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In vitro Propagation and Mass multiplication of *Dalbergia latifolia* Roxb: A Vulnerable Tree Species from Eastern Ghats, Tamil Nadu, India

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Abstract
Many medicinal and economic important tree species are considered as endangered or threatened owing to rapid agricultural and urban development, deforestation and indiscriminate collection, so the tissue culture technique is the most effective tool for mass propagation and conservation of these rare and endangered medicinal plants. The family Fabaceae (alternatively known as the Leguminosae) is one of the largest families of flowering plants, consisting of 730 genera and over 19,400 species. *Dalbergia latifolia* Roxb. is Vulnerable tree species from Eastern Ghats. It is one of the most important timber species, yields the famous Indian rose wood or black wood of commerce. As the wood takes fine polish, it is extensively used in furniture and cabinet work. The wood is also used for decorative, carving, ornamental ply-boards, and wheels of gun carriages, ammunition boxes and temple cars. Ply-boards from Indian rose wood satisfy aircraft specifications. The leaves are used as fodder and parts of the tree are reported to be useful as stimulant and appetiser and also in dyspepsia, diarrhoea and leprosy. Oil from seeds is used as an antidote to poison. The *in vitro* propagation studies of *Dalbergia latifolia* is a vulnerable and economically important tree species. Due to its vast economic and medicinal importance, the plant has been over exploited from the wild very fast. The conservation of this vulnerable species is mandatory. Therefore, effective strategies to be developed for the production of viable saplings of this multipurpose plant species by using plant tissue culture technique. The present results recommend that BAP (1.5 mg/l) induced optimum shoot proliferation. IBA at 3.0 mg/l concentration was optimum for root induction from shoots. Rooted shoots were successfully transferred to field conditions with 80% survival. This protocol may be applicable to conserve the other RET plant species.

Keywords: *Dalbergia,* vulnerable, eastern ghats, *in vitro* propagation, conservation

1. Introduction

Many medicinal and economic important tree species are considered as RET species owing to rapid agricultural and urban development, deforestation and indiscriminate collection. So, the tissue culture technique is the most effective method for mass propagation and conservation of these RET medicinal plants (Fay, 1992; Hassan *et al*., 2011) [15, 18]. Several techniques have been adopted for *in vitro* propagation. Among them, some are general techniques such as preparation of nutrient medium, aseptic manipulation, maintenance of culture and some are specific techniques such as organ culture, callus culture, organogenesis, embryogenesis, suspension culture, anther and pollen culture, plant protoplast culture, embryo culture etc. Advances in the area of plant tissue culture for the production of secondary metabolites by callus culture have made it possible for the increased yield of a wide variety of pharmaceuticals such as alkaloids, terpenoids, steroids, saponins, phenolics and flavonoids (Ramachandra and Ravishankar, 2002) [34].

The family Fabaceae is one of the largest families of flowering plants, consisting of 730 genera and over 19,400 species. The genus *Dalbergia* is placed under the subfamily Faboideae containing 274 International Legume Database and Information Service accepted species distributed all over the world, especially in the tropical and subtropical regions. Most of the *Dalbergia* tree species are widely used as timber purpose and are valuable because of their decorative and fragrant wood (Chopra *et al.*, 1980) [10]. Many *Dalbergia* species are used in traditional system of medicines all over the world in the treatment of various ailments like diarrhea, leucoderma, dyspepsia, dysentery, syphilis, gonorrhea, stomach ache, leprosy, eye diseases, scabies, pain and ringworm (Khare, 2007; Kazembe *et al*., 2012) [21, 22].

Dalbergia latifolia Roxb. Is one of the most important timber species, yields the famous Indian rose wood or black wood of commerce. As the wood takes fine polish, it is extensively used in furniture and cabinet work. The wood is also used for decorative, carving, ornamental ply-boards and wheels of gun carriages, ammunition boxes and temple cars. Ply-boards from Indian rose wood satisfy aircraft specifications. The leaves are used as fodder and parts of the tree are reported to be useful as stimulant and appetiser and also in dyspepsia, diarrhoea and leprosy. Tannins extracted from the bark are used for a number of medicinal purposes. Oil from seeds is used as an antidote to poison. The plant is a good shade tree in coffee plantations (Bourdillon, 1908) [8].

