CLONAL PROPAGATION OF SELECTED PLUS TREES OF INDIAN ROSEWOOD (*DALBERGIA LATIFOLIA* ROXB.) THROUGH TISSUE CULTURE

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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
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1995

DECLARATION

I hereby declare that this thesis entitled ***Clonal propagation of selected plus trees of Indian rosewood (Dalbergia latifolia Roxb.) through tissue culture*** *is a bonafide record of research work done by me during the course of research and that this 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.*

Place : Vellanikkara

Date : 25-11-1995

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Certified that this thesis entitled ***Clonal propagation of selected plus trees of Indian rosewood (Dalbergia latifolia Roxb.) through tissue culture*** *is a record of research work done by Sri.* ***C.S. KANNAN****, 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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We, the undersigned members of the Advisory Committee of Sri. ***C.S. KANNAN****, a candidate for the Degree of* ***Master of Science in Forestry****, agree that this thesis entitled* ***Clonal propagation of selected plus trees of Indian rosewood (Dalbergia latifolia Roxb.) through tissue culture*** *may be submitted by Sri.* ***C.S. KANNAN*** *in partial fulfilment of the requirement for the Degree.*

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To
Parvathi Warrier,
My Grandmother
With Love . . . .

GRATITUDE

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Strange and great are the ways of **THE POWER**.
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CONTENTS

ABBREVIATIONS

ABA	-	Abscisic acid
AC	-	Activated charcoal
ADS	-	Adenine sulphate
$AgNO_3$	-	Silver nitrate
BA	-	Benzyl adenine
CCC/Cycocel	-	Chloro Choline Chloride
CH	-	Casein hydrolysate
cv	-	Cultivar
CW	-	Coconut water
2,4-D	-	2,4-Dichlorophenoxy acetic acid
Na_2EDTA	-	Sodium salt of ethylene diamine tetra acetic acid
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acid
Kin	-	Kinetin
L-G	-	L-Glutamine
M	-	Molar
µ*M*	-	Micromolar
MS	-	Murashige and Skoog (1962) medium
N	-	Normal
NAA	-	Naphthalene acetic acid
PG	-	Phloroglucinol
v/v	-	Volume by volume
w/v	-	Weight by volume
WPM	-	Lloyd and McCown's (1980) Woody Plant Medium
2-ip	-	N-6-(2-isopentyl) adenine

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Introduction

INTRODUCTION

Increase in world population and rise in demand for tree products, especially wood, has necessitated the growing of more forests with improved quality stock. Afforestation programmes thus, should go hand in hand with tree improvement programmes to ensure predictable economic returns. Tree improvement by selection and breeding through conventional techniques, however, are too slow because of the long life cycle of the trees. *In vitro* clonal propagation of selected genotypes is a much faster means of implementing tree improvement than producing improved seeds through seed orchards or controlled pollination programmes.

Tree legumes, an important component of tropical forest ecosystem, in general are greatly under exploited. Rosewood (*Dalbergia latifolia* Roxb.) is one of the most valuable timbers belonging to this group and has been in the world market for centuries commanding high prices in national and international trade.

Dalbergia latifolia is a large deciduous or nearly evergreen tree with a full rounded crown and a cylindrical and fairly straight bole, varying in size according to locality (Troup, 1921). It belongs to the family Fabaceae and sub family Papilionoideae. The species is found in the sub Himalayan tract from Oudh eastwards, Bihar, Orissa and Central, Western and Southern India.

Indian rosewood ranks among the finest woods for furniture and cabinet work. It is well known in Europe and America where its chief use is in the pianoforte trade. It is also a valuable decorative wood suitable for carving and ornamental plyboards and veneers. The wood can be used for construction purposes and for making agricultural and musical instruments. Carefully selected and manufactured Indian rosewood plyboards satisfy aircraft specifications (C.S.I.R., 1952).

Plant parts of *D. latifolia* and oil from the seeds have medicinal value (Nair, 1986). The tree yield gum and the bark contain about two per cent tannin. The leaves of this species are relished by cattle and goats. It can be grown in coffee plantations as a shade tree (C.S.I.R., 1952). The soil binding and nutrient enriching capacity through nitrogen fixation makes the species ideal for the various forestry practices like agroforestry, farm forestry, social forestry etc.

Plantation programmes in rosewood face many problems, in particular the inadequate supply as well as poor quality of seedlings. Seed propagation is not satisfactory in this species as the percentage of germination is very low (Sita and Swamy, 1992). Moreover, seeds lose their viability in relatively short time (Troup, 1921). Hence micropropagation is attaining considerable attention for obtaining large number of genetically

pure elite populations using *in vitro* methods. The possibility of producing adequate quantity of plantlets of known superior genotypes with uniform quality can be assured through this method of propagation. With unprecedented progress in biotechnology during the past two decades, coupled with the continually increasing demand for wood and tree products throughout the world, one can predict with certainty that asexually produced trees will contribute a significant portion of forest planting stock in the future.

Dalbergia mainly being an Asian species has been neglected by the resourceful research workers of the European countries. Indeed rosewoods have only been given little attention even by our scientists. Already techniques have been standardised for the enhanced release of axillary buds of *Dalbergia latifolia* using explants from saplings or young trees (Mahato, 1992). However, no success has yet been reported by using explants directly taken from mature trees which is a felt need for production of clonal progenies of proven genotypes.

The present study therefore, was focused on the formulation of a protocol for *in vitro* clonal propagation of selected plus trees of Indian rosewood.

Review of Literature

REVIEW OF LITERATURE

The potential benefits of clonal planting stock in reforestation programmes have long been recognised. It has been estimated that at least about ten per cent increase in gain can be expected from planting selected clonal propagules rather than selected seed families (Kleinschmit, 1974). The best commercial application of tissue culture in forest tree species has been the production of true to type progenies at a very rapid rate compared to the conventional methods (Levy, 1981). Micro-propagated plants are reported to grow faster and mature earlier than their seed propagated progenies (Vasil and Vasil, 1980). It is unfortunate that in most of the tree species the application of tissue culture technique as a propagation and breeding tool is not fully achieved. However, considerable progress has been made over the last two decades on micropropagation methods for forest trees. *In vitro* production of clonal progenies has been achieved in many of the softwood species. Some of the recent reports of success in micropropagation of these species are available in *Pinus pinaster* (David *et al.*, 1982), *Cedrus deodara* (Bhatnagar *et al.*, 1983), *Pinus nigra* (Kolveska-pletikapis *et al.*, 1983), *Euphedra foliata* (Bhatnagar and Singh, 1984), different species of *Picea* (Arnold and Erikson, 1986; Roberts *et al.*, 1992), *Pinus radiata* (Smith, 1986; Horgan, 1987), *Juniperus oxycedrus* (Gomez and Segura, 1994), hybrid larch (Larix x Leptoeuropaea) (Lelu *et al.*, 1994), *Pinus eldarica* (Sen *et al.*, 1994), *Sequoia sempervirens* (Sul and Korban, 1994) etc.

Success in micropropagation of a number of broadleaved tree species has also been achieved. A detailed review of the recent literature on *in vitro* propagation of hardwood species is presented below:

2.1. Micropropagation

2.1.1. *Acacia* species

Induction of shoot buds in *Acacia nilotica* under *in vitro* conditions in MS medium incorporated with IAA (0.1–10.0 mg l^{-1}) was achieved by Mathuri and Chandra (1983). Ahmad (1990) noticed that nodal segments from seedlings of *Acacia mangium* could give rise to plantlets when cultured on MS containing 0.5 mg l^{-1} BA. The capacity for the shoot morphogenesis in internodal segments of *Acacia holosericea* and *A. saligna* were demonstrated by Jones *et al.* (1990) through indirect organogenesis. He also reported that *A. divenosa, A. holosericea, A. salicina, A. sclerosperma* and *A. saligna* showed enhanced release of axillary buds in MS medium. Shoot growth and rooting of *Faidherbia (Acacia) albida* was obtained on MS basal medium amended with BA at 10^{-7} *M* and NAA at 10^{-7} *M* but rooting was most successful on medium supplemented with 10^{-7} *M* NAA alone (Ruredzo and Hanson, 1993).

2.1.2. *Adenanthera pavonia*

Bejoy and Hariharan (1993) achieved *in vitro* propagation of *Adenanthera pavonia* using cotyledonary nodes as explant. Best shoot multiplication rate of 5-6 shoots per cotyledonary node explant was obtained on MS basal medium containing 30 g l^{-1} sucrose, 7 mg l^{-1} BA, 1.0 mg l^{-1} kinetin and 8 g l^{-1} agar under 16 hour photoperiod. Excised shoots rooted within 4-5 weeks when incubated on half strength MS medium with 20 g l^{-1} sucrose, 2.0 mg l^{-1} IBA and 0.5 mg l^{-1} NAA.

2.1.3. *Albizia* species

Sinha and Mallick (1993) reported that multiple shoot formation from segments of *in vitro* seedlings of *Albizia falcataria* was seen in MS media supplemented with BA (4.4-8.9 μ*M*). Sankhla *et al.* (1994) carried out micropropagation of *Albizia julibrissin* and found that seedling explants when cultured on MS medium containing B_5 vitamins, 3 per cent sucrose, 0.25 per cent phytagel and thidiazuron 0.1-10 μ*M* induced shoot formation. It was also found that callus or shoot formation did not occur without TDZ. *In vitro* propagation of *Albizia lebbeck* was obtained when seedling explants were cultured on MS medium with kinetin and BA (Chimmala, 1994).

2.1.4. *Anogeissus pendula*

Joshi *et al.*(1991) observed multiple shoot formation from excised seedling segments of *Anogeissus pendula* through indirect organogenesis. Cotyledon segments were noticed to have a higher organogenetic potential than epicotyl segments, when cultured in MS supplemented with 1.0 mg l^{-1} BA and 0.1 mg l^{-1} IAA.

2.1.5. *Anthocephalus indicus*

Hoque *et al.* (1992) successfully micro-propagated *Anthocephalus indicus*. The best response for multiple shoot regeneration was obtained by cotyledon node culture on MS medium with 1.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA and shoot tip culture with 0.5 mg l^{-1} BA and 0.5 mg l^{-1} kinetin. Half strength MS with 0.2 mg l^{-1} IBA was optimal for root induction.

2.1.6. *Azadirachta indica*

Ramesh and Padhya (1990) obtained adventitious shoot buds when excised leaf discs of neem (*Azadirachta indica*) were cultured on Wood and Braun's medium supplemented with Kin and BA. Each isolated bud grown on medium containing gibberellic acid (GA_3) developed into a healthy shoot. Successful shoot proliferation and plant formation was achieved when stem nodes and stem segments taken from mature trees of *Azadirachta indica* were cultured in MS medium supplemented with 0.5 μ*M* thidiazuron (TDZ) and 0.5 μ*M* NAA (Yasseen, 1994).

2.1.7. Bamboos

It has been calculated that 10,000 plantlets can be obtained from single seedling in a year through *in vitro* propagation of *Dendrocalamus strictus, Bambusa arundinaceae* and *B. vulgaris* (Nadgir *et al.*, 1984). *Dendrocalamus strictus* embryos started callusing in B_5 medium having 2,4-D at 10.30 µ*M*. The callus on subculture gave rise to somatic embryos and germinated in the same medium (Rao and Rao, 1988). Multiple shoots were produced from axillary buds of 54 species from 15 genera of bamboo, cultured on MS medium containing 22.0 µ*M* BA. Rooting occured in media containing 2.7-5.4 µ*M* NAA (Prutpongse and Gavinlertvatana, 1992).

Plant regeneration via somatic embryogenesis was achieved in callus culture derived from nodal explants of *in vitro* grown seedlings and excised mature zygotic embryos of *Bambusa vulgaris, Dendrocalamus giganteus* and *D. strictus* on half strength MS culture medium with 0.5 mg l^{-1} kinetin, 2.0 mg l^{-1} 2,4-D, 10 mg l^{-1} adenine sulphate and 3 per cent (w/v) sucrose, incubated in the light or in the dark (Rout and Das, 1994). Somatic embryos germinated into normal plants and were transferred to soil with 95 per cent success.

2.1.8 *Bauhinia* species

Successful micropropagation of 15-18 year old *Bauhinia purpurea* trees through callus culture has been reported by Kumar (1992). Stem segments cultured in MS with 10 µ*M* 2,4-D developed callus, which regenerated into plantlets with 2-3 leaves in the same basal medium supplemented with 5 µ*M* kinetin. *In vitro* propagation protocol was developed for *Bauhinia variegata* from explants taken from mature trees (Mathur and Mukunthakumar, 1992).

2.1.9. *Betula* species

Vijayakumar *et al.* (1990) achieved successful *in vitro* propagation of *Betula uber*, an endangered species. Pseudo terminal buds of *B. uber* when placed in medium containing 0.6 and 0.05 mg l^{-1} of BA and IAA respectively opened in four to five days and produced upto three leaves in one week. The plantlets regenerated have been field planted. *In vitro* propagation of various *Betula* species has been reported (Arnold *et al.*, 1994). It was observed that root initiation, root elongation and root branching were increased by moderate concentrations of cupric chloride.

2.1.10. *Caesalpinia pulcherima*

Rohman *et al.* (1993) observed that axillary bud from *Caesalpinia pulcherima* cultured on MS medium containing NAA and BA/kinetin exhibited shooting. The greatest number of roots were produced on medium containing IAA and cytokinin.

2.1.11. *Casuarina* species

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

2.1.12. *Cephalotaxus harringtonia*

Establishment of callus cultures from stem explants of *Cephalotaxus harringtonia* cultured on MS medium supplemented with 4.5 µ*M* 2,4-D and 0.05 µ*M* kinetin has been reported by Wickremesinhe and Arteca (1993). Transfer of callus on to a hormone free medium gave rise to both shoots and roots.

2.1.13. *Dalbergia latifolia*

Callus mediated shoot bud formation and rooting of shoots in *Dalbergia latifolia* Roxb. has been reported (Rao, 1986; Sita *et al.*, 1986). Devi and Nataraja (1987) noticed the response of *in vitro* produced seedlings to callus mediated organogenesis on MS medium containing 1-2 mg l^{-1} BA. Successful induction of multiple shoots from excised hypocotyl segments and shoot tips of *in vitro* germinated seedlings of Indian rosewood on MS supplemented with cytokinins and auxins has been achieved by Rai and Chandra (1989). Indian rosewood could be regenerated from callus tissue of cambial origin (Kumar *et al.*, 1991). Mahato (1992) succeeded in getting multiple shoots from nodal segments of Indian rosewood (12 year old tree as well as seedlings of two and a half year old) on MS supplemented with Kin and BA 0.5 mg l^{-1} each. Both Woody Plant Medium (WPM) and Murashige and Skoog (MS) medium were found to be suitable for the primary culture establishment from the explants. *In vitro* propagation of elite trees of Indian rosewood was attempted taking root suckers as explant source (Sita and Swamy, 1992) using the three approaches namely, organogenesis, somatic embryogenesis and induction of multiple shoots from axillary meristems. Successful plantlet regeneration of Indian rosewood from leaf disc cultured on MS (¾ reduced major elements) supplemented with 5.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA has been achieved by Sita and Swamy (1993).

2.1.14. *Dalbergia sissoo*

Rapid propagation of *Dalbergia sissoo* was achieved through *in vitro* technique by the proliferation of axillary buds from 30 to 40 year old plants on MS medium supplemented with Kin 2-5 mg l^{-1}, IBA 0.1-0.5 mg l^{-1} and BA 2-5 mg l^{-1} (Chauhan *et al.*, 1994). Chimmala (1994) also successfully micropropagated *Dalbergia sissoo* using MS medium amended with 1.75 mg l^{-1} kinetin and 2.5 mg l^{-1} BA.

2.1.15. *Delonix regia*

Delonix regia was micropropagated successfully by Hoque *et al.* (1992). The best response for multiple shoot regeneration was obtained by cotyledon node culture on MS medium with 1.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA and shoot tip culture with 0.5 mg l^{-1} BA and 0.5 mg l^{-1} kinetin. Half strength MS with 0.2 mg l^{-1} IBA was optimal for root induction.

