficient [10] for pairwise comparisons based on the quantity of shared bands produced by the primers using the NTSYS-PC (Numerical Taxonomy System), version 2.1.

3. Results and Discussion

3.1. Seed Germination and Shoot Multiplication

For the optimization of the shoot bud multiplication medium, germinated seeds (**Figure:1d**) were used. In the present study, a greater than 90% seed germination rate of *S. tumbuggaia* was achieved using half strength MS medium lacking plant growth regulators as earlier reported [2]. Leaching of phenolics into the culture medium posed a crucial problem during the early stage of the seedling establishment. Therefore, to minimize the lethal browning of explants caused by phenolics compounds, antioxidant ascorbic acid (100 mg l^{-1}) and phenolics absorbent activated charcoal (0.02% w/w) were added to the seed germination medium. However, the addition of this antioxidant and phenolics absorbent had little effect over browning of seeds but effectively reduced the leaching of phenolics in culture medium. *In vitro* seed germination in Dipterocarpaceae was earlier reported by few workers [18,32, 33] who studied seedling germination in four genera of Dipterocarpaceae using BAP as a key regulator. However, *in vitro* seed germination and establishment protocols using half strength MS medium devoid of plant growth regulators were found to be a considerably improvement and less time consuming for Dipterocarpaceae [12,21,33]. The effectiveness of cytokinins (BAP, TDZ, and KN) alone and/or in combination was assessed for axillary shoot bud induction, shoot elongation, and proliferation from shoot tip explants cultured on half strength MS medium (**Figure:1e,f**). The concentration and combinations of cytokinins used influenced the average number of shoots per explants as well as the mean shoot length. Different concentrations of BAP, TDZ, and KN when used alone and/or in combination were found to be significant different for shoot multiplication, numbers of shoots per explant, and

shoot length response (**Tables 1 and 2**). Individually, supplementation of BAP (4.44 μM), TDZ (6.81μM), and KN (13.92μM) resulted in a maximum of 75.6, 66.6, and 55% multiplication response, respectively (**Table 1**). However, when these plant growth regulators were tested in double combinations, 6.66μM BAP+0.45μM TDZ and 13.3μM BAP+0.54μM NAA, the double combinations induced a maximum of 89.5 and 73.8% multiplication response with 4.60 ± 0.30 shoots per explant and 4.80 ± 0.44 cm shoot length and 4.25 ± 0.77 shoots per explant and 4.41 ± 0.41 cm shoot length, respectively (**Figure:1g; Table 2**). Similar results have been reported in several studies performed with *Bougainvillea glabra* and *Catha edulis* [8, 34]. In the study done by Jain and Chaturvedi [12] with *Shorea robusta*, BAP, when used alone, was found effective for *in vitro* proliferation of shoot cultures. Whereas, few workers reported that IAA enhances *in vitro* proliferation of *Shorea roxburghii*, but with limited success [21, 32]. In these studies, BAP alone at 4.44μM induced a 75.6% multiplication response, however, it was found less effective when compared to BAP (6.66μM) and TDZ (0.45μM) used in combination with 89.5% multiplication response observed in the present study.