The species is of great commercial importance because of its high-value timber. The heartwood is very hard, weighing

about 850 kg/m3. It is fragrant and decorative and is used to make premium-quality furniture, panelling and veneers. The species is nitrogen fixing and popular agroforestry species. During the first three years the trees are inter-planted with rice, maize, beans or cassava and later, when the canopies begin to close, they are under-planted with shade-tolerant crops like coffee, turmeric and ginger. In other systems it is grown with fruit trees like mango, annona, jackfruits and guava. The nitrogen-rich foliage is locally an important source of fodder.

In this connection, the *in vitro* propagation studies of *Dalbergia latifolia* vulnerable and economically important tree species. Due to its vast economic and medicinal importance, the plant has been over exploited from the wild very fast. The conservation of this vulnerable species is mandatory. Therefore, studies on *in vitro* propagation of *Dalbergia latifolia* have been framed to conserve the species from extinction.

2. Materials and Methods

2.1 Materials

Table 1: IUCN status of *Dalbergia latifolia*

Name of the taxa	Threat status (IUCN 2017.2)	Distribution
Dalbergia latifolia Roxb.	Vulnerable A1cd ver 2.3	India (Andhra Pradesh, Karnataka, Sikkim, Tamil Nadu, Uttar Pradesh); Indonesia (Jawa); Nepal

2.2 Collection and Preparation of explants

The explants (shoot tip and nodal parts) of *Dalbergia latifolia* were collected from the campus of Sri Kaliswari College, Sivakasi, Virudhunagar Dist (Fig 1-A). The collected explants were washed thoroughly under running tap water 15 minutes then they were washed in an agitated solution of liquid detergent for 5 minutes. The disinfected materials were removed by rinsing the material with sterilized cooled distilled water for 3-5 times.

2.3 Surface sterilization

The shoot tip and nodal parts of *Dalbergia latifolia* were surface sterilized with 0.1% Mercuric chloride for 5 minutes. Then, subsequently washed with sterile distilled water. The explants were again sterilized with 4% Sodium hypochlorite for 3 minutes and rinsed with sterile distilled water. The materials were again surface sterilized with ethanol (70%) for 3 minutes. The sterilized explants finally washed with sterile distilled water and the explants cut the tip end with help of a sterilized blade.

2.4 Preparation of medium

The basal medium consists of mineral salts and organic nutrients (Murashige and Skoog, 1962) [26] with vitamins were used for the present study. For convenience, throughout this chapter MS medium with MS salts vitamins is being referred as MS medium.

For the medium preparation, 30g/l of sucrose and 8g/l of agar was also added as carbon source and gelling agent respectively with the MS medium chemical composition. The pH of the medium was adjusted into 5.8 using 0.1% HCl and 0.1% NaOH and warmed until the solution was clear. Along with boiled medium, growth hormones like BAP and Kn were added at different concentrations (0.5-2.0 mg/l) and dispensed into the culture vessels. The medium was sterilized in an autoclave at 121ºC for 15 minutes. The sterilized culture vessels with MS medium supplemented with different hormones were transferred to the laminar air flow chamber for the successful sterilization. The explants were carefully inoculated in the culture vessels with MS medium along with growth hormones without any microbial contamination. In addition, special care was taken to stimulate the initiation of growth by providing proper atmospheric temperature (25 ± 2ºC) and light intensity of 2500-3000 lux by cool fluorescent lamp. After 30-45 days, the shoot development from the culture was observed carefully and number of shoots and length of shoots developed from the explants were also observed.

2.5 Rooting of the shoots

Auxillary shoots developed in culture in the presence of cytokinin generally lack roots. To obtain full plant, the developed shoots were transferred to a rooting medium combined with different concentration of IAA and IBA (1.0-5.0 mg/l). A half strength of MS medium is found better for rooting of shoots in large number of plantlets.

2.6 Transplantation and acclimatization

The rooted plants were gently washed with sterile double distilled water to remove adhering medium completely without causing any damage to the root. The regenerates were transplanted in the culture medium combined with sand, coco peat and soil with 1:1:1 ratio. The most essential requirement for the successful transplantation is to maintain the plants under a very high humidity (90-100%). For the first 10-15 days, the regenerated plantlets were kept under mist chamber covered with clear plastic sheets with some small holes poked in the plastic cover sheet for air circulation. Inside the culture vessel, the humidity is high and thus, the natural protective covering of outside is not fully developed. During this, regenerates attains ability to synthesis more food and developed circular covering. Plants were maintained under shade and are then ready to use in open nursery for successful reintroduction programme.