2.1.16. *Eucalyptus* species

Ashok (1985) reported success in *in vitro* propagation of *Eucalyptus citriodora.* Activated charcoal increases shoot development in this species. Kumar and Ayyappan (1987) used juvenile tissues of *in vitro* germinated seedlings of *E. camaldulensis* and produced multiple shoot on MS supplemented with BA, adenine sulphate and sodium dihydrogen phosphate.

In vitro shoot growth and rooting were obtained for *E. globulus* (Bennett *et al.*, 1994). He suggested that alternating cytokinins (BA and Kin) in multiplication media showed better growth than cultures in which either of the cytokinins was used continuously or both were used in an equimolar mixture.

2.1.17. *Eugenia jambolina*

Seedling explants of *Eugenia jambolina* were cultured on MS and B_5 media for morphogenetic response and induction of callus culture. The regeneration ability and shoot bud production was efficient on MS medium with 1.75 mg l^{-1} kinetin and 2.5 mg l^{-1} BA. The isolated induced shoots were rooted on the same medium with 1.5 mg l^{-1} NAA (Chimmala, 1994).

2.1.18. *Fagus sylvatica*

An *in vitro* shoot multiplication system was established for juvenile beech (*Fagus sylvatica*) tissues and plantlets were regenerated (Vieilez *et al.*, 1993).

2.1.19. *Ficus* species

Shoot tip explants of *Ficus lacor* saplings could be induced to form shoots at a rate of 20-30 in MS containing 1.0 mg l^{-1} BA, 1.0 mg l^{-1} kinetin and 1000 mg l^{-1} casein hydrolysate (Amatya and Rajbhandary, 1990). Rapid propagation of *Ficus religiosa* was

achieved *in vitro* from juvenile nodal explants cultured on MS medium supplemented with 2-5 mg l^{-1} BA, 0.5-2 mg l^{-1} IBA and 1-2 mg l^{-1} adenine sulphate (Deshpande *et al.*, 1994).

2.1.20. *Fraxinus* species

Hammatt and Ridout (1992) obtained plantlets from embryo derived cotyledonary nodes of common ash (*Fraxinus excelsior*). The addition of thidiazuron (TDZ) to MS salts and vitamins, instead of BAP increased both the culture weight and the proportion of common ash embryo hypocotyl explants that produced adventitious shoots and resulting shoots were rooted in half strength WPM with 1.0 mg l^{-1} IBA (Tabrett and Hammatt, 1992). Micropropagation of *Fraxinus augustifolia* from mature and juvenile plant material has been achieved (Perez-Parron *et al.*, 1994).

2.1.21. *Hevea brasiliensis*

Mascarenhas *et al.* (1982) observed that terminal buds from 10-20 year old *Hevea brasiliensis* when cultured on MS medium along with 0.5 mg l^{-1} kinetin, 2.0 mg l^{-1} BA, 2000 mg l^{-1} casein hydrolysate, 0.1 mg l^{-1} calcium pentathenate and 0.1 mg l^{-1} biotin induced three shoots per explant. Datta and Datta (1985) reported that shoots were developed from nodal explants on MS medium containing 1.0 mg l^{-1} BA whereas multiple shoots could be induced with 2.0 mg l^{-1} BA. Somatic embryogenesis was initiated

using fragments of the inner seedcoat from immature seed of rubber (Montoro *et al.*, 1995) which were cultured on a callogenisis medium comprising macronutrients, micronutrients and vitamins of carron MH medium with 30 µ*M* silver nitrate, 234 µ*M* sucrose, 4.4 µ*M* 3,4-D, 4.44 µ*M* kinetin and 2 g l^{-1} gelrite. It was found that high concentration of calcium led to friable calli but only low concentration of calcium allowed embryo development.

2.1.22. *Kalmia latifolia*

Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) was reported by Lloyd and McCown (1980). It has been worked out that at least 7000 shoots can be obtained per one sq.ft of culture shelf space per year.

2.1.23. *Leucaena leucocephala*

Successful *in vitro* multiplication for clonal propagation of *Leucaena leucocephala* through axillary bud culture was reported by Datta and Datta (1985), Goyal *et al.*(1985) and Hoque *et al.* (1992). Cotyledon node culture on MS medium with 1.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA and shoot tip culture with 0.5 mg l^{-1} BA and 0.5 mg l^{-1} kinetin gave the best result (Hoque *et al.*, 1992).

2.1.24. *Malus* species

Somatic embryogenesis has been reported in apple (Paul *et al.*, 1994). Efficient propagation occurred when cotyledons of immature zygotic embryos of apple (Malus x domestica Borkh. Golden Delicious) were plated on medium with 6 mg l^{-1} NAA in the dark and transferred to medium with 0.5 mg l^{-1} BA and 0.05 mg l^{-1} NAA in light. Plantlets were formed after two month cold treatment on half-strength plant growth factor-free MS medium.

2.1.25. *Melia azedarach*

Best response from nodal explants of *Melia azedarach* was obtained on MS medium containing 0.5 mg l^{-1} kinetin and 0.5 mg l^{-1} BA (Dhingra *et al.*, 1991).

2.1.26. *Morus alba*

Rapid clonal multiplication of mulberry plant could be obtained by culturing the axillary buds in MS with 2.0 mg l^{-1} BA (Tewary and Rao, 1990).

2.1.27. *Parkinsonia aculeata*

Mathur and Mukunthakumar (1992) developed *in vitro* propagation protocols for *Parkinsonia aculeata* from explants taken from mature trees.

2.1.28. *Populus* species

Direct and indirect bud regeneration from root, internode, leaf lamina or petiole explants of poplar (*Populus trichocarpa x deltoides* cv. Hunnegem) were tried (Jehan *et al.*, 1994). Efficient and rapid regeneration was obtained after *in vitro* culture on MS medium containing 0.5 µ*M* NAA and 1 µ*M* BA. It was also possible to obtain indirect shoot regeneration following callus induction on MS medium containing 10 µ*M* 2,4-D or NAA with subsequent sub culture in presence of 10 µ*M* NAA and 5 µ*M* BA.

2.1.29. *Prosopis* species

In vitro shoot multiplication of *Prosopis glandulosa* through indirect organogenesis could be obtained in a variety of combinations involving different media and growth regulators (Jang *et al.*, 1988). Colleman and Ernst (1990) successfully regenerated shoot cultures of *Prosopis deltoides* from internodal stem explants. Shoot formation from root segments of *Prosopis alba x P. grandidenta* cultured in WPM containing zeatin was reported by Son and Hall (1990).

Shoot formation from nodal explants of elite trees of *Prosopis cineraria*, on MS medium containing NOA and NAA at 3.0 mg l^{-1} each was achieved (Kackar *et al.*, 1991). Nandwani and Ramawat (1991) noticed multiple shoot formation from nodal

explants of *P. juliflora* on MS medium with cytokinin and auxin. Shoots could be rooted on MS medium containing NAA or IBA. Callus cultures of *P. tamarugo* were established from hypocotyl and cotyledon explants on MS medium containing 2.0 mg l^{-1} NAA and 0.2 mg l^{-1} BA (Nandwani and Ramawat, 1992). Regeneration from callus was observed when it was subcultured to MS containing 5.0 mg l^{-1} BA.

2.1.30. *Pterocarpus* species

Among the genus *Pterocarpus, P. indicus* was the first one to be attempted for micropropagation. Lee and Rao (1980) however, could only obtain callus in their experiments. Patri *et al.*(1988) experimenting on *P. santalinus* observed that shoots could be obtained through callus culture in $\frac{1}{4}$ MS supplemented with 3.0 mg l^{-1} BA and 40 mg l^{-1} adenine. Sita *et al.* (1992), however, could enhance the rate of multiplication and produce rooted plantlets of red sanders. Multiple shoot formation from axillary buds of *Pterocarpus marsupium* was obtained on WPM supplemented with 2.0 mg l^{-1} kinetin and 0.1 mg l^{-1} IAA (Santhoshkumar, 1993).

2.1.31. *Santalum album*

Endosperm tissues of *Santalum album* when cultured on MS amended with 2,4-D (1-2 mg l^{-1}), kinetin (0.1-0.2 mg l^{-1}), BA (0.5-2 mg l^{-1}) and NAA (1.0 mg l^{-1}) induced callus formation followed by differentiation (Sita *et al.*, 1980).

2.1.32. *Sesbania* species

Sesbania grandiflora could be micropropagated by using hypocotyl and cotyledon segments (Shankar and Ram, 1990 and Hoque *et al.*, 1992). Callus of *Sesbania bispinosa* could be obtained from both cotyledon and mature leaf explants in MS containing BA at 0.5 mg l^{-1} and 2,4-D at 2.0 mg l^{-1}. Callus could be later regenerated into shoot in MS medium supplemented with 2.0 mg l^{-1} BA and 15 per cent (v/v) coconut milk (Sinha and Mallick, 1991).

2.1.33. *Stewartia malacodendron*

Surface sterilized shoot tips and nodal explants from two year old container grown seedlings of *Stewartia malacodendron*, an endangered woody species were established on WPM supplemented with 4.44 µ*M* BA and solidified with 0.8 per cent Agar (Gillis and Kane, 1994).

2.1.34. *Syzygium cumini*

Multiple shoots could be induced from shoot tip explants of *Syzygium cumini* seedlings (Yadav *et al.*, 1990). The authors used MS medium supplemented with 0.23-8.9 µ*M* of BA singly or in combination with auxins (NAA, IBA or IAA) at the range of 0.12-1.0 µ*M*.

2.1.35. *Tamarindus indicus*

Mascarenhas *et al.* (1987) observed successful *in vitro* propagation of tamarind when explants from nearly all parts of seedlings were cultured on MS having cytokinins and auxins. Shoot tip cultures in tamarind on MS with 0.5-5.0 mg l^{-1} BA was effective for shoot induction (Kopp and Nataraja, 1990). Optimal culture conditions for high frequency plant regeneration from excised cotyledons of tamarind were established by Jaiwal and Gulati (1991).

2.1.36. *Tectona grandis*

The successful induction of multiple shoot formation from terminal buds of 100 year old teak trees was achieved by Gupta *et al.* (1980). Over 500 plants could be produced from a single bud of a selected elite tree in MS containing kinetin (0.15 mg l^{-1}) and BA (0.15 mg l^{-1}).

2.1.37. *Vateria indica*

Different routes like enhanced release of axillary buds, organogenesis and embryogenesis were attempted for Malabar white pine (*Vateria indica*) by Divatar (1994). Out of the various growth regulator combinations tried, only 2-ip and IBA could support bud break and shoot production. Moderate callusing was obtained from leaf and internodal segments on MS and half strength MS media supplemented with growth regulators

2-ip + 2,4-D (in MS media) and 2-ip + IBA (in half strength MS). However, the calli did not respond to organogenesis or embryogenesis.

2.1.38. *Quercus* species

Manzanera and Pardos (1990) reported successful micropropagation from juvenile and adult oak (*Quercus suber*). It was observed that the explants from old and young sources differed greatly in their optimum culture conditions. Somatic embryogenesis and plant regeneration have been noticed in *Quercus acutissima* (Kim *et al.*, 1994).

2.1.39. *Ziziphus* species

Kim and Lee (1988) reported that axillary buds of *Ziziphus* cv. Geumsung showed best shoot and root growth when 500 mg l^{-1} activated charcoal was added to half strength MS with 0.5 mg l^{-1} BA. However, cv. Bokjo responded best to 1000 mg l^{-1} activated charcoal in the above medium.

2.2. Controlling factors in micropropagation

2.2.1. Culture medium

Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of the culture media (Razdan, 1993). Although the basic requirements of cultured plant tissues

are similar to those of whole plants, in practice, nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species. A proper medium should contain not only adequate quantity of major plant nutrients like salts of nitrogen, potassium, calcium, phosphorus, magnesium and sulphur as well as minor nutrients like salts of iron, manganese, zinc, boron, copper, molybdenum and cobalt, but also a carbohydrate usually sucrose, trace amounts of organic compounds like vitamins, amino acids and plant growth regulators. Some cultures perform well with the addition of undefined organic compounds like coconut water, fruit juice, yeast extract and casein hydrolysate. Media compositions are formulated considering specific requirements of a particular culture system. For example, some tissues show better response on solid medium while others prefer a liquid medium.

2.2.1.1. Basal media

Recently, the complexity of *in vitro* nutrients has been considered in a more unified fashion. The earlier media were characterised by a low overall concentration of inorganic ions, especially those of potassium and nitrate, and by providing nitrogen solely in the form of nitrate. Later, the media developed for germination of orchid seeds were similar, except that they contained ammonium ions (Knudson, 1946; Vacin and Went, 1949). Composition of different media has been developed by

different workers and named after them such as, Gautheret (1942), White (1943), Hildebrandt *et al.* (1946), Nitsch (1951), Heller (1953), Reinert and White (1956), Murashige and Skoog (1962), Linsmaier and Skoog (1965), Gamborg *et al.* (1968), Schenk and Hildebrandt (1972) etc.

A medium specially designed for tree species is the Woody Plant Medium (WPM) of Lloyd and McCown (Lloyd and McCown, 1980). Compared with MS, it is low in ammonium, nitrate, potassium, chloride and high in sulphate. B_5 medium originally designed for cell suspension or callus cultures, has, with modifications, proved valuable for protoplast culture (Gamborg and Shyluk, 1981). The SH medium is similar to B_5, but with slightly higher levels of mineral salts. The medium designated N_6 was developed for cereal anther culture and is used with success in other types of cereal tissue culture also (Chu, 1978). The E_1 medium supports rapid growth of cells for embryogenesis and for the culture of protoplasts (Gamborg *et al.*, 1983). In experiments on anther culture the medium devised by Nitsch and Nitsch (1969) is frequently used. After 1980, the most popular media have been DCR (Gupta and Durzan, 1985) and WPM (Lloyd and McCown, 1980) especially for woody species. Success in employing these various media in all probability lies in the fact that the ratios as well as the concentrations of nutrients nearly match the optimum requirement with regard to the growth and differentiation of respective cell or tissue systems (Razdan, 1993).

2.2.1.2. Growth regulators

Growth regulators are organic compounds (other than a nutrient) which in small amounts promote, inhibit or qualitatively modify growth and development (More, 1979). There are several recognised classes of plant growth substances such as auxins, cytokinins, gibberellins, ethylene, abscissins and the hypothetical compounds like florigen and anthesins. The growth, differentiation and organogenesis of tissues become feasible only on the addition of one or more of these classes of hormones to a medium. The most important factor in successful tissue culture is the addition of plant growth regulators (Krikorian, 1982).

Skoog and Miller (1957) proposed the concept of hormonal control of organ formation. Their classic experiments on tobacco pith cultures showed that root and bud initiation were conditioned by a balance between auxin and cytokinin. High concentration of auxin promoted rooting whereas proportionately more cytokinin initiated bud or shoot formation. Unequal proportion of auxin and cytokinin led to unorganised growth of the tissue.

Following the discovery of auxin by Went (1926) and its chemical characterisation by Kogl *et al.* (1934), several workers found out its use in cell cultures for purposes like callus induction, rooting etc. Media are supplemented with various

auxins such as 1H-indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA), 1H-indole-3-butyric acid (IBA), 2,4-dichlorophenoxy acetic acid (2,4-D), naphthoxy acetic acid (NOA), 4-chlorophenoxy acetic acid (4-CPA), 2,4,5-trichloro phenoxyacetic acid (2,4,5-T), 2-methyl-4-chlorophenoxy acetic acid (MCPA), 4-amino-3,5,6-trichloro picolinic acid (Picloram) and 3,6-dichloro-2-methoxy benzoic acid (Dicamba).

Cytokinins are adenine derivatives which are mainly concerned with cell division, modification of apical dominance and shoot differentiation in the tissue culture. The most frequently used cytokinins are 6-Furfuryl-aminopurine (kinetin), N-6-Benzyl-adenine (BA), 6-(4-hydroxy-3-methyl-trans-2-butanyl-amino) purine (zeatin) and N-6-(2-isopentyl) adenine (2-ip).

