Micropropagation of Indian laurel (*Calophyllum inophyllum*), a source of anti-HIV compounds

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An efficient protocol for *in vitro* micropropagtion of *Calophyllum inophyllum* (Linn.), an evergreen littoral tree, through multiple shoot formation from seed explants was developed. *In vitro* germination of the seeds was standardized on Woody Plant Medium (WPM) hormone free and/or supplemented with 6-benzylaminopurine (BAP; 2.22 μM) and on half or full strength MS medium. Multiple shoot formation was achieved on WPM supplemented with BAP (2.22–44.00 μM) and thidiazuron (TDZ; 0.91–4.54 μM) from the decapitated seedling explants. The maximum multiple shoots, 20.9 per explant were induced on TDZ (0.91 μM) after two subcultures. Elongated shoots of size >4.0 cm were obtained on all media combinations with an average of 2.2–8.7 per explant. Elongation of the stunted shoots induced on BAP and TDZ was done on half strength WPM without any growth hormones. The elongated shoots on half WPM and/or full strength WPM supplemented with indole-3-butyric acid (2.46–24.60 μM) alone or in combination with BAP (2.22 μM) resulted in 52% rooting with 1–5 roots per rooted plant. The micropropagated plants were acclimatized successfully with 77% survival rate after five weeks. These plants were planted in the institute campus for *ex situ* conservation, where 72% plants are showing good growth and development.

Keywords: *Calophyllum inophyllum*, *ex situ* conservation, micropropagation, seed.

CALOPHYLLUM inophyllum Linn. [Guttifereae (Clusiaceae)], commonly known as 'Indian laurel' or 'Alexandrian laurel' is a broad leaved evergreen tree (Figure 1 *a*) occurring as a littoral species along the beach crests, although sometimes occurring inland[1]. It is known to have cancer chemopreventive agents[2], coumarins and xanthones with antimicrobial activity[3]. The oil has various medicinal uses in rheumatism, skin diseases, joint pains and haemorrhage[4–6]. The aqueous extracts of the root bark and leaves are used as a cicatrisant, and those of the fruit have analgesic properties and are used in treatment of wounds and herpes[7]. Oil is also used as luminant, lubricant, for soapmaking, etc. The timber is used for beams, furniture, railway carriages and shipbuilding[8]. *Calophyllum* species are gaining importance as a source of anti-HIV medicines. The inophyllums and (+)-calanolide isolated from *C. inophyllum* L. and *C. lanigerum* Miq[9–13] showed strong activity against human immunodeficiency virus type-1 (HIV-1). *C. inophyllum* possesses potential threat due to decline in the population because of various biotic[14] and abiotic factors. The fruit being a drupe, has a hard endocarp with long dormancy period and low rate of germination. Being a littoral species, the seeds are taken away in the tidal water thereby limiting the propagation rate. Conventional propagation via vegetative cuttings is not practised due to difficulty in rooting[15] in almost all species of *Calophyllum* and immediate protective measures are essential for the continued existence of the genus[16]. Tissue culture technology would be a useful tool for overcoming these limitations and accelerate mass propagation of this important medicinal tree.

Here, we report a reliable method for *in vitro* multiplication and micropropagation of *C. inophyllum* using mature seed as explant, followed by successful plantlet growth, development and *ex situ* conservation.

Mature fruits of *C. inophyllum* Linn. (Figure 1 *b*) were collected from Harne village (17°49′63″N, 73°05′65″E; 2 m altitude) near Dapoli, Ratnagiri along the coast of Maharashtra, in the fruiting season during the second week of March 2003.

