IN VITRO PROPAGATION OF DALBERGIA LATIFOLIA ROXB. THROUGH TISSUE CULTURE

BY
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THESIS

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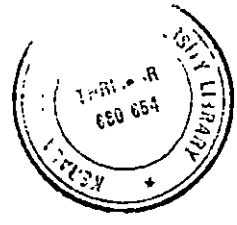

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I hereby declare that this thesis entitled "*In vitro* propagation of *Dalbergia latifolia* Roxb. through tissue culture" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship, or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled "*In vitro* propagation of *Dalbergia latifolia* Roxb. through tissue culture" is a record of research work done independently by Shri. Khages Chandra Mahato under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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LIST OF ABBREVIATION

1.	BA	Benzyle aminopurine
2.	2, 4-D	Dichloro - phenoxyacetic acid
3.	IAA	Indole - 3 - acetic acid
4.	IBA	Isopentyle adenine
5.	μM	Micromolar
6.	NAA	Napthalene acetic acid

INTRODUCTION

1. INTRODUCTION

With only two per cent of the world's total forest area and almost 15 per cent of its human and livestock populations, India faces a critical inbalance in its forest resource base. Deforestation of natural forests is occuring at an alarming rate of 1.5 million ha per year. With only 14 per cent of the total geographical area covered by closed canopy forest, severe shortages in food, fodder, fuelwood, and timber requirements can be anticipated in the near future unless remedial measures are taken up on a far footing. Extensive reforestation programme and stepping up our efforts on social and agroforestry sectors together with measures to create an awareness of the dire need for such activities in terms of ecological considerations.

Availability of sufficient quantity of planting materials of superior genotypes is the pre-requisite for an extensive reforestation programme. Plantations of tropical species with traditional stock of poor rating cause substantial economic losses. Any reforestation programme, thus, should go hand-in-hand with tree improvement programme to ensure the genetic superiority of the planting material. Production of clones through vegetative propagation of selected 'plus' trees is the simplest, most economical and least time consuming method of tree improvement. The preferance of vegetative propagation of trees, for establishing forestry plantations as opposed to the use of seed, has alreday been established and is attributed to the

maintainance of genetic superiority of mother trees through asexual propagation (Murashiage, 1974; Smith, 1986). However, conventional methods of vegetative propagation can rarely be used in forest trees as most of the economically important species are not amenable to this method of propagation, and where it is feasible, the number of seedlings that can be produced will be quite insufficient to meet the massive planting requirements.

One of the ways to overcome the above problem is to resort to micropropagation of selected genotypes through tissue culture. The primary objective of the microculture of woody plants through tissue culture has been to generate large number of propagules with predictable and desirable characters (McCown and Mc Crown,1987). Among the different approaches to in vitro propagation, induced proliferation of meristem, namely, shoot apex or axillary buds to produce multiple shoots that can eventually be rooted in a very short time seems to be applicable in most cases (Minocha, 1980).

Rosewood (Dalbergia latifolia Roxb.) is a nearly evergreen tree of moist deciduous forests. In India, it is distributed widely from North to South and East to West. The timber of this species is very hard and close grained and is mainly used for furniture, doors, windows and also veneer making. Very large trees are still available in the Kerala forests which fetch fancy prices up to Rs. 1,00,000 per cubic meter. This tree has got a

place in social forestry or man made forestry programmes also because of its soil binding and nutrient enriching capacity through nitrogen fixation. It is noticed that rosewood seeds have poor viability. Even in optimal condition, a maximum of sixty per cent germination can be expected. The capacity for coppicing as well as propagation by sucker is low in this species. Under these circumstances, production of large number of good quality seedlings is a limiting factor for plantation programmes involving rosewood. Studies on the *in vitro* propagation of *Dalbergia latifolia* Roxb. through tissue culture were taken up with the objective of standardising techniques for the production of clonal plantlets through micropropagation in the above context.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

During the last three decades, there has been a progressive upsurge of interest in the use of tissue culture for the propagation of crop plants. The culture of plant tissue is not a particularly new science. In fact, as early as 1893, Rechinger described the formation of callus on isolated fragments of stems and roots. Haberlandt (1902), the father of plant tissue culture was the first to culture plant tissues under *in vitro* condition on a nutrient medium. However, his attempts were unsuccessful because he selected a very simple medium which lacked growth regulators. The early development of tissue culture technique owes to the relentless efforts of many pioneering investigators including White (1934), Gautheret(1939), Nobecourt(1939), Miller *et al.* (1956), Reinert (1958), Steward *et al.* (1958), Bergmann(1960) and Vasil and Hilderbrandt (1965, 1967).

The best commercial application of tissue culture techniques has been in the production of true to type plants at a very rapid rate compared to conventional methods (Levy,1981). Tissue culture plants are reported to grow faster and mature earlier than their seed propagated progenies (Vasil and Vasil,1980). Multiplication of plants through tissue culture can occur through enhanced formation of axillary shoots followed by rooting of individual shoots and production of adventitious shoots either directly from the explant or through the intermediate stage of

callus, followed by rooting of individual shoots and also by somatic cell embryogenesis (Murashige, 1974; 1977).

In the first route meristems like shoot tips or axillary buds are cultured which assume genetic uniformity of the progeny to a great extent (Rao and Lee, 1986). This method is adopted commercially in various crop species for rapid clonal multiplication. The second route which is callus mediated somatic organogenesis, is not recommended for clonal propagation, but may be ideal for variant line selection. Somatic embryogenesis, the third route is limited to a few species but results in the most rapid mode of plant production (Evans et al., 1981). All these culture systems are achieved in different media constituted by a judicious combination of chemicals, hormones and other growth regulators which will differ with the crop species and purpose of culture. The greatest success of tissue culture has been achieved in herbaceous horticultural species (Hu and Wang, 1983). All species in which organogenesis and plant formation can be achieved in vitro may not be suited for large scale clonal propagation (Vasil and Vasil, 1980). In some species, the process is too expensive, the rate of multiplication slow and the mortality of plants on field planting higher.

Literature, on in vitro culture of plant tissues is very extensive and has been recently compiled in the form of reviews and books (Murashige, 1974, 1978; David and Thomas, 1979; Thomas

et al., 1979; Bajaj, 1986; Bonga and Durzan, 1987; Mascarenhas and Muralidharan, 1989).

Tissue culture technique has not been exploited extensively in trees so far. However, there are many reports of successful tissue culture from coniferous trees such as Pinus pinaster (David et al., 1982), Cedrus deodara, Pinus roxburgii (Bhatnagar et al., 1983), Pinus nigra (Kolveska-pletikapis et al., 1983), Euphedra foliata (Bhatnagar and Singh, 1984) and Pinus radiata (Horgan, 1987) in addition to a few broad leaved species.

A comprehensive review of the work carried out on the in vitro propagation of broad leaved tree species is presented here.

Success in micropropagation of different species has been reported by various workers. Indu Mathuri and Chandra (1983) has noticed induction of shoot bud of Acacia nilotica under in vitro conditions. Explants when cultured on MS medium with IAA (0.1 - 10 mg l^{-1} were found to produce root and shoots in 10-15 days. Mittal et al. (1989) has reported multiple shoots formation from excised axillary bud explant of Acacia nilotica on MS medium containing coconut water (CW) 50 per cent and BA 10^{-6} M.

Grellier et al. (1984) has stated that multiplication of Betula pendula and Betula pubescens was achieved on MS basal medium containing low level of cytokinins. Pseudo terminal buds of B. uber when placed on medium supplemented with 0.6 and 0.05

mg l^{-1} of BA and IAA respectively opened in four to five days and produced up to three leaves in a week (Vijayakumar et al., 1990).

Aboel-nil (1987) reported that the in vitro propagation method has been found successful in Casuarina species, namely, C. cunninghamiana, C. glauca and C. equisetifolia. Callus was induced from juvenile and mature stem segment explants on MS medium supplemented with 0.5 μM 2 ip and NAA. Buds were regenerated from callus tissue and stem segment explants on MS medium containing BA at 2.2-11.0 μM combined with IAA at 0.5 μM.

Callus mediated shoot bud formation and rooting of shoots in Dalbergia latifolia Roxb. has been reported (Rao, 1986; Lakshmi Sita et al., 1986). Response of in vitro produced seedling to callus mediated organogenesis on MS medium containing 1-2 mg l^{-1} BA has also been reported by Sudha Devi and Nataraja (1987). Successful induction of multiple shoots on excised hypocotyl segments and shoot tips of in vitro germinated seedling of Indian rosewood on MS supplemented with cytokinins and auxins has been achieved by Rai and Chandra (1989).

Multiple shoots from nodal segments of mature trees of Dendrocalamus strictus, Bambusa arundinacea and B. vulgaris were obtained by Nadgir et al. (1984) when explants were cultured on MS medium containing BA (0.02 mg l^{-1}) + CW (five per cent). The plantlets have been rooted and transferred to the field. It has been calculated that 10,000 plantlets can be obtained from single

seedling in a year through this method of cloning. Dendrocalamus strictus embryos start callusing soon after inoculation on B_5 medium supplemented with 2,4-D at 10-30μ M. The callus on subculture gives rise to somatic embryos and the germination of embryos take place on same medium (Rao and Rao, 1988).

Ashok (1985) has reported success in in vitro propagation of Eucalyptus citridora. He has suggested that activated charcoal increase the shoot development in this species. Juvenile tissues of in vitro germinated seedlings of E. camaldulensis showed multiple shoot production on MS supplemented with BA, adenine sulphate and sodium dihydrogen phosphate (Kumar and Ayyappan, 1987). According to Gupta and Mascarenhas (1987) the nodal explants of Eucalyptus species, namely, E. camaldulensis, E. tereticornis and E. globulus produced multiple shoot on MS medium containing kinetin (0.2 mg l^{-1}), BA (0.3 mg l^{-1}), calcium pantothenate (0.1 mg l^{-1}) and biotin (0.1 mg l^{-1}). Maximum multiple shoot induction from axillary shoot bud of E. tereticornis was noticed on modified MS medium supplemented with BA at 0.1 mg l^{-1} and NAA at 0.1 mg l^{-1} (Das and Mitra, 1990).

Mascarenhas et al. (1982) carried out micropropagation of Hevea brasiliensis and found out that terminal buds from 10-20 year old trees when cultured on MS medium along with 0.5 ppm kinetin, 2.0 ppm BA, 200 ppm casein hydrolysate, 0.1 ppm calcium pantothenate and 0.1 ppm biotin induced three shoots/explant.

Differentiation of embryoids from the anther derived callus was achieved by transfer of fresh callus initiated on MS basal medium containing 0.1-10 ppm 2,4-D and 1.5 ppm kinetin. It was found that embryoids appeared only when high levels of sucrose (10 per cent) were incorporated in both the callus initiation and differentiation media (Paranjothy, 1987).

Commercially feasible micropropagation of mountain laurel (Kalmia latifolia) was reported by Lloyd and McCown (1980). For multiplication, shoot tip explants placed initially in liquid woody plant medium (WPM) supplemented with 4-16μM 2 ip produced axillary shoots by one to two months. These new shoots were excised from the original explant and placed on the same WPM solidified medium. Shoots were rooted in 100 per cent peat medium in a warm (30° to 35°C), high humidity chamber under 24 hour cool white flourescent light (30 $Em^{-2}Sec^{-1}$). Hormone treatment did not appear to be necessary for root induction. Once rooting occurred, the plants were treated as seedlings in normal nursery production programmes after hardening in green house. It has been calculated that at least 7000 shoots can be obtained per one sq. ft of culture shelf space per year.

In Leucaena leucocephala, for tissue culture propagation from two to three meter tall trees, lateral bud explants were cultured on MS medium with BA (3.0 mg l^{-1}) and NAA (0.05 mg l^{-1}). This method was found to be best suited for shoot multiplication

in four to five weeks (Goyal *et al.*, 1985). Datta and Datta (1985) reported that shoots were developed from nodal explants on MS medium supplemented with 1 mg l^{-1} BA, whereas multiple shoots on MS having 2 mg l^{-1} BA.

In vitro shoot multiplication of *Populus glandulosa* via callus was obtained on MS, WPM or GD (Gresshof and Doy's) medium with 3.0 mg l^{-1} zeatin and 1.0 mg l^{-1} NAA in MS, or 1.0-13.0 mg l^{-1} zeatin and 0.5-1.0 mg l^{-1} NAA in WPM. Shoot induction from callus was optimum on MS with 1.0 mg l^{-1} zeatin and 0.5 mg l^{-1} NAA, or 1.0 mg l^{-1} IAA and on WPM with 3.0 mg l^{-1} zeatin and 0.5 mg l^{-1} NAA (Jang *et al.*, 1988).

Patri *et al.* (1988) demonstrated that nodal and terminal cuttings derived from aseptic seedlings of *Pterocarpus santalinus* produced shoots on MS medium (1/4 salt) plus BA (3 μM) plus adenine (0.4 μM).

Callus induction followed by differentiation was reported by Lakshmi Sita *et al.* (1980) in *Santalam album* from endosperm tissue collected from fresh green fruit. The explants were cultured on MS medium supplemented with 2,4-D (1.0-2.0 mg l^{-1}), kinetin (0.1-0.2 mg l^{-1}), BA (0.5-2.0 mg l^{-1}) and NAA (1.0 mg l^{-1}). Callus initiated from these culture was routinely maintained on MS medium supplemented with 1 mg l^{-1} 2,4-D.

Scott *et al.* (1988) reported that embryos of *Shorea roxburghii* gave good shoot development in liquid medium containing 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} BA.

In vitro propagation of tamarind has been achieved from all explants of aseptic seedlings (namely, stem, leaf, root and cotyledon) when cultured on MS medium supplemented with kinetin (0.2), BA (0.5), NAA (0.5) and biotin (0.1) mg l^{-1} (Mascarenhas *et al.*, 1987). Four to five shoots developed from hypocotyl, one from nodal and three from shoot tip explants. Kopp and Nataraja (1990) reported shoot tip cultures in *Tamarindus indica* on MS media containing BA (0.5-5 mg l^{-1}).

Gupta *et al.* (1980) reported the successful induction of multiple shoot formation from excised terminal buds of 100 year old elite teak trees. Performance of these *in vitro* trees are being studied in the field (Mascarenhas *et al.*, 1987). They have suggested that over 500 plants can be produced from a single bud of a selected tree on MS medium containing kinetin (0.15 mg l^{-1}) and BA (0.15 mg l^{-1}).

Axillary buds of zyzyphus (ziziphus) cv. Geumsung showed the best shoot and root growth after 8 weeks when 500 mg l^{-1} activated charcoal was added to half strength Murashige and Skoog medium with 0.5 mg l^{-1} BA. However, cv. Bokjo responded best to 1000 mg l^{-1} activated charcoal in the above medium (Kim and Lee, 1988).

2.1. Factors influencing success of *in vitro* propagation

2.1.1. Explant

As a rule, larger the size of explant, the more rapid the growth and the greater are the rates of survival (Hussey, 1983). When tissues are cut, the cut surfaces turn brown due to the oxidation of phenols in the damaged cells (Monaco *et al.*, 1977). If the explant size is small, the cut surface:volume ratio is high and there will be difficulty in the survival of the explant. In meristem culture for virus elimination, explants of length 0.1-0.5 mm are used (Hussey, 1978).

Within any plant, tissues differ in their degree of determination and thus their ability to undergo morphogenesis. Buys *et al.* (1966) suggested different nutrients for different explants.

The youngest and less differentiated tissues are found in plant meristems and the culture of this tissue has been successful in a wide range of species (Hughes, 1981). During the maturation process of tissues several physiological changes occur which may influence the *in vitro* behaviour of the explants (David, 1982). In general, young tissues have a higher degree of morphogenic competence than older tissues. Rao (1986) and Lakshmi Sita *et al.* (1986) found that callus could be induced from young tissues of *Dalgergia latifolia* Roxb. but not from the mature

tissues. Pierik and Steegman (1975) also observed that the ability of Rhododendron stem segments to produce roots decreased with increasing age of the stem. Durzan (1984) reported that tissues can be rejuvenated or at least invigorated by acclimatizing plants to in vitro conditions before explants are taken. Quoirin et al. (1974) reported that elongated shoots rooted more easily than small shoots on their medium.

2.2. Surface sterilization

The objective of surface sterilization is to remove all the microorganisms present on the explant with minimum damage to the plant part. Explants for surface sterilization are usually cut to a size larger than that of the final explant and after sterilization they are trimmed to smaller size and transferred to the medium (Hussey, 1979).

Fungal and bacterial contamination in plant tissue culture are very common. To check these problems, fungicides and antibiotics are used either as surface sterilant or medium additives. However, most of the systemic fungicides and some antibiotics inhibit growth of the plant cultures. Dodds and Roberts (1985) suggested to avoid the use of antibiotics for sterilization since they or their degradation products may be metabolised by plant tissues with unpredictable results. However, Davey et al. (1980) suggested that the application of

antibiotics like streptomycin, ampicillin or nystatin may be done when the material is infected with known bacterial or fungal contaminants. Several workers have reported the use of various fungicides in cultures for reducing fungal contamination (Brown et al., 1982; Shields et al., 1984).

The most commonly used surface sterilant is an aqueous solution of sodium hypochlorite. A dilution of 10 per cent (v/v) is normally effective for the purpose, particularly when it is mixed with a surfactant like teepol or similar liquid detergent. Sodium hypochlorite being toxic to plant cells, it is necessary to wash the treated tissue twice or thrice with sterile distilled water (Hu and Wang, 1983). Both concentration and time of treatment can be increased or reduced according to the need. Concentration ranging from 1.0 per cent (Minocha, 1980) to 10.0 per cent (Kuo and Tsay, 1977) have been used. In case of mercuric chloride, Lakshmi Sita et al. (1986) used 0.1 per cent concentration for 10-12 minutes for sterilization of explant taken from seedlings of Dalbergia latifolia Roxb. Alcohol alone or in combination with other surface sterilants has been used for sterilization (Bonga, 1982). Maroti and Levi (1977) reported that it was better to rinse first with ethanol (45 per cent) for three minutes followed by a 10 minutes bleach treatment (5.0-10 per cent) and finally three rinses with sterile water.