It was recently discovered that N, N' diphenyl-urea (DPU), N-2-chloro-4-pyridyl-N-phenyl urea (CPPU), N-phenyl-N'-1,2,3-thiadiazol-5-yl urea (thidiazuron or TDZ) and other derivatives of diphenyl urea show the cytokinin type activity (Pierik, 1989). Thidiazuron (TDZ) is among the most active cytokinin-like substances for woody plant tissue culture. It facilitates efficient micro-propagation of many recalcitrant woody species. Sankhla *et al.*(1994) observed that callus and shoot formation did not occur without TDZ in *Albizia julibrissin*. The concentrations at which TDZ is most effective are 10 to 1000 times lower than

other plant growth regulators. The high cytokinin activity and positive response of woody species to TDZ have established it as among the most active cytokinins for *in vitro* manipulation of many woody species (Huetteman and Preece, 1993).

Gibberellins and abscisic acid are occasionally used in tissue culture. Of the over 20 known gibberellins, GA_3 is the most commonly used. It promotes the growth of cell cultures at low density, enhances callus growth and induces dwarf or stunted plantlets to elongate. Abscisic acid (ABA) in the culture medium either stimulates or inhibits the callus growth depending on the species (Razdan, 1993). Sankhla *et al.* (1993) reported that gibberellin biosynthesis inhibitors increased shoot formation in *Albizzia julibrissin*, conversely, GA_3 decreased shoot formation, indicating that modification of gibberellin status can have a strong impact on the number of shoots formed.

Ethylene is also an important growth hormone and has an important influence on many aspects of *in vitro* regeneration. But it is also clear that we cannot at present describe a specific role or roles for ethylene in tissue culture which can be applied at a general species-wide level (Biddington, 1992).

2.2.1.3. Other medium supplements

Several supplements of uncertain and variable composition are now common in use. Adenine, adenine sulphate, casein hydrolysate, yeast extract, peptone, coconut water, banana homogenate, orange juice, tomato juice etc. are some of the complex substances added to the media. In tissue culture the success achieved with the use of coconut milk (5 to 20 per cent) has been significant. Similarly, potato extract has been found as a suitable medium for anther culture (Razdan, 1993). Coconut water is reported to be having myo-inositol (Pollard *et al.*, 1965). Adenine and its more soluble form adenine sulphate enhance growth and organ formation due to their cytokinin like activity (Skoog and Tsui, 1948). Amino acids are routinely added to the media as they provide an immediately available source of nitrogen and their uptake can be much more rapid than that of inorganic nitrogen in the same medium (Simpkins *et al.*, 1970). Polyphenolic compounds like phloroglucinol in the medium too has been found to have a beneficial role in organogenesis and growth (Hunter, 1979; Mallika *et al.*, 1992).

Addition of activated charcoal in tissue culture media may have beneficial or harmful effects. Growth, rooting, organogenesis and embryogenesis are reported to be stimulated in a wide variety of species and tissues (Wang and Huang, 1976) and

inhibited in certain others on the other hand (Fridborg and Eriksson, 1975). It may also prevent browning of tissues (Tisserat, 1979). Harmful effects of activated charcoal include binding of plant growth regulators and other metabolites (Weatherhead *et al.*, 1978). Scott *et al.* (1990) has reported that activated charcoal added to liquid MS reduced IAA and IBA concentrations by over 97 per cent.

2.2.1.4. Carbon energy sources

Sucrose is the most commonly used carbon energy source for the plant tissue culture. Most of the workers have used 20 to 30 g l^{-1} sucrose in the medium. Glucose and fructose may be substituted in some cases, but most other sugars are reported to be very poor (George and Sherrington, 1984). Marino *et al.* (1993) reported that shoot proliferation rate was increased with sorbitol as the carbon energy source than with sucrose in apricot (*Prunus armeniaca*). The sugar-alcohol sorbitol (D-glucitol) has proven the most effective carbon source for *in vitro* proliferation of the apple rootstocks *(Malus robusta* Rehd. No.5) (Pua and Chong, 1984) and *Malus pumila* M.9 (Welander *et al.*, 1989).

2.2.1.5. Vitamins

It is necessary to supplement the medium with required vitamins and amino acids to achieve the best growth of the tissue. Thiamine (B_1), nicotinic acid (B_3), pyridoxine (B_6), calcium pentothenate (B_5) and myo-inositol are used more often. Of these, thiamine is the basic vitamin required by all cells and tissues. Linsmaier and Skoog (1965) demonstrated that, most vitamins are not essential for callus growth in tobacco. Pyridoxine, biotin and nicotinic acid could be deleted from the medium without serious impact on growth.

2.2.2. Explant

2.2.2.1. Explant size and its position on the mother plant

The type of the explant varies with each plant species and the most suitable one should be determined for each species (Skirvin, 1980). Norton and Norton (1986) studied the effects of explant length (2.5 to 20 mm), axillary bud number (0 to 6), presence or absence of apex and explant derivation (top, middle or base of plant canopy) in *Prunus* and *Spirea*. The number of shoots formed after four weeks increased with the explant length and decreased with the number of buds present. Explants taken from the top of the canopy produced most shoots, but removal of the apex did not affect the shoot number. If the explant size is small, the cut surface : volume ratio is high and there will be difficulty in the survival of the explant (Hussey, 1978).

As a rule, larger the size of the explant, the more rapid the growth and greater are the rates of survival (Hussey, 1983). However, larger the explant size more will be the chance of it harbouring contaminant micro-organisms.

2.2.2.2. Age of the explant

Generally, the more juvenile the specimen, the easier it is to propagate vegetatively. Often some parts of the tree may be mature, or senescent, while other portions still display juvenile characteristics. If we are faced with a recalcitrant *in vitro* culture from which true to type vegetative propagation does not materialize, it probably is wise to first develop methods for vegetative propagation from highly juvenile material (Bonga, 1982). Slow growth, low propagation rate and weak *in vitro* performance of mature explants as compared to juvenile shoots has been discussed in different tree species like pear (Maarri *et al.,* 1986), *Corylus avellana* (Messeguer and Mele, 1987; Rodriguez *et al.,* 1988), *Artocarpus heterophyllus* (Rajmohan and Kumaran, 1988), *Dalbergia latifolia* (Sita *et al.,* 1980 and Rao, 1986) etc. The transfer into soil of rooted plantlets is more critical in the micropropagation process of mature trees than with plantlets regenerated from seedlings in case of sycamore maple (Hanus and Rohr, 1987). However, *Azadirachta indica* (Yasseen, 1994), *Dalbeigia sissoo* (Chauhan *et al.,* 1994),

Tecotona grandis (Gupta *et al.*, 1980) and *Artocarpus heterophyllus* (Amin and Jaiswal, 1993) have been cloned *in vitro* successfully taking nodal and terminal bud explants from mature trees.

2.2.2.3. Season of collecting the explants

Season of collecting the explant was also found to influence the success of plant tissue culture. Yu (1991) observed that the test material taken after 10 continuous rainy days had a contamination rate of cent per cent and that taken after 15 continuous sunny days had a low contamination rate of 20 per cent in *Litchi chinensis*. Season is a very important factor for mature tree tissue culture. Spring (March-April) was the best season to initiate tissue culture from mature trees. At least 95 per cent aseptic shoot cultures were obtained and buds flushed within 10 to 12 days as compared to 5 to 6 weeks during other seasons for *Corylus avellana* (Messeguer and Mele, 1987). Amin and Jaiswal (1993) reported that November to January was the best season for initiation of cultures from field grown trees of *Artocarpus heterophyllus*.

2.2.2.4. Genotype

Response of *in vitro* culture vary between plant species, and even in a species it may vary between varieties. There were large differences in the capacity of the explants from different

selections of mature *Eucalyptus marginata* trees to survive in culture (McComb and Bennet, 1982). Genotype specific effects have been reported for *Sequoia sempervirens* (Sul and Korban, 1994).

2.2.2.5. Surface sterilization

The explants collected may harbour a lot of micro-organisms which when inoculated on to a nutrient medium, contaminante the entire *in vitro* system. The objective of surface sterilization is to remove all the micro-organisms, present on the explant with minimum damage to the plant part (George and Sherrington, 1984). Ethyl alcohol, mercuric chloride, chlorine water, bromine water, silver nitrate, sodium hypocholorite, calcium hypochlorite, commercial bleach etc. are some of the common surface sterilants used in tissue culture. Maroti and Levi (1977) advanced a view wherein explants are surface sterilised in a series of steps involving a rinse in ethyl alcohol (45 per cent v/v) for three minutes followed by a 10 minute bleach treatment (0.5-10 per cent w/v) and finally three rinses in distilled water. In case of *Dalbergia latifolia* use of mercuric chlorite (0.1 per cent w/v) for 10-15 minutes has been demonstrated to be effective (Sita *et al.*, 1986; Sita and Swamy, 1992 and Mahato, 1992).

2.2.2.6. Systemic contaminants

Micro organisms present on the outer surface can be eliminated by surface sterilization treatments, but those existing within the internal tissues cannot be removed, and they cause latent contamination, which is a serious problem associated with woody plant tissue culture. Ten systemic micro organisms (bacteria and yeasts) were isolated from stem sections of *ex vitro* grown rubber plants (Wilson and Power, 1989). Growing stock plants under controlled conditions and regularly spraying the plants with systemic and contact fungicides can reduce or avoid the contamination problem to certain extent (Mallika *et al.*, 1992).

Several workers have reported the use of various fungicides in the culture medium for reducing fungal contamination (Brown and Sommer, 1982; Shields *et al.*, 1984). Dodds and Roberts (1985) suggested to avoid the use of antibiotics for sterilization since they or their degradation products may be metabolized by plant tissues with unpredictable results.

A new chemical sterilant, diethylpyrocarbonate (DPC), when incorporated to the media killed all the contaminating microbes. The plant cells cultivated on media treated with DPC (1-2 g l^{-1}) did not show changes in their basic characteristics (Macek *et al.*, 1994). Parfitt and Almehdi (1994) reported that

contamination was controlled through use of a two per cent carbon dioxide atmosphere, increased light level to promote photosynthesis and elimination of all carbon sources from the substrate in case of *Pistachio* species.

2.2.2.7. Explant exudations

A serious problem generally faced in culturing adult tissues from woody species is the oxidation of phenolic substances leached out from the cut surface of the explant which turns the medium dark brown leading to the death of explant (Razdan, 1993). Polyphenol interference in culture establishment of different woody plant species was reported by several workers (Anderson, 1975; Lloyd and McCown, 1980; Amin and Jaiswal, 1988 and Gill and Gill, 1994). Gill and Gill (1994) reported that explants taken during the months of November-April resulted in exudation of minimum phenols and the maximum release of phenols during May-June and September-October in *Eucalyptus.*

2.2.3. Culture environment

Physical conditions as well as pH of the medium, light, temperature and relative humidity of the culture room, season of culturing etc. all have been found to play a profound influence on the rate of growth and extent of differentiation exhibited by the cultured cells.

Importance of physical condition on culture is evident in case of *Dalbergia latifolia* cultures (Mahato, 1992). Shake cultures in liquid medium gave a performance far superior to semisolid media.

Bonga (1982) remarked that pH of the medium is usually set at about 5.0 for liquid cultures and at 5.8 for semi-solid. Leifert *et al.* (1994) established the effect of short term pH reduction in culture medium on the prevention of survival and establishment of contaminating microbes in a *Delphinium* species. Shoots grown at pH 5.6 were all visibly contaminated within eight subcultures while 65 per cent of shoots grown on pH 3.5 were visibly free of contamination after 13 subcultures. This method may be applied to large scale commercial propagation.

Light requirement for differentiation involve a combination of several components, namely, intensity, quality and duration (Murashige, 1974). The optimum day light period required is 16 hours for a wide range of plants.

Yeoman (1986) reported that the environmental temperature of the species at the original habitat should be taken into consideration during the *in vitro* culture also. However, most tissue culture experiments are done at a temperature of around 25±2°C, with successful results.

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.

2.2.4. Rooting of *in vitro* produced shoots

In vitro produced shoots can be rooted either through *in vitro* methods itself or through *ex vitro* methods. Often where shoot multiplication was induced on full strength MS medium, the salt concentration was reduced to half (Garland and Slotz, 1981; Zimmerman and Broome, 1981) or a quarter (Skirvin and Chu, 1979) for rooting. Lowering nitrogen content has been observed to promote rooting (Rucker, 1982).

Generally, auxin favours root formation. Among the auxins, NAA has been the most effective one for induction of rooting (Ancora *et al.*, 1981). Sometimes a combination of auxins may give a better response (Gupta *et al.*, 1980). Root initiation, root elongation and root branching were increased by moderate concentrations of cupric chloride (Arnold *et al.*, 1994) in birch. Activated charcoal has got profound influence on rooting of shoots *in vitro*, It may absorb toxic substances as well as residual cytokinin in the medium there by improving root regeneration and development (Jaiswal and Amin, 1987).

Rooting of shoots *in vitro* and *in vivo* has been reported by the simple, highly efficient and more economical methodology by Yeoman (1986), Schwarz *et al.* (1988) and Vijayakumar *et al.* (1990). This is achieved by using vermiculite, sand and peat singly or in combination in tubes or pots as the rooting medium and maintaining it in high humid conditions.

2.2.5. Hardening and planting out

Tissue culture plantlets are very tender and their transfer from the artificial environment of the culture vessel to the free living existence of a green house or similar environment and their establishment outside culture conditions is of great importance. Nutrition of the plantlets during the acclimatization phase is species dependant. Success in acclimatization depends upon not only the post-transfer conditions but the pre-transfer culture conditions also (Ziv, 1986). Schackel *et al.* (1990) indicated that stomata of apple shoots do not close after being removed from the culture. Such non functional leaves will have to be hardened during this phase for achieving success in planting out.

2.3. Economics of plantlet production *in vitro*

Economic conditions assume great importance in any commercial attempt of mass multiplication of plants. The high rate of multiplication, less space and time requirement and year

round production are some of the important factors that attribute to profitable production of plantlets through *in vitro* techniques. The estimated unit cost of producing one jack plantlet was Rs.9.09 (Rajmohan, 1985) and one rosewood plantlet was Rs.4.50 (Mahato, 1992). Results of the analysis of *in vitro* plantlet 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 (Hasnian and Cheliak, 1986).

The investment in commercial tissue culture business will depend to a large extent on cost of the laboratory set-up, type of plant to be propagated and the skill involved. Countries like India could provide an incentive in attracting alien investment in establishing laboratories for commercial tree micropropagation (Razdan, 1993). It can be envisaged that eventually the unit cost of tissue culture propagation of economically important species will be considerably diminished through plant regeneration from somatic embryos (Sohal and Srivastava, 1994) and switching the rooting stage from an *in vitro* step to an *ex vitro* one. Another approach followed now-a-days is to automate micropropagation at its various stages. In this connection bioreactors are being used for large scale multiplication of somatic embryos, shoots and bulbs. Robots have also been used for micro-propagation work (Razdan, 1993).

Though micropropagation is being increasingly used for large scale production of many fruit, nut and ornamental species, reports on clonal propagation of tropical tree species at a commercial scale are not frequent in the literature. Trees especially the leguminous one had been resisting the attempts for micropropagation till very recent time. The research works currently going on in the field of *in vitro* regeneration of tropical trees are not keeping pace with the requirement. A continued increase in the use of micropropagation for woody plants is anticipated.

Materials and Methods

MATERIALS AND METHODS

The materials used and the methods followed for the study on clonal propagation of selected plus trees of Indian rosewood (*Dalbergia latifolia* Roxb.) through tissue culture, done at the College of Forestry, Vellanikkara during 1993 to 1995 are presented here.

3.1. Source materials

3.1.1. Plus trees

A survey was carried out to identify the candidate trees of rosewood in Vellanikkara and nearby areas. Three plus trees (Plates 1, 2 and 3) were selected based on the morphological and biometric observations from the identified candidate trees in the field. Details of the selected plus trees are given in Table 1.

3.1.2. Root suckers

Few root suckers of the plus tree TR-1 were identified and selected. The other two trees were not having root suckers. The selected root suckers were sprayed with a mixture of a systemic fungicide Bavistin 50 per cent WP (Carbendazim) and a contact fungicide Indofil M-45 (Mancozeb) 0.3 per cent daily in order to reduce the chances of contamination in the cultures.