For *in vitro* germination, mature fruits were decoated mechanically to remove the hard, stony endocarp and the seeds (Figure 1 *c*) were washed thoroughly with liquid detergent solution (Labolene 0.1%, v/v; Qualigens, India) as surfactant for 5 min and then washed with tap water (× 5), followed by washing with antiseptic (Savlon 10%, v/v; Johnson and Johnson Ltd, India). The seeds were then rinsed thoroughly with double distilled-water (DDW) (× 3) and treated with insoluble polyvinylpyrrolidone (0.1%, w/v; Sigma, USA) for 30 min and later by antifungal agent (Bavistin, 1% w/v; BASF, India) for 30 min. Seeds were again rinsed with DDW (× 5). All further treatments were carried out under sterile conditions in a laminar airflow chamber. The seeds were rinsed in ethyl alcohol (70%, v/v; Merck, India) for 15–20 s, washed with sterile distilled water (SDW) (× 3), followed by mercuric chloride (0.1%, w/v; Qualigens, India) for 5 min and finally washed thoroughly with SDW (× 7). Seeds were presoaked in SDW and/or gibberellic acid (GA_3; 0.058 μM) and/or heated at 35°C and then presoaked in GA_3 (0.058 μM), for 24 h prior to inoculation. For germination, four types of media were tested, viz. hormone-free Woody Plant Medium[17] (WPM) and/or supplemented with 6-benzylaminopurine (BAP; 2.22 μM) and half strength and full strength Murashige and Skoog[18] (MS) medium without any growth regulators. All the media were supplemented with sucrose (2%, w/v) and pH was adjusted to 5.6–5.8 with 0.1 N NaOH solution. All the media were gelled with 0.80% agar (w/v, Qualigens). Growth regulators were incorporated into the media prior to autoclaving. The media were autoclaved at 1.05 kg cm^{-2} and 121°C for 20 min before use. One seed was inoculated per glass cul-

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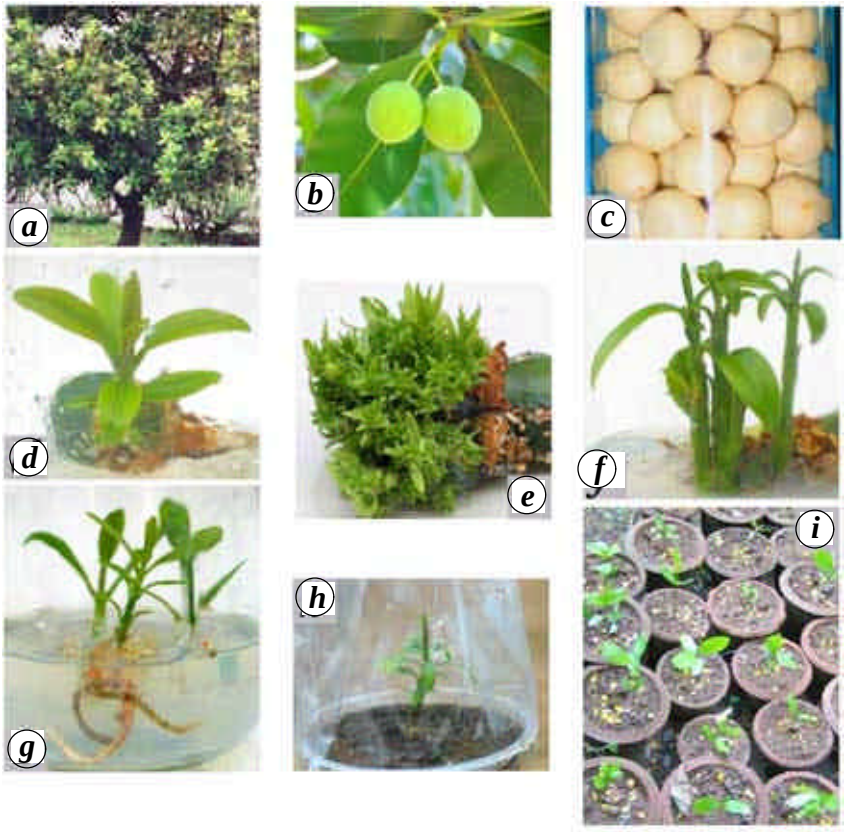

Figure 1. ***a***, Tree of *Calophyllum inophyllum* Linn. at Harne. ***b***, Globose drupe fruits. ***c***, Seeds removed after breaking the hard, stony endocarp. ***d***, *In vitro* germinating seed on WPM full strength medium (45 days after inoculation). ***e***, Proliferation of multiple shoots from a single decapitated seedling after 7 weeks of culture on WPM medium containing 0.91 μM TDZ. ***f***, Elongated multiple shoots of size >4.0 cm. ***g***, Well-rooted plantlets prior to transfer to potting mixture. ***h***, Acclimatized plant 3 weeks after transfer to greenhouse. ***i***, *In vitro* propagated plantlets 4 months after transfer to the pots.

ture bottle (Laxbro, India), with 50 seeds per treatment. The cultures were incubated at 25 ± 1°C under cool white fluorescent light (16/8 h photoperiod, 35 μmol m^{-2} s^{-1}; Philips, India). The frequency of seed germination was scored after 25 days based on five replications for each experiment.