4. Rooting and Acclimatization

The frequency of root induction together with the number of roots per shoot and root length were recorded after 7d of culture and were significantly affected by the hormone concentration used (**Table 3**). When the shoot cultures were placed on full strength MS medium devoid of any plant growth regulator, the rooting response was not obtained. The addition of different concentrations of auxins influenced the rate of root induction along with the number of roots per shoot. Of the plant growth regulators tested, IBA was found to be more effective for root induction compared to IAA and NAA. The fortification of half strength MS medium with IBA at concentrations of 0.22 and 0.44 μM increased the root induction rate up to 75% with 5.11 to 5.81 roots per shoot, respectively. Similarly, IBA at concentrations of 0.22 and 0.44 μM produced 5.10 to 6.10 cm root length, respectively. Increasing IBA concentration (0.672 to 2.02) only induced swelling in the lower region of shoot cultures and failed to induce a rooting response when supplemented in either half and full strength MS medium. An IBA concentration of 2.24μM was found the most suitable for root induction, with an 85% rooting response, 6.48 ± 0.58 roots per shoot, and 6.55 ± 0.36 cm root length within a 30-d culture period (**Figure:1h**). Supplementation of IAA failed to provide a root induction response except at 2.85μM concentration with half strength MS medium, which induced a 50% rooting response with 6.06 ± 0.67 roots per shoot and 4.23 ± 0.16 cm root length response. The addition of NAA also did not provide an effective rooting response and resulted in only 30 to 50% root induction.

Based on these observations, IBA was selected as a suitable rooting plant growth regulator for *S. tumbuggaia*. Results obtained from earlier studies carried out with *S. robusta*, *Shorea leprosula* and *Dipterocarpus alatus* and *Dipterocarpus intricatus* supports these findings,

where a higher concentration of IBA alone and in combination with IAA was associated with an increased rate of root induction and establishment [1,12,18].The observations presented in these studies also support and indicate a considerably better effectiveness of lower NAA concentrations for root induction.

4.1. Genetic Fidelity Testing of Micropropagated Plants

The rooted plantlets generated from seedlings of *S. tumbuggaia* appeared morphologically similar to the plantlets obtained from the explant collection site. To authenticate the retention of genetic stability of *in vitro* generated multiple shoot cultures, ISSR molecular marker-based genetic assessment was performed. The ISSR marker assessment technique for genetic assessment provides a benefit over the dependency on morphological and chemical traits to confirm the genetic fidelity of *in vitro* raised plants [7,22]. The ISSR technique produces more stable products than Random Amplification of Polymorphic DNA (RAPD) although it is as simple as RAPD in operations. Thus, ISSR is generally used to study population genetics, taxonomy, and phylogeny in several plant species [38,39]. A total of nine randomly selected multiple shoot cultures derived from different seeds collected from the same mother plant with the mother plant were subjected to ISSR profiling with10 UBC-ISSR primers. Results obtained from ISSR profiling showed that *in vitro* raised plantlets and mother plants have very little genetic disparity after *in vitro* proliferation. A total of 24 monomorphic and 6 polymorphic bands were scored ranging from 250 to 1500 bp, with an average of three bands per primer (**Table 4; Figure. 2**). Studies completed with *Ochreinauclea missionis* (4), *Ceropegia spiralis* (6), *Moringa peregrine* (16), and *Ceropegia evansii* (5) showed similar results, where *in vitro* raised plants were found to be similar to their mother plant.

These results indicate true to type character of the regenerated plantlets. However, the possibility of genetic variations arising during the *in vitro* process cannot be ruled out because tissue culture techniques are known to induce somaclonal variations in regenerated or micropropagated plants, due to stress induced by high concentrations of growth regulators and the fast rate of sub-culturing. Therefore, testing of clonal fidelity of regenerants remains one of the most important prerequisites for plant tissue culture generated plants [17] especially in trees and woody shrubs with long rotation cycles. These results indicate the genetic stability and homogeneity among the generated progenies.

5. Conclusion

The present study constitutes the first report wherein an efficient *in vitro* propagation protocol developed in *S. tumbuggaia* was utilized for greenhouse establishment and conservation of this highly endangered tree species. It is pertinent to highlight that the members of Dipterocarpaceae are generally considered non-amenable to *in vitro* tissue culture and therefore, there are only a few reports on tissue culture of *S. roxburghii* and *S. robusta* [12,