Table 1 Biometric observations on the selected plus trees of rosewood*

Tree No.	Location	Total height (m)	Clean bole height (m)	Girth (m)	Total volume (m^3)	Clean bole volume (m^3)	Age (approx)
TR-1	Kerala Agricultural University Campus, Vellanikkara	19.42	9.29	1.52	2.499	1.537	Above 60 years
TR-2	Kerala Agricultural University Campus, Vellanikkara	19.32	5.76	1.12	1.350	0.517	Above 50 years
TR-3	Farmer's field near NBPGR, Vellanikkara	20.02	8.70	1.22	1.660	0.927	Above 60 years

* Total height as well as clean bole height was measured using an optical clinometer and girth at breast height with a measuring tape. Volume was worked out using the formula, basal area x corresponding height x form factor. The values given for form factor were 0.7 and 0.9 for total and clean bole height, respectively.

3.1.3. Young trees

To know the difference in *in vitro* response of juvenile and adult materials, few young (eight to ten year old) trees were also used as source material for explants. These trees were also given the prophylactic spraying with fungicides as in the case of root suckers.

3.2. Collection and preparation of explant

3.2.1. Collection of stem segments

Stem segments of approximately 30-40 cm with 12-15 nodes were excised from the selected plus trees using a sharp blade attached to a long bamboo pole and brought to the laboratory as quickly as possible. For the young trees and root suckers, a sharp razor blade was used for the purpose.

3.2.2. Preparation of explant

The cuttings were defoliated and washed free of dust in running tap water using a detergent. After drying the segments in blotting paper, they were swabbed with cotton dipped in 70 per cent (v/v) ethanol. The stem segments were then cut into nodal or internodal segments of size 1.5 cm (approximately) using a sharp sterile blade.

It was not possible to give the fungicidal spray to the plus trees due to their large size. Therefore, in order to control the contamination in cultures, nodal explants collected from the plus trees were immersed in the fungicidal solution used for spraying the root suckers and young trees.

3.3. Treatments done for elimination or neutralisation of polyphenols

a. Soaking the explants in distilled water for three hours before culturing

b. Soaking the explants in solution of 150 mg l^{-1} ascorbic acid

c. Soaking the explants in solution of 150 mg l^{-1} citric acid

d. Soaking the explants in solution of ascorbic acid and citric acid both at 150 mg l^{-1}

e. Addition of ascorbic acid at the rate of 150 mg l^{-1} in the culture medium

f. Addition of citric acid at the rate of 150 mg l^{-1} in the culture medium

g. Addition of ascorbic acid and citric acid, both at 150 mg l^{-1}

h. Addition of polyvinylpyrrolidone at the rate of 7 g l^{-1} in the culture medium

i. Addition of 150 mg l^{-1} citric acid, 150 mg l^{-1} ascorbic acid and 7 g l^{-1} polyvinylpyrrolidone in the culture medium.

j. Addition of activated charcoal at the rate of 0.25-1.00 g l^{-1} in the culture medium

k. Placing the cultures in the dark for first 8-10 days of culturing initial explants

l. Subculturing the explant at short intervals during the initial phase of development and

m. A combination of all these methods

3.4. Surface sterilisation

Pre-treated explants were washed with sterile water and then moved to a *Klenzaids* laminar air flow cabinet, which was made contamination free using a UV lamp. The working table and

sides of the laminar flow were thoroughly wiped with absolute alcohol. The sterilants used were mercuric chloride (0.1 per cent w/v) and freshly prepared chlorine water. For all the treatments the explants were immersed in the given chemical for the stipulated period (12 and 5 minutes for mercuric chloride and chlorine water, respectively) with occasional stirring. The explants after surface sterilisation were removed from the chemical and rinsed thrice in sterilised distilled water to remove traces of sterilant sticking to the surface. The explants then were spread on to a sterile petri plate lined with sterile blotting paper, for drying.

3.5. Media

The basic media used for the study included Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980). The chemical composition of the media are given in Table 2. The basic media were supplemented with plant growth regulators and other additives in the different experiments.

3.5.1. Growth regulators

Different concentrations of auxins (2,4-D, IAA, NAA and IBA) and cytokinins (kinetin, 2-ip and BA) individually or in combination were incorporated in the media at various stages of culture.

Table 2 Chemical composition of Murashige and Skoog (MS) and Woody Plant Medium (WPM)

Compound	Quantity (mg l^{-1})	
	Murashige and Skoog (MS)	Woody Plant Medium (WPM)
INORGANIC		
Ammonium nitrate	1650.0	400.0
Boric acid	6.2	6.2
Calcium chloride - 2 hydrate	440.0	96.0
Calcium nitrate - 4 hydrate	0	556.0
Cobalt chloride - 6 hydrate	0.025	0
Copper sulphate - 5 hydrate	0.025	0.025
Ferrous sulphate - 7 hydrate	27.8	27.8
Manganese sulphate - 1 hydrate	22.3	22.3
Magnesium sulphate - 7 hydrate	370.0	370.0
Na_2EDTA - 2 hydrate	37.3	37.3
Potassium dihydrogen phosphate	170.0	170.0
Potassium iodide	0.83	0
Potassium nitrate	1900.0	0
Potassium sulphate	0	990.0
Sodium molybdate - 2 hydrate	0.25	0.25
Zinc sulphate - 7 hydrate	8.6	8.6
ORGANIC		
Inositol	100.0	100.0
Nicotinic acid	0.5	0.5
Thiamine Hcl	0.1	0.1
Pyridoxine Hcl	0.5	0.5
Glycine	2.0	2.0
OTHERS		
Sucrose (in per cent w/v)	3.0	2.0
Agar (in per cent w/v)	0.7	0.7

½ MS denotes half the amounts of the inorganic constituents per litre.

3.5.2. Other supplements

Adenine sulphate, casein hydrolysate, cycocel, phloroglucinol, L-glutamine, silver nitrate, coconut water and activated charcoal were tried at different levels.

3.5.3. Anti oxidants

In order to prevent blackening or browning of the medium, citric acid (150 mg l^{-1}) ascorbic acid (150 mg l^{-1}) or a combination of the two (150 mg l^{-1} each) were added to the media.

3.5.4. Absorbants

Activated charcoal (0.25 to 1.00 per cent w/v) or polyvinylpyrrolidone (0.7 per cent w/v) were added to the media to absorb the inhibitory compounds like polyphenols.

3.6. Media preparation

The chemicals used for preparation of 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 by dissolving the required quantity of the chemical in distilled water and were stored in amber coloured bottles under

refrigerated conditions. The stock solutions of nutrients were prepared freshly every four weeks and that of vitamins, amino acids and growth regulators every week.

Specific quantities of the stock solutions of the chemicals were pipetted out into a 1000 ml beaker which was rinsed with distilled water. Sucrose and inositol were added fresh and dissolved. Required quantities of growth regulators and other supplements were also added and the solution was made upto the required volume. The pH of the solution was adjusted to the range 5.5 to 5.8 (using 1 *N* NaOH or 1 *N* HCl) with a pH meter. Agar was then added to the medium and the final volume made upto 1000 ml.

The solution was then boiled for melting the agar by keeping in a microwave oven. Twenty ml each of the melted media was poured hot to the oven dried culture tubes (150 x 25 mm), which were previously washed, rinsed in distilled water and dried. Borosil brand glasswares were used in the study. The tubes with the medium were then tightly plugged with cotton plugs and autoclaved for 15-20 minutes at 15 psi pressure and 121°C temperature (Dodds and Roberts, 1985). After sterilization, the culture tubes were stored in an air-conditioned culture room for further use.

3.7. Inoculation procedure

Inoculation was carried out under strict aseptic condition in a laminar air flow cabinet. Sterilised forceps, petriplates, blotting paper, surgical blades and distilled water were used.

To inoculate the explants to the culture medium, the cotton wool plug of the culture tubes was removed and the tube neck was first flamed over a gas burner kept in the chamber. The sterile explants were quickly transferred into the medium using sterile forceps. The neck of the culture tube was again flamed and the cotton wool plug replaced.

3.8. Culture conditions provided

The cultures were incubated in a culture room provided with cool white fluorescent (Philips) lamps to give a light intensity of 2000 lux for 16 hours light period or in complete darkness for the initial eight to ten days of culture to overcome the phenol problem. The temperature was maintained at 27±2°C.

3.9. Experiments done using explants from young trees

3.9.1. Enhanced release of axillary buds

Enhanced release of axillary buds was tried for eight to ten year old young trees. Eight media combinations which were found to be most favourable for *in vitro* propagation from nodal

explants of young trees of Indian rosewood (Mahato, 1992) were selected for the study. Details of the media used are presented in Table 3.

Silver nitrate at the rate of 5.0 mg l^{-1} was added to the media to check the callus growth.

3.9.2. Storage of explants

Defoliated stem segments of about 30-40 cm length, sealed in polythene cover were stored in refrigerator at approximately 5°C for one or two days. Explants prepared from the refrigerated stem segments were used for culture in order to see the effect of storage on *in vitro* growth.

3.9.3. Rooting

3.9.3.1. *In vitro* rooting

In vitro produced shoots were kept for rooting in half strength MS medium supplemented with one per cent (w/v) activated charcoal and incubated in light. The cut end of individual shoots were dipped in 1000 mg l^{-1} IBA made up in alcohol for two seconds before keeping for rooting.

Table 3 Media combinations tried for enhanced release of axillary buds of young trees of rosewood

Basal media	Cytokinin (mg l^{-1})	Auxin (mg l^{-1})
MS	BA 0.25	Nil
MS	BA 2.00	Nil
WPM	BA 0.25	Nil
WPM	BA 2.00	IAA 0.10
WPM	Kin 1.00	IAA 0.10
WPM	Kin 2.00	IAA 0.10
WPM	2-ip 0.50	Nil
WPM	2-ip 1.00	Nil

3.9.3.2. *Ex vitro* rooting

A trial was also attempted to get rooting under *in vivo* condition providing high humidity (90 per cent RH). The cut ends of individual shoots were treated with 1000 mg l^{-1} IBA made up in alcohol. They were transferred to small pots with sterilised sand. The shoots were covered by polythene bag for 15-20 days.

3.9.3.3. Planting out

In vitro produced plantlets were planted out from the culture tube following a series of acclimatization processes. After proper root development under *in vitro* the plantlets were taken out from the 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 plantlets were then transferred to sterilized sand contained in small pots.

3.9.3.4. Acclimatization

Control of temperature and humidity during acclimatization was achieved by covering the transplanted plantlets with transparent plastic covers and keeping the pots under shade. Plants were nourished with MS nutrient solution (with macro and micro nutrients at half strength) having a pH 5.8 on alternate days. 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 and the plantlets were treated like any other containerised seedlings.

3.10. Experiments done using explants from plus trees

3.10.1. Enhanced release of axillary buds

Nodal explants, pre-treated to overcome the phenol and contamination problem were kept for the enhanced release of axillary buds. Table 4 show the different media combinations tried. Subculturing was done as and when required.

3.10.2. Difference in response between genotypes

To know the difference in *in vitro* response between the three selected plus trees, nodal explants were cultured separately on MS medium supplemented with BA 2.0 mg l^{-1}.

3.10.3. Rooting *in vitro*

Shoots excised from the shoot proliferating culture were used for *in vitro* rooting. Shoots after giving the pulse treatment were kept for rooting on half strength MS medium supplemented with one per cent (w/v) activated charcoal and incubated in light.

Table 4 Treatments tried for enhanced release of axillary buds of plus trees of rosewood

Basal medium	Treatment
MS	BA (0.25, 0.50, 2.00, 3.00 & 5.00 mg l^{-1}) alone and along with IAA (0.10 mg l^{-1})
MS	Kin (1.00, 2.00, 3.00 & 5.00 mg l^{-1}) alone and along with IAA (0.10 mg l^{-1})
MS	2-ip (0.25, 0.50, 1.00, 3.00 & 5.00 mg l^{-1}) alone and along with IAA (0.10 mg l^{-1})
MS	AS (1.00, 4.00 & 8.00 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
MS	CH (100, 500 & 1000 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
MS	CCC (1.00, 3.00 & 5.00 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
MS	PG (200 & 500 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
MS	L-G (1000 & 1500 mg l^{-1}) along with BA (2.00 mg l^{-1})
MS	CW (10 & 20%) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
MS	AC (0.25 & 1.00%) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
WPM	BA (0.25, 0.50, 2.00, 3.00 & 5.00 mg l^{-1}) alone and along with IAA (0.10 mg l^{-1})
WPM	Kin (1.00, 2.00, 3.00 & 5.00 mg l^{-1}) alone and along with IAA (0.10 mg l^{-1})
WPM	2-ip (0.25, 0.50, 1.00, 3.00 & 5.00 mg l^{-1}) alone and along with IAA (0.10 mg l^{-1})
WPM	AS (1.00, 4.00 & 8.00 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
WPM	CH (100, 500 & 1000 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
WPM	CCC (1.00, 3.00 & 5.00 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
WPM	PG (200 & 500 mg l^{-1}) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
WPM	CW (10 & 20%) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})
WPM	AC (0.25 & 1.00%) alone and along with Kin (1.00 mg l^{-1}) and IAA (0.10 mg l^{-1})

3.11. Experiments done using explants from root suckers of the plus tree TR-1

3.11.1. Enhanced release of axillary buds

Nodal segments from the identified root suckers of the plus tree TR-1 were used for axillary bud release. Different media combinations tried are presented in Table 5.

3.11.2. Rooting *in vitro*

Shoots excised from the shoot proliferating culture were used for *in vitro* rooting. Shoots given with the pulse treatment were kept for rooting, on half strength MS medium supplemented with one per cent (w/v) activated charcoal and incubated in light.

3.11.3. Callus mediated organogenesis

Internodal segments from the root suckers were used for callus culture. The basal medium MS supplemented with BA (0.5 or 1.0 mg l^{-1}), NAA (1.0 or 2.0 mg l^{-1}), 2,4-D (3.0 or 4.0 mg l^{-1}) and coconut water (10 or 15 per cent) was used for callus induction. For maintenance, calli were routinely subcultured on the same medium. Small pieces of these calli were cut and cultured on MS medium amended with BA (3.0 mg l^{-1}) and NAA (0.5 mg l^{-1}).

Table 5 Media combinations tried for enhanced release of axillary buds of root suckers of rosewood plus tree TR-1

Basal medium	Treatment
MS	BA (1.00 mg l^{-1}) and NAA (0.05 mg l^{-1})
MS	BA (1.00 mg l^{-1}) and Kin (1.00 mg l^{-1})
MS	BA (1.00 mg l^{-1}), Kin (1.00 mg l^{-1}) and L-G (1000 or 1500 mg l^{-1})

3.12. Observations

Each trial was carried out with seven tubes replicated four times. Observations were recorded at an interval of four days after inoculation. All the data were calculated based on cultures that remained uncontaminated after the required period of incubation. The following observations were recorded from various experiments.

a. Number of cultures uncontaminated

Number of cultures free from contamination were expressed as a percentage of total number of culture.

b. Number of explants showing bud initiation

Number of cultures showing bud initiation were expressed as a percentage of total number of surviving cultures. A culture was said to have bud initiation when the dormant axillary bud has just emerged out.

c. Time taken for bud initiation

The time (in days) taken for bud initiation was noted.

d. Number of explants showing bud expansion

A culture was said to have bud expansion when, the bud just emerged showed further signs of growth. Number of cultures showing bud expansion were expressed as a percentage of total number of surviving cultures.

e. Time taken for bud expansion

The time (in days) taken for bud expansion was recorded.

f. Number of explants with shoot development

Number of explants with shoot development were expressed as a percentage of total number of surviving cultures.

g. Average number of shoots per culture

Average number of shoots per culture was expressed as average of the number of shoots produced in the different replications.

h. Average shoot length

This was expressed as average of all the shoots produced in the different replications.

i. Number of explants that produced leaves

Number of cultures that produced leaves were expressed as a percentage of total surviving cultures.

j. Number of cultures that showed callusing at nodal base

The number of cultures that had callusing at base were expressed as a percentage of total surviving cultures.

k. Number of cultures that showed internodal callusing

The number of internodal segments that showed callusing were expressed as a percentage of total surviving cultures.

l. Callus index

Observations were recorded for callus induction, growth rate and morphology. Callus index (CI) was worked out as below:

$$CI = P \times G$$

where P is the percentage of callus initiation and G is the growth score.