The *in vitro* germinated seedlings were removed from the culture bottles under sterile conditions in a laminar airflow chamber. Decapitation (of shoot and root) was done using sterile surgical blade. The decapitated seedlings (one per culture bottle) were then inoculated on hormone free WPM and/or WPM supplemented with BAP (2.22–44.00 μM) or thidiazuron (TDZ; 0.91–4.54 μM). The culture conditions were similar to the germination experiment. The numbers of multiple shoots induced were scored after every 20 days from the day of inoculation to a period of 60 days. The explants on all the combinations of media were subcultured twice during this period at an interval of 20 days. The multiple shoots induced on BAP (2.22–8.90 μM) and TDZ (0.91–4.54 μM) elongated well on the same medium. The stunted shoots induced on higher concentrations of BAP (13.30–44.00 μM) and some from TDZ (0.91–4.54 μM) were elongated on half strength WPM without any growth regulators. Well-elongated shoots were shifted to half and full strength WPM alone or supplemented with indole-3-butyric acid (IBA; 2.46–24.60 μM), alone or in combination with 2.22 μM BAP for root induction. For all the treatments, a minimum of 50 shoots with three replicates was maintained. The rooted shoots were acclimatized in sterilized mixture of soil, cocoa peat and sand (1 : 2 : 1) under greenhouse conditions for four weeks and later transferred to earthen pots for further growth and development in the nursery. These micropropagated plants were planted after 3–5 months in the institute campus in an attempt for their *ex situ* conservation. Pits of size 50 cm^3 were made at the plantation sites, which were filled with garden soil, and farmyard manure (1 : 1). Plants were carefully transplanted to the pits from the earthen pots; 72% of the plants are showing good growth and development.

Analysis of variance (ANOVA) was done by completely randomized block design (CRBD) using Agrobase 99 software for all the experiments and the angular transformation values were derived according to Snedecor and Cochran[19].

Germination of seeds was observed after 10–15 days of incubation on all media combinations. Without phytohor-

Table 1. Influence of BAP and TDZ on multiple shoot induction from decapitated seedling explant

Medium	Hormone	Concentration (μM)	Average no. of multiple shoots after incubation (days)		
			20	40 S1	60 S2
WPM	–	–	1.2	2.3	4.8
	BAP	2.22	3.6	9.7	12.0*
		4.40	1.3	5.1	8.1
		8.90	2.0	4.9	6.4
		13.30	3.5	6.3	9.8
		22.19	3.8	8.8	12.7*
		44.00	6.9	11.6	13.3*
	TDZ	0.91	6.9	15.0	20.9**
		2.27	4.4	10.1	15.0**
		4.54	3.6	6.6	13.1*

LSD (P = 0.05) = 6.97; LSD (P = 0.01) = 9.24.
*,**Significant at 5% and 1% level respectively; S1, S2, Subcultures one and two.

mones, seed germination on MS basal medium was poor (24–42%) and slow compared to WPM basal medium with/or without hormones, which showed good and faster germination (36–78%).