2.3. Culture medium

2.3.1. Basal medium

A wide variety of plant tissue and cell culture media have been reported. The earliest and widely used basic media were White (1943) and Heller (1953) . Since 1960, however, most researchers have been using MS (Murashige and Skoog, 1962), B_5 (Gamborg et al., 1968) or SH (Schenk and Hildebrandt, 1972) media. But after 1980, the most popular media are DCR (Gupta and Durzan, 1985) and WPM (Lloyd and McCown, 1980) especially for woody species. The MS salt composition is used very widely, particularly if the desired objective is plant regeneration. The B_5 medium has been used for cell and protoplast culture (Gamborg et al., 1981). The SH medium is similar to B_5, but with slightly higher levels of mineral salts.

Another medium designated as N_6 (Chu, 1978) was developed for cereal anther culture and becoming recognised as a suitable medium for tissue culture of cereal crops.

2.3.2. Growth regulators

The most important factor in successful tissue culture is the addition of growth regulators (Krikorian, 1982). But no universal ratio of auxin and cytokinin has so far been developed for root and shoot induction. However, Hempel (1979) concluded

that in majority of cases, callus growth was supported by auxin. Hasegawa (1980) also reported that high concentration of auxin may not only inhibit axillary budbreaking but also induce callus formation.

For axillary shoot proliferation, cytokinin has been utilised to overcome the apical dominance of shoot and to enhance the branching of lateral buds from leaf axils (Murashige, 1974).

Some other growth regulators, like gibberellins and abscisic acid have some times shown dramatic effects on overcoming bud dormancy and achieving organogenesis (Borkowska and Habdas, 1982 and Yostuya et al., 1984).

2.3.3. Carbon and energy sources

Sucrose is the main carbon energy source for most of the plant tissue culture. Glucose and fructose may be substituted in some cases, but most other sugars are reported to be very poor (George and Sherrington, 1984).

2.3.4. Vitamins

The most common vitamins which are used in plant tissue culture are pyridoxine, nicotinic acid, biotin, riboflavin, folic acid and thiamine. Among these, thiamine is very essential and usually added in plant tissue culture media at levels of 0.1 ppm to 1.0 ppm. Thorpe and Patel (1984) has reported that thiamine

is the most often added vitamin, followed by nicotinic acid and pyridoxine.

2.3.5. Other organic compounds

For successful growth of tissues and organs, addition of complex organic compounds to the basic medium is reported by Conger (1981). Some of these are casein hydrolysate, coconut water, yeast, malt extract and orange and tomato juice. Many researchers feel that when such compounds are added to the basic medium, they have little control over the experiments and it has therefore been recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981).

The discovery of myoinositol in coconut water (CW) by Pollard et al. (1961), led to the inclusion of inositol in the plant tissue culture media. However, in the presence of CW addition of inositol may not be much beneficial. Coconut water is reported to be promoting growth and differentiation in Datura embryos (Van Overbeek et al., 1941) and in callus mediated organogenesis of Dalbergia latifolia Roxb. (Lakshmi Sita et al., 1986).

The addition of activated charcoal (AC) to the culture media may have beneficial effect. AC is reported to prevent browning in cultured tissues (Tisserat, 1979). Effects of AC may be attributed to three factors such as darkening of the medium

(Proskaur and Berman, 1970) adsorption of inhibitory compounds (Wang and Huang, 1976) and adsorption of plant growth hormones from the medium (Weatherhead et al., 1978). Activated charcoal is usually added between 0.2 and three per cent.

2.4. Culture conditions

The culture conditions play an important role in the success of tissue culture. The physical form of medium, pH, light, temperature, relative humidity and time of the year etc. play an important role in in vitro growth and differentiation.

The physical form of the medium, that is, whether in the solid or liquid state is important for organogenesis. The pH of the plant tissue culture medium is an important variable in the culture medium. Plant cells in culture require an acidic pH and an initial pH of 5.5 to 5.8 is optimum (Gamborg and Shyluk, 1981). Bonga (1982) reported that the pH of the medium is usually set at about 5.0 for liquid formulations and at about 5.8 for agar-gel media.

Light requirement for differentiation involve a combination of several components, namely, intensity, quality and duration. An optimum combination of these is necessary for certain photomorphogenic events. According to Murashige (1977), the optimum day light period is 16 hours for a wide range of plants.

Yeoman (1986) reported that the usual environmental temperature of the species concerned should be taken into account. However, most tissue cultures are grown successfully at temperatures around 25±2°C. In general, tropical species need higher temperatures.

Relative humidity is rarely a problem except in arid climates, where rapid drying occurs. Hu and Wang (1983) reported that air humidity is infrequently controlled and when it is controlled, 70 per cent has been found to be the most frequent setting.

It has been noticed that tissues taken from field grown plants are not equally amenable to tissue culture conditions throughout the year. Borrod (1971) determined that wood from a chestnut clone formed callus the best in March and the worst in December, whereas, another clone showed just reverse response.

2.5. Genotype

The genotype of the plant chosen for propagation also influence response in culture. Genotype specific effects have been reported for Anthurium andraeanum and A. scherzerianum (Pierik and Steegman, 1976).

2.6. Root induction

Since auxin is essential for root initiation, majority of rooting media contains auxin. The concentration of rooting

hormone required is often critical to provide sufficient stimulus to initiate roots while preventing the excessive formation of callus (Yeoman, 1986). Sometimes, a combination of auxins may give better response (Gupta et al., 1980). The root elongation phase has been found to be very sensitive to auxin concentration. High concentrations of auxin inhibited root elongation (Thimann, 1977).

Most of the researchers have reported that in vitro rooting can successfully be achieved by reducing salt concentrations in the media, particularly in MS, B_5 and WPM, which contain high salt concentrations. Abundant rooting was observed when the salt concentration was reduced to one-half or one-third in the medium (Lane, 1979). But in such cases, sometimes poor top growth resulted (Gupta et al., 1981).

Yeoman (1986), Schwarz et al. (1988) and Vijayakumar et al. (1990) have advocated in vivo rooting approach which may provide a simple, highly efficient and more economical methodology for completing the process of regeneration. They took shoots over five mm in length and planted them in plastic pots containing a mixture of peat, vermiculite and sand in ratio of 4:2:1. The shoots were maintained in a high humidity environment and watered daily. During the first two weeks, a water solution containing 15μM NAA was administered four times at equally spaced intervals to promote rooting. Twenty per cent of shoots rooted after eight weeks.

2.7. Acclimatization and planting out of plantlets

Acclimatization is one of the important phases of micro-propagated plants. The success in acclimatization depends upon not only post-transfer conditions but also the pre-transfer culture condition (Ziv, 1986). Vitrification conditions adversely affect the survival of plantlets *in vivo*.

Hu and Wang (1983) suggested a period of humidity acclimatization for the newly transferred plantlets to make them adapted to the external environment. The survival of plantlets depended upon the vigourous growth and newly produced leaves at the time of planting out (Sutter *et al.*, 1985). Rajmohan (1985) reported the use of plastic microscope covers for maintaining 90-100 per cent relative humidity and obtained 55-60 per cent survival of *in vitro* produced jack plantlets.

2.8. *In vitro* cytological changes

Larkin and Scowcroft (1981) reported structural and numerical changes in chromosomes in association with *in vitro* regeneration plantlets. Numerical changes have been observed in callus cultures of several crops (Rice, 1982; Evans and Sharp, 1983). Long term cultures resulted in tissue culture induced variability in chromosome number in both the callus and in the plants regenerated from it. The origin of chromosomal instability of cultured tissue has been attributed to endoreduplication

(Partanen, 1973), nuclear fusion (Mitra and Steward, 1961) as well as abnormal spindle formation (Sunderland, 1973).

But in case of apical meristem culture, such variations are rare (Ancora et al., 1981). According to Bonga (1982) the meristematic line, consisting of specific cells in more or less fixed position exercise a strict control over the mitotic events. Any mutated cell in a multicellular shoot apex will mostly form only limited areas of tissue which will finally be eliminated from the meristematic region (Broertjes and Van Harten, 1978).

2.9.Economic considerations

In attempting to make direct economic comparisons between different systems of producing plantable seedlings for reforestation is always confronted with making certain assumptions within the limits of recognised constraints. In order to obtain some reasonably reliable comparisons between seedlings produced via tissue culture and those produced by conventional nursery techniques three broad aspects are considered namely, (1) capital outlay in the form of land, building, facilities and equipment; (2) total direct production costs includings supervisory personnel, labour, taxes, insurance, supplies and expendibles, equipment operation, lighting operation and (3) the potential value of the product produced within a given period, in forestry, the yield of forest products at the end of the rotation (Brown and Sommer, 1982).

The economic analysis of mass production of tissue culture plants points to the fact that a high rate of multiplication is essential to lower down the total cost production. Anderson et al. (1977) and De Fossard (1981) has also stressed on high multiplication rate for cost reduction. Rajeevan and Pandey (1986) reported that the in vitro rooting and hardening off is the most expensive stages. Some other problems may also arise namely, labour intensive, insufficient rooting, delayed growth due to poor root function and damage to roots during planting (Maene and Debergh, 1983).

However, in several cases, the reported cost of tissue culture plants is much less than the price of clonal plants produced by conventional methods in India. Chaturvedi and Sinha (1979) reported that in Dioscorea floribunda tissue culture process offers very fast rate of propagation, which is not possible by the conventional methods.

Rajmohan (1985) reported that by in vitro axillary bud proliferation techniques in jack, on an average 65.38 numbers of plantlets could be produced per year from a single explant. The unit cost of producing one jack plantlet including one month hardening was found to be Rs 9.09 in comparison to Rs 8.00 the cost of a jack fruit graft.

Results of the analysis of in vitro plantlets production show that cost of tissue culture plantlets will be relatively

high, but this cost will be more than compensated by savings realized by reduced rotation age (Hasnain et al., 1986).

It is clear from above cited review that success in micropropagation of tropical trees is not satisfactory. The research workers have been able to achieve plantlet formation in only a few tropical tree species. For routine propagation of desirable genotypes of tropical species, a lot of efforts are still needed.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study, namely, in vitro propagation of Dalbergia latifolia Roxb. through tissue culture was carried out in the College of Forestry of Kerala Agricultural University, Vellanikkara, Thrissur during 1989-1992. The materials and methods used for the experiment are described in detail in the sections to follow.

3.1. Explant

Nodal and internodal segments collected from mature (12 year old) tree as well as seedlings (two and a half year old) of Dalbergia latifolia Roxb. available in the main campus of the University were used as explants in the present study.

3.1.1. Collection and preparation of explants

Explants collected from field grown trees harboured lot of fungus and other microbes. To check the contamination in culture the lower branches of the tree were sprayed with the mixture of a systemic fungicide Bavistin 50 per cent WP (Carbendazim) and the contact fungicide Dithane M-45 (Mancozeb), 0.3 per cent each once in 10-15 days.

Stem segments of approximately 25-30 cm with 10-12 nodes obtained from the mature tree and those of length 10-15 cm from the seedlings were carefully excised using sterilized surgical blades or scalpel and brought to the laboratory as quickly as

possible. The leaves were removed and stem segments washed thoroughly in tap water so as to remove all the dust and fungicide adhering to them. After drying them using sterilized blotting paper pieces they were swabbed with cotton dipped in absolute alcohol.

3.1.2. Surface sterilization

Surface sterilization was carried out under perfect aseptic conditions in a Laminar Flow air cabinet. The stem segments were cut into nodal segments of 1.0-1.5 cm and put into sterilant. The different sterilants used are listed in Table 1. In all cases the explants were kept immersed in the chemical for the required period. The explants, after surface sterilization, were rinsed thrice with sterile distilled water to remove traces of the sterilant from the surface.

Another experiment was conducted to estimate the optimum size of the explant for enhanced release of axillary buds and to select the ideal material for callus mediated organogenesis. The details are given in Table 2.

3.2. Culture media

The basic media used in the present study were Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and woody plant (WPM) medium (Lloyd and McCown, 1980). The composition of the media are given in Table 3. The basic media were supplemented

Table 1. Sterilants used, their concentration and duration of treatment for *in vitro* culture of *Dalbergia latifolia* Roxb.

Sterilants	Concentration (%)	Duration (minutes)
Chlorine water	-	3-15
Mercuric chloride	0.1	3-15
Ethyl alcohol	95.0	3-12

Table 2. Explants tried for *in vitro* multiplication of *Dalbergia latifolia* Roxb.

Method of multiplication	Explant
1. Enhanced release of axillary buds	Nodal and internodal segments (measuring <0.5 cm, between 0.5 cm and 1.0 cm and > 1.0 cm in length) taken from 25-30 cm long branches.
2. Callus production (for callus mediated organogenesis)	Nodal and internodal segment as described above

Table 3. Composition of Murashige and Skoog and Woody Plant Medium.

A. Murashige and Skoog basal medium

Constituents	mg l^{-1}	Constituents	mg l^{-1}
	Inorganic constituents		
Major constituents		Minor constituents	
NH_4NO_3	1650	H_3BO_3	6.2
KNO_3	1900	$MnSO_4 \cdot 4H_2O$	22.3
$CaCl_2\ 2H_2O$	440	$ZnSO_4 \cdot 7H_2O$	8.6
$MgSO_4 \cdot 7H_2O$	370	KI	0.83
KH_2PO_4	170	$Na_2MoO_4 \cdot 2H_2O$	0.25
		$CuSO_4 \cdot 5H_2O$	0.025
		$CoCl_2 \cdot 6H_2O$	0.025
		Na_2EDTA	37.3
		$FeSO_4\ 7H_2O$	27.8
	Organic constituents		
Pyridoxine	0.5		
myo-inositol	100		
Glycine	2.0		
Thiamine-Hcl	0.1		
Nicotinic acid	0.5		

(Contd.....)

Table 3 (Contd.....)

B. Woody plant medium

Constituents	mg l^{-1}	Constituents	mg l^{-1}
	Inorganic constituents		
Major constituents		Minor constituents	
NH_4NO_3	400	H_3BO_3	6.2
KH_2PO_4	170	$CuSO_4 \cdot 5H_2O$	0.25
$MgSO_4 \cdot 7H_2O$	370	$MnSO_4 \cdot H_2O$	22.3
K_2SO_4	990	$ZnSO_4 \cdot 7H_2O$	8.6
$Ca(NO_3)_2 \cdot 4H_2O$	556	$Na_2MoO_4 \cdot 2H_2O$	0.25
$CaCl_2 \cdot 2H_2O$	96	Na_2 EDTA	37.3
		$FeSO_4 \cdot 7H_2O$	27.8
	Organic constituents		
Pyridoxine	0.5		
myo-inositol	100		
Glycine	2.0		
Thiamine-HCl	1.0		
Nicotinic acid	0.5		

with different cytokinins, auxins, vitamins, casein hydrolysate, coconut water etc. in the different experiments.

3.2.1. Media preparation

The chemicals used for preparing various media were of analytical grade from British Drug House (BDH), SISCO Research Laboratories (SRL), Merck or Sigma. Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the media. Stock solutions of major and minor nutrients were prepared first, by dissolving the required quantity of chemicals in double glass distilled water and stored under refrigerated conditions in amber coloured bottles. The stock solution of nutrients were prepared fresh every four weeks and that of vitamins, amino acids and phytohormones prepared fresh every week. Specific quantities of the stock solution of chemicals were pipetted out into a 500 ml beaker. Sucrose and inositol were added fresh and dissolved. Then the volume made up to about 500 ml by adding double glass distilled water. The pH of the solution was checked with a pH indicator paper and adjusted to 5.8 using 1.0 per cent NaoH/HCl. To prepare semi-solid medium, good quality agar was added and the final volume made up. Agar was then melted by keeping the solution in a water bath maintained at a temperature of 90-95°C. In case of liquid medium, agar was avoided. The medium was poured into culture tubes (15x2.5 cm)/conical flasks (250 ml) which were washed thoroughly, rinsed in distilled water and oven dried. Corning brand culture tubes and conical flasks were used.

Sterilization of media was done for 15-20 minutes at a pressure of 1.1 kg/cm^2 (121°C). After sterilization, the culture tubes/flasks were stored in an air-conditioned culture room.

3.3. Shoot induction

3.3.1. Standardisation of medium supplements

Studies were conducted to determine the effects of various growth regulators on shoot induction, growth and multiple shoot production from axillary buds. Details of the initial experiments conducted using WPM and MS media are presented in Table 4. For standardising the basal proliferation media detailed studies were conducted using different levels of growth regulators (Table 5) and compounds other than growth regulators (Table 6).

3.3.2. Standardisation of physical conditions

In order to study the effect of the physical conditions of culture on shoot induction, growth and multiple shoot formation in axillary bud explants, an experiment was conducted where liquid and semi solid medium with varying pH and incubation under different light intensities were tried. The details of the treatments are given in Table 7.

Table 4. Details of initial experiment for the culture establishment from primary nodal segments of Dalbergia latifolia Roxb.

Basal medium	Growth of regulators
WPM/MS	Nil
	kinetin 1.0 ppm.
	kinetin 1.0 ppm + IAA 0.1 ppm
	kinetin 1.0 ppm + IBA 0.1 ppm

Table 5. Trials on the induction and elongation of axillary shoots by different levels of growth regulators in cultures of Dalbergia latifolia Roxb. using primary nodal segments as explants.

Basal medium	Treatment
WPM/MS	1. 3x2 Combinations of kinetin (1.0, 2.0, 3.0 ppm) and IAA (0.0, 0.1 ppm)
WPM/MS	2. 3x4 combinations of kinetin (1.0, 2.0, 3.0 ppm) and IBA (0.1, 0.5, 0.01, 0.05 ppm)
WPM/MS	3. Five levels of BA (0.25, 0.5, 1.0, 2.0, 3.0 ppm) alone
WPM/MS	4. 2x1 combinations of BA (1.0, 2.0,4.0 ppm) and IAA (0.1 ppm)
WPM	5. 2x2 combinations of BA (1.0, 2.0 ppm) and NAA (0.1, 0.5 ppm)
WPM	6. 2x1 combinations of BA (1.0, 2.0 ppm) and 2,4-D (0.1 ppm)
WPM/MS	7. 2 ip (0.5, 1.0, 2.0, 3.0 ppm) alone and with IAA 0.1 and 0.5 ppm
WPM	8. Different level of Gibberellic acid (0.5, 1.0, 2.0 ppm) alone or with BA and kinetin (0.5 ppm each)

Table 6. Trials on the induction and elongation of axillary shoots by compounds other than growth regulator in cultures of Dalbergia latifolia Roxb. using primary nodal segments as explants.