Scoring was made based on the spread of the calli and a maximum score of four was given for those that have occupied the whole surface of the media within four weeks of culture period in culture tube.

m. Number of rooted cultures

Number of cultures that produced roots were expressed as a percentage of total surviving cultures.

n. Number of acclimatized plantlets

Number of acclimatized plantlets were expressed as a percentage of total plantlets kept for acclimatization.

3.13. Statistical analysis

Data were analysed by applying techniques for analysis of variance for CRD (Snedecor and Cochran, 1967) wherever necessary. Arc sine transformed values were used for the analysis for the parameters expressed as percentage. Treatment means were compared using Duncan's multiple range test at 5 per cent level of significance.

Results

RESULTS

This chapter includes the results obtained from the various experiments conducted for the clonal propagation of selected plus trees of Indian rosewood (*Dalbergia latifolia* Roxb.) through tissue culture carried out during 1993-95 at College of Forestry, Vellanikkara.

4.1. Enhanced release of axillary buds of young trees

4.1.1. Effect of fungicides in controlling the contamination rate

Total loss of all the cultures due to contamination occurred when the explants were collected from the unsprayed mother trees.

An initial wiping of explants with 70 per cent ethanol, followed by mercuric chloride treatment (0.1 per cent mercuric chloride for 12 minutes) alone was found to be ineffective in controlling the contamination. A prophylactic spraying with the mixture of a contact and a systemic fungicide, Indofil M-45 (Mancozeb) and Bavistin (Carbendazim), respectively, both at 0.3 per cent given to the mother plants, however, considerably reduced the rate of contamination. Cultures totally free of contamination were obtained during the months of December and January with cent per cent culture establishment.

Abnormal leaf development from explants in culture without shoot proliferation was noticed when the mother trees were sprayed everyday with the fungicides (Plate 4). Reducing the frequency of fungicidal application at an interval of two days successfully eliminated this abnormality without giving rise to any increase in contamination rate.

4.1.2. Polyphenol exudation

Phenol problem was practically absent with only a maximum of five per cent of the cultures showing light browning. Therefore, no treatment was given to overcome browning of the medium.

4.1.3. Effect of various media combinations

Data pertaining to the effect of the eight media combinations tried for the enhanced release of axillary buds of young trees of rosewood are given in Table 6 and Figures 1, 2 and 3.

4.1.3.1. Bud initiation and bud expansion

All the cultures showed bud initiation when WPM containing IAA (0.1 mg l^{-1}) was supplemented with kinetin (1.0 or 2.0 mg l^{-1}) and these two combinations were found statistically superior to all the other treatments. Bud initiation percentage was 89.3 for the treatment

Table 6 Effect of various media combinations on bud initiation, bud expansion and shoot proliferation in nodal explants from young trees of rosewood*

Treatment (Growth regulators in mg l^{-1})	Percentage of cultures showing		Period (days) for culture establishment		Percentage of cultures developing shoots	Mean number of shoots per culture	Mean shoot length (cm)	Mean number of leaves
	Bud initiation	Bud expansion	Bud initiation	Bud expansion				
MS+BA 0.25	89.30 b♠	78.60 b	6.30 b	8.20 b	78.60 b	2.00 b	3.30 c	2.00 bcd
MS+BA 2.00	82.10 d	64.30 d	6.30 b	8.20 b	64.30 d	3.50 a	3.00 c	1.50 d
WPM+BA 0.25	85.70 c	71.40 c	7.10 a	10.20 a	71.40 c	1.00 c	3.00 c	2.50 bc
WPM+BA 2.00 + IAA 0.10	60.70 e	42.60 f	7.10 a	10.20 a	42.60 f	1.00 c	2.50 c	2.00 cd
WPM+Kin 1.00 + IAA 0.10	100.00 a	100.00 a	5.10 c	7.10 c	100.00 a	2.50 b	7.80 a	4.50 a
WPM+Kin 2.00 + IAA 0.10	100.00 a	100.00 a	6.10 b	10.20 a	100.00 a	2.00 b	5.50 b	3.00 b
WPM+2-ip 0.50	64.30 e	53.60 e	6.10 b	8.20 b	53.60 e	1.00 c	6.00 b	3.00 b
WPM+2-ip 1.00	64.30 e	64.30 d	6.20 b	8.10 b	64.30 d	1.00 c	5.50 b	3.00 b
SEM	0.71	0.84	0.12	0.08	0.84	0.20	0.33	0.31
CV(%)	2.1	2.7	3.7	1.9	2.7	23.3	14.5	22.8

* All treatments replicated four times with seven cultures
Culture period - six weeks

♠ Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05)

MS + BA 0.25 mg l^{-1} and was superior to the treatment WPM + BA 0.25 mg l^{-1} (85.7 per cent). Addition of BA along with IAA at 2.0 and 1.0 mg l^{-1}, respectively, to WPM was found statistically on par with the combination of WPM + 2-ip (0.5 or 1.0 mg l^{-1}) and were found inferior to all other treatments (Table 6 and Figure 1).

Least number of days for bud initiation (5.1) was noticed when WPM was amended with 1.0 mg l^{-1} kinetin along with 0.1 mg l^{-1} IAA. This combination was superior to all other treatments (Table 6). Treatments MS + BA 0.25 or 2.0 mg l^{-1}, WPM + Kin and IAA (2.0 and 0.1 mg l^{-1}, respectively) and WPM + 2-ip 0.5 or 1.0 mg l^{-1} were on par and were significantly inferior to the combination of WPM + Kin 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}. Incorporation of BA at the rate of 0.25 mg l^{-1} alone or 2.0 mg l^{-1} BA along with 0.1 mg l^{-1} IAA to WPM recorded the maximum number of days (7.1) for bud initiation and these two combinations were significantly inferior to all others.

Kinetin (1.0 or 2.0 mg l^{-1}) along with 0.1 mg l^{-1} IAA in WPM resulted in cent per cent bud expansion and was significantly superior to all other treatments (Plate 5). On the other hand WPM supplemented with BA and IAA at the rate of 2.0 and 0.1 mg l^{-1}, respectively, gave the least value (42.6 per cent) for bud expansion and was significantly inferior to all other treatments (Table 6).

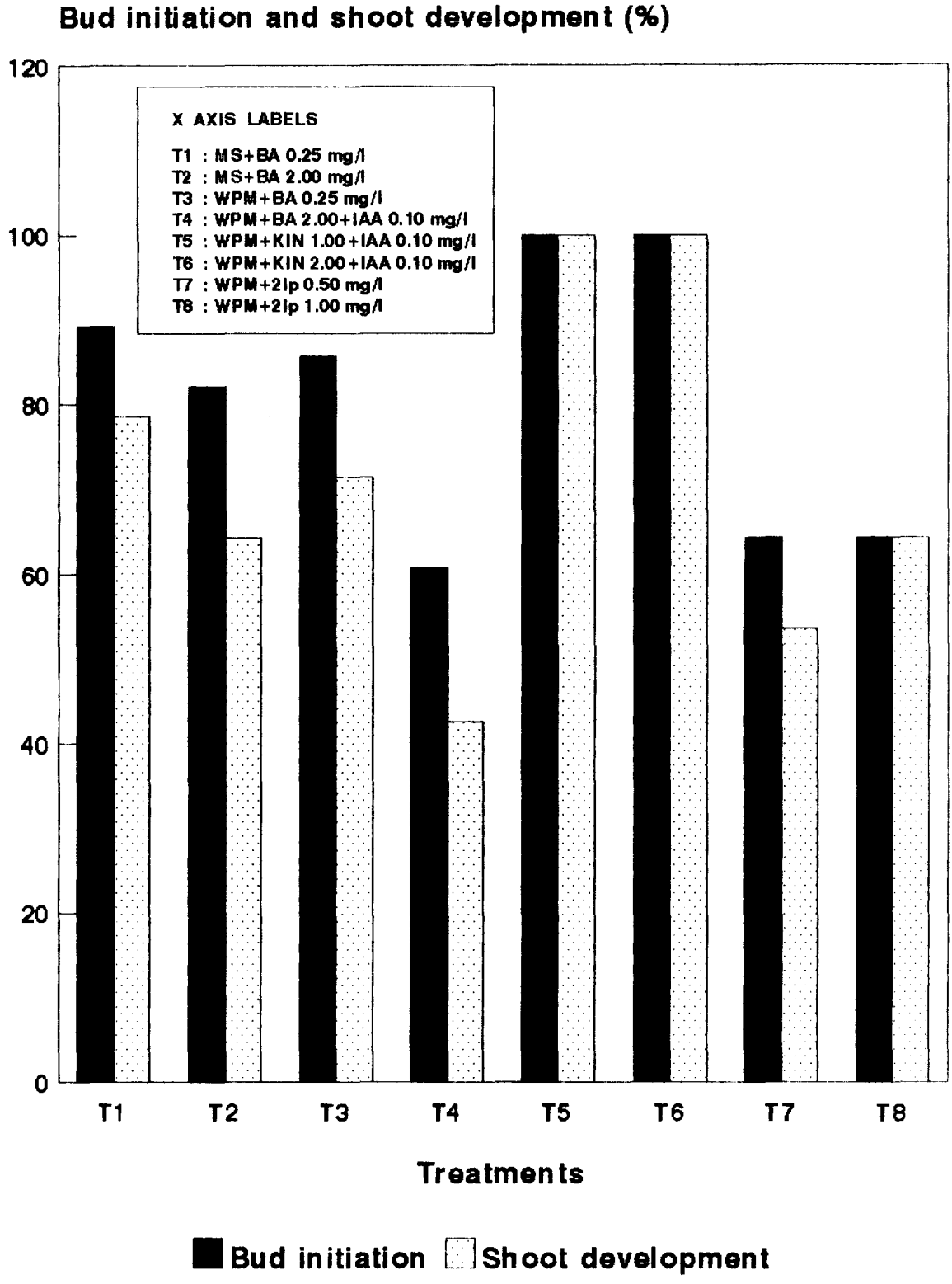


Fig.1 Effect of various treatments on bud initiation and shoot development in nodal explants from young trees of rosewood

Combination of kinetin 1.0 mg l^{-1} and IAA 0.1 mg l^{-1} in WPM recorded the minimum number of days (7.1) for bud expansion and was significantly superior to all other treatments (Table 6). Maximum number of days (10.2) for bud expansion was registered when the above medium was supplemented with BA 0.25 mg l^{-1} alone, BA 2.0 mg l^{-1} along with IAA 0.1 mg l^{-1} or kinetin and IAA at the rate of 2.0 and 0.1 mg l^{-1}, respectively.

4.1.3.2. Shoot proliferation

Data on the effect of various media combinations on *in vitro* shoot proliferation of explants from young trees of *Dalbergia latifolia* are given in Table 6 and Figure 1.

Axillary buds cultured in WPM containing 1.0 or 2.0 mg l^{-1} kinetin along with 0.1 mg l^{-1} IAA gave the maximum value for shoot development (100 per cent) and these combinations were found significantly superior to all others. Treatment WPM + BA 2.0 mg l^{-1} + IAA 0.1 mg l^{-1} gave the least value (42.6 per cent) for shoot proliferation and was found inferior to all other treatments.

The highest number of shoots per culture (3.5) was obtained (Plate 6) by the addition of 2.0 mg l^{-1} BA to MS medium and this was significantly superior to all other treatments.

The combination, WPM + Kin 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} resulted in an average shoot number of 2.5 which was on par with WPM supplemented with kinetin 2.0 and IAA 0.1 mg l^{-1} as well as MS medium supplemented with BA 0.25 mg l^{-1}. All the other treatments gave single shoots only (Figure 2).

The shoot length was maximum (7.8 cm) in WPM amended with kinetin and IAA at 1.0 and 0.1 mg l^{-1}, respectively, and was significantly superior to all other combinations. On the other hand WPM supplemented with BA and IAA at 2.0 and 0.1 mg l^{-1}, respectively, recorded the minimum shoot length (2.5 cm) and was on par with treatments MS + BA 0.25 or 2.0 mg l^{-1}, WPM + BA 0.25 mg l^{-1} and WPM + Kin 2.0 mg l^{-1} + IAA 0.1 mg l^{-1}. Addition of 2.0 mg l^{-1} kinetin along with 0.1 mg l^{-1} IAA or 2-ip (0.5 or 1.0 mg l^{-1}) to WPM produced shoots having an average length of 5.5, 6.0 and 5.5 cm, respectively, and were par. Data are presented in Table 6 and Figure 3.

Considering the mean number of leaves produced per shoot in a period of six weeks after culture, combination of WPM + Kin and IAA at 1.0 and 0.1 mg l^{-1}, respectively, was significantly superior to all other treatments (Table 6 and Figure 2). The minimum number of leaves was produced by MS + BA 2.0 mg l^{-1} (1.5) and was on par with MS + BA 0.25 mg l^{-1} and WPM + BA 2.0 + IAA 0.1 mg l^{-1}. Addition of

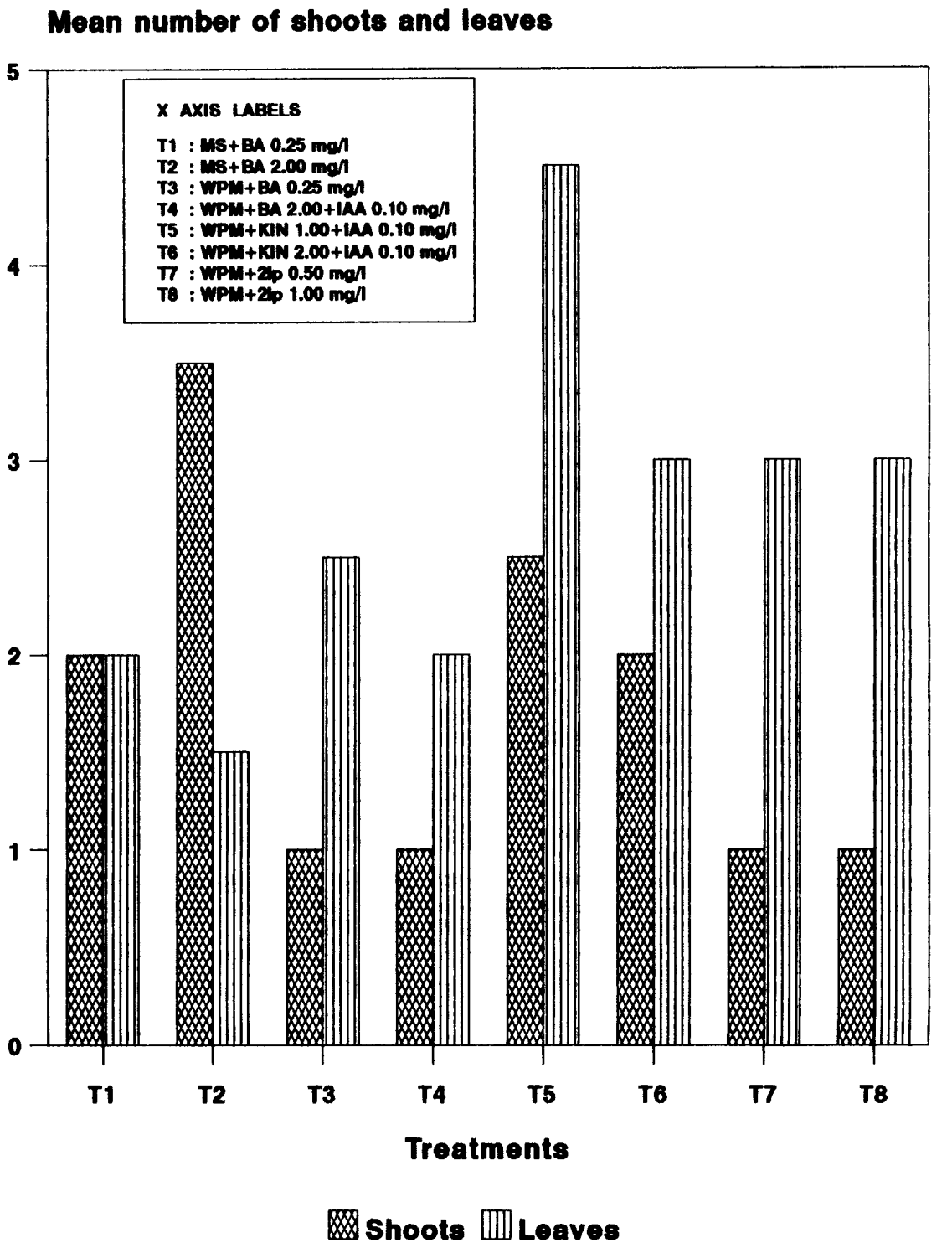


Fig.2 Effect of various treatments on mean number of shoots and leaves in nodal explants from young trees of rosewood