Presoaking facilitated leaching of phenolics from the seeds, prevented browning and caused swelling of seeds, thereby hastening the germination process by three weeks, compared to the seeds which were not presoaked prior to inoculation (used as control). The best germination 78% (significant at P = 0.01) was observed when seeds were soaked for 24 h prior to inoculation on WPM basal medium without any growth hormones (Figure 1 *d*). However, with increase in presoaking time to 36 or 48 h, there was no further increase in the germination percentage. Moreover, it resulted in contamination of cultures. Therefore, for seedling establishment 24 h presoaked seeds were used. On an average, all seeds germinated within 10–15 days. However, growth was slow and it took about 25–30 days to develop into seedlings of 5–8 cm size. Well-germinated seedlings (size >5.0 cm) were decapitated and inoculated on hormone-free WPM and/or WPM supplemented with BAP (2.22–44.00 μM) or TDZ (0.91–4.54 μM) for multiple shoot induction. The induction of multiple shoots was observed 10–15 days after inoculation. Observations for multiple shoot induction were taken at 20 days intervals up to a period of 60 days (Table 1). More multiple shoots were induced on 2.22, 22.19, and 44.00 μM BAP compared to other concentrations (4.40, 8.90 and 13.30 μM). Low concentration of TDZ (0.91 μM) induced equal number of shoots as that of higher concentration of BAP (44.0 μM) after 20 days of incubation. The same trend of response continued for 60 days. After 60 days of incubation, the maximum number of multiple shoots observed was 20.9 per explant with TDZ (0.91 μM; Figure 1 *e*). All concentrations of BAP induced multiple shoots from 6.4 to 13.3 per explant. ANOVA showed that BAP (2.22, 22.19 and 44.00 μM) and TDZ (4.54 μM) were significant at 5% level, while TDZ (0.91 and 2.27 μM) was found to be significant at 1% level. On the basis of statistical analysis, it appears that TDZ is significantly better than BAP for multiple shoot induction, since it induced greater number of shoots at much lower concentrations.

Though more multiple shoots were induced at high concentrations of BAP, the number of elongated shoots of size greater than 4.0 cm (Figure 1 *f*) was significantly higher at lower concentrations of BAP (2.22 and 4.40 μM). Generally, longer shoots are produced at lower BAP concentrations, whereas more shoots are induced with higher BAP concentration[20]. However, TDZ even at very low concentration (0.91 μM) induced more multiple shoots. Also, the proportion of elongated shoots of size >4.0 cm was significantly higher (4.9–7.2 per explant) compared to BAP (Table 2). The possible reason is its auxin and cytokinin-like activity[21]. Maximum number of elongated shoots, 8.7 per explant (significant at P = 0.01), was noticed on WPM supplemented with BAP (2.22 μM). Transfer of the stunted shoots induced on BAP/TDZ to half strength WPM without any growth hormones showed considerable elongation in about 30–40 days.

The developing shoots of more than 4.0 cm size were excised and used for root induction. These shoots were cultured on half and/or full strength WPM supplemented with IBA (2.46–24.60 μM) alone or in combination with BAP (2.22 μM). Combination of half WPM supplemented with IBA (2.46–24.60 μM) had no effect on root induction. Shoots induced on BAP-containing medium induced rooting in 8–12 days, with 1–5 roots per explant. However, shoots induced on TDZ rooted only after two passages of one-month duration each on half strength WPM supplemented with IBA (2.46–24.60 μM) alone or in combination with BAP (2.22 μM). The high carry-over effect of TDZ has been well documented in the literature[22,23]. In all the combinations, rooting was observed with 14–52% frequency (Table 3; Figure 1 *g*). Maximum rooting (52%) was observed on WPM supplemented with IBA (2.46 μM) alone. On the basis of ANOVA, it was observed that full strength WPM and/or WPM supplemented with IBA (2.46–24.60 μM) was significantly superior to half strength

Table 2. Average number of elongated shoots induced with different hormone concentrations

Medium	Hormone	Concentration (μM)	Average no. of elongated shoots (>4.0 cm) after incubation (days)		
			20	40 S1	60 S2
WPM	–	–	0.4	1.2	2.2
	BAP	2.22	1.8	6.1	8.7**
		4.40	0.8	5.1	6.2*
		8.90	0.6	1.5	4.9
		13.30	1.0	2.1	3.7
		22.19	0.7	2.0	2.7
		44.00	1.3	1.9	2.3
	TDZ	0.91	3.1	6.3	7.2*
		2.27	2.1	5.6	6.4
		4.54	2.7	3.8	4.9

LSD (P = 0.05) = 3.77; LSD (P = 0.01) = 5.00.
*,**Significant at 5% and 1% level respectively; S1, S2, Subcultures one and two.