Basal medium	Supplement	
	compoud	concentration
1. MS	Adenine sulphate	1, 2, 3, 4, 5, 6 7, 8 ppm
2. MS + BA 0.25 ppm	Casein hydroly-sate	100, 500, 1000 ppm
3. MS + BA 1.0 ppm	NaH_2PO_4, $2H_2O$	0.17 g l^{-1}
4. MS	Glyphosate	0.17 ppm
5. MS + BA 0.25 ppm	Coconut water	10 and 15 per cent
6. MS/WPM + BA 1.0 ppm	Activated charcoal	1000 mg l^{-1}
7. ½MS/½WPM + BA 1.0 ppm	Activated charcoal	500 mg l^{-1}
8. 2MS/2WPM + BA 1.0 ppm		

Table 7. Physical conditions of culture tested on axillary shoo induction, growth and multiple shoot formation in axillar bud culture of Dalbergia latifolia Roxb.

Explant	Physical condition			
	Semi solid medium	liquid medium	pH of medium	Light intensities during incubation
Nodal segment	0.8 per cent agar	shaking with subculturing in 12 h intervals	4.7, 5.0, 5.2, 5.5, 5.8, 6.0.	0, 500, 1000, 2000 Lux for 16 h

To establish axillary bud cultures in liquid for shoot growth and proliferation, sterilized explants were initially incubated in liquid WPM along with 2 ip (1.6 ppm). The cultures were sub-cultured in every 12 h period. After three and a half days of initial incubation, they were transferred to stable WPM supplemented with 2 ip (1.6 ppm) for shoot initiation and growth.

3.4. Establishment of continuous culture

In order to achieve this, two methods were tried.

1. The multiple shoots produced from the explant were excised individually and the original explant sub-cultured to fresh medium.

2. The multiple shoots were removed and cut into nodal segments of 6-7 mm. These nodal segments were cultured on fresh MS/WPM medium supplemented with different growth regulators and compounds as given in Table 8.

3.5. Root induction

3.5.1. *In vitro* rooting

Shoots (three and five cm) excised from the shoot proliferating culture were used for *in vitro* rooting. Rooting was tried in half strength MS/WPM salts with supplements and vermiculite/vermiculite-sand mixture. Effects of various medium supplements and light conditions during root induction were studied. The details of the trial are given in Table 9.

3.5.2. *In vivo* rooting

A trial was also attempted to get rooting under *in vivo* condition providing high humidity (90 per cent RH) and cool temperature ($24\pm2^{\circ}C$). The cut end of individual shoots were treated with 1000 ppm IBA made up in alcohol. They were transferred to small pots with vermiculite alone or vermiculite + sand (1:1). The shoots were covered by polythene bag and kept air tight for 15-20 days.

Table 8. Trials on establishment of continuous culture of Dalbergia latifolia Roxb. using nodal segments from primary cultures.

Basal medium MS and WPM

Basal media	Medium supplements (ppm)
WPM/MS	1. Adenine sulphate (0.5, 1.0)
	2. BA (0.25, 0.5, 1.0)
	3. kinetin (0.5, 1.0)
	4. 2 ip (0.5, 1.0)
	5. BA (0.5) + kinetin (0.5)
	6. BA (0.5) + kinetin (0.5) + NAA (0.5)
	7. BA (0.5) + kinetin (0.5) + $AgNo_3$ (0.5)
	8. BA (0.5) + kinetin (0.5) + GA_3 (0.5)
	9. BA (0.5) + 2 ip (0.5)
	10. BA (0.5) + 2 ip (0.5) + NAA (0.5)
	11. BA (0.5) + 2 ip (0.5) GA_3 (0.5)

Table 9. Trials on rooting of *in vitro* produced shoots of *Dalbergia latifolia* Roxb.

Basal medium	Treatment (under Dark, 500, 1000 and 2000 lux)
1. ½MS/½WPM	1. IBA (0.25, 0.5, 1.0, 2.0 ppm)
	2. IAA (0.25, 0.5, 1.0, 2.0 ppm)
	3. NAA (1.0, 2.0 ppm)
	4. Biotin + IAA Combination
	5. 2 x 2 combinations of IBA and IAA (1.0, 2.0 ppm)
	6. Phloroglucinol, IAA and IBA combinations
	7. Activated charcoal (1.0 per cent) + pulse treatment with IBA (1000 ppm)
2. Full MS + AC (1.0 per cent)	1. Pulse treatment with IBA (1000 ppm)
3. Vermiculite + Sand (1:1)	1. Pulse treatment with IBA (1000 ppm)
4. Vermiculite alone	1. Pulse treatment with IBA (1000 ppm)

3.6. Planting out and acclimatization

In vitro produced plantlets were planted out from the test tube following a series of acclimatization processes. After proper root development under *in vitro* the plantlets were taken out from flasks or tubes in such a way that no damage was caused to the root system. The roots were washed gently under running tap water to remove the adhering medium. The roots were dipped in 0.2 per cent Bavistin solution for five minutes and the plantlets transferred to potting mixture contained in small pots.

3.6.1. Standardisation of potting mixture

Different potting mixtures were used to select the best one for maximum survival and establishment of the plantlets. The following potting mixtures were used after autoclaving at a pressure of 1.1 kg cm^2 (121 °C) for 25 minutes.

i. Soilrite
ii. Vermiculite alone
iii. Vermiculite + sand (1:1)
iv. Sand + soil (1:1)
v. Sand + Soil (0.5:1.5)

The potting mixtures were filled in small pots with a hole, for facilitating drainage. Plants were nourished with MS/WPM nutrients solution (with macro and micro nutrients at half strength) having a pH 5.8 at weekly intervals.

3.6.2. Control of temperature and humidity

Control of temperature and humidity during acclimatization was achieved by covering the transplanted plantlets with transparent plastic covers and keeping the pots under shade. This was done based on the observations made by Rajmohan (1985). Spraying of cold water into the covers at an interval of four to five hours using a hand sprayer with fine mist nozzle enable to maintained high humidity (90-100 per cent RH) and mild temperature ($25\pm3^{\circ}C$) inside the plastic cover.

3.6.3. Removal of polythene bag or cover

After 15-20 days, when the plants showed initial signs of establishment in the pot, the polythene bag was removed for one to two hours daily. Gradually the time was increased. After 20-25 days of initial planting, the polythene bag was totally removed. After 40 to 45 days of initial planting the individual plantlets were transferred to larger polythene bags (22.5 x 10 cm) containing a homogeneous mixture of equal proportions of sand, soil and FYM. At this stage, the plantlets were treated like any other containerised seedlings.

3.7. Field planting

The plantlets became ready for field planting by about four months growth in the polybag. Three such plantlets were

transferred to the field in the middle of May (1991) in front of College of Forestry, Vellanikkara, Thrissur.

3.8. Economics of mass multiplication of Dalbergia latifolia Roxb. clones through tissue culture techniques

The cost of production of Dalbergia latifolia Roxb. plantlets using shoot bud explants was worked out based on the facilities available in tissue culture laboratory where this work was carried out. Presently, this laboratory has a potential of maintaining 5000 cultures for multiplication as well as rooting and 23,000 plantlets for hardening. One Scientist (Rs 2800/month) and one Technician (Rs 1200/ month) were considered necessary for lab work. Based on the rate of culture establishment, rate of multiplication, rooting response of shoots, survival of the plantlets and the maximum capacity of laboratory, the number of plantlets that can be produced per year from 75 initial explants was estimated. In the estimation the total cost of building, equipment, glassware, chemicals, miscellaneous items and labour cost was distributed over the years according to their potential/durability. The cost of production of one Dalbergia latifolia Roxb. plantlets including the expenses of hardening period, was finally worked out.

RESULTS

4. RESULTS

The results of various experiments carried out to standardise the optimum culture conditions for production of maximum number of clonal seedlings of Dalbergia latifolia Roxb. through tissue culture method are presented below.

4.1. Explant choice and sterilization

4.1.1. Explant choice

Results of the trial on screening various explants of Dalbergia latifolia Roxb. for initiating bud release and callus production are presented in Table 10. Preliminary results indicated that the nodal segments one cm or more in length was the most suitable for initiating enhanced release of shoot buds. In the initial trial, 60 per cent nodal segments (one and a half cm) initiated shoot release within 10 to 15 days of culture. Explants measuring more than one and a half cm length were more prone to contamination under culture conditions than were the smaller ones.

Trials to induce callusing (for callus mediated organogenesis) in various explants as listed in Table 2 were quite effective. The percentage of compact callus which could be used for organogenesis, however, was low.

Table 10. Response of various explants of Dalbergia latifolia Roxb. on initiating shoot bud release and callusing (Average of two replications with 12 cultures per replication)

Response	Explants showing response (%)				
	Nodal segment*			Internodal segment*	
	<0.5 cm length	1.0 cm length	1.5 cm length	0.5 cm length	>1.0 cm length
1. Shoot bud release[a]	25	45	60	0	0
2. Callusing[b] (friable callus)	-	-	60	-	65
3. Callusing[b] (Compact green callus)	-	-	15	-	15

* - Rest of explants as per Table 2 failed to show any response

0 - No Response

- - Not tried

a - Culture medium as per Mallika et al. (1990)

b - Culture medium as per Lakshmi Sita et al. (1986)

4.1.2. Seasonal influence on explant contamination and culture establishment

Seasonal influence on contamination rate and culture establishment in the nodal and internodal segments of Dalbergia latifolia Roxb. is presented in Table 11. In general, prophylactic spraying of the mother trees with fungicides greatly reduced fungal contamination of the explants. While the unsprayed material showed 90-100 per cent contamination throughout the year, explants from sprayed mother trees showed only about 15 per cent contamination. The maximum culture establishment was found during January to March.

4.1.3. Surface sterilization of explants

Since the field grown material is known to be heavily infested with microbes and spores, special care was taken for surface sterilization of the explants. Effect of various sterilizing agents on nodal and internodal segments of Dalbergia latifolia Roxb. are presented in Table 12. Chlorine water was found to be least effective. Mercuric chloride (0.1 per cent) for 12 minutes was found to the most effective sterilizing agent. Increasing the treatment time led to the death of explants. Ethyl alcohol was not an effective sterilant in Dalbergia latifolia Roxb.

Table 11. Influence of explant collecting season on contamination rate and culture establishment from sprayed and unsprayed mother trees of Dalbergia latifolia Roxb (Average of three replications with 24 cultures per replicatlion)

Basal medium: WPM + 1.0 ppm kinetin + 0.1 ppm IAA

Month	Contamination (%)		Culture establishment (%)
	sprayed	unsprayed	
January	10	90	70
Ferbruary	10	97	70
March	10	97	60
April	5	90	50
May	5	95	50
June	15	90	55
July	20	100	55
August	15	100	50
September	15	90	40
October	15	90	45
November	10	100	45
December	10	90	45

Culture period - three weeks.

Table 12. Effect of various sterilizing agents on the explants from *Dalbergia latifolia* Roxb.

Sterilizing agent	Time (min)	Total number of explants inoculated	No. of explants infected	No. of explants not infected	
				Living	Dead
Chlorine water	3	15	15	0	0
	5	15	15	0	0
	7	15	15	0	0
	10	15	13	2	0
	12	15	12	3	0
Mercuric chloride (0.1 per cent)	3	15	15	0	0
	5	15	15	0	0
	7	20	12	8	0
	10	20	10	10	0
	12	20	2	18	0
	14	20	1	17	2
Ethyl alcohol (95.0 per cent)	3	10	10	0	0
	5	10	10	0	0
	10	20	9	5	6
	12	20	8	5	7

4.2. Initial basal medium for culture establishment

The results of the preliminary study on the effects of different culture media and medium supplements on bud sprout from nodal segments of Dalbergia latifolia Roxb. are presented in Table 13. The results indicated that WPM supplemented with kinetin (1.0 ppm) and IAA (0.1 ppm) exhibited more than 75 per cent bud sprout within 10 days (Plate 1). In the visual rating of growth response, the above combination was found to perform better than others. In both MS and WPM media without any growth substances the explants failed to induce bud sprouting.

4.3. Bud proliferation and multiple shoot production

4.3.1. Standardisation of basal proliferation medium

In order to further refine the establishment medium and arrive at a suitable basal proliferation medium (BPM), detailed trials were conducted with different levels of cytokinins (kinetin, BA and 2 ip), auxins (IAA, IBA,NAA and 2,4-D) and gibberellin (GA_3) in MS and WPM (semi-solid and liquid) medium using nodal and internodal segments as initial explants and also with the shoots excised from initial cultures of the establishment medium. The results of these trials are presented below.

Table 13. Effect of different culture media and medium supplements in the initial culture establishment from primary nodal segments of Dalbergia latifolia Roxb.

Medium	Supplements	Growth* response
1. MS	-	+
	Kinetin 1.0 ppm	+
	Kinetin 1.0 ppm + IAA 0.1 ppm	++
	Kinetin 1.0 ppm + IBA 0.1 ppm	++
2. WPM	-	+
	Kinetin 1.0 ppm	++
	Kinetin 1.0 ppm + IAA 0.1 ppm	+++
	Kinetin 1.0 ppm + IBA 0.1 ppm	++

* Data based on 24 observations.

\+ Explants remain green without growth for a period of four weeks and show no symptoms of response.

++ Explants remain green without growth for a period of one week and show symptoms of drying.

+++ Explants remain green without growth for three to four days and afterward show growth

4.3.1.1. Cytokinins

Data on the independent influence of cytokinins (kinetin BA and 2 ip) and their combined effect with auxins (IAA, IBA,NAA and 2,4-D) on the number of axillary shoots induced per initial explant and the number of fairly elongated shoots produced after four weeks of culturing are presented in Tables 14 and 15.

Addition of kinetin to woody plant medium in varying doses (1.0, 2.0, 3.0 ppm) in the absence of auxins, did not induce shoot proliferation in the primary shoot explants. (Table 14). However, application of kinetin in conjunction with IAA/IBA stimulated shoot proliferation and shoot elongation. Furthermore, relatively lower levels of IAA (0.1 ppm) and IBA (0.01 and 0.05 ppm) with kinetin (1.0 to 3.0 ppm) induced higher percentage of bud sprout and proliferation. The response was maximum (80 per cent) in combination of 1.0 ppm kinetin and 0.1 ppm IAA. A tendency for production of more than one bud sprout was noticed in the medium supplemented with kinetin 2.0 and 3.0 ppm along with IAA 0.1 ppm or IBA 0.05 ppm. At higher levels of kinetin and IAA the shoots exhibited a tendency for vitrification .

The effect of BA, with and without auxins, was studied in another trial in which BA was tried in five levels ranging from 0.25 to 3.0 ppm (Table 14). Even in the absence of auxins, the effect of BA was remarkable in inducing axillary shoot in

Table 14. Effect of different culture medium supplements on the culture establishment from primary nodal segments of Dalbergia latifolia Roxb. in WPM.

Cytokinin	Concentration (ppm)	Auxin	Concentration (ppm)	% of explant showing shoot proliferation	% of explant with multiple shoot	No. of shoots/ explant	No. of leaves/ shoot	longest shoot (cm)
kinetin	1.0-3.0	-	-	0	0	0	-	-
	1.0	IAA	0.1	80	0	1	6	7
	2.0		0.1	55	14	2	4	5
	3.0		0.1	30	14	2	4	5
	1.0	IBA	0.1,0.5	0	0	0	-	-
	1.0		0.01	40	0	1	2	3
	2.0		0.01	35	0	1	2	3
	3.0		0.01	30	0	1	1	2
	1.0		0.05	35	0	1	2	3
	2.0		0.05	32	14	2	2	3
	3.0		0.05	30	14	2	1	2
BA	0.25	-	-	70	0	1	3	4
	0.5	-	-	65	0	1	3	3
	1.0	-	-	60	0	1	2	4
	2.0	-	-	30	0	1	2	3
	3.0	-	-	20	0	1	2	2
	1.0	IAA	0.1	50	0	1	2	2
	2.0		0.1	40	21	6	2	2
	1.0	NAA	0.1	40	0	1	2	3
	2.0		0.1	35	0	1	2	3
	1.0		0.5	34	14	3	1	3
	2.0		0.5	30	21	4	1	2
	1.0	2,4-D	0.1	35	14	2	1	3
	2.0		0.1	30	14	6	1	3
2 ip	0.5	-	-	70	0	1	6	7
	1.0	-	-	65	14	2	5	6
	2.0	-	-	60	21	5	5	6
	3.0	-	-	45	14	4	4	3
	2.0	IAA	0.1	55	21	5	5	5
	2.0		0.5	45	14	5	4	5

All treatments replicated three times with 14 cultures.
Culture period - five weeks.

Table 15. Effect of different culture medium supplements on the culture establishment from primary nodal segments of Dalbergia latifolia Roxb. in MS media.

Cytokinin	Concentration (ppm)	Auxin	Concentration (ppm)	% of explant showing shoot proliferation	% of explant with multiple shoot	No. of shoots/ explant	No. of leaves/ shoot	longest shoot (cm)
kinetin	1.0-3.0	-	-	0	0	0	-	-
	1.0,2.0	IAA	0.1	0	0	0	-	-
	1.0,2.0	IBA	0.1	0	0	0	-	-
	1.0	IAA	0.01	12	0	1	2	4
	2.0		0.05	8	0	1	1	3
	1.0	IBA	0.01	10	0	1	2	3
	2.0		0.05	5	0	1	1	2
BA	0.25	-	-	75	0	1	2	4
	0.5	-	-	70	0	1	2	5
	1.0	-	-	65	20	2	2	3
	2.0	-	-	60	25	20	1	4
	3.0	-	-	50	21	7	1	3
	4.0	-	-	35	14	4	1	2
	5.0	-	-	0	0	0	-	-
	1.0	IAA	0.1	55	0	1	2	3
	2.0		0.1	50	21	3	2	3
	2.0		0.01	55	21	15	2	3
2 ip	0.25	-	-	7	0	1	2	2
	0.5	-	-	5	0	1	1	2
	1.0	-	-	5	0	1	1	1
	2.0	-	-	5	0	1	1	1
	1.0	IAA	0.1	5	0	1	1	1
	1.0		0.05	5	0	1	1	1

All treatments replicated three times with 14 cultures.
Culture period - five weeks.

primary nodal segment-particularly at its lower levels (0.25, 0.5, 1.0 ppm). Supplementing the medium with BA along with different levels of auxins (IAA, NAA and 2,4-D) generally was found to induce multiple shoot production. Combination of BA (2.0 ppm) with IAA or 2,4-D (0.1 ppm) produced upto six shoots from a single explant (Plate 2). However, in these combinations, the number of explants showing bud sprout was comparatively less.