3. Results

In the present study, the explants of *Dalbergia latifolia* were micropropagated and the multiple shoot initiation was obtained within 45 days of culture. Shoot and root development was obtained in the full strength MS medium. The pH adjusted to 5.8 was optimum for the shoot multiplication, root induction and subsequent regeneration. Culture room with continuous light from fluorescent tube was maintained at a constant temperature of 25 ± 2ºC and 80 ± 5% relative humidity.

3.1 Shoot proliferation

For inoculation the explants were further trimmed and extra leaves were removed and made into suitable sizes. After cutting the explants into suitable size, 5-6mm shoot tip explants, 20-25mm long nodal and internodal explants were

inoculated on MS medium supplemented with different concentration (0.5-2.5 mg/l) of BAP and Kn. Multiple shoots were initiated from all of the explants after 4 weeks of culture and all explants were free from microbial contamination. The successfully formed shoots were excised individually from proliferated explants and further cultured on same medium to increase the number of shoots (Fig 1-B & C).

3.2 Effect of plant growth regulators

Number of newly initiated shoot buds depends on the growth regulator concentration and type of cytokinin (BAP and Kinetin) used. The synergetic effect of BAP (1.5 mg/l) induced 6.49 ± 0.82 mean number of shoots and 7.03 ± 0.11 mean length of shoots was obtained from Shoot tip explants of *Dalbergia latifolia*. The number of shoot developed or organogenesis was highest (10.62 ± 0.19) in internodal with 9.25 ± 0.41 mean length of shoots in explants treated with 1.5 mg/l of BAP. The frequency of organogenesis was highest in nodal explants treated with 1.5 mg/l of BAP (Table 2; Fig 1- E & F; Fig 2- B & C).

The mean number of shoot developed was highest (5.94 ± 0.19) with mean length of shoots 9.12 ± 0.56 in 1.5 mg/l of Kinetin. In 1.5 mg/l (Kn) concentration, the number of shoot organogenesis was highest 9.24 ± 0.47 in internodal with mean length of 7.38 ± 0.52 shoots in internodal explant. The frequency of nodal organogenesis was highest in nodal explants treated with 1.5 mg/l of Kinetin (Table 2; Fig 1-D; Fig 2-A).

Fig 1

Fig 2

3.3 Rooting

In vitro multiplied shoots were carefully removed from the culture medium and washed thoroughly with distilled water to remove the excess amount of medium and transfer to the medium with different concentrations (1.0-5.0 mg/l) of IAA and IBA for rooting. Among the various growth regulators tested, IBA at 3.0 mg/l showed the best results, where 5.72 ± 0.19 mean number of roots and 7.73 ± 0.52 mean number of root length were initiated after 15 days of culture. It shows the complete plantlets with elongated shoot and root systems ready to transfer to the soil (Table 3).

Among the growth regulators tested, IAA at 4.0 mg/l also showed good results, whereas 5.69 ± 0.42 mean number of roots and 5.02 ± 0.37 mean number of root length were initiated after 15 days of culture. It shows the complete plantlets with elongated shoot and root systems ready to be transferred to the soil. IAA at lower concentration has produced poor number of roots at culture condition (Table 3).

3.4 Hardening process

The regenerated healthy rooted shoots of *Dalbergia latifolia* were used for hardening. The plantlets were removed from the culture tubes with the help of a forceps and were rinsed with tap water for removing the agar medium carefully to avoid damage. The plantlets were then transplanted into plastic cups containing sand + soil + coco peat (1:1:1). The pots were covered with holed polythene bags for about 2-3 weeks and were carefully sprayed with water and shifted to the glasshouse for hardening of plantlets. The minimum and maximum temperatures of the glasshouse at the time of transplantation were 18°C and 25°C respectively. The relative humidity of the glasshouse was around 70-80%. The plantlets were watered daily (Fig 2-D).