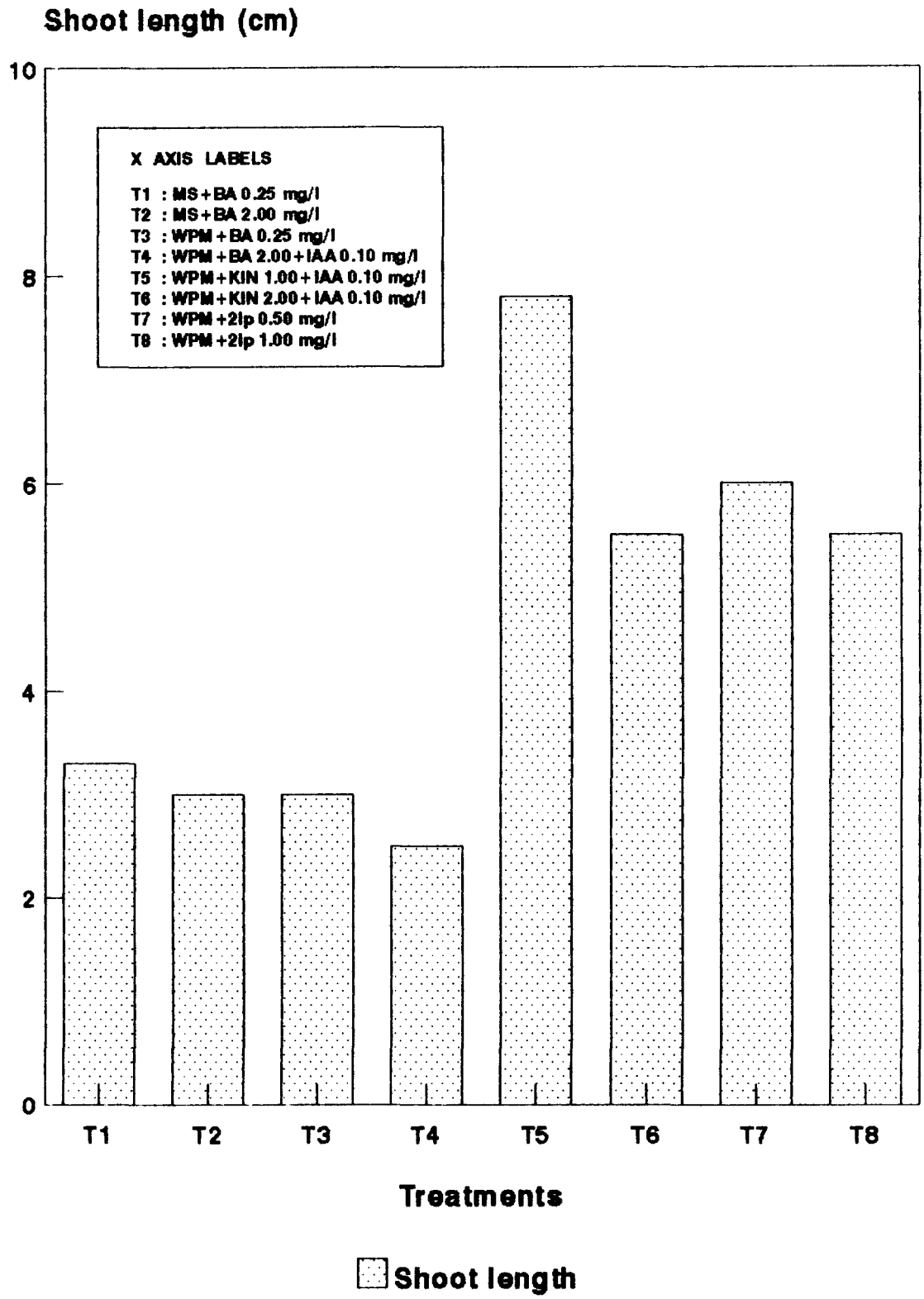


Fig.3. Effect of various treatments on mean shoot length in nodal explants from young trees of rosewood.

kinetin 2.0 and IAA 0.1 mg l^{-1} as well as 2-ip 0.5 or 1.0 mg l^{-1} in WPM produced an average of three leaves per shoot and was found to be on par with WPM or MS medium supplemented with BA 0.25 mg l^{-1}.

4.1.4. Effect of storage of explants

Explants stored in refrigerator for one or two days failed to show any response either in WPM + Kin 1.0 + IAA 0.1 mg l^{-1} or WPM + Kin 2.0 + IAA 0.1 mg l^{-1}. All the cultures dried after three weeks of inoculation.

4.2. Callus induction in explants from young trees

All the treatments resulted in inducing callus at the base of the explants. Percentage of callusing was 100 for all the combinations. However, the nature of callus exhibited variation depending on the media combinations. Details of callus induction are given in Table 7. Among the various treatments tried WPM supplemented with BA, at levels of 0.25 mg l^{-1} alone and 2.0 mg l^{-1} along with IAA at 0.1 mg l^{-1} induced profuse callusing with white and light brown friable calli, respectively. All the other treatments produced white compact callus except light brown callus in MS + BA 2.0 mg l^{-1}. Addition of silver nitrate at 5.0 mg l^{-1} level did not exhibit any influence on inhibiting callus induction. However, callus growth was not found to inhibit the growth of the cultures in any way.

Table 7 Effect of various media combinations on callus induction in nodal explants from young trees of rosewood*

Treatment (Growth regulators in mg l^{-1})	Percentage of cultures showing callusing at the base	Quantity of callus	Callus morphology
MS+BA 0.25	100.00	Medium	White compact
MS+BA 2.00	100.00	Medium	Light brown compact
WPM+BA 0.25	100.00	Profuse	White friable
WPM+BA 2.00+ IAA 0.10	100.00	Profuse	Light brown friable
WPM+Kin 1.00+ IAA 0.10	100.00	Medium	White compact
WPM+Kin 2.00+ IAA 0.10	100.00	Medium	White compact
WPM+2-ip 0.50	100.00	Medium	White compact
WPM+2-ip 1.00	100.00	Medium	White compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

4.3. Root induction in *in vitro* produced shoots from nodal explants of young trees

4.3.1. *In vitro* rooting

Addition of activated charcoal (one per cent) to half strength MS medium and pulse treatment to excised shoot by IBA (1000 mg l^{-1}) was found successful in rooting (Plate 7). This treatment gave 95 per cent success. As regard to the nature of roots produced, they were white and thin with an average length of 4 cm. Slight callusing was observed at the basal cut end of the shoot.

4.3.2. *Ex vitro* rooting

Shoots transferred to sterilised sand in cups after IBA (1000 mg l^{-1}) treatment failed to produce roots.

4.3.3. Direct rooting

Direct shoot and root formation (Plates 8 and 9) was noticed when the nodal explants from young trees were cultured on WPM supplemented with kinetin 1.0 or 2.0 mg l^{-1} along with 0.1 mg l^{-1} IAA. Percentage of cultures showing direct rooting was 14.3.

4.4. Planting out and acclimatization

Sixty five per cent of the plantlets survived when they were planted out in sand. The polythene covers from the planted out plantlets were removed after one month (Plates 10 and 11) and afterwards they were treated like normal containerised seedlings. The plantlets which had shown direct rooting failed to survive when planted out.

4.5. Enhanced release of axillary buds of plus trees - explants taken directly from the trees

4.5.1. Effect of Fungicidal treatment in controlling contamination

Culture contamination was cent per cent when the explants were collected from field grown trees which were not given any prophylactic spraying. Immersing the explants in fungicides for one hour, however, considerably reduced the rate of explant contamination. The percentage of contaminated cultures was reduced to as low as 20 during the months of March and April with proper combination of surface sterilization procedure.

4.5.2. Surface sterilization

An initial wiping of the fungicide treated explants with 70 per cent ethanol, followed by immersion in 0.1 per cent mercuric chloride for a period of 12 minutes gave good result with only 20 per cent of the culture showing contamination along with a culture survival of 80 per cent. Chlorine water was found to be totally ineffective for controlling contamination.

4.5.3. Phenolic exudation

Browning of the medium was noticed in all the cultures when the explants were inoculated without taking measures to control the phenolic exudation. All the browned cultures died (Plate 12) after a period of three weeks of inoculation. No individual treatment was found successful in controlling the polyphenol interference. However, a combination of methods tried was found to be very effective in preventing exudation and oxidation of polyphenols with a maximum of 80 per cent of the cultures without browning. The treatment combination included soaking the explants in a solution of ascorbic acid and citric acid both at 150 mg l^{-1} for three hours and incorporation of 150 mg l^{-1} each of ascorbic acid and citric acid and 0.7 per cent polyvinylpyrrolidone (PVP) in the culture medium. It was observed that browning intensity increased with increasing concentration of BA.

4.5.4. Difference in response between genotypes

Each genotype responded differently when explants from the three selected plus trees were cultured on the same medium viz., MS + BA 2.0 mg l^{-1} (Table 8 and Figure 4). The plus tree TR-1 recorded the maximum value of 42.9 per cent for bud initiation, bud expansion and shoot proliferation and was significantly superior to the results obtained for the other two trees. Percentage of bud initiation, bud expansion and shoot proliferation were 21.4 and 17.9 for the trees TR-2 and TR-3, respectively, which were found statistically on par. Average shoot length was 4.0 cm for TR-1 which again was found superior to that of the other two genotypes. However, cultures from all the three trees induced callusing at the base. The plus tree TR-1 was selected due to its superiority in response for further studies.

4.5.5. Effect of plant growth regulators

Explants remained green without growth for a period of four weeks and showed no symptoms of growth when cultured on to basal MS medium or WPM (Plates 13 and 14). In order to find out the effect of plant growth regulators on bud break and shoot proliferation from axillary buds of plus trees, the media were supplemented with different levels of cytokinins viz., BA, kinetin and 2-ip independently and in combination with the auxin IAA (0.1 mg l^{-1}).

Table 8 Difference in culture response between the selected plus trees of rosewood in MS medium supplemented with BA at 2.0 mg l^{-1}*

Tree No.	Percentage of cultures showing		Period (days) for culture establishment		% of cultures developing shoots	Number of shoots per culture	Mean shoot length (cm)	% of cultures showing callusing at the base
	Bud initiation	Bud expansion	Bud initiation	Bud expansion				
TR-1	42.9 a♠	42.9 a	7	13	42.9 a	1.0	4.0 a	100
TR-2	21.4 b	21.4 b	7	14	21.4 b	1.0	2.5 b	100
TR-3	17.9 b	17.9 b	9	16	17.9 b	1.0	2.0 b	100
SEM	0.89	0.89			0.89		0.20	
CV (%)	5.7	5.7			5.7		14.4	

* All treatments replicated four times with seven cultures
Culture period - Six weeks

♠ Means with the same letter are not significantly different by Duncan's multiple range test (P - 0.05)

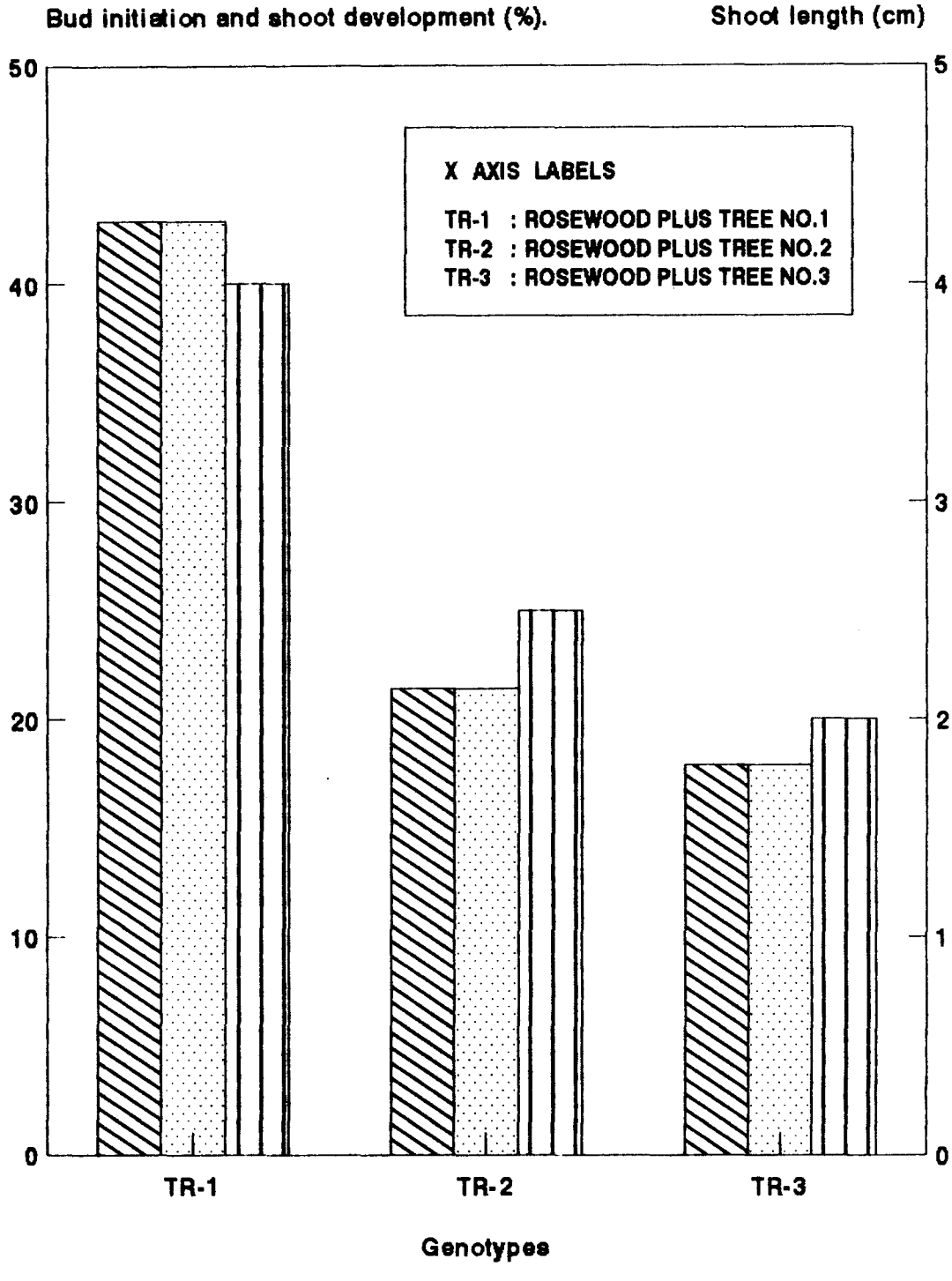


Fig.4. Difference in culture response between the selected plus trees of rosewood in the medium, MS+BA 2.0 mg/l.

4.5.5.1. Effect of BA and IAA on bud initiation, bud expansion and shoot proliferation in MS medium

Data on the effect of different concentrations of BA alone and in combination with IAA supplemented to MS medium on bud initiation, bud expansion and shoot proliferation are presented in Table 9 and Figure 5.

Axillary bud explants cultured in MS medium containing BA and IAA at 2.0 and 0.1 mg l^{-1}, respectively, resulted the highest value (46.4 per cent) for bud initiation (Plate 15) bud expansion (Plate 16) and shoot proliferation (Plate 17). This was found on par with MS + BA 2.0 mg l^{-1}. Combinations, MS + BA 0.25 mg l^{-1} and MS + BA 0.25 mg l^{-1} + IAA 0.1 mg l^{-1} were found the second best treatments with 14.3 per cent of the cultures showing bud initiation bud expansion, and shoot proliferation. The explants did not respond to MS medium supplemented with 0.50, 3.0 and 5.0 mg l^{-1} BA alone and BA 5.0 mg l^{-1} along with IAA 0.1 mg l^{-1}.

Only one shoot per culture was produced by the different responding treatments. A maximum shoot length of 4.0 cm (Plate 18) was obtained with BA at 2.0 mg l^{-1}. All the other responding treatments produced an average shoot length of 1.0 cm each.