Effect of 2 ip was tested at four levels (0.5, 1.0,2.0, 3.0 ppm) with and without auxin IAA (0.1, 0.5 ppm) (Table 14). In the absence of auxins, the lower levels of 2 ip (0.5 and 1.0 ppm) exhibited maximum shoot induction (up to 70 per cent). While these cultures produced one to two shoots per culture, 2 ip 2.0 ppm with or without the auxin IAA produced up to five shoots per explant (Plate 3). Rooting was noticed in the one and a half month old primary cultures in medium containing 2 ip with or without IAA (Plate 4).

Cytokinin supplements were tried in MS media also to find out their effects on shoot proliferation and multiple shoot production. The data is presented in Table 15. The combinations tried were different concentrations of kinetin, BA and 2 ip either with or without auxins IAA and IBA. In case of kinetin 1.0-3.0 ppm alone or in combination with IAA/IBA 0.1 ppm the primary nodal segments remained green for three to four weeks without showing any symptom of bud sprout. When the auxin IAA/IBA was reduced to 0.01/0.05 ppm and combined with 1.0 and

2.0 ppm kinetin bud sprout could be achieved. However, the percentage of explants with shoot bud proliferation was very low (around 10 per cent).

The effect of BA, alone or along with auxins, was investigated in which seven levels (0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 ppm) of BA were tried with and without auxin IAA (0.1 and 0.01 ppm). All concentrations of BA tried in the experiment except 5.0 ppm were found to induce bud sprout and shoot proliferation in the explants. A maximum of 75 per cent explants responded to the medium supplemented with BA 0.25 ppm. When BA was applied in combination with different levels of IAA it tended to reduce shoot induction and growth. Multiple shoot production was exhibited by explants cultured in all these combinations and a maximum number of 20 shoots per explant was produced in medium supplemented with BA 2.0 ppm (Plate 5).

Supplementing the medium MS with 2 ip alone or in combination with IAA was not found to promote bud sprout and proliferation in Dalbergia latifolia Roxb.

Nodal and internodal segments were excised from a seedling which was sprayed weekly to run off with BA 200 ppm solution produced up to 20 shoots per explant in about 45 per cent of the cultures in MS medium along with BA 0.25 ppm.

4.3.1.2. Auxins

With constant levels of cytokinins the effect of different concentrations of auxins was found to be remarkable with regard to shoot induction and growth in the primary nodal explants (Table 16). The response varied greatly between the different levels of IAA, IBA, NAA and 2,4-D. In general, the lower concentration of auxins were found to be favouring shoot induction. The maximum response (80 per cent) was shown by explants cultured in woody plant medium with IAA (0.1 ppm) and kinetin (1.0 ppm). However, the number of shoots per explant was highest (six) in culture with 2,4-D (0.1 ppm) as the auxin supplement along with BA (2.0 ppm) in WPM.

4.3.1.3. Gibberellin

The effect of GA_3, either with or without BA, was found to be unfavourable for shoot induction both in primary nodal segments and shoot explants from culture. All the levels of GA_3 tried (0.5, 1.0, 2.0 ppm) failed to induce axillary budbreak. However, in a combination of BA (1.0, 2.0 ppm) and GA_3 (0.5 ppm), a small percentage of the primary nodal segments exhibited slight induction of shoot bud growth. The combined effect of GA_3, BA and kinetin (0.5 ppm) was also not appreciable in inducing shoot growth in both the explants.

Table 16. Effect of auxins (IAA, IBA, NAA and 2,4-D) on shoot induction in primary nodal segments of Dalbergia latifolia Roxb.

Basal media	Auxin	Concentration (ppm)	Cytokinin	Concentration (ppm)	% of explant showing shoot proliferation	No. of shoots/ explant
WPM	IAA	1.0	kinetin	1.0	0	0
		0.5		1.0	30	1
		0.1		1.0	80	1
		1.0	2 ip	2.0	0	0
		0.5		2.0	45	2
		0.1		2.0	55	5
	IBA	0.5,0.1	kinetin	1.0	0	0
		0.05		1.0	35	1
		0.01		1.0	40	1
	2,4-D	1.0	BA	2.0	10	1
		0.5		2.0	20	1
		0.1		2.0	30	6
MS	IAA	1.0	kinetin	1.0	0	0
		0.1		1.0	0	0
		0.01		1.0	12	1
	IBA	0.1	kinetin	1.0	0	0
		0.05		1.0	30	1
		0.01		1.0	10	1
	NAA	1.0	BA	2.0	20	1
		0.5		2.0	30	5
		0.1		2.0	30	3

All treatments replicated two times with 14 cultures.
Culture period - four weeks.

4.3.2. Standardisation of medium supplements

The effect of various compounds, other than growth regulators, on bud proliferation, growth and multiple shoot formation from nodal explants of Dalbergia latifolia Roxb. was studied. The effect of adenine sulphate, casein hydrolysate (CH), coconut water (CW) and glyphosate are presented in Table 17.

4.3.2.1. Adenine sulphate

Adenine sulphate, a cytokinin related compound was found to favour shoot induction in the primary nodal segments (Table 17). Doses from 2 to 8 ppm did not show any marked difference in budbreak, while higher concentrations tended to increase the multiple shoot production. Budbreak and proliferation were not markedly influenced when the media was supplemented with BA along with adenine sulphate. The number of shoots per explant, however, was highest in the above combination . The leaf number was found to be maximum (six) with 1 to 3 ppm adenine sulphate as the medium supplement.

4.3.2.2. Casein hydrolysate, coconut water and glyphosate

With respect to casein hydrolysate (CH), all the levels tested (100,500, 1000 mg) with basal media MS + 0.25 ppm BA exhibited more or less similar response to shoot proliferation. This compound substantially enhanced the number of shoots in the

Table 17. Effect of adenine sulphate, casein hydrolysate, coconut water and glyphosate, on shoot induction and multiple shoot formation in primary nodal segements of *Dalbergia latifolia* Roxb.

Treatment		% of explant showing shoot proliferation	% of explant with multiple shoot	No. of shoots/ explant	No. of leaves/ shoot	Longest shoot (cm)
Basal medium : MS						
Adenine sulphate (ppm)	1.0	40	0	1	6	6
	2.0	50	0	1	6	5
	3.0	50	21	2	6	6
	4.0	55	21	3	5	6
	5.0	55	21	4	4	5
	6.0	55	21	4	4	5
	7.0	60	25	4	4	4
	8.0	55	28	6	4	4
Adenine sulphate B.A (1.0)	8.0 +	60	25	7	4	4
Basal media : MS + 0.25 ppm BA						
MS + 0.25 ppm BA		75	0	1	2	4
Casein hydrolysate						
	100 mg l^{-1}	55	35	15	1	5
	500 mg l^{-1}	52	42	20	1	5
	1000 mg l^{-1}	50	39	25	1	5
Coconut water	10 per cent	40	35	7	3	3
,,	15 per cent	40	32	7	3	3
Glyphosate 0.17 ppm		65	21	2	4	5

All treatments replicated two times with 14 cultures.
Culture period - four weeks.

primary nodal segments. Maximum number of shoots were produced at 1000 mg l^{-1} (25 per explant).

Coconut water at 10 and 15 per cent in the basal medium also could induce budbreak and shoot growth. In both concentrations the number of shoots formed was seven per explant. A single dose (0.17 ppm) of glyphosate tried in the basal medium recorded budbreak and shoot growth in 65 per cent of the explants (Plate 6). This compound was, however, not very favourable for multiple shoot production.

The shoot growth is a function of shoot length and number of leaves. Casein hydrolysate in general, tended to increase the shoot length but the number of leaves was less in comparison to glyphosate and CW. Both 10 and 15 per cent supplement of CW showed no variation in the growth rate of shoots.

4.3.2.3. Inorganic components of MS, WPM media and effect of activated charcoal (AC)

Results of a trial to study the influence of the strength of inorganic salts and the effect of activated charcoal (AC) in MS and WPM on their shoot induction, growth and multiple shoot formation potentialities in nodal segments of Dalbergia latifolia Roxb. are presented in Table 18.

Decreasing (½) or increasing (x2) the inorganic salts in MS was found to reduce the shoot regeneration potential of the

Table 18. Effect of inorganic salts and activated charcoal on shoot induction, growth and multiple shoot formation from primary nodal segments of Dalbergia latifolica Roxb. in WPM and MS media.

Basal medium include:1.0 ppm, BA

Treatment	% of explant showing shoot proliferation	% of explant with multiple shoot	No of shoots/ explant	No. of leaves/ shoot	longest shoot (cm)
MS	65	20	2	2	3
½MS Salts	30	0	2	2	4
2x MS Salts	10	0	1	2	2
MS +NaH_2PO_4, $2H_2O$($0.17g\ l^{-1}$)	50	35	6	1	5
MS + AC (500 mg l^{-1})	50	0	1	3	5
MS + AC (1000 mg l^{-1})	25	0	1	2	4
½MS + AC (500 mg l^{-1})	20	0	1	2	3
WPM	60	0	1	2	4
½WPM Salts	40	0	1	2	2
2x WPM Salts	10	0	1	1	1
WPM+NaH_2Po_4, $2H_2O$ ($0.17g\ l^{-1}$)	10	0	2	2	3
WPM + AC(500 mg l^{-1}	10	0	1	1	2

All treatments replicated two times with 14 cultures.
Culture period - four weeks

medium. While in full MS salts, 65 per cent of the explants responded to budbreak and shoot growth, it was only 30 and 10 per cent with (½) MS and (x2) MS, respectively. However, the number of shoots per explant and leaves per shoot were not much affected by the variation in salts concentration. Optimum concentration of salts was full MS with respect to the maximum shoot length also. Sodium dihydrogen orthophosphate was found to increase the number of shoots per explants to six and the number of cultures with multiple shoot to 50 per cent though this salt registered a reduction in the number of explants showing response to budbreak and shoot growth. Activated charcoal did not enhance the potentialities of the medium. Lower concentration (500 mg l^{-1}) of AC with full MS produced shoots in 50 per cent of the cultures, while other combinations were still inferior to this.

Full salt concentration in the WPM was found to be superior to all other combinations tried. Activated charcoal (500 mg l^{-1}) as well as sodium dihydrogen orthoposphate reduced the shoot regeneration and growth potentialities of this medium.

4.3.2.4. Carbon sources

The effect of sucrose as carbon/engergy source on shoot induction, growth and multiple shoot formation in primary nodal segments of Dalbergia latifolia Roxb. are given in Table 19.

Number of explants showing shoot proliferation as well as multiple shoots were found to be influenced by varying levels of

Table 19. Effect of Sucrose on shoot induction, growth and multiple shoot formation in primary nodal segments of Dalbergia latifolia Roxb.

	Basal medium include 2.0 ppm BA				
Treatment	% of explant showing shoot proliferation	% of explant with multiple shoot	No. of shoots/ explant	No. of leaves/ shoot	longest shoot/ (cm)
MS + Sucrose 1.0 per cent	55	20	15	1	3
MS + Sucrose 2.0 per cent	60	20	20	2	4
MS + Sucrose 3.0 per cent	60	30	15	3	5
WPM +Sucrose 1.0 per cent	15	0	1	2	2
WPM +Sucrose 2.0	30	0	1	2	3
WPM +Sucrose 3.0 per cent	10	0	1	2	2

All treatments replicated two times with 14 cultures.

Culture period - five weeks.

sucrose. In both the media, sucrose at two per cent level induced higher shoot growth percentage and number of explant with multiple shoots, when compared to one per cent level. Sucrose concentration had a remarkable influence on shoot length also. Length of the longest shoot in MS and WPM culture was maximum (five and three cm respectively) at three per cent and two per cent levels of sucrose. As far as number of leaves per shoot is concerned, maximum leaves were produced by three per cent level in MS medium.

4.4. Standardisation of physical conditions

4.4.1. Culture medium

The effects of physical conditions of the culture medium on axillary shoot induction, growth and multiple shoot formation in nodal segments of Dalbergia latifolia Roxb. are presented in Table 20.

Variation on culture response was noticed between the physical states of the media. Explants cultured in liquid medium with shaking for three and a half days and sub-culturing after every 12 h showed maximum response. Hundred per cent of such explants responded to culturing by showing shoot proliferation (Plate 7). An interesting observation was that these cultures could be maintained up to six and a half months with subsequent sub-culturing. This is against two to three months life

Table 20. Effect of physical conditions of the medium on shoot induction, growth and multiple shoot formation in primary nodal segments of Dalbergia latifoia Roxb.

Basal medium :WPM + 2 ip 1.6 ppm

Treatment	Life of explant	% of explant showing shoot proliferation	% of explant with multiple shoot	No. of shoots/ explant	No. of leaves/ shoot	longest shoot (cm)
Semi-Solid: agar 0.8 per cent	3 sub-culture (3 months)	60	21	4	5	6
Liquid: With shaking for 3½days (with sub-culturing after 12 h)	12 sub-culturing (6½ months)	100	100	3	6	7
Liquid: With shaking for 3½days (with out sub-culture)	4 sub-culture (2 months)	60	34	2	5	6
Liquid: With out shaking	0	0	0	0	0	0

All treatments replicated two times with 14 cultures.
Culture period - four weeks.

expectancy of the primary culture in semi-solid medium or liquid medium without sub-culturing during shaking. With respect to length of longest shoot and leaf numbers, maximum values (seven cm and six respectively) were registered by liquid culture whereas, in case of semi-solid medium the respective values were six cm and five.

4.4.2. Light

The effect of light intensity on shoot induction, growth and multiple shoot formation in primary nodal segments of Dalbergia latifolia Roxb. is presented in Table 21.

Light at higher intensities (1000 lux, 2000 lux) tended to increase the per cent of explants showing shoot proliferation. Early bud sprouting was noticed at 1000 and 2000 lux light intensities and were superior to 500 lux. The shoot length as well as leaf numbers were found to increase gradually by increasing the light intensities. Maximum shoot length (seven cm) as well as leaf number (six) were recorded in 2000 lux light intensities.

In general 2000 lux light was found to be the most favourable with regard to all the parameters tested.

4.4.3. pH

A preliminary trial on the influence of different pH levels of the medium on the induction and growth of shoot in primary

Table 21. Effect of light intensities on shoot induction, growth and multiple shoot formation in primary nodal segments of Dalbergia latifolia Roxb.

Basal medium: WPM + kinetin 1.0 ppm + IAA 0.1 ppm.

Treatment	Time taken for sprouting (days)	% of explant showing shoot proliferation	No. of leaves/ shoot	longest shoot (cm)
500 lux	15-20	65	3	4
1000 lux	10-15	70	5	5
2000 lux	7-10	80	6	7
No light	15-25	40	2	2

All treatments replicated two times with 14 cultures.
Culture period - four weeks.

nodal segments indicated that the levels varying from 4.7 to 5.8 had not much influence except pH 4.7. A pH 4.7 reduced the number of bud sprouting from 80 per cent to 70 per cent in basal medium WPM + kinetin 1.0 ppm + IAA 0.1 ppm. The post-autoclave pH values of the medium (after about 48 hours) indicated a drop in the values to a tune of 0.2 to 0.5. Again, when the pH of the test media were observed after four weeks of culturing, it was found that initial pH levels of 5.0 and 5.2 had gone down further to 4.5 and 4.6 respectively and all others (ie. 5.5 and 5.8) had stabilized at 5.1 $\pm$ 0.1 level.

4.5. Callus mediated Organogenesis

The results of trials conducted to standardise a suitable basal medium for the induction and growth of callus using primary nodal and internodal segments are presented in Table 22. Callusing was noticed with all the explants in all the media tried. However, the rate of induction, growth and especially the nature of callus varied depending on the media composition. Large amount of friable callus was obtained on media supplemented with auxins alone in about five to six weeks. However, friable callus thus obtained was unsuitable for shoot induction. A green compact fast-growing callus was obtained when MS media was supplemented with NAA (3.0/5.0 ppm) and BA (1.0 ppm) along with or without 2,4-D (1.0/2.0 ppm) (Plate 8). Pieces of these calli were separated and cultured on media supplemented with BA

Table 22. Data on callus induction and organogenesis using primary nodal and internodal segments of Dalbergia latifolia Roxb. in MS media.

Sl. No.	Callusing			Organogenesis	
	media supplements	quantity of callus	nature of callus	media supplements	results
1.	2,4-D or NAA 3.0 ppm	profuse	friable green	-	-
2.	NAA 5.0 ppm + 2,4-D 1.0 ppm + BA 1.0 ppm + CW 10 per cent	medium	compact green	BA 3.0 ppm + NAA 1.0 ppm	+
3.	NAA 3.0 ppm + BA 1.0 ppm	light	Compact green	BA 3.0 ppm +NAA 1.0 PPM	+
4.	NAA 5.0 ppm + 2,4-D 1.0 ppm + CW 15 per cent	profuse	Compact green	BA 3.0 ppm + NAA 0.5 ppm	+
5.	NAA 5.0 ppm + BA 1.0 ppm + CW 15 per cent	profuse	Compact green	BA 3.0 ppm + NAA 0.5 ppm	++
6.	2,4-D 2.0 ppm + BA 1.0 ppm	medium	Compact green	BA 3.0 ppm + NAA and 2,4-D (0.5 ppm)	+

\- Not tried
\+ No regeneration obtained
++ Regeneration noticed in some cultures.

(3.0 ppm), NAA (0.5/1.0 ppm) along with or without 2,4-D (0.5 ppm). No regeneration was noticed till the end of four to five weeks except in media supplemented with BA (3.0 ppm) and NAA (0.5 ppm). However, in this medium also regeneration was noticed only in a few cultures.