21, 32, 35] with limited success. These limited *in vitro* studies with genus *Shorea* also make it difficult to understand the different aspects of *in vitro* proliferation and conservation and therefore, more extensive research work is needed. During the collection of seeds, there were very few trees of *S. tumbuggaia* in the Tirumala Hills, which is supposed to be the biggest genetic source of this rare species [2]. Unfortunately, viable attempts were not made for *in vitro* establishment and proliferation of this endangered tree. In light of the obtained results, the conclusion was made that half strength MS medium without plant growth regulator provides efficient seed germination. The maximum explant regeneration, shoot buds per explants and shoot length, was obtained when shoot tips were cultured on MS medium supplemented with 6.66 µM BAP and 0.45µM TDZ. IBA at a 2.24µM in MS medium was found to be effective for root induction and elongation. Furthermore, the ISSR primers used in the study produced more monomorphic bands compared to polymorphic bands in the regenerated plants. Thus, the present study generated valuable information with regard to the efficacy of ISSR primers to analyze the genetic stability of regenerated plants of *S. tumbuggaia* that can be used to screen *in vitro* produced and conserved plants. The established procedure can be effectively used for seed-based *in vitro* shoot proliferation and conservation of elite genotypes and to provide high quality plants for field cultivation and commercial farming with minimal risk of producing somaclonal variants.

6. Figures

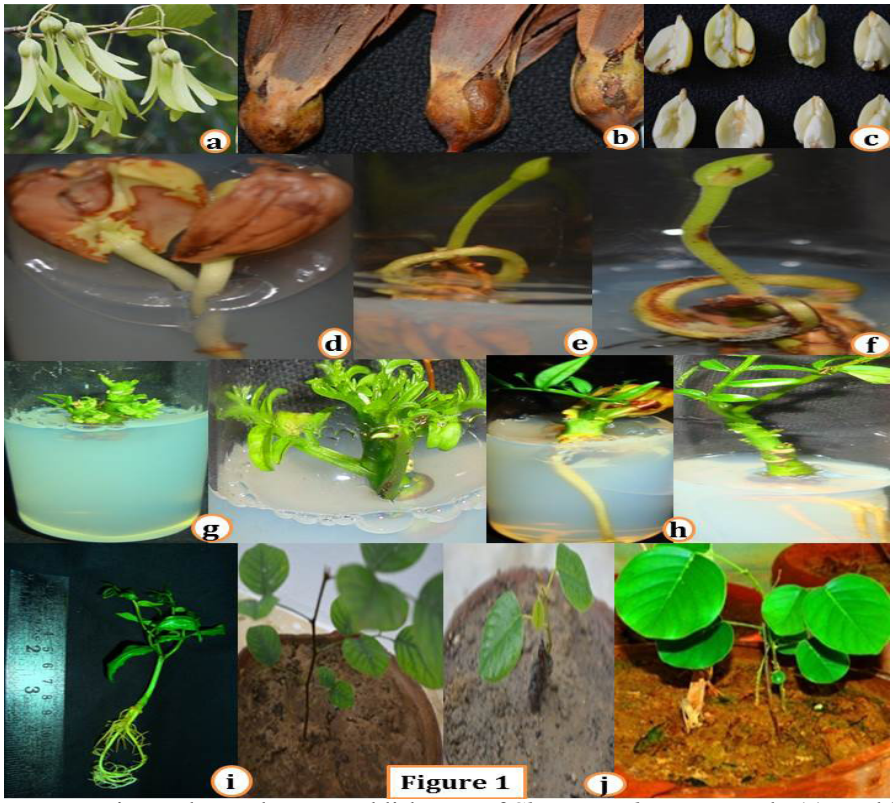

Figure 1. *In vitro* micropropagation and greenhouse establishment of *Shorea tumbuggaia* Roxb. (a) Healthy, young hanging winged fruits on plant. (b) Mature fruits. (c)Seeds removed after breaking the hard seed coat. (d–f) Germinating seeds (on half strength MS medium; (d) 4th day, (e)10thday, (f) 15thday).g Multiple shoot induction and axillary bud proliferation (on MS +6.66µMBAP + 0.45µM TDZ). (h)Root induction (after 14th day on half
MS +2.24µM IBA). (i) Mature plantlets with roots (2 mo old). (j) Greenhouse established plantlets. MS medium (Murashige and Skoog 1962) without plant growth regulators. IBA indole acetic acid, TDZ thidiazuron.