Table 2: *In vitro* shoot multiplication of *Dalbergia latifolia* on MS medium with different growth regulators

Concentration of growth hormone mg/L	Mean no. of shoot/explants		Mean length of shoots (cm)	
	Shoot tip	Nodal	Shoot tip	Nodal
BAP (Benzyl amino purine)				
0.5	2.65±0.19	4.71±0.50	4.07 ± 0.31	5.31± 0.29
1.0	4.18±0.31	7.31 ± 0.25	5.87 ± 0.39	6.15 ± 0.43
1.5	6.49±0.82	10.62 ± 0.19	7.03 ± 0.11	9.25 ± 0.41
2.0	5.23±0.72	6.14 ± 0.48	4.09 ± 0.51	5.91 ± 0.38
2.5	4.28±0.45	4.01 ± 0.29	2.98 ± 0.28	4.76 ± 0.14
Kn (Kinetin)				
0.5	3.78 ± 0.31	5.01 ± 0.24	3.17 ± 0.45	3.63 ± 0.71
1.0	4.17 ± 0.52	5.83 ± 0.74	5.05 ± 0.14	4.18 ± 0.13
1.5	5.94 ± 0.19	9.24 ± 0.47	9.12 ± 0.56	7.38 ± 0.52
2.0	4.90± 0.61	6.23 ± 0.24	6.10 ± 0.52	4.12± 0.31
2.5	3.71 ± 0.73	4.89 ± 0.23	4.57 ± 0.701	3.43 ± 0.12

± Standard error

Table 3: Effect of growth hormones on average rooting of *in vitro* developed root of *Dalbergia latifolia*

Growth regulators mg/L	Mean number of roots	Mean root length (cm)
IBA		
1.0	1.46±0.25	1.06±0.63
2.0	3.19±0.63	1.75±0.05
3.0	5.72±0.19	7.73±0.52
4.0	4.76±0.36	5.53±0.09
5.0	5.05±0.17	3.81±0.41
IAA		
1.0	2.45±0.10	1.32±0.36
2.0	3.61±0.21	2.40±0.42
3.0	3.89±0.38	4.13±0.35
4.0	5.69±0.42	5.02±0.37
5.0	3.56±0.31	2.01±0.60

± Standard error

4. Discussion

In vitro propagation of mature trees employing vegetative explants has been a difficult task and lagging behind that of herbaceous plants due to various factors, like juvenility vs. maturity, inherent slow growing habit, exogenous and endogenous infection, presence of phenolic compounds, long complex life cycles, great genetic variations, etc. (Bonga and Durzan, 1986; Durzan, 1985; Zimmerman, 1985; Bajaj, 1991 and 1997) [7, 14, 42, 3]. It is well established that *in vitro* propagation of plant species is influenced by several factors, like genotype, age and source of initial tissue/organ which in turn are related to their endogenous hormonal status (George, 1993) [16].

In the present study confirmed the sterilization procedure adopted resulted in 90% aseptic and responsive cultures. The effectiveness of 0.1% HgCl, 4% Sodium hypochlorite and ethanol (70%) in surface disinfection of explants of tree species has earlier been reported in *Quercus robur* (Puddephat *et al*., 1997) [32], *Citrus lemon* (Rathore *et al.,* 2007) [35]. In present study, the shoot regeneration of *Dalbergia latifolia* was observed without growth regulators but the percentage of shoot regeneration was less as compared to shoots regenerated by media supplemented with different concentration of cytokinins (BAP and Kinetin). Similar observation were made by Paal *et al.* (1981) [31]. In present study the shoot multiplication protocol for *Dalbergia latifolia* was developed by culturing explants on MS medium with different concentration of BAP and Kn. Development of shoot initiated after 7 days (BAP) and 10 days (Kn) of inoculation.

Dewan *et al.* (1992) [12] reported that BAP 8.84 µM +IAA 5.7 µM was found to be most prolific combination of the treatments with regard to number of shoots, leaves and length of axillary shoots of *in vitro* established culture. Among the two cytokinins (BAP and Kinetin), BAP was found to be most effective cytokinin in inducing multiple shoot formation. However, a combination of an auxin IAA with BAP augmented multiplication of shoots. The synergetic effect of auxin along with cytokinins on shoot multiplication and shoot bud induction has been reported by several workers (Ajithkumar and Seeni, 1998; Kaur *et al*., 1998; Nodye *et al*., 2003; Bhatt and Dhar, 2004; Chand and Singh, 2004) [2, 20, 29, 6, 9].