4.6. Continuous culture for micropropagation

An experiment was conducted to establish continuous culture. When the shoots from the primary explants were cut and the original explant cultured in MS medium with kinetin and BA 0.5 ppm each, new shoots were formed (Plate 9). Repeated sub-culturing up to three cycles, every time removing the fresh shoots, exhibited new shoot production (Plate 10).

With the objective of producing a continuous culture almost all combinations of treatments given in Table 14 and 15 were tried by taking nodal segments from the primary culture. Except in lower concentrations of BA/kinetin, the explants were suppressed by callusing. Data on growth response of nodal explants taken from primary cultures in MS/WPM medium containing different combination of cytokinins are presented in Table 23. While BA, kinetin and 2 ip by themselves were not very favourable for these explants a combination of BA and kinetin 0.5 ppm each produced up to seven shoots per explant in about 93 per cent of the cultures (Plate 11). Combination of BA and 2 ip (0.5 ppm

Table 23. Effect of medium supplements in continuous culture of Dalbergia latifolia Roxb. using nodal segments from primary cultures

Treatment (ppm)		WPM				MS			
		No. of explant with multiple shoot (%)	No. of shoots/ explant	No. of leaves/ shoot	longest shoot (cm)	No. of explant with multiple shoot (%)	No. of shoots/ explant	No. of leaves/ shoot	longest shoot (cm)
Adenine sulphate	0.5	10	2	4	5	12	2	5	4
	1.0	15	2	3	4	15	2	3	3
BA	0.25	10	2	5	4	10	2	4	4
	0.5	15	2	5	3	20	2	5	3
	1.0	10	2	3	2	15	2	4	3
kinetin	0.5	15	2	5	5	15	2	4	4
	1.0	10	2	2	3	10	2	3	3
2 ip	0.5	10	2	3	4	10	3	4	4
	1.0	5	2	2	3	5	2	2	3
* BA (0.5) + kinetin (0.5)		85	6	4	4	93	7	6	4
BA (0.5) + kinetin (0.5) + NAA (0.5)		45	2	3	3	45	3	3	3
BA (0.5) + kinetin (0.5) + $AgNO_3$ (0.5)		35	2	2	2	40	2	2	3
BA (0.5) + kinetin (0.5) + GA_3 (0.5)		30	2	2	3	35	2	2	2
BA (0.5) + 2 ip (0.5)		70	3	3	3	75	4	3	3
BA (0.5) + 2 ip (0.5) + NAA (0.5)		35	2	2	3	30	2	2	2
BA (0.5) + 2 ip (0.5) + GA_3 (0.5)		25	2	2	2	20	2	2	3

All treatments replicated three times (except*) with 14 cultures.
* Treatments replicated six times with 14 cultures.

each) was the next best which induced bud sprout in 75 per cent of the cultures each producing an average of four shoots.

4.7. Root Induction

4.7.1. *In vitro* rooting

4.7.1.1. Standardisation of basal media and medium supplements

Results of the trial conducted to find out the suitability of WPM and MS medium to standardise the concentration of auxins in the basal medium for rooting of *Dalbergia latifolia* Roxb. shoots produced *in vitro* are presented in Table 24.

Semi-solid WPM/MS containing half strength of inorganic salts and full strength of organic growth factors was found to be more suited than full strength medium (semi-solid) for rooting of *Dalbergia latifolia* Roxb. shoots produced *in vitro*. Rooting in terms of the number of roots produced per shoot, length of longest root and number of days required for root initiation was relatively better in the former medium.

Of the different levels of IBA, IAA and NAA tried for rooting of shoots, IBA (1.0 and 2.0 ppm) recorded the best results in terms of root per shoot and length of the longest shoot. At the lower concentration (0.25/0.5 ppm) IBA and IAA did not produce any root. In general, roots produced by IBA were normal and attained a length of four and a half cm within a

Table 24. Effect of different rooting treatments on *in vitro* produced shoots of *Delbergia latifolia* Roxb.

Treatment	Nature of root	No. of roots/ shoot	lenght of longest root (cm)	Time needed for rooting (days)	Environment (26±2°C)	Rooting (%)
1. Basal media ½MS/½WPM						
IBA 0.25, 0.5 ppm	0	0	0	0	0	0
IAA 0.25, 0.5 ppm	0	0	0	0	0	0
IBA 1.0, 2.0 ppm	Normal	4-5	4.5	15-20	Diffuse light (500 lux)	30-35
IAA 1.0, 2.0 ppm	Normal	3-4	3.5	20-25	Diffuse light (500 lux)	35-40
NAA 1.0, 2.0 ppm	"	2-3	3.0	15-20	Diffuse light (500 lux)	5-10
IAA 1.0, 1.5 ppm + Biotin 0.2 ppm	Normal	4-5	3.5	20-25	Diffuse light (500 lux)	30-35
IBA 1.0 ppm + IAA 1.0 ppm	Normal	4-5	4.0	20-25	Diffuse light (500 lux)	30-35

(Contd.....)

Table 24 (Contd.....)

IBA 1.0, 2.0 ppm + NAA/ IAA 1.0, 2.0 ppm + Phloroglucinol 126 ppm	Thick, stubby unbranched resembling tubers	3-4	4.0	15-20	Diffuse light (500 lux)	30-40
IBA 1.0, 2.0 ppm	Normal	2-3	4.0	20-25	Bright light (1000-3000 lux)	20-25
*Activated charcoal(AC) 1.0 per cent + Two Second basal dip to excised shoot in IBA (1000 ppm;made in alcohol)	Normal	5-6	5.5	10-15	Bright light (1000-3000 lux)	80-90
2. *Vermiculite+Sand (1:1) + Two second basal dip to excised shoot in IBA (1000 ppm; made in alcohol)	Normal	2-3	6	12-15	Bright light (1000-3000 lux)	90-100
3. *Vermiculite (alone) + Two second basal dip to excised shoot in IBA (1000 ppm; made in alcohol	Normal	2-3	6	12-15	Bright light (1000-3000 lux)	90-100
4. Full MS+AC(1.0 per cent) Two Second basal dip to excised shoot in IBA (1000 ppm; made in alcohol)	Normal	2-3	4.0	25-30	Bright light (1000-3000 lux)	50-60

All tratments replicated three times (except *) with 14 cultures.
* Treatments replicated five times with 14 cultures.

period of 15-20 days. The effect of varying levels (0.25, 0.5, 1.0, 2.0 ppm) of IAA on rooting of shoots was also good except in the case of 0.25 and 0.5 ppm. At the levels of 1.0 and 2.0 ppm of IAA, normal roots attaining a length of three and a half cm within a period of 20-25 days were produced. In both the cases ie. IAA and IBA, slight callusing was observed at the basal cut end of the shoot. The effect of varying levels of NAA (1.0/2.0 ppm) on rooting of shoots was very poor and at the highest level of NAA (2.0 ppm) only callusing was observed at the basal cut end of the shoot. In general, the influence of NAA on rooting of Dalbergia latifolia Roxb. shoots produced in vitro was not very satisfactory.

The effect of IBA in combination with IAA/NAA either along with or without phloroglucinol were also studied. It was found that IBA combined with IAA, produced similar results as IBA alone whereas IAA/NAA (1.0 ppm) when combined with phloroglucinol (126 ppm) the rooting nature was changed into thick, stubby, unbranched and tuberous (Plate 12).

Addition of activated charcoal (AC) to the culture media and pulse treatment to excised shoot by IBA (1000 ppm) was found to remarkably influence the initiation of roots as well as the number of roots produced per shoot. Addition of activated charcoal to the media increased the percentage (80-90 per cent) of rooting. As far as the nature of roots produced in AC containing medium is concerned, they were white and thin and the

root length exceeded five cm (ie. five and a half cm) in a period of 10-15 days (Plate 13).

Pulse treatment of IBA (1000 ppm) to the excised shoot and then transfer to vermiculite alone or with sand in culture tube was found to be the best rooting procedure. Nearly 100 per cent of the shoots produced roots within 12-15 days without any symptoms of callus production at the cut ends. As far as the nature of roots are concerned, they were normal resembling natural roots (Plate 14).

4.7.1.2. Standardisation of light

The intensity of light provided during the initial stage of rooting was found to influence the performance of rooting. In a combination ½MS/½ WPM along with IBA 1.0/2.0 ppm under diffused light produced 30-35 per cent rooting while this combination at bright light produced only 20-25 per cent rooting (Table 24).

4.7.2. *In vivo* rooting

Shoots transferred to vermiculite alone or vermiculite + sand (1:1) in pots after IBA (1000 ppm) treatment exhibited rooting (Plate 15). Sixty per cent of such shoots rooted in vermiculite under *in vivo* conditions. In vermiculite + sand medium, the rooting was around 25 per cent.

4.8. Planting out and acclimatisation

In vitro rooted plantlets were removed from culture, roots washed and dipped in Bavistin 0.2 per cent and transferred to small pots which were kept covered with polythene bags (Plate 16, 17).

4.8.1. Rooting method, light intensity and potting medium

Rooting method (*in vitro*/*in vivo*), light intensites, during rooting and the potting medium used while planting out influenced the acclimatisation and survival of plantlets. The data are presented in Table 25.

The survival of *in vitro* rooted plantlets after planting out varied depending on light intensities during rooting period. While higher intensity of light (3000 lux) recorded 90 per cent of survival, plantlets rooted at 500 lux light recorded only 65 per cent survival after planting out. The survival of planted out plantlets was maximum (95 per cent) when rooting was done under *in vivo* condition.

Among the different potting media tried for planting out the rooted plantlets, soilrite was found to be the best where 90 per cent of the plantlets survived. The least suitable potting medium was mixture of sand and soil.

Table 25. Influence of rooting method, light coditions and potting medium on the survival of plantlets of *Dalbergia latifolia* Roxb. after planting out.

Treatment	Survival of plantlets after planting out (%)
A. Rooting method and light condition (Data from plantouts in soilrite)	
1. *In vitro* rooting in vermiculite + sand for 25 days under 3000 lux	90
2. *In vitro* rooting in vermiculite + sand for 25 days under 500 lux	65
3. *In vivo* rooting in vermiculite (alone)for 25 days under shade	95
B. Potting medium (Data from plantouts of A.1)	
1. Soilrite	90
2. Vermiculite alone	55
3. Vermiculite + sand (1:1)	50
4. Sand + Soil (1:1)	10
5. Sand + Soil (½:1.5)	10

All treatments replicated two times with 14 cultures.

4.8.2. Survival and growth during acclimatisation

Data on the survival and growth rate of plantlets during acclimatisation are presented in Table 26. The polythene cover from the planted out plantlets was removed at three weeks (Plate 18). During this early stages of acclimatisation a mortality of 10 per cent was observed. After about six weeks the plantlets were transferred to polybag containing potting mixture (Plate 19) and at this stage about five per cent mortality was encountered. Afterwards up to 16 weeks the plantlets did not show any variation in their survival rate. Once the plantlets were transferred to polybags their growth rate showed steady increase as indicated by the increment in height, root length and number of leaves.

4.8.3. Field Planting

Three plants were transferred to the field in May, 1991 (Plate 20). All these plants are performing well. The average height of these plants recorded on 1.2.92 was 200 cm. At the time of writing this thesis 49 plantlets are ready for field planting.

4.9. Economics of mass multiplication of Dalbergia latifolia Roxb. through tissue culture technique

The cost of production of clonal plantlets of Dalbergia latifolia Roxb. (using nodal segment as explants and adopting the

Table 26. Comparative study of survival and growth of plantlets during acclimatisation (Data from *in vitro* rooting at 3000 lux and planting out in soilrite).

Period after planting out	Survival* (%)	Height* (cm)	Longest root length**(cm)	No. of leaves/ plant
1. 3 weeks	90	7	4	8
2. 6 weeks (Transfer to polythene bag 22.5 x 10 cm size)***	85	12	5	10
3. 8 weeks	85	20	6	10
4. 12 weeks	85	40	12	25
5. 16 weeks	85	80	35	25

* - Average of 50 (fifty) observations.

** - Average of three observations.

*** - Planting medium: Ordinary potting mixture containing sand, soil, FYM (1:1:1).

axillary bud release technique) was worked out based on the facilities of the plant tissue culture laboratory, in the Department of Plantation Crops, College of Horticulture, Kerala Agricultural University, Vellanikkara (Table 27). At present this laboratory has a potential of maintaining 5000 cultures for multiplication and 5000 cultures for *in vitro* rooting. About 23,000 plants can be hardened in the glass house attached to the College. One Scientist (Rs. 2800/month) and one Technician (Rs. 1200/month) are considered necessary for this work. The programme can be initiated with 75 explants. The duration for culture establishment is two weeks; for shoot proliferation - five weeks; for *in vitro* rooting - three weeks; for hardening - three weeks and acclimatisation under green house conditions a minimum two weeks. At the 17^{th} week, two cycles can be introduced into the production system wherein a portion (5000 shoots) of the total multiple shoots produced (32,676 shoots) can be diverted for *in vitro* rooting and the remaining portion recycled for shoot proliferation. Based on the capacity of the laboratory, rate of culture establishment (93 per cent), rooting response (90 per cent) and the survival of the plantlets (85 per cent), an estimated number of 22,950 plantlets per year can be produced. The total cost involved per year works out to Rs.84651.00 , the cost of building, equipment, glasswares, chemicals and miscellaneous items having been distributed over the years according to their potentials/ durability. The cost of production of one *Dalbergia latifolia* Roxb. plantlet including

Table 27. Economics of production of Dalbergia latifolia Roxb. through tissue culture.

I. COST OF PRODUCTION

Maximum capacity for the production of plantlets using the physical facilities of the existing Tissue Culture lab. : 5000 cultures for shoot multiplication 5000, cultures for rooting and 23000 plantlets for hardening.

Item of Expenditure		Expenditure per year (Rs.)
A. Physical		
1. Glass wares	Rs. 40000/5 years	8000.00
2. Chemicals	Rs. 12000/5 years	2400.00
3. Wooden racks - 5 Nos.	Rs. 6300/20 years	315.00
4. Fluorescent tubes (63 Nos.) and fittings	Rs. 7000/5 years	1400.00
5. Autoclave	RS. 3000/20 years	1500.00
6. Laminar Flow Cabinet - 1 No.	Rs. 40000/10 years	4000.00
7. Air conditioner - 1 No.	Rs. 24000/15 years	1600.00
8. Hot air oven - 1 No.	Rs. 7000/10 years	700.00
9. Balance- 1 No .	Rs. 5000/20 years	500.00
10. pH meter - 1 No.	Rs. 4000/10 years	400.00
11. Refrigerator - 1 No.	Rs. 7000/15 years	466.00
12. Water bath - 1 No.	Rs. 5000/20 years	250.00
13. Heating mantles - 1 No.	Rs. 600/5 years	120.00
14. Double glass distillation unit - 1 set.	Rs. 8000/10 years	800.00

(Contd.....)

Table 27 (Contd...)

15. Cotton, inoculation aids foils, alcohol etc.	Rs. 1500/year	1500.00
16. Pots and potting mixture	Rs. 5000/year	5000.00
17. Humidity maintainance devices	Rs. 4000/year	300.00
18. Electricity charges, maintainance charges and miscellaneous items	Rs. 5000/year	5000.00
19. Building and furnishings	Rs. 120000/50 years	2400.00
B. Salary (Technical staff)		
1. Salary of one Scientist (Rs. 2800/- p.m)	Rs. 33600/year	33600.00
2. Salary of one Technician (Rs. 1200/- p.m)	Rs. 14400/year	14400.00
C. Labourer cost		
1. Cost of one labourer (Rs. 50/day)		18000.00
Total		102651.00

(Contd.....)

Table 27 (Contd.....)

II. NUMBER OF PLANTLETS PRODUCED PER YEAR

No. of weeks	Culture establi-shment	Shoot pro-liferation	Rooting	Hardening	Plantlets after one month hardening
2	75	150			
7		976			
12		5860			
17		32676	5000		
20				4500	
22		27900	5000		
25				4500	3825
27		27900	5000		
30				4500	3825
32		27900	5000		
35				4500	3825
37		27900	5000		
40				4500	3825
42		27900	5000		
45				4500	3825
47		27900	5000		
50				4500	3825
52		27900	5000		
		Total			22950

(Contd.....)

Table 27 (Contd....)

III. COST OF SINGLE PLANTLETS (Rs.)	
1. Cost of production (Initial culture to one month hardening)	3.69
2. Cost of maintenance till planting in the main field	0.78
TOTAL	4.47

one month's hardening is estimated to be Rs. 3.69. Maintenance cost of plantlets till they are planted out in the main field is worked out to Rs. 0.78 per plantlet. Thus the total cost of production of one plantlet from explant to field planting stage is estimated to be Rs. 4.47.

DISCUSSION

5. DISCUSSION

Because of growing population and dwindling availability of non-renewable resources, the demand for wood and wood products is expected to rise over the next few decades. Unless adequate number of planting materials are concurrently made available at the most appropriate planting season, large scale reforestation programmes cannot be carried out successfully. Traditional propagation methods cannot be expected to meet this challenge. Clonal progenies of plus trees are far superior to seedling progenies to quantum leaps in the production and productivity of important tree crops. Mass propagation of selected genotypes by tissue culture offers commercial application for the production of true-to-type plants at a very rapid rate. In vitro propagation is also useful for evaluating the growth performance of genotypes, with respect to cold hardiness, disease resistance and growth and quality tracts. In the present study, tissue culture techniques have been employed for clonal propagation of Dalbergia latifolia Roxb. through micropropagation. Attempts were also made for standardising techniques for inducing callus mediated organogenesis.

5.1. Explant choice

As a general rule, larger the size of explant, greater the rate of survival (Hussey, 1983). When tissues are cut, the cut surfaces turn brown due to oxidation of phenols in the damaged cells (Monaco et al., 1977). If the explant size is small, the

cut surface : volume ratio is high and there will be difficulty in the survival of the explant. Legrand and Mississo (1986) and Flynn et al. (1990) reported a positive correlation between explant size and growth rate response in cocoa. In the present study it has been found that nodal segments of 1.5 cm responded better under culture conditions. Internodal segments showed only callusing. This suggested that there was definite type of explant predetermination with respect to their morphogenetic potential.