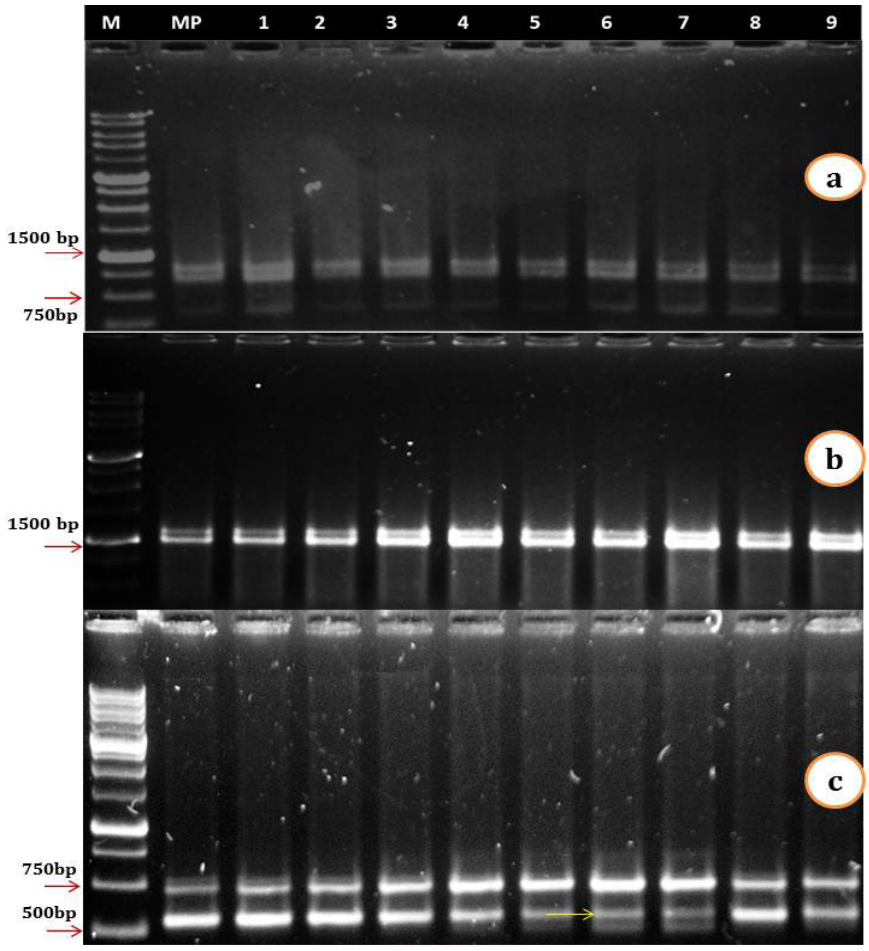

Figure 2

Figure 2. Amplified DNA bands generated from ISSR primer-based profiling of *in vitro* raised *Shorea tumbuggaia* Roxb. plantlets after seven sub-culture passages. (a)UBC-818. (b)UBC-808. (c) UBC-822.M molecular marker (1 kb DNA, RTU, Biochem Life Sciences, India), MP mother plant. Lanes 1–9 are randomly selected *in vitro* grown plants. Pink arrow represents band size of DNA marker; yellow arrow represents polymorphic bands.