In the present study, the synergetic effect of BAP (1.5 mg/l) induced 6.49 ± 0.82 mean number of shoots and 7.03 ± 0.11 mean length of shoots was obtained from Shoot tip of *Dalbergia latifolia*. The number of shoot developed was highest (10.62 ± 0.19) in internodal with 9.25 ± 0.41 mean length of shoots in explants treated with 1.5 mg/l of BAP. The mean number of shoot developed was highest (5.94 ± 0.19) with mean length of shoots 9.12 ± 0.56 in 1.5 mg/l of kinetin. In 1.5 mg/l (kn) concentration, the number of shoot organogenesis was highest 9.24 ± 0.47 in internodal with mean length of 7.38 ± 0.52 shoots in internodal explant. The frequency of organogenesis was highest in shoot tip and nodal explants treated with 1.5 mg/l of BAP compare than Kn.

BAP responded best for shoot formation in other leguminous trees species where BAP induced shoot multiplication has been reported are *Acacia koa* (Skolmen and Mapes, 1976) [36],

Dalbergia sissoo (Mukhopadhayay and Mohan Ram, 1981) [25], *Albizzia lebbeck* (Upadhayay and Chandra, 1983) [40] *Leucaena leucocephala* (Dhawan and Bhojwani, 1985; Nangia and Singh, 1996) [13, 28], *Prosopis juliflora* (Nandwani and Ramawat, 1991) [27], *Prosopis laevegata* (Gonzalez *et al*., 2007) [17]. Higher concentration of auxins did not supported better results as compared lower concentration of the same. Among all treatments of auxins, IAA was found to be more effective as compared to NAA and 2,4-D as also reported by Sudha Devi and Natraja (1987) [39] in *Dalbergia latifolia.* MS basal medium supplemented with IAA and NAA was reported to induce rooting from *in vitro* shoots (Soni, 2010) [37]. Barve and Mehta (1993) [5] obtained better root induction on combinations of IAA, IBA, and NAA. Kant *et al.* (2010) [19] achieved rooting by transferring of regenerated shoots to White's medium without hormones and high concentration of activated charcoal.

In the present study confirmed that IBA at 3.0 mg/l showed the best results, whereas 5.72 ± 0.19 mean number of roots and 7.73 ± 0.52 mean number of root length were initiated after 15 days of culture. Among the growth regulators tested, IAA at 4.0 mg/l also showed good results, whereas 5.69 ± 0.42 mean number of roots and 5.02 ± 0.37 mean number of root length were initiated after 15 days of culture. IBA at 3.0 mg/l showed the best results compare than IAA. The earlier report has been supported the current results IBA has been shown to be very effective in root induction in various species of tropical trees including *Eucalyptus grandis* (Macrae and van Staden, 1990) [23], *Syzygium cuminii* (Yadav *et al*., 1990) [41], *Litchi chinensis* (Das *et al*., 1999) [11], *Garcinia indica* (Malik *et al*., 2005) [24] and *Terminalia arjuna* (Pandey *et al*., 2006) [30]. IBA was found to be more effective to induce rooting with reduced rate of callus. The efficiency of IBA on rooting has been reported for several medicinal plants like *Chlorophytum borivilianum* (Purohit *et al*., 1994) [33], *Aloe polyphylla* (Abrie and Van Staden, 2001) [1]. Medium fortified with IBA and NAA induced long roots while IAA derived roots were comparatively shorter.

In most of the micropropagation protocols, hardening is a prerequisite for successful establishment of regenerated plants. *In vitro* raised plantlets were very sensitive and delicate because of controlled condition during culture period. Tissue cultured plants lose their water rapidly when moved to the external conditions. Polythene bags used for initial maintenance of humidity were removed and plants were allowed to remain in plastic cups for another 4 days before they were transferred to large pots and irrigated with tap water. The hardened and acclimatized plants were then directly planted in the field. In *Hemidesmus indicus*, 80% of the plants transferred directly into the nursery were lost, while those hardened for 2-4 weeks showed 94% survival (Sreekumar *et al*., 2000) [38]. The hardened plants of *Dalbergia latifolia* showed a good survival rate of about 90%.

5. Conclusion

The *In vitro* propagation method was developed to conserve a vulnerable tree species of *Dalbergia latifolia*. The present results recommend that BAP (1.5 mg/l) induced optimum shoot proliferation. IBA at 3.0 mg/l concentration was optimum for root induction from shoots. This protocol may be applicable to conserve the other RET plant species.

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