Incidence of fungal infection in cultures of field explants is a serious problem in tree micropropagation. Doublin (1984) observed that with explants collected from the field the percentage of infection was over ninety, regardless of the procedure used for sterilization of the explants. Fungicidal spraying of mother trees have been suggested earlier by Legrand and Mississo (1986) as a method of reducing culture contamination. Results of the present study have indicated that prophylactic spraying with the systemic fungicide Bavistin (Carbendazim) and the contact fungicide Dithane M-45 (mancozeb) could bring down the contamination of explants greatly. The incidence of explant contamination, however, depended on the season of collection also. During rainy season the contamination rate was generally high. Among the different surface stemilants tried for the explants collected from sprayed trees, 0.1 per cent mercuric chloride for 12 minutes was found to be the most effective and least toxic in the present study. Increasing the treatment time resulted in mortality of explants.

Seasonal influence on the physiological state of the tree and its effect on culture establishment has already been well documented (Borrod, 1971, Seabrook et al., 1976; Yang, 1977). In Dalbergia latifolia Roxb. also, budbreak in the explants appears to be influenced by the season. The period between January to March was found to be most ideal for culture establishment.

5.2. Budbreak and shoot proliferation

Axillary buds are usually present in the axils of each leaf and every bud has a potentiality to develop into a shoot. In nature, these buds are normally dormant due to apical dominance. The application of cytokinin to the axillary buds can overcome the apical dominance effect (Murashige, 1974). The multiplication of axillary shoots can be substantially enhanced by providing suitable cytokinin at an appropriate concentration either with or without auxins. The direct effect of cytokinin is believed to be an enhancement of cell division. The effect of cytokinin in tissue culture may vary according to the particular compound used, the type of culture and the variety of plant from which it was derived (George and Sherrington, 1984). In the present study, it has been found that kinetin was incapable of inducing budbreak both in WPM and MS media while, addition of the cytokinin BA or 2 ip to any of these media could induce shoot proliferation. Lower levels of BA (0.25 ppm) was more effective in both the media. A gradual reduction in bud proliferation was

recorded by increasing levels by BA. Shoot development from nodal explants of a number of tree species in media supplemented with BA has been reported earlier (Grellier et al., 1984; Goyal et al., 1985; Datta and Datta, 1985; Kim and Lee, 1988; Mittal et al., 1989). Axillary bud proliferation induced by BA while kinetin was without effect has been noticed by Vieitez and Vieitez (1980) in chestnut.

The naturally occuring cytokinin, 2 ip has been reported to be more effective than BA or kinetin in a number of species like Rhododendron (Anderson, 1975), blueberry (Cohen, 1980) and garlic (Bhojwani, 1980 a). This cytokinin was, however, ineffective in inducing shoot multiplication for hybrid willow (Bhojwani, 1980 b) and white clover (Bhojwani, 1981). In our studies addition of 2 ip to MS and WPM medium was found to favour shoot proliferation in explants of Dalbergia latifolia Roxb. The response varied greatly depending on the medium. While lower levels of 2 ip (0.25 ppm) in WPM induced budbreak and shoot development in about 70 per cent of tree explants, the same concentration in MS could induce only seven per cent shoot development. Higher levels of 2 ip in both the media were found to be less favourable. The role of 2 ip in shoot induction has been indicated in Kalmia latifolia (Lloyd and McCown, 1980) and in cocoa (Mallika et al., 1990).

The effect of different cytokinins tried in the present experiment on multiple shoot induction was markedly different. Higher concentration 2 ip in WPM induced up to five shoots per

explant, while in MS a single shoot was always formed. The situation was reversed when BA was used. Up to a maximum of 20 shoots per explant was produced by BA 2.0 ppm supplemented to MS. The multiple shoot formation tendency was completely absent in WPM supplemented with BA, though a large proportion of the explants did respond to culture by showing budbreak and shoot proliferation. The use of 2 ip in micropropagation through multiple shoot production has been established already in blueberry (McComb, 1978; Zimmerman and Broome, 1980).

The general concept given by Skoog and Miller (1957) that organ differentiation in plants is regulated by an interplay of the two groups of hormones cytokinin and auxin, is found to be universally applicable. According to this hypothesis, the nature of organogenic differentiation is determined by the relative concentration of two hormones. A higher cytokinin to auxin ratio promotes shoot formation and higher auxin to cytokinin ratio favours root differentiation. The interactions of cytokinin and auxin, however, are more complex and more than one combination of two substances is likely to produce optimum results.

Among the different combinations of cytokinins and auxins tried in the present study using WPM and MS medium, the maximum frequency of shoot proliferation (80 per cent) was recorded in WPM with kinetin 1.0 ppm and IAA 0.1 ppm (Table 14). This hormone combination in MS was completely ineffective in shoot induction (Table 15). Much lower levels of IAA/IBA along with

kinetin exhibited some amount of response when used in MS medium. While kinetin alone either in WPM or MS medium did not induce shoot formation, additional supplements with auxin IAA in WPM and IBA in MS made the medium very efficient. Such effect of auxins has been explained as a result of the ability of auxins to nullify the suppressive effect of cytokinin concentration on axillary shoot growth (Lundergan and Janick, 1980).

The most effective combination of kinetin and IAA in WPM was 1.0 ppm and 0.1 ppm, respectively. In WPM auxin IAA seems to be more favourable while in MS IBA combinations are better than combinations of IAA.

Among the different cytokinins, BA is the cheapest and combinations of BA with different auxins have been found to be very effective in organogenesis in a number of plant species (Aboel-nil, 1987; Jang et al., 1988; Scott et al., 1988; Rai and Chandra, 1989; Vijayakumar et al., 1990). In Dalbergia latifolia Roxb. none of the BA + auxin combination could surpass the organogenetic potentialities of BA alone in MS medium. Different levels of NAA/IAA with BA in this medium produced 20-55 per cent shoot proliferation while BA by itself could produce up to 75 per cent proliferation. In WPM also BA supplements were superior to combination of BA with any of the auxins tried (Table 14).

The natural cytokinin 2 ip + IAA combinations did not show any beneficial effect over 2 ip alone both in WPM and MS medium.

While IAA 0.1 and 0.5 ppm with 2 ip 2.0 ppm in WPM reduced the shoot regeneration potential, this combination in MS did not change the medium quality at all.

Some of the cytokinin-auxin combinations were found to favour multiple shoot production. In WPM, the maximum number of shoots per explant was six in combinations of BA 2.0 ppm with IAA 0.1 ppm and BA 2.0 ppm with 2,4-D 0.1 ppm. The 2 ip-IAA combinations did not affect the number of shoots in comparison to 2 ip alone. In MS medium some of the BA+IAA combinations did show multiple shoot production tendency but this potentiality was maximum for the medium with BA 2.0 ppm alone.

Gibberellins (GA_3) form another class of plant growth substance which can influence growth and development in a variety of ways. When GA_3 is added to culture media, it often produces effects which are similar to those of auxins. According to Krishnamoorthy (1981) GA_3 treatment enhances the levels of endogenous auxins in the plants. In the present study addition of GA_3 to the culture medium was highly unfavourable in axillary shoot induction and growth. Inhibitory effects of GA_3 in culture has been reported earlier by Heide (1969) in begonia. It is presumed that the enhancement of endogenous auxin level in the explant in the presence of GA_3 might have suppressed the shoot proliferation potentialities of the media.

The possible growth regulatory effect of adenine has been exploited in tissue culture also. The addition of adenine sulphate

at concentrations from 1.0 to 8.0 ppm in MS medium has been found to induce shoot proliferation of Dalbergia latifolia Roxb. A synergistic effect of adenine on cytokinins has been suggested by Nitsch et al. (1967). However, such an effect was not noticed in the present study. Adenine sulphate 8.0 ppm with BA 1.0 ppm did not show any marked difference in shoot growth when compared with adenine sulphate alone. With respect to number of shoots per explant, a dose-dependent relationship could be envisaged. The maximum number of shoots per explant (seven) was recorded in MS medium supplemented with adenine sulphate and BA. The favourable effects of adenine at 8.0 ppm level in inducing multiple shoot may be due to its promotive effects caused by counteracting the inhibitory action of auxins on shoot bud release. However, it has not been established that supplements of nucleotides are necessary for plant tissue cultures (Gamborg and Shyluk, 1981).

Supplementing tissue culture medium with amino acids has been tried by various workers. Casein hydrolysate (CH) which is a mixture of all the amino acids present in the original protein is used as a source of amino acids. Murashige and Skoog (1962) observed that casein hydrolysate allowed vigorous organ development over a broad rang of IAA and kinetin in MS media. In the present investigation, casein hydrolysate 100, 500, 1000 mg l^{-1} supplemented to MS containing BA 0.25 ppm registered a reduction in shoot proliferation. However, this amino acid supplements seem to have a definite influence on the

production of multiple shoot. Up to 25 shoots per explant were observed in media supplemented with CH 1000 mg l^{-1} (Table 17). Multiple shoot production tendency of CH has been reported earlier by Mascarenhas et al. (1982) in Hevea brasiliensis.

Coconut water (CW) contains a number of cell division factors and free amino acids (Shantz and Steward, 1952). Addition of 10 or 15 per cent CW in MS supplemented with BA 0.25 has induced shoot proliferation of Dalbergia latifolia Roxb. to the extent of 40 per cent. This media supplement has also shown multiple shoot production. The major disadvantage with CW was the production of large amount of callus on cut ends of the explant leading to the supression of shoot growth and elongation. Both shoot growth and multiple shoot production induced by medium containing CW has been reported in a number of tree species (Nadgir et al., 1984; Mittal et al., 1989; Rai and Chandra, 1989). The favourable effects of CW in the promotion of growth and differentiation of excised tissues and organ have been attributed to the presence of cytokinins and gibberellin like substances in it (Straus and Rodney, 1960). Gamborg and Shyluk (1981), however, has cautioned that the use of complex substances in the medium may adversely affects the growth of tissues and organ under culture.

Glyphosate is a general purpose post-emergence weed-killer which is highly translocated within plant tissues. It has been reported that glyphosate can induce adventitious shoot production from explants under in vitro conditions. Application of 0.17

mg l^{-1} in culture medium of alfalfa, spraying 200 to 500 mg l^{-1} of the chemical to the mother plant of lowbush blueberry or stem dip of craneberry in a 1025 mg l^{-1} glyphosate solution for 30 seconds, were found to induce multiple shoot formation (George and Sherrington, 1984). A single dose of 0.17 ppm glyphosate added to MS + BA 0.25 ppm in the present study has been found to induce only up to two shoots per explant in about 21 per cent of the culture.

In an attempt to find out the effect of different concentrations of inorganic salts on shoots proliferation in Dalbergia latifolia Roxb. WPM/MS supplemented with BA 1.0 ppm was compared with their half (½) and doubled (x2) concentrations of salts. For both the media tried, the full concentrations of inorganic salts were found to be the optimum and the lower and higher levels showed reduction in explant response. The shoot growth was unaffected by medium with half salt concentration. However, by doubling (x2) the salts in MS and WPM the shoot growth was drastically reduced, probably due to the toxic effects of higher levels of salts. In an earlier report by Patri et al. (1988) MS medium with 1/4 salt concentration supplemented with BA (3μm) plus adenine (0.4μM) increased shoot induction in nodal and internodal segments of Pterocarpus santalinus. Addition of inorganic salt to the basal media in the form of sodium dihydrogen orthophosphate has been reported to enhance multiple shoot production in Eucalyptus camaldulensis (Kumar and

Ayyappan, 1987). In the present study also addition of $NaH_2PO_4.2H_2O$ 0.17 g l^{-1} to MS supplemented with BA 1.0 ppm produced up to six shoots per explant (Table 18). However, these shoots showed symptoms of drying starting from the tip in a period of two weeks after initiation.

Finely divided activated charcoal (AC) has frequently been added to media at different stages of tissue culture. AC is not a growth regulator in the strict sense, but its capacity to adsorb a wide range of compounds has made AC a valuable medium supplement. In tree micropropagation, AC is of great use as it can adsorb inhibitory compounds secreted from explants. It has been reported that AC can promote morphogenesis also. Kim and Lee (1988) reported that addition of AC 500 mg l^{-1} in half strength MS media containing BA 0.5 mg l^{-1} showed the best shoot and root growth from axillary buds of zyzyphus. Addition of AC 500 and 1000 mg l^{-1} to MS or WPM supplemented with BA 1.0 ppm in the present study, however, registered a reduction in percentage of shoot proliferation. In MS media this reduction seems to be dose dependent, AC 1000 mg l^{-1} showing maximum influence. In WPM the effect of AC was more drastic (Table 18). It is suggested that AC can exhibit an inhibitory effect on morphogenesis probably due to adsorption of growth regulators in the media (George and Sherrington, 1984). Evidences of removal of growth regulator and inhibitory compound by AC from the medium by adsorption have been recorded already (Wang and Huang, 1976, Weatherhead et al., 1978).

A suitable source of carbon energy is generally required for growing tissues under *in vitro* condition. The selection of sucrose as the most suitable energy source for cultures was the result of many comparisons between possible alternatives. Sucrose has almost invariably been found to be the best carbohydrate, although glucose is generally found to support growth well. In cardamom, both sucrose and glucose were found to be ideal carbon energy sources and osmoticum for supporting the growth of shoot bud cultures (Reghunath, 1989). In *Dalbergia latifolia* Roxb., two per cent level of sucrose was found to be ideal for shoot induction as well as multiple shoot production both in MS and WPM. This requirement may be related to the specific carbohydrate metabolism through which water relations and endogenous phytohormones are regulated. Gamborg *et al.* (1974) has suggested that sucrose concentrations affect the efficiency of nitrate and ammonium ion in the medium and the effect of cytokinin on cell division may also be dependent on the sugar availability.

In an attempt to standardise the physical condition of the media, it has been found that liquid cultures using WPM supplemented with 2 ip 1.6 ppm with shaking for three and half days along with sub-culturing after every 12 hours remarkably increased the budbreak and shoot proliferation. All the explants under culturing exhibited shoot growth with an average of three shoots per explant. The growth rate of the shoots also was

superior to the rest of the conditions tried. The shoots when excised and the original explant sub-cultured to the same medium, new shoots were produced within two weeks. This could be repeated up to six months with sub-culturing once in 20 days. The advantage of liquid cultures has been reported earlier in Cattleya, Cephalotus follicularis and most of the bromeliads (Murashige, 1974). The success of a micropropagation programme is mainly governed by the rate of multiplication and realisation of maximum number of shoots within the shortest possible time. High rate of multiple shoot production has been reported in Kalmia latifolia (Lloyd and McCown, 1980) using agitating liquid medium by slow (120 rpm) rotation and continuous sub-culturing after 12 h at initial stages for seven days. Maintaining the cultures under continuous shaking at initial stage, poses certain practical difficulties. Failure of the shaker even for a short period may cause considerable damage to the cultures due to the resulting anaerobic condition. In the present investigations it was found that the period of continuous shaking could be reduced to three and half days from seven days without much reduction in the multiplication rate at initial stage.

The present observation of the positive effect of shake culture in Dalbergia latifolia Roxb. thus suggests that this method can be effectively utilised for large scale propagation of the species through micropropagation.

Light requirement for differentiation involve a combination of several factors, namely, intensity, quality and duration. An optimum combination of these is necessary for certain photomorphogenic events. According to Murashige (1977), the optimum day light period is 16 hours for a wide range of plants. Shoot tips explants are normally maintained in an illuminance of about 500 to 3000 lux for micropropagation (George and Sherrington, 1984). In the present study it has been found that sprouting from axillary buds of Dalbergia latifolia Roxb. is maximum (80 per cent) at a light intensity of 2000 lux. Lower intensities of light (500 and 1000 lux) exhibited a delayed and reduced response. The growth rate of shoots were also maximum at a light intensity of 2000 lux. At lower levels of light as well as in dark, the explants showed callusing tendency.

The pH of culture medium is an important variable in tissue culture. Plant cells in culture require an acidic pH and an initial pH of 5.5 to 5.8 is optimum (Gamborg and Shyluk, 1981). Bonga (1982) reported that the pH of the medium is usually set at about 5.0 for liquid formulations and at about 5.8 for agar-gel media. Among the different levels of pH ranging from 4.7 to 5.8, tried in the present study only the lower pH (4.7) had recorded a reduction in bud sprouting. No difference in the response of the explant could be envisaged in the other pH levels. The initial levels of pH 5.0 and 5.2 had gone down to 4.5 and 4.6 respectively and pH levels 5.5 and 5.8 had stabilized at 5.1 ± 0.1 after four weeks of culturing.

Callus mediated organogenesis is an alternate method of micropropagation. Wherever applicable, this is often the fastest method of shoot multiplication and has been suggested as a potential method of cloning plant species (Murashige, 1974, 1977). The most serious objection against the use of callus culture is the possible genetic instability of their cells. However, callus mediated organogenesis as a method of clonal propagation has been reported in several tree species. In Dalbergia latifolia Roxb. this method has been successfully used by Rao (1986), Lakshmi Sita et al. (1986), Sudha Devi and Nataraja (1987) and Rai and Chandra (1989) using explants from seedlings or cotyledonary tissues. Callusing could be induced in nodal and internodal segments using MS media supplemented with different combinations of 2-4,D, NAA, BA and coconut water (Table 22). Compact green callus obtained from the different combinations were cultured on MS with BA 3.0 ppm and NAA 1.0 ppm. Shoot regeneration could be observed only in the callus derived from a combination of NAA 5.0 ppm, BA 1.0 ppm and CW 15 per cent in MS medium. These shoots however, did not show satisfactory growth. According to Lakshmi Sita et al. (1986), the above media composition was ideal for callus mediated organogenesis from nodal and internodal segments of five year old Dalbergia seedling. In the present studies, however, organogenesis was found to be difficult probably due to the age of the mother tree or due to the variation in amino acid composition of coconut water used as suggested by Gamborg and Shyluk (1981).