7. Tables

Table 1: Effect of different plant growth regulators on multiple shoot induction in ***Shorea tumbuggaia***

PGR concentration (µM)	Morphogenic responses (Mean ± SE)		
	Multiplication response (%)[x]	No. of shoots/explant[x]	Shoots length (cm)[x]
BAP 2.22	50.3 ± 1.21^{bc}	3.08 ± 0.19^{bcd}	3.36 ± 0.15^{a}
BAP 4.44	75.6 ± 1.92^{j}	4.19 ± 0.29^{fg}	4.66 ± 0.37^{c}
BAP 6.66	68.3 ± 1.23^{ij}	3.88 ± 0.35^{def}	4.08 ± 0.48^{abc}
BAP 8.88	67.3 ± 1.32^{hi}	4.66 ± 0.22^{g}	4.50 ± 0.48^{c}
BAP 11.1	60.3 ± 1.54^{efgh}	3.97 ± 0.28^{efg}	4.41 ± 0.39^{c}
BAP 13.3	62.6 ± 1.44^{fghi}	3.58 ± 0.26^{ef}	4.41 ± 0.51^{abc}
TDZ 2.27	ns	ns	ns
TDZ 4.54	58.3 ± 1.22^{efg}	2.08 ± 0.19^{a}	4.66 ± 0.18^{c}

TDZ 6.81	66.6 ± 1.45^{ghi}	4.05 ± 0.35^{fg}	4.08 ± 0.26^{abc}
TDZ 9.08	58.3 ± 1.43^{cdef}	3.16 ± 0.29^{cde}	4.66 ± 0.31^{c}
TDZ 11.35	60.3 ± 1.67^{efg}	3.66 ± 0.22^{def}	4.50 ± 0.41 ^{c}
TDZ 13.6	48.3 ± 1.47^{b}	3.91 ± 0.28^{efg}	4.41 ± 0.37^{bc}
KN 2.32	50.0 ± 1.49^{bc}	ns	ns
KN 4.64	50.0 ± 1.49^{ac}	ns	ns
KN 6.96	44.6 ± 1.33^{ab}	ns	ns
KN 9.28	50.3 ± 1.39^{c}	2.93 ± 0.25^{abc}	4.16 ± 0.39^{abc}
KN 11.6	50.3 ± 1.39^{bc}	2.71 ± 0.14^{abc}	4.83 ± 0.50^{c}
KN 13.92	55.0 ± 1.32^{cde}	2.58 ± 0.19^{ab}	3.58 ± 0.25^{ab}

Analysis of variance (ANOVA)

Source of variation	**DF**	**Mean sum of squares**		
		Multiplication response (%)	**No. of shoots/explant**	**Shoots length (cm)**
Treatments	18	1369.8*	28.32*	23.1*
Error	36	5.164	0.056	0.084

Means (±SE) within a column followed by the same letter are not significantly different at p≤0.05 PGR plant growth regulators, IBA indole butyric acid, IAA indole acetic acid, NAA naphthalene acetic acid, ns no response, ½MS half strength, MS full strength MS medium (Murashige and Skoog 1962)^{x} Data were scored after three passages (2 wk each) on MS medium with given plant growth regulator concentrations*p<0.05

Table 2: Effect of different hormonal combination on multiple shoot induction in *Shorea tumbuggaia*

PGR concentration (µM)	**Morphogenic responses (Mean ± SE)**		
	Multiplication response (%)^{x}	**Average number of shoots/explant^{x}**	**Shoots length (cm)^{x}**
BAP 2.22 + TDZ 0.454	64.4 ± 1.2^{ab}	1.41 ± 0.12^{ab}	3.56 ± 0.44^{ab}
BAP 4.44 + TDZ 0.454	65.5 ± 1.6^{ab}	2.83 ± 0.53^{bcd}	4.91 ± 0.34^{c}
BAP 6.66 + TDZ 0.454	89.5 ± 2.1^{c}	4.60 ± 0.30^{fg}	4.80 ± 0.44^{c}
BAP 8.88 + TDZ 0.454	75.0 ± 1.9^{bc}	2.34 ± 0.36^{abc}	4.05 ± 0.56^{abc}
BAP 11.1 + TDZ 0.454	58.3 ± 1.5^{ab}	1.25 ± 0.19^{a}	4.75 ± 0.51^{c}
BAP 13.3 + TDZ 0.454	60.0 ± 1.3^{ab}	1.34 ± 0.15^{a}	3.65 ± 0.28^{ab}
BAP 2.22 + NAA 0.536	56.5 ± 1.2^{ab}	3.26 ± 0.53^{cdef}	4.66 ± 0.38^{c}
BAP 4.44 + NAA 0.536	65.0 ± 1.5^{ab}	4.19 ± 0.49^{defg}	4.33 ± 0.29^{bc}