Table 3. *In vitro* root induction in shoots of size >4.0 cm

Medium	Hormone concentration (μM)		Mean per cent rooting	No. of plantlets obtained	Per cent acclimatization
	IBA	BAP			
Half WPM	–	–	14 (21.59)	10	71.40
	2.46	2.22	40 (39.23)**	32	80.00
	4.90	2.22	34 (35.26)**	26	76.47
	9.80	2.22	27 (31.30)**	22	78.57
	14.70	2.22	23 (28.65)**	20	83.33
	24.60	2.22	18 (24.57)*	14	77.77
WPM	–	–	16 (23.04)	12	75.00
	2.46	–	52 (45.76)**	42	80.76
	4.90	–	41 (39.42)**	36	85.71
	9.80	–	29 (32.15)**	22	73.33
	14.70	–	43 (40.59)**	34	77.27
	24.60	–	41 (39.61)**	30	71.42

LSD (P = 0.05) = 2.85; LSD (P = 0.01) = 3.87.
*,**Significant at 5% and 1% level respectively.
Figures in parenthesis are angular transformation values of percentage of response.

WPM and/or half WPM supplemented with IBA (2.46–24.60 μM) and BAP (2.22 μM).

The rooted plantlets were transferred to sterilized potting mixture of soil, cocoa peat and sand (1 : 2 : 1) and acclimatized in a greenhouse with temperature of 25 ± 2°C, 80% relative humidity, and with 77% survival rate after a period of five weeks (Figure 1 *h*). The well developed and hardened plants after 8 weeks were transferred to earthen pots containing a mixture of garden soil and farmyard manure (1 : 1) for further growth and development (Figure 1 *i*) and finally planted in the institute campus.

Nair and Seeni[15] reported *in vitro* multiplication of *C. apetalum* Willd. using mature tree explants on MS medium supplemented with BAP (8.8 μM). The multiplication ratio was 1 : 2/3. The present work is on *in vitro* propagation of *C. inophyllum* Linn., a tree species with immense medicinal importance (especially in AIDS chemotherapy). Thus efforts have been made for the *ex situ* conservation of this threatened medicinal tree. The above protocol can be used for mass propagation of *C. inophyllum*, since the success rate of vegetative propagation is low and is usually not practised.

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Influence of northeasterly trade winds on intensity of winter bloom in the Northern Arabian Sea

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Chlorophyll and wind pattern retrieved from remote sensing data have been used to study biological activity in the oceanic waters of Northern Arabian Sea (NAS) during February–March 2002–05. Occurrence of algal bloom in these waters during this period was noticed with the help of ship observations in the past. The same was detected from OCEANSAT I/OCM with time series chlorophyll images for January–March 2000. Occurrence of this bloom was later re-confirmed using OCM data in the subsequent years also. The time-series chlorophyll images established that the bloom develops every year during February–March. This period happens to coincide with the presence of northeasterly trade winds over the NAS. Two ship cruises were conducted with the help of research vessels FORV *Sagar Sampada* (SS-212 during 26 February–7 March 2003 and SS-222 during 21 February–11 March 2004) during this period at the bloom site. The aim was species identification of the bloom and to study various environmental parameters associated with the bloom. Two diverse situations in the context of biological activity were observed while collecting *in situ* data in 2003 and 2004. Distribution of the bloom was found uniform over a large area and concentration of phytoplankton was relatively higher in 2003. Compared to this, it was observed during the same period in 2004 that phytoplankton was distributed in scattered and small patches and its concentration was relatively less. Corresponding to this observation, it was noticed from the ship data that wind strength was significantly weaker and the oceanic waters were less turbulent in 2004 compared to the same in 2003. In light of this elementary observation, an attempt was made to observe variations in the wind pattern during 2003 and 2004 using QuikSCAT/SeaWinds scatterometer data. It could be established that occurrence of the bloom as well as the observed inter annual variability in chlorophyll pattern were coupled with prevailing trade winds. It was found that density of surface water increased (inversion) during this period, which could result in convective action and the observed bloom. The vertical density gradient revealed an increasing pattern with increase in wind speed. Moreover, it was observed that response of chlorophyll to acting wind force is delayed by one to two weeks. This led to an important inference that wind can be treated as a precursor to predict

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