Table 9 Effect of BA and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of rosewood in MS medium*

Treatment		Percentage of cultures showing		Period (days) for culture establishment		Percentage of cultures developing shoots	Mean number of shoots per culture	Mean shoot length (cm)
BA (mg l^{-1})	IAA	Bud initiation	Bud expansion	Bud initiation	Bud expansion			
0.25	0	14.30 b♠	14.30 b	7.00	13.00	14.30 b	1.00	1.00
0.50	0	0	0	Nil	Nil	0	0	0
2.00	0	42.90 a	42.90 a	7.00	13.00	42.90 a	1.00	4.00
3.00 & 5.00	0	0	0	Nil	Nil	0	0	0
0.25	0.10	14.30 b	14.30 b	7.00	13.00	14.30 b	1.00	1.00
0.50	0.10	7.10 c	7.10 c	7.00	13.00	7.10 c	1.00	1.00
2.00	0.10	46.40 a	46.40 a	7.00	13.00	46.40 a	1.00	1.00
3.00	0.10	7.10 c	7.10 c	7.00	13.00	7.10 c	1.00	1.00
5.00	0.10	0	0	Nil	Nil	0	0	0
SEM		0.92	0.92			0.92		
C.V.(%)		6.9	6.9			6.9		

* All treatments replicated four times with seven cultures
Culture period - six weeks

♠ Means with the same letter are not significantly different by Duncan's multiple range test (P - 0.05)

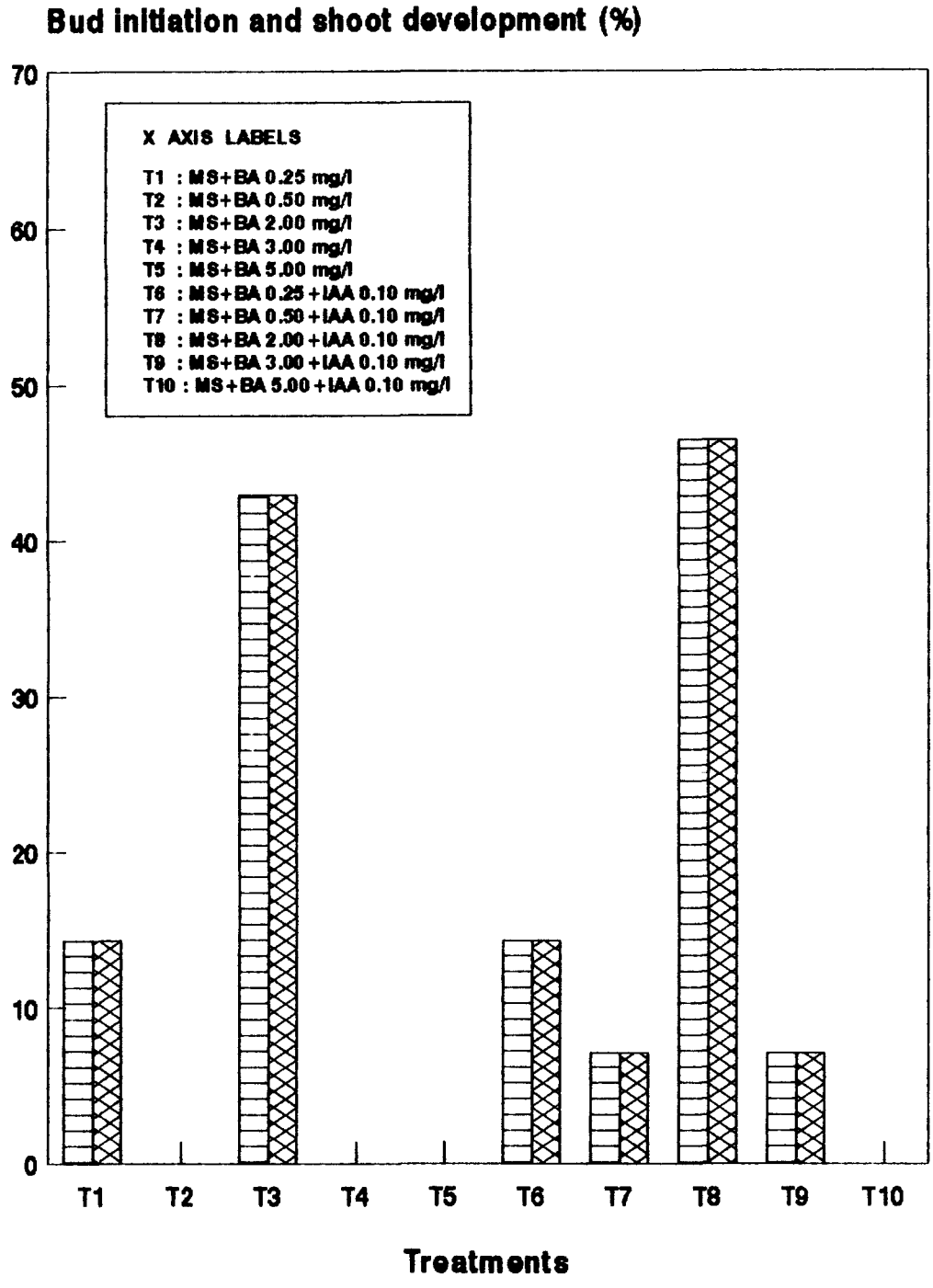


Fig 5. Effect of BA and IAA on bud initiation and shoot development in nodal explants from the plus tree of rosewood in MS medium.

All the combinations (Plate 18) failed to induce leaf production. However, leaf initials could be observed on the shoots formed.

4.5.5.2. Effect of BA and IAA on callus induction in MS medium

All the treatments induced callusing at the base of the culture (Table 10). Benzyl adenine at lower concentrations (0.25 and 0.50 mg l^{-1} alone and along with IAA 0.1 mg l^{-1}) produced light brown compact callus. Higher levels of BA alone and along with IAA resulted in giving dark brown compact callus (Plate 19).

4.5.5.3. Effect of BA and IAA on bud initiation, bud expansion and shoot proliferation in WPM

Table 11 and Figure 6 show the data relating to the effect of BA and IAA on culture establishment and shoot proliferation in WPM.

Combinations of BA at 0.25 and 2.0 mg l^{-1} along with IAA 0.1 mg l^{-1} gave 35.7 per cent of the cultures showing bud initiation. Bud initiation percentage was 14.3 when WPM was supplemented with BA 0.50 mg l^{-1}. All the other combinations failed to respond.

Table 10 Effect of BA and IAA on callus induction in nodal explants from the plus tree of rosewood in MS medium*

Treatment BA (mg l^{-1})	IAA	% of cultures showing callusing at the base	Quantity of callus	Callus morphology
0.25	0	100.00	Light	Light brown compact
0.50	0	100.00	Light	Light brown compact
2.00	0	100.00	Medium	Dark brown compact
3.00	0	100.00	Medium	Dark brown compact
5.00	0	100.00	Medium	Dark brown compact
0.25	0.10	100.00	Medium	Light brown compact
0.50	0.10	100.00	Medium	Light brown compact
2.00	0.10	100.00	Medium	Dark brown compact
3.00	0.10	100.00	Medium	Dark brown compact
5.00	0.10	100.00	Medium	Dark brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

Table 11 Effect of BA and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of rosewood in WPM*

Treatment		Percentage of cultures showing		Period (days) for culture establishment		Percentage of cultures developing shoots	Number of shoots per culture	Mean shoot length (cm)
BA (mg l^{-1})	IAA	Bud initiation	Bud expansion	Bud initiation	Bud expansion			
0.25	0	35.70	7.10	12.00	20.00	7.10	1.00	1.00
0.50	0	14.30	0	12.00	Nil	0	0	0
2.00	0	35.70	0	12.00	Nil	0	0	0
3.00 & 5.00	0.10	0	0	Nil	Nil	0	0	0
0.25 & 0.50	0.10	0	0	Nil	Nil	0	0	0
2.00	0.10	35.70	14.30	12.00	20.00	14.30	1.00	2.50
3.00 & 5.00	0.10	0	0	Nil	Nil	0	0	0

* All treatments replicated four times with seven cultures
Culture period - Six weeks

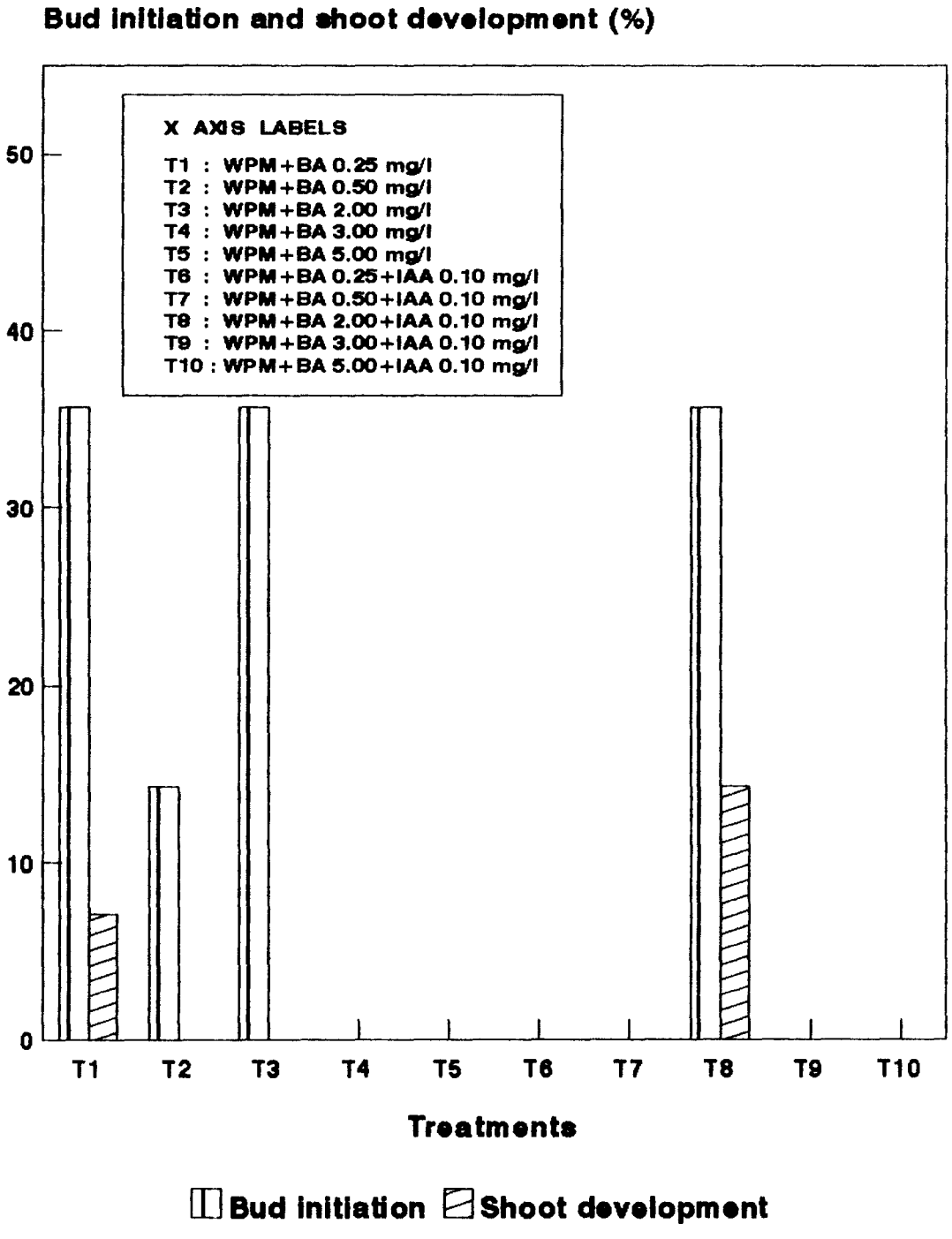


Fig.6. Effect of BA and IAA on bud initiation and shoot development in nodal explants from the plus tree of rosewood in WPM .

With regard to bud expansion and shoot proliferation, WPM supplemented with BA at 0.25 mg l^{-1} and BA along with IAA (2.0 and 0.1 mg l^{-1}, respectively) only gave positive results. Time taken for bud initiation and bud expansion was 12 and 20 days, respectively, by the responding treatments.

Mean number of shoots per culture was one only. The treatment, WPM + BA 2.0 + IAA 0.1 mg l^{-1} produced the longest shoot having 2.5 cm length. Leaf expansion was absent (Plate 20).

4.5.5.4. Effect of BA and IAA on callus induction in WPM

Callus induction was noticed for all the combinations tried. However, the percentage of cultures showing callusing, the intensity of callusing and the callus morphology varied depending on the treatment. Callusing intensity was found to increase with the addition of auxin (Table 12). Addition of BA and IAA at 2.0 and 0.1 mg l^{-1}, respectively, resulted in giving cent per cent callusing at the base of the cultures. Low levels of BA (0.25 and 0.5 mg l^{-1} alone and along with IAA 0.1 mg l^{-1}) produced light brown and compact callus where as higher levels of BA alone and along with the auxin gave dark brown and compact callus.

Table 12 Effect of BA and IAA on callus induction in nodal explants from the plus tree of rosewood in WPM*

Treatment		% of cultures showing callusing at the base	Quantity of callus	Callus morphology
BA (mg l^{-1})	IAA			
0.25	0	85.70	Low	Light brown compact
0.50	0	89.30	Low	Light brown compact
2.00	0	89.30	Low	Dark brown compact
3.00	0	85.70	Low	Dark brown compact
5.00	0	85.70	Low	Dark brown compact
0.25	0.10	92.90	Medium	Light brown compact
0.50	0.10	89.30	Medium	Light brown compact
2.00	0.10	100.00	Medium	Dark brown compact
3.00	0.10	89.30	Medium	Dark brown compact
5.00	0.10	92.90	Medium	Dark brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

4.5.5.5. Effect of kinetin and IAA on bud initiation, bud expansion and shoot proliferation in MS medium

Data obtained from the study of the influence of kinetin alone or in combination with IAA in MS medium are tabulated in Table 13. Addition of kinetin at concentrations of 1.0 or 2.0 mg l^{-1} resulted in bud initiation in 21.4 per cent of the cultures in both the combinations. These treatments, however, failed to show bud expansion and further shoot proliferation. The only successful treatment was MS + Kin 3.0 mg l^{-1}, showing bud initiation, bud expansion and shoot proliferation in 14.3 per cent of the cultures. Average shoot length was 1.0 cm with only one shoot per culture. None of the combinations produced leaves. All levels of kinetin tried along with 0.1 mg l^{-1} IAA also failed to induce any culture success during this study.

4.5.5.6. Effect of kinetin and IAA on callus induction in MS medium

Table 14 show the effect of kinetin and IAA on callus induction in nodal explants from the plus tree of rosewood in MS medium. All the combinations were found successful with cent per cent of the cultures producing callus at the base. The auxin IAA was found beneficial in increasing the quantity of the callus. Pale yellow and compact callus was formed in the treatments with kinetin at 1.0 and 2.0 mg l^{-1} alone and in combination with IAA 0.1 mg l^{-1}. The rest of the treatments produced light brown and compact callus.

Table 13 Effect of kinetin and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of rosewood in MS medium*

Treatment		Percentage of cultures showing		Period (days) for culture establishment		Percentage of cultures developing shoots	Number of shoots per culture	Mean shoot length (cm)
Kin (mg l^{-1})	IAA	Bud initiation	Bud expansion	Bud initiation	Bud expansion			
1.0 & 2.0	0	21.4	0	14.0	Nil	0	0	0
3.0	0	14.3	14.3	14.0	18	14.3	1.0	1.0
5.0	0	0	0	Nil	Nil	0	0	0
1.0, 2.0, 3.0 & 5.0	0.1	0	0	Nil	Nil	0	0	0

* All treatments replicated four times with seven cultures
Culture period - Six weeks

Table 14 Effect of kinetin and IAA on callus induction in nodal explants from the plus tree of rosewood in MS medium*

Treatment		% of cultures showing callusing at the base	Quantity of callus	Callus morphology
Kin (mg l^{-1})	IAA			
1.0	0	100.0	Light	Pale yellow compact
2.0	0	100.0	Light	Pale yellow compact
3.0	0	100.0	Light	Light brown compact
5.0	0	100.0	Light	Light brown compact
1.0	0.1	100.0	Medium	Pale yellow compact
2.0	0.1	100.0	Medium	Pale yellow compact
3.0	0.1	100.0	Medium	Light brown compact
5.0	0.1	100.0	Medium	Light brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

4.5.5.7. Effect of kinetin and IAA on bud initiation, bud expansion and shoot proliferation in WPM

Data on the trials conducted to evaluate the effect of kinetin and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of *Dalbergia latifolia* in WPM are presented in Table 15 and Figure 7.