BAP 6.66 + NAA 0.536	60.0 ± 1.5^{ab}	4.16 ± 0.36^{defg}	4.08 ± 0.33^{abc}
BAP 8.88 + NAA 0.536	55.6 ± 1.8^{ab}	3.83 ± 0.32^{defg}	3.16 ± 0.20^{a}
BAP 11.1 + NAA 0.536	55.6 ± 1.4^{ab}	4.33 ± 0.72^{efg}	3.83 ± 0.36^{ab}
BAP 13.3 + NAA 0.536	73.8 ± 1.9^{bc}	4.25 ± 0.77^{efg}	4.41 ± 0.41^{bc}
BAP 2.22 + KN 0.464	ns	ns	ns
BAP 4.44 + KN 0.464	48.3 ± 1.1^{ab}	2.12 ± 0.32^{abc}	ns
BAP 6.66 + KN 0.464	46.3 ± 1.1b^{ab}	3.75 ± 0.17^{cde}	ns
BAP 8.88 + KN 0.464	56.6 ± 1.4^{bab}	4.83 ± 0.20^{g}	4.25 ± 0.30^{bc}
BAP 11.1 + KN 0.464	70.6 ± 1.6^{bab}	4.66 ± 0.22^{cg}	3.91 ± 0.33^{abc}
BAP 13.3 + KN 0.464	68.3 ± 1.5^{ab}	4.75 ± 0.21^{fg}	4.16 ± 0.32^{bc}

Analysis of variance (ANOVA)

Source of variation	**DF**	**Mean sum of squares**		
		Multiplication response (%)	**Average number of shoots/explant**	**Shoots length (cm)**
Treatments	18	48.193*	4.819*	6.562*
Error	36	3.744	0.187	0.084

Means (±SE) within a column followed by the same letter are not significantly different at p≤0.05 PGR plant growth regulators, BAP 6-benzylaminopurine, TDZ thidiazuron, NAA naphthalene acetic acid, KN kinetin, ns no response, ^{x} Data were scored after three passages (2 wk each) on MS medium with given plant growth regulator concentrations, *p<0.05

Table 3: Effect of different auxins on *in vitro* rooting of micropropagated shoot cultures of *Shorea tumbuggaia*

Medium used	**PGR concentration (µM)**	**Morphogenic responses (Mean ± SE)**		
		Rooting response (%)^{x}	**No. of roots/ shoot^{x}**	**Root length (cm)^{x}**
½ MS	No PGR	48.0 ± 1.12^{ef}	3.12 ± 0.12^{ab}	4.62 ± 0.25^{bc}
MS	No PGR	ns	ns	ns
½MS	IBA0.224	40.0±1.11^{c}	5.11 ± 0.22^{ef}	5.10 ± 0.20^{cd}
½ MS	IBA 0.448	75.0 ± 1.21^{h}	5.81 ± 0.45^{gh}	6.10 ± 0.10^{ef}
½ MS	IBA 2.24	85.5 ± 1.29^{i}	6.48 ± 0.58^{i}	6.55 ± 0.36^{f}
½ MS	IBA 4.48	46.6 ± 1.12^{de}	4.17 ± 0.13^{cd}	5.40 ± 0.32^{d}
MS	IBA 0.224	55.0 ± 1.23^{g}	5.06 ± 0.31^{efg}	5.27 ± 0.25^{d}
MS	IBA 0.448	56.6 ± 1.35^{g}	5.22 ± 0.41^{efg}	6.43 ± 0.38^{f}
MS	IBA 2.24	55.0 ± 1.39^{g}	4.61 ± 0.25^{de}	4.49 ± 0.13^{bc}