One of the pre-requisites for a commercially feasible and economically viable micropropagation programme is a constant supply of *in vitro* propagules. This can be achieved by producing a continuous culture system where shoots can be regularly harvested and put for further cultural operations. In order to achieve this objective, experiments were conducted to standardise the proper medium composition which will be ideal for producing continuous culture using the *in vitro* shoots from the primary cultures. Combinations of cytokinins and auxins which were favourable for shoot induction in primary cultures were not suitable for explants from these cultures. Most of them induced callusing in the explants. However, lower concentrations of cytokinins favoured shoot proliferation. Multiple shoot production being an important aspect of continuous culture, different combinations of growth regulators were tried with this objective. Lloyd and McCown (1980) reported that 2 ip has a potentiality for inducing multiple shoots in explants derived from the primary cultures. This cytokinin at 0.5 and 1.0 ppm in WPM and MS media induced only up to two shoots per explant in five per cent of the cultures (Table 23). Better results were obtained by combining different cytokinins like BA and kinetin or BA and 2 ip. MS medium supplemented with BA and kinetin 0.5 ppm each was found to be the most ideal in multiple shoot production from nodal segments of primary shoot derived from *Dalbergia latifolia* Roxb. This combination in WPM and MS medium gave up to six and seven shoots per explant, respectively. This finding

seems to be of great significance as it can be utilised for making available a constant supply of shoots from a limited number of primary cultures initially originated from field explants.

5.3. Rooting

Auxins are essential for root initiation and majority of the rooting media contain this growth regulator. The concentration of rooting hormone required is often critical to provide sufficient stimulus to initiate roots, while preventing the excessive formation of callus (Yeoman, 1986). Sometimes, a combination of auxin may give better response (Gupta *et al.*, 1980). The root elongation phase has been found to be very sensitive to auxin concentration. High concentrations of auxin inhibited root elongation (Thimann, 1977).

Most of the researchers have reported that *in vitro* rooting can successfully be achieved by reducing salt concentrations in the media, particularly in MS, B_5 and WPM, which contain high salt concentrations. Abundant rooting was observed when the salt concentration was reduced to one-half or one-third in the medium (Lane, 1979). But in such cases, sometimes poor top growth resulted (Gupta *et al.*, 1981).

In the present study lower concentrations (0.25, 0.5 ppm) of IBA or IAA did not induce rooting when combined with half strength MS or WPM (Table 24). Supplementing with any of these

auxins at 1.0 or 2.0 ppm induced rooting in 30 to 40 per cent of the shoots. Comparable results have been reported by Lakshmi Sita et al. (1986) in this species. Auxin NAA induced rooting in 5 to 10 per cent of the shoots, but the roots were thick stubby and unbranched. The vitamin biotin in combination with IAA did not show any beneficial effect on rooting. A combination of IBA and IAA also did not enhanced rooting potentiality of the medium. The positive effect of phlorogluginol (PG)- a phenolic compound found in the xylem sap of apple- has been established on rooting under in vitro conditions (Jones, 1976; McComb, 1978; James and Thurbon 1979; James, 1979; Jones and Hopgood, 1979; Garland and Stoltz, 1981). However, a large number of other workers who have compared the responses of rosaceous fruit trees in media with or without PG found this compound to be without advantage or even inhibitory (Quoirin et al., 1977; Singha, 1980; Zimmerman and Broome, 1981). The stimulatory effect of PG is suggested to be genotype-specific (Jones and Hopgood,1979; Zimmerman and Broome, 1981). Addition of phloroglucinol (126 ppm) along with IBA + NAA/IAA did not increase the percentage of rooting in comparison to medium containing auxins alone in the present study. Moreover, the roots formed in the medium containing PG were thick, stubby, tuberous and unbranched.

Rooting in in vitro shoots of Dalbergia latifolia Roxb. could readily be achieved by a root inductive auxin treatment (IBA 1000 ppm) followed by culturing in an auxin free medium

(Table 24). The rooting percentage was maximum (90-100 per cent) when vermiculite + sand (1:1) or vermiculite alone was used as the rooting medium. With respect to time taken for rooting as well as the length of roots also, this treatment was found to be the most effective. However, the number of roots were maximum in a medium containing activated charcoal 1.0 per cent.

Debergh and Maene (1981) pointed out that rooting under *in vitro* was the most labour intensive part of micropropagation. *In vivo* rooting of the shoots produced through culture has been advocated by different workers (Yeoman, 1986; Schwarz *et al.*, 1988; Vijayakumar *et al.*, 1990). This can be achieved by providing suitable planting medium as well as humidity condition necessary for avoiding possible desiccation of the shoots. *In vivo* rooting provides facilities for simultaneous hardening and rooting of the shoots so that the mortality of plantlets while planting out from the rooting medium can be reduced to a great extent. In the present investigation shoots excised from cultures where given a pulse treatment of IBA 1000 ppm and transferred to vermiculite alone or vermiculite + sand (1:1) in small plastic cups or pots and provided 100 per cent humidity condition. Shoots transferred to vermiculite exhibited 60 per cent rooting while a mixture of vermiculite + sand supported rooting in about 25 per cent of the shoots. These roots were comparable to the *in vitro* (vermiculite) produced roots with respect to their number and growth. Hundred per cent *in vivo* rooting has been achieved in

peat and 1:1 peat/perlite under high humidity conditions (greater than 80 per cent) in Kalmia latifolia and Betula platyphylla, respectively (McCown and Amos, 1979; Lloyd and McCown, 1980).

5.4. Hardening and planting out

Hardening the in vitro plantlets so as to make them adapt to the outside environment is a critical process due to their anatomical and physiological peculiarities. On transplanting excessive water loss from the plants had been recorded due to the improper development of cuticle and slowness of stomatal response to water stress (Brainered and Fuchigami, 1981; Fabbri et al., 1984). The problem may be aggravated if the vascular connection between roots and shoot is improper. A period of humidity acclimatization was considered necessary for the newly transferred plantlets to adapt to the outside environment, during which the plantlets undergo morphological and physiological adaptation enabling them to develop typical terrestrial plant water control mechanism (Hu and Wang, 1983, Sutter et al., 1985). In the present studies, high relative humidity (90 to 100 per cent) was maintained during the initial period of planting out with the help of polythene covers and intermittant cold water sprays. After two weeks, the covers were lifted partially for short intervals to make the plantlets hardened with respect to lower relative humidity. Gradually within another one week the cover could be completely removed. Murashige (1977) had successfully adopted similar methods of covering plantlets with

plastic tent or glass followed by misting for the first two weeks in soil subsequently removing the cover in a gradual process. During the period under high humidity the plants developed their stomatal control mechanism which helps them to reduce excessive water loss when planted out.

Addition of inorganic nutrients to the potting mixture is essential for the normal growth of the potted plants (Brown and Sommer, 1982). Application of 5-10 ml nutrient solution containing MS organic salts at half concentration (pH of 5.8) at weekly intervals enhanced the survival and promoted normal growth of the plantlets once they were planted out from the rooting medium. Rajmohan (1985) and Reghunath (1989) also reported the use of a nutrient solution similar to the above for the successful nurture of *in vitro* raised jack and cardamom plantlets respectively.

The success in acclimatization depends not only upon the post-transfer conditions but also the pre-transfer culture conditions (Ziv, 1986). Among the different *in vitro* methods tried, rooting in vermiculite + sand (1:1) for 25 days under 3000 lux light recorded the maximum survival of plantlets (90 per cent) after planting out (Table 25). The positive role of rooting under high light intensities on plant survival has been established earlier by Murashige (1977). Among the different potting media attempted, soilrite has been found to be the most suitable in comparison to vermiculite, sand, soil and their

combinations. The better performance of soilrite may be attributed to its ability to maintain optimum moisture status at the same time providing sufficient aeration. The maximum survival of plantlets after planting out (95 per cent) was recorded for plantlets rooted under in vivo conditions.

5.5. Economics

A tissue culture method, to become commercially feasible, has to assure a reasonable percentage of plant survival in the field. The growth and yield of such plants should also be better than that of seedlings or other conventional materials of propagation. Dalbergia latifolia Roxb. takes 60 years to reach rotation. Hence, in the present investigations a detailed field study could not be undertaken on the comparative performance in various stages of growth. However, certain preliminary observations were made on the initial establishment and growth of tissue culture plants till the age of four months (Table 26). It has been shown that the growth parameters such as height of the plant, length of root and numbers of leaves per plant increased substantially once the plantlets were transferred to polythene bags containing a potting mixture of sand, soil and FYM (1:1:1). After 16 weeks an average height of 80 cms has been recorded which is well comparable with an ordinary containerised seedling of Dalbergia latifolia Roxb.

Mass production of plantlets through micropropagation can be economically viable only if it is cost effective. In order to

achieve this, methodology should be devised so that sufficient number of plantlets from genetically superior parent source be made available at a very reasonable cost. As a result of the investigations carried out the methodology suggested to achieve the above objective is to start primary cultures from nodal segments of elite trees in WPM (semi-solid) supplemented with kinetin 1.0 ppm + IAA 0.1 ppm.

Seventy five primary cultures established in the above medium provides an average of three nodal segments per culture. These when subjected to continuous culture in MS medium supplemented with kinetin and BA 0.5 ppm each will give an average of six healthy shoots per culture. They can be subjected to another cycle of continuous culture so that at the 17th week a set of 5000 shoots can be kept for rooting and 5000 cultures for shoot proliferation. Simultaneous hardening of rooted plantlets along with repeated cycles of short proliferation and rooting can give rise to a total of about 23000 plantlets in one year. Considering the production and maintenance cost, the cost of production of one plantlets works out to Rs. 4.47 (Table 27).

The cost of production of plantlets can probably be brought down by increasing the number of plantlets produced. The production and maintenance facilities become limiting factors in this context. The protocol developed as a result of the present investigation provides possibility for production of unlimited number of plantlets provided adequate laboratory facilities as well as hardening and acclimatization facilities are available.

SUMMARY

The present investigation was carried out at the College of Forestry, Vellanikkara during 1989-92 with the objective of standardising the techniques of micropropagation of Dalbergia latifolia Roxb. using a 12 year old mother tree through tissue culture. The salient findings are presented below:

1. The suitable primary explants for initiating enhanced release of axillary buds were the nodal segments measuring a length of 1.5 cm.

2. A prophylactic spraying of the mother tree with the Bavistin and the contact fungicide Dithane M-45 remarkably reduced the contamination per cent of the explants.

3. The most ideal period for collecting explants from the field was found to be January-February during which period the rate of contamination was less and culture establishment was maximum.

4. Treating the explants with mercuric chloride (0.1 per cent) for 12 minutes was found to be the best surface sterilization method.

5. Both WPM and MS media were found to be suitable for culture establishment from primary nodal segments as well as nodal segments excised from primary culture.

6. In the case of primary nodal segments, WPM + kinetin 1.0 ppm + IAA 1.0 ppm was found to be the best for single shoot initiation whereas MS + BA 2.0 ppm and MS + BA 0.25 ppm + CH 1000 mg l^{-1} was better for multiple shoot production.

7. Auxins (IAA, IBA, NAA, 2,4-D) did not influence the shoot multiplication rate. However, the growth of cultures (in terms of the length of the longest shoot and the leaf number) was improved by lower levels of auxins tried.

8. Adenine sulphate at higher levels (6 ppm) supplemented to MS medium induced multiple shoots production.

9. Casein hydrolysate in the basal proliferation media (BPM) was found suitable in terms of the rate of shoot multiplication from primary nodal segments.

10. No beneficial effect was obtained when coconut water (CW) was included in BPM, because of the high callusing tendency.

11. Half (x½) or double (x2) strength of WPM/MS was found unsuitable for shoots of Dalbergia latifolia Roxb. Leaf shedding was noticed in (x½) strength, whereas in (x2) reduced sprouting and tip drying of shoots were observed.

12. Sucrose 2.0 per cent was ideal as carbon energy source and osmoticum for supporting the growth of shoots in culture. Reducing their quantity to 1.0 per cent was unfavourable in terms of shoot multiplication rate and growth. Increasing

the quantity to 3.0 per cent did not improve the proliferation rate.

13. Physical state of the culture medium had remarkable influence on the life of primary nodal segments. Compared to semi-solid cultures, liquid cultures with shaking at initial period and then transferred of explants to static liquid medium produced shoots upto 6 months.

14. Light intensity of 2000 lux, for 16 h followed by 8 h dark was found suitable for culture in both semi-solid and liquid medium.

15. The optimum initial pH of the medium was between 5.5 and 5.8

16. Induction and growth of callus was supported by MS medium containing NAA 3.0 ppm + BA 1 ppm or NAA 5.0 ppm + 2,4-D 1.0 ppm + CW 15 per cent or NAA 5.0 ppm + BA 1.0 ppm + CW 15 per cent.

17. For establishment of continuous culture by repeatedly using nodal segments from primary cultures of Dalbergia latifolia Roxb. MS + kinetin 0.5 ppm + BA 0.5 ppm was found to be the most ideal.

18. For in vitro rooting of shoots, pulse treatment with IBA (1000 ppm) to individual shoot and then transfer to ½MS or ½WPM containing 1.0 per cent activated charcoal or

vermiculite either with or without sand was found to be the best method.

19. In vivo rooting was also achieved by pulse treatment of IBA to excised shoots and culturing in vermiculite medium.

20. Soilrite was identified as the best medium for planting out the plantlets, supporting 90 per cent survival.

21. Transparent plastic covers were suitable as humidity maintenance devices, maintaining high relative humidity (RH) supporting 85 per cent of plantlet survival. Occasional spraying of cold water (12 $\pm$ 2° C) enabled to maintain high RH and mild temperature inside the plastic cover.

22. The height and length of longest root of tissue culture produced seedlings after 16 weeks were 80 and 35 cm, respectively.

23. The height of plantlet in the field after 8 months (2.1.92) was recorded to be 200 cm.

24. The cost of production of one tissue culture plantlet, including hardening was worked out to Rs 4.47.

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* not seen the original.

PLATES

Plate 1 Bud sprout in WPM Supplemented with kinetin 1.0 ppm and IAA 0.1 ppm.

Plate 2 Multiple shoot production in medium WPM supplemented with BA 2.0 ppm and IAA or 2,4-D 0.1 ppm.

Plate 3 Multiple shoot production in WPM supplemented with 2 ip 2.0 ppm.

Plate 4 Rooting of primary culture in WPM supplemented with 2 ip 2.0 ppm.