MS	IBA 4.48	40.0 ± 1.19^{c}	3.68 ± 0.26^{bc}	5.45 ± 0.10^{d}
½ MS	IAA 0.285	ns	ns	ns
½ MS	IAA 0.570	ns	ns	ns
½ MS	IAA 2.85	50.0 ± 1.30^{ef}	6.08 ± 0.67^{hi}	4.23 ± 0.16^{b}
½ MS	IAA 5.70	ns	ns	ns
MS	IAA 0.285	ns	ns	ns
MS	IAA 0.570	ns	ns	ns
MS	IAA 2.85	ns	ns	ns
MS	IAA 5.70	ns	ns	ns
½ MS	NAA 0.268	45.0 ± 1.24^{d}	2.68 ± 0.26^{a}	5.45 ± 0.19^{d}
½ MS	NAA 0.536	35.0 ± 1.09^{b}	5.67 ± 0.23^{fgh}	4.45 ± 0.16^{bc}
½ MS	NAA 2.63	30.0 ± 1.03^{a}	3.08 ± 0.24^{ab}	3.45 ± 0.16^{a}
½ MS	NAA 5.36	ns	ns	ns
MS MS	NAA 0.268 NAA 0.536	50.3 ± 1.21^{f} 40.0 ± 1.12^{c}	5.51 ± 0.15^{fgh} 4.32 ± 0.28^{d}	6.35 ± 0.24^{f} 5.74 ± 0.23^{de}
MS	NAA 2.63	39.3 ± 1.05^{c}	3.45 ± 0.56^{b}	4.26 ± 0.14^{b}
MS	NAA 5.36	ns	ns	ns

Analysis of variance (ANOVA)

Source of variation	**DF**	**Mean sum of squares**		
		Rooting response (%)	**No. of roots/shoot**	**Root length (cm)**
Treatments	26	2174.715*	18.419*	21.453*
Error	52	12.439	0.056	0.040

Means (±SE) within a column followed by the same letter are not significantly different at p≤0.05 PGR plant growth regulators, IBA indole butyric acid, IAA indole acetic acid, NAA naphthalene acetic acid, ns no response, ½MS half strength, MS full strength MS medium (Murashige and Skoog 1962)^{x} Data were scored after three passages (2 wk each) on MS medium with given plant growth regulator concentrations*p<0.05

Table 4. Primer sequences and number of scored monomorphic and polymorphic bands produced by ISSR markers in *in vitro* raised *Shorea tumbuggaia*

Primer used scored per plant	**Primers sequences (5′—3′)**	**Annealing temperature (°C)**	**No. of monomorphic bands**	**No. of polymorphic bands scored per plant**	**Band range (bp)**
UBC 807	$(AG)_8T$	44.6	3	1	350–1000
UBC 808	$(AG)_8C$	47.1	2	0	1500–1700
UBC 809	$(AG)_8G$	47.1	3	1	250–1000
UBC 810	$(GA)_8T$	44.6	2	1	500–1500
UBC 811	$(GA)_8C$	47.1	2	0	250-1500
UBC 812	$(GA)_8A$	44.6	3	1	350-1000
UBC 814	$(CT)_8A$	44.6	2	1	500–1000

UBC 815	$(CT)_8G$	44.6	2	1	350–1500
UBC 818	$(CA)_8G$	44.6	2	0	750–1500
UBC 822	$(TC)_8A$	46.1	3	1	250–750

Average bands per primer 3.0

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