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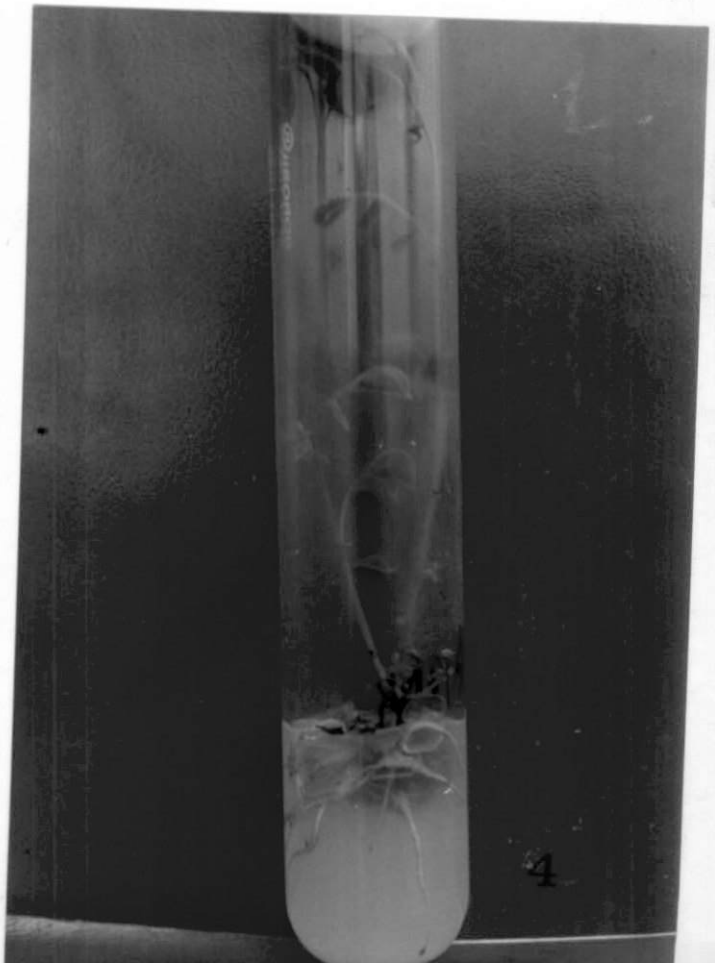
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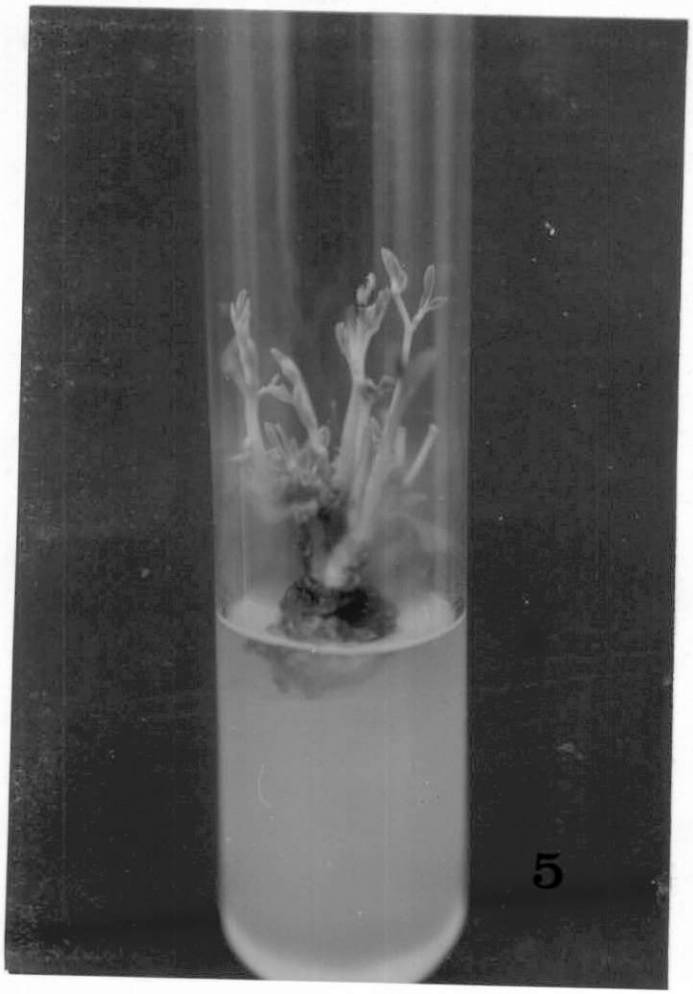
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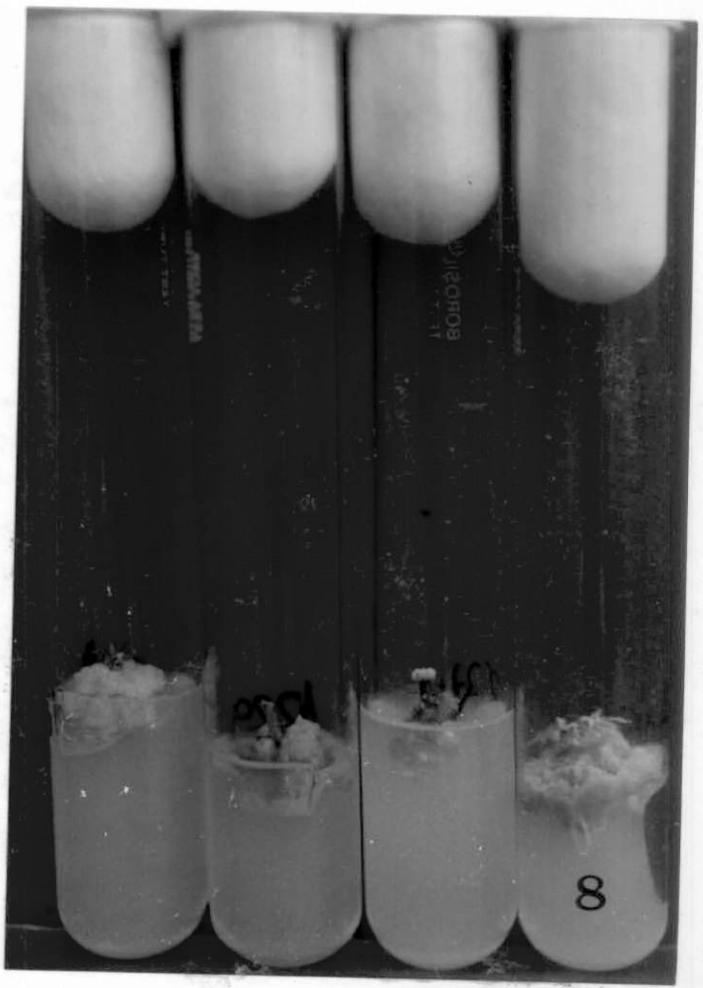
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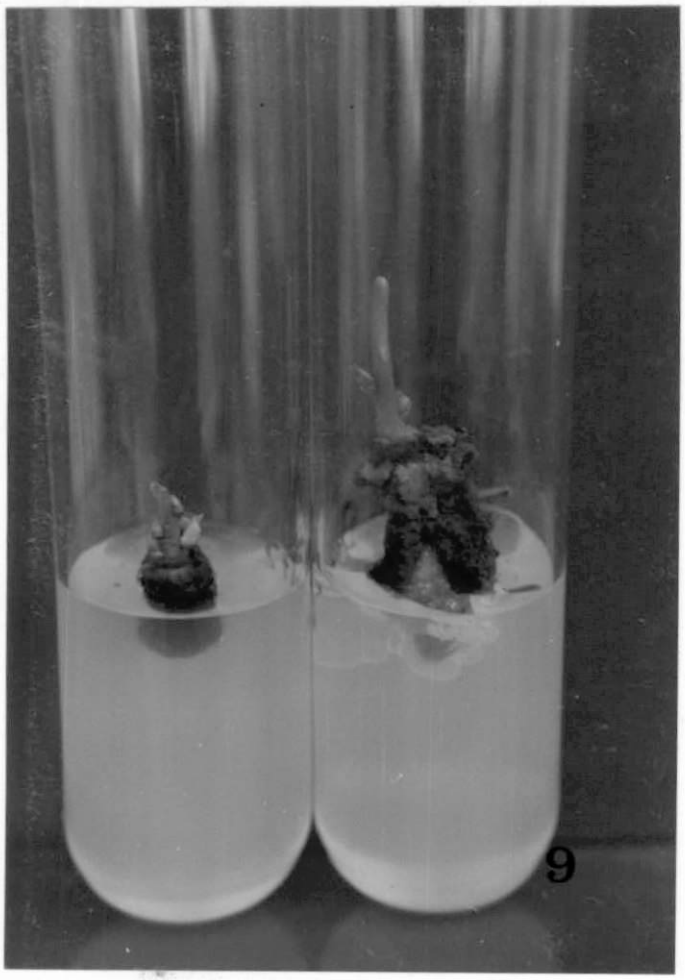

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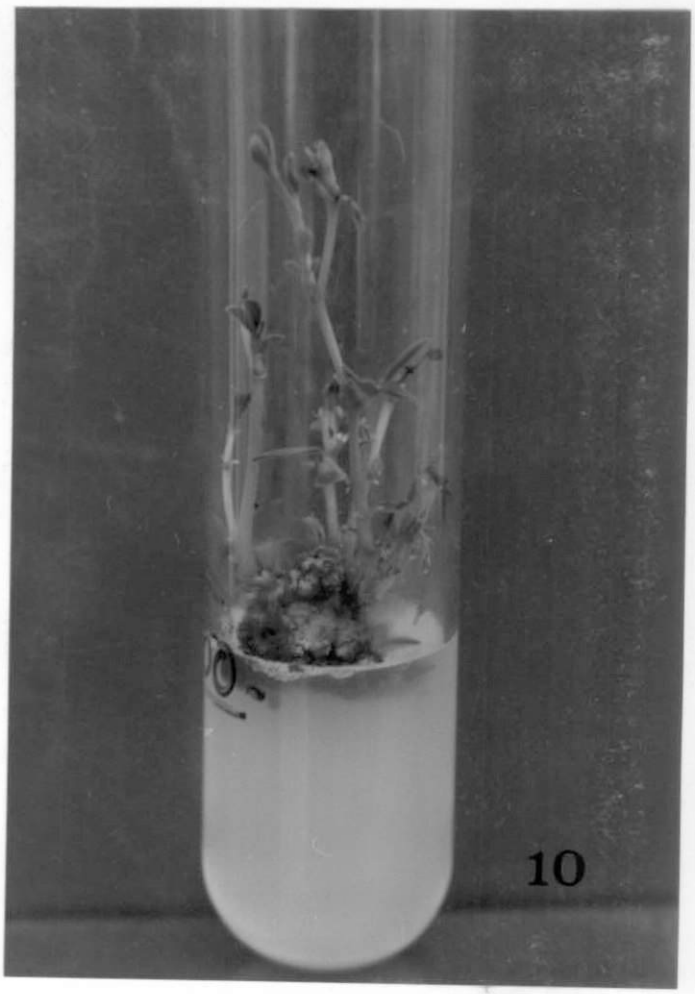

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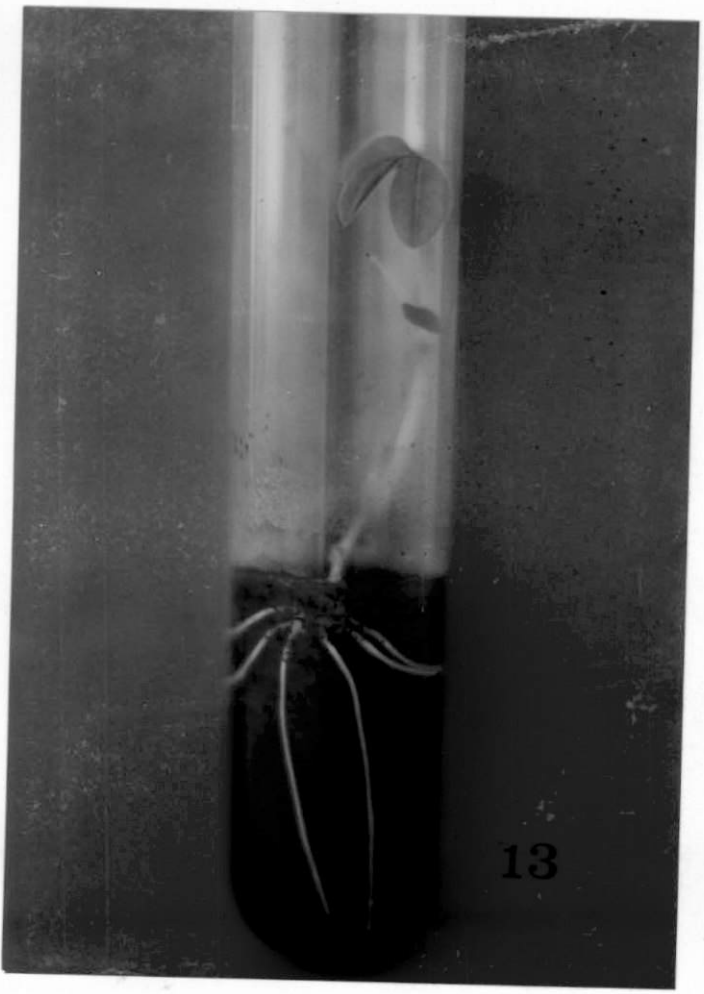
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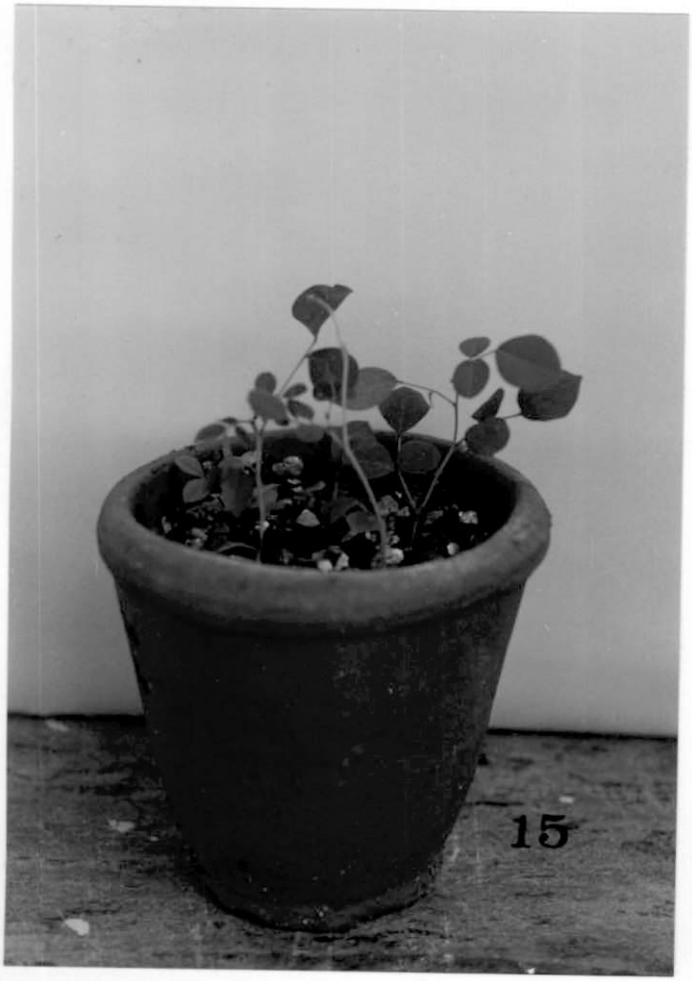
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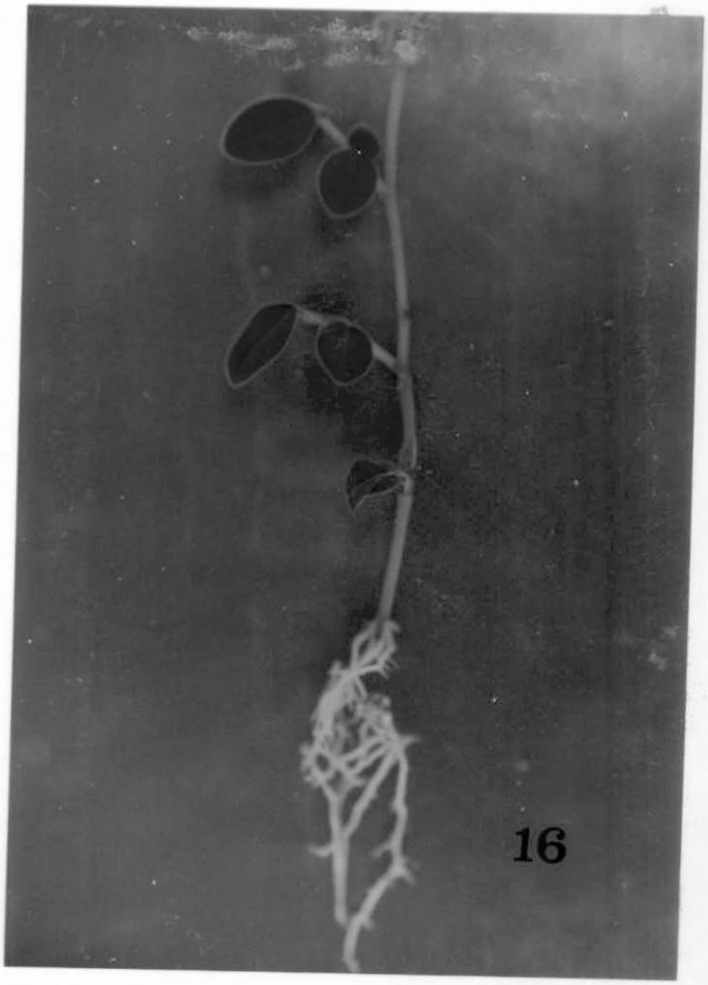
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Plate 17 Plantlet planted out in pot with soilrite (in hardening period).

Plate 18 Plantlet three week after planting out.

Plate 19 Six week old plantlet transferred to ploybag with potting mixture.

Plate 20 Six month old plantlet in the field.


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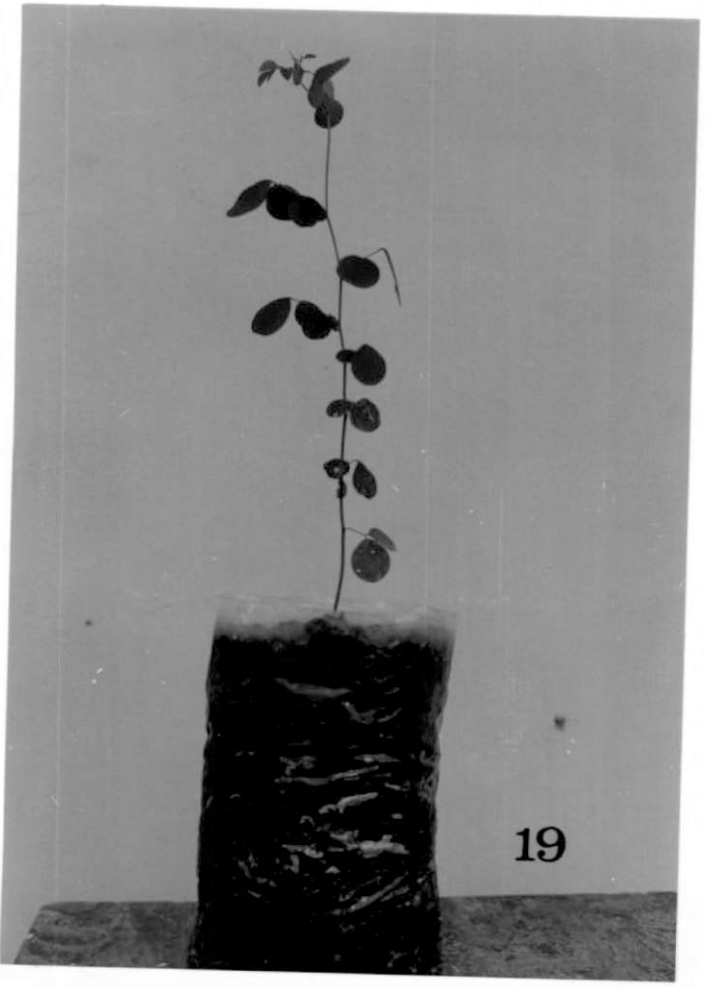
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IN VITRO PROPAGATION OF DALBERGIA LATIFOLIA ROXB. THROUGH TISSUE CULTURE

BY
KHAGES CHANDRA MAHATO

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Science in Forestry

Kerala Agricultural University

COLLEGE OF FORESTRY
Faculty of Agriculture
VELLANIKKARA - THRISSUR

1992

ABSTRACT

In investigations carried out in the College of Forestry, Vellanikkara during 1989-92, it was found that nodal segments of 1.5 cm length were ideal as the explants. Prophylactic spraying of the mother tree with the systemic fungicides Bavistin and the contact fungicide Dithane M-45 coupled with surface sterilization of explants with mercuric chloride 0.1 per cent for 12 minutes fully controlled contamination of the culture. Both woody plant medium (WPM) and Murashige and Skoog (MS) medium were found to be suitable for the primary culture establishment from the explants. While WPM supplemented with kinetin 1.0 ppm and IAA 0.1 ppm was most suitable for inducing healthy single shoots in about 80 per cent of the explants, MS along with BA 2.0 ppm or BA 0.25 ppm and CH 1000 mg l^{-1} induced maximum number of multiple shoots (up to 25). Among the various media supplements tested, adenine sulphate was found to be capable of inducing multiple shoots and CH increased the rate of shoot multiplication. Coconut water did not show any beneficial effects. Liquid cultures with shaking at initial periods prolonged the life of the primary culture up to six months with continuous production of shoots. Continuous culture was developed using nodal segments of shoots derived from the primary cultures. The most suitable medium for this was found to be MS supplemented with kinetin and BA 0.5 ppm each.

The best *in vitro* rooting was achieved by resorting to a pulse treatment of the shoots with IBA (1000 ppm) and culturing them in vermiculite or vermiculite + sand medium. Up to 100 per cent rooting could be achieved by this method. *In vivo* rooting was obtained by transferring the shoots after IBA treatment to vermiculite under high humidity conditions. Planting out and hardening of the *in vitro* rooted plantlets was carried out in soilrite. Up to 90 per cent survival could be achieved. The hardened plantlets were acclimatized in polythene bags with ordinary potting mixture and after 16 weeks they were field planted. The cost of production of one plantlet including hardening was worked out to Rs 4.47.