Among the various combinations tried, only one with kinetin 2.0 and IAA 0.1 mg l^{-1} was found effective in giving bud initiation and bud expansion along with shoot formation. Bud expansion and shoot proliferation was only 14.3 per cent though the bud initiation percentage was 50. However, the time taken for bud initiation and bud expansion was just seven and ten days respectively. The longest shoot measured a length of 1.5 cm with one shoot per culture. No leaf formation was noticed. Kinetin at 2.0, 3.0 and 5.0 mg l^{-1} alone did not show any response.

4.5.5.8. Effect of kinetin and IAA on callus induction in WPM

White compact callus was produced by all the levels of kinetin alone or in combination with IAA (Table 16). Addition of auxin was found to increase the amount of callus produced. WPM + Kin 2.0 mg l^{-1} + IAA 0.1 mg l^{-1} gave cent per cent callusing. Combinations of kinetin 3.0 and 5.0 mg l^{-1} along

Table 15 Effect of kinetin and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of in rosewood WPM*

Treatment		Percentage of cultures showing		Period (days) for culture establishment		Percentage of cultures developing shoots	Number of shoots per culture	Mean shoot length (cm)
Kin (mg l^{-1})	IAA	Bud initiation	Bud expansion	Bud initiation	Bud expansion			
1.0	0	7.1 d♠	0	12.0 b	Nil	0	0	0
2.0, 3.0 & 5.0	0	0	0	Nil	Nil	0	0	0
1.0	0.1	14.3 c	0	7.0 c	Nil	0	0	0
2.0	0.1	50.0 a	14.3	7.0 c	10.0	14.3	1.0	1.5
3.0	0.1	35.7 b	0	7.0 c	Nil	0	0	0
5.0	0.1	14.3 c	0	14.0 a	Nil	0	0	0
SEM		1.28		0.45				
CV (%)		9.0		9.5				

* All treatments replicated four times with seven cultures
Culture period - Six weeks

♠ Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05)

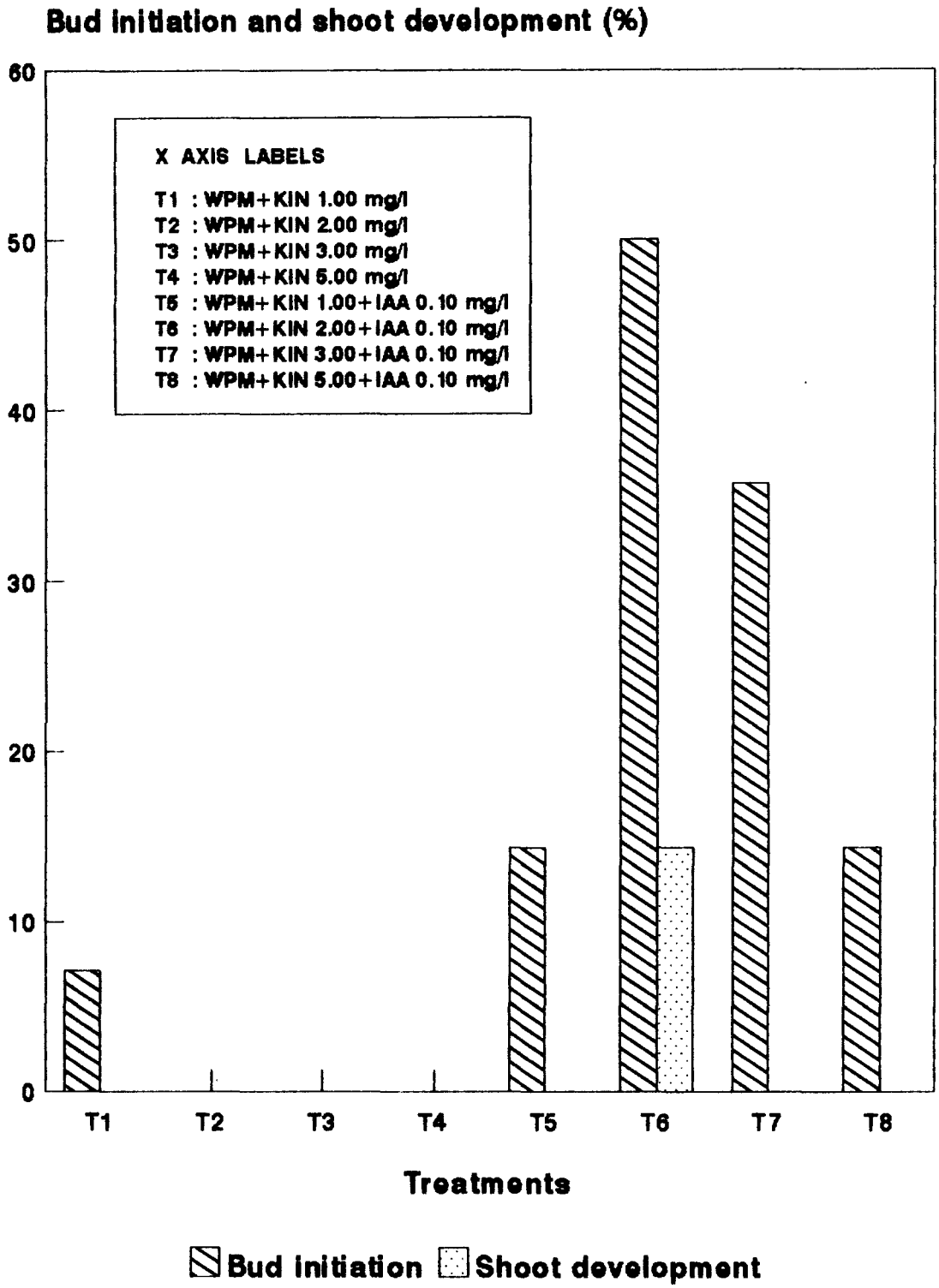


Fig.7. Efect of KIN and IAA on bud initiation and shoot development in nodal explants from the plus tree of rosewood in WPM .

Table 16 Effect of kinetin and IAA on callus induction in nodal explants from the plus tree of rosewood in WPM*

Treatment		% of cultures showing callusing at the base	Quantity of callus	Callus morphology
Kin (mg l^{-1})	IAA			
1.0	0	28.6	Light	White compact
2.0	0	35.7	Light	White compact
3.0	0	35.7	Light	White compact
5.0	0	57.1	Light	White compact
1.0	0.1	42.9	Medium	White compact
2.0	0.1	100.0	Medium	White compact
3.0	0.1	92.9	Medium	White compact
5.0	0.1	92.9	Medium	White compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

with the auxin exhibited the second highest value of 92.9 per cent each. The lowest value for callusing (28.6) was noticed with the addition of 1.0 mg l^{-1} kinetin in the medium.

4.5.5.9. Effect of 2-ip and IAA on bud initiation bud expansion and shoot proliferation in MS medium

Growth performance of the cultures due to the effect of 2-ip and IAA in MS medium is presented in Table 17 and Figure 8.

Percentage of bud initiation in the various combinations tried varied from zero to 42.9 per cent. MS medium with 2-ip 1.0 mg l^{-1} registered the maximum bud initiation and it was significantly superior to all other treatments. However, just half of the cultures only had shown further bud expansion and shoot development. The mean shoot length observed was 1.5 cm with only one shoot per culture (Plate 21). Leaf expansion was not noticed. Only 7.1 per cent of the cultures developed shoots when 2-ip 0.5 mg l^{-1} was added to the medium. The average shoot length was 0.75 cm. Time taken for bud expansion was 14 and 21 days, respectively, in these treatments. All the other combinations failed to exhibit bud expansion and shoot proliferation.

Table 17 Effect of 2-ip and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of rosewood in MS medium*

Treatment		Percentage of cultures showing		Period (days) for culture establishment		Percentage of cultures developing shoots	Number of shoots per culture	Mean shoot length (cm)
2-ip (mg l^{-1})	IAA	Bud initiation	Bud expansion	Bud initiation	Bud expansion			
0.25	0	14.30 c♠	0	10.00	Nil	0	0	0
0.50	0	28.60 b	7.10	10.00	21.00	7.10	1.00	0.75
1.00	0	42.90 a	21.40	10.00	14.00	21.40	1.00	1.50
3.00 & 5.00	0	0	0	Nil	Nil	0	0	0
0.25, 0.50 & 1.00	0.10	0	0	Nil	Nil	0	0	0
3.00 & 5.00	0.10	7.10 d	0	10.00	Nil	0	0	0
SEM		1.37						
CV(%)		9.8						

* All treatments replicated four times with seven cultures
Culture period - Six weeks

♠ Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05)

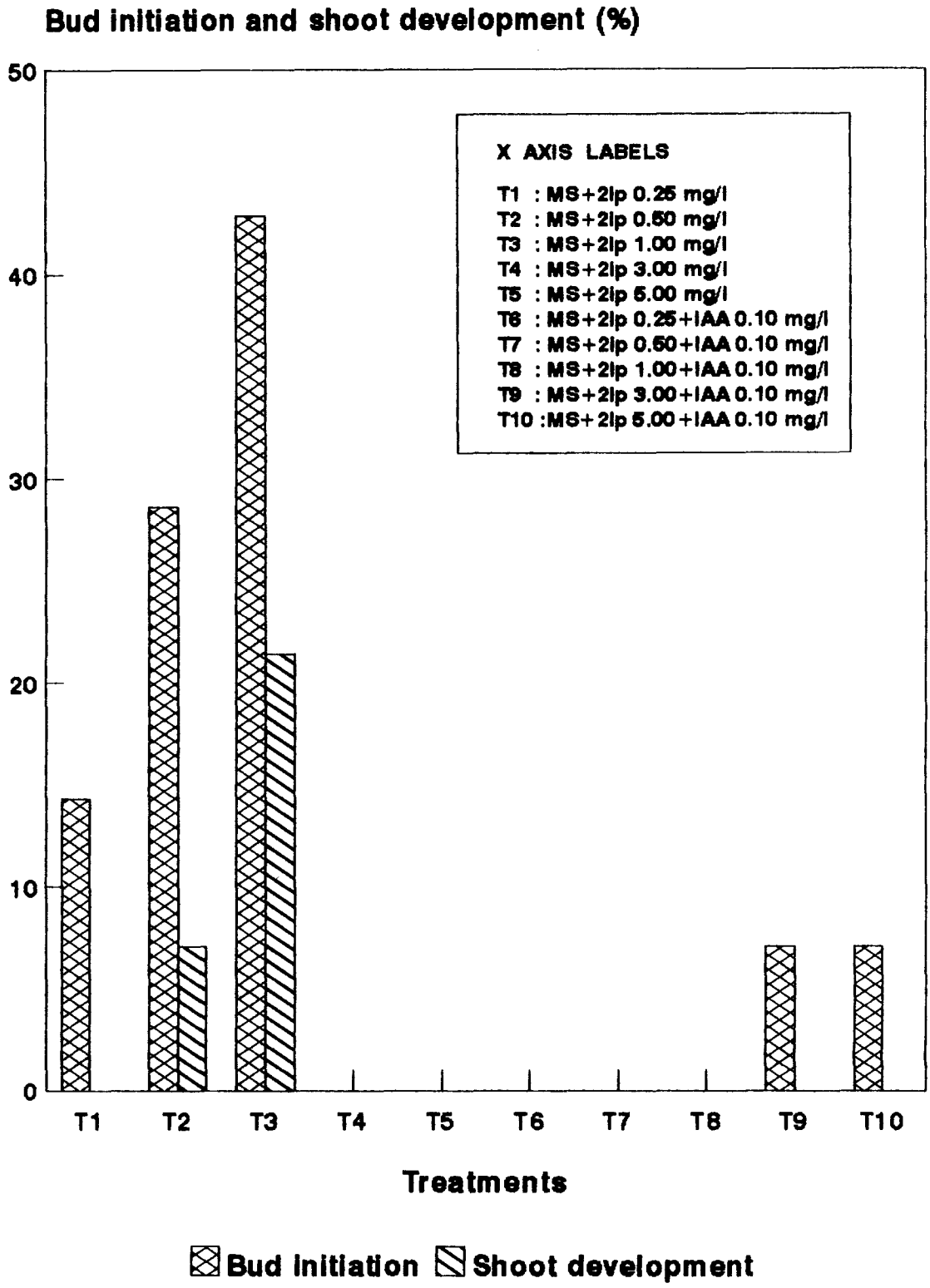


Fig.8. Effect of 2ip and IAA on bud initiation and shoot development in nodal explants from the plus tree of rosewood in MS medium.

4.5.5.10. Effect of 2-ip and IAA on callus induction in MS medium

The calli induced by the treatments without auxin were compact in nature and pale yellow in colour (Table 18). Callus production was enhanced by the addition of IAA at 0.1 mg l^{-1} with light brown compact callus. All the cultures responded positively for the induction of callus with cent per cent success in the treatment MS + 2-ip 5.0 + IAA 0.1 mg l^{-1}.

4.5.5.11. Effect of 2-ip and IAA on bud initiation, bud expansion and shoot proliferation in WPM

Data recorded on the effect of 2-ip and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of rosewood in WPM are presented in Table 19 and Figure 9.

Among the various levels of 2-ip, 1.0 mg l^{-1} was found the best treatment in terms of bud initiation bud expansion and shoot proliferation (42.9, 21.4 and 21.4, respectively). Percentage of shoot proliferation was 7.1 when 2-ip at 0.25 or 0.50 mg l^{-1} was incorporated to WPM. Mean number of shoots per culture was one with an average length of 1.5 cm for the combinations with 2-ip 0.25 and 1.0 mg l^{-1} and 1.0 cm for 0.5 mg l^{-1}. No leaves were formed in any of the treatments. Other levels of 2-ip tried alone as well as all levels of 2-ip along with IAA 0.1 mg l^{-1} could not induce bud expansion or shoot growth.

Table 18 Effect of 2-ip and IAA on callus induction in nodal explants from the plus tree of rosewood in MS medium*

Treatment 2-ip (mg l^{-1})	IAA	% of cultures showing callusing at the base	Quantity of callus	Callus morphology
0.25	0	71.40	Light	Pale yellow compact
0.50	0	78.60	Light	Pale yellow compact
1.00	0	85.70	Light	Pale yellow compact
3.00	0	64.30	Light	Pale yellow compact
5.00	0	85.70	Light	Pale yellow compact
0.25	0.10	92.90	Medium	Light brown compact
0.50	0.10	92.90	Medium	Light brown compact
1.00	0.10	92.90	Medium	Light brown compact
3.00	0.10	92.90	Medium	Light brown compact
5.00	0.10	100.00	Medium	Light brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

Table 19 Effect of 2-ip and IAA on bud initiation, bud expansion and shoot proliferation in nodal explants from the plus tree of in rosewood WPM*

Treatment		Percentage of cultures showing		Period (days) for culture establishment		Percentage of cultures developing shoots	Number of shoots per culture	Mean shoot length (cm)
2-ip (mg l^{-1})	IAA	Bud initiation	Bud expansion	Bud initiation	Bud expansion			
0.25	0	7.10 c♠	7.1	10.00	14.00	7.10	1.00	1.50
0.50	0	28.60 b	7.1	10.00	21.00	7.10	1.00	1.00
1.00	0	42.90 a	21.4	9.00	14.00	21.40	1.00	1.50
3.00 & 5.00	0	0	0	Nil	Nil	0	0	0
0.25	0.10	7.10 c	0	9.00	Nil	0	0	0
0.50 & 1.00	0.10	0	0	Nil	Nil	0	0	0
3.00	0.10	7.10 c	0	10.00	Nil	0	0	0
5.00	0.10	0	0	Nil	Nil	0	0	0
SEM		1.03						
CV(%)		8.6						

* All treatments replicated four times with seven cultures
Culture period - Six weeks

♠ Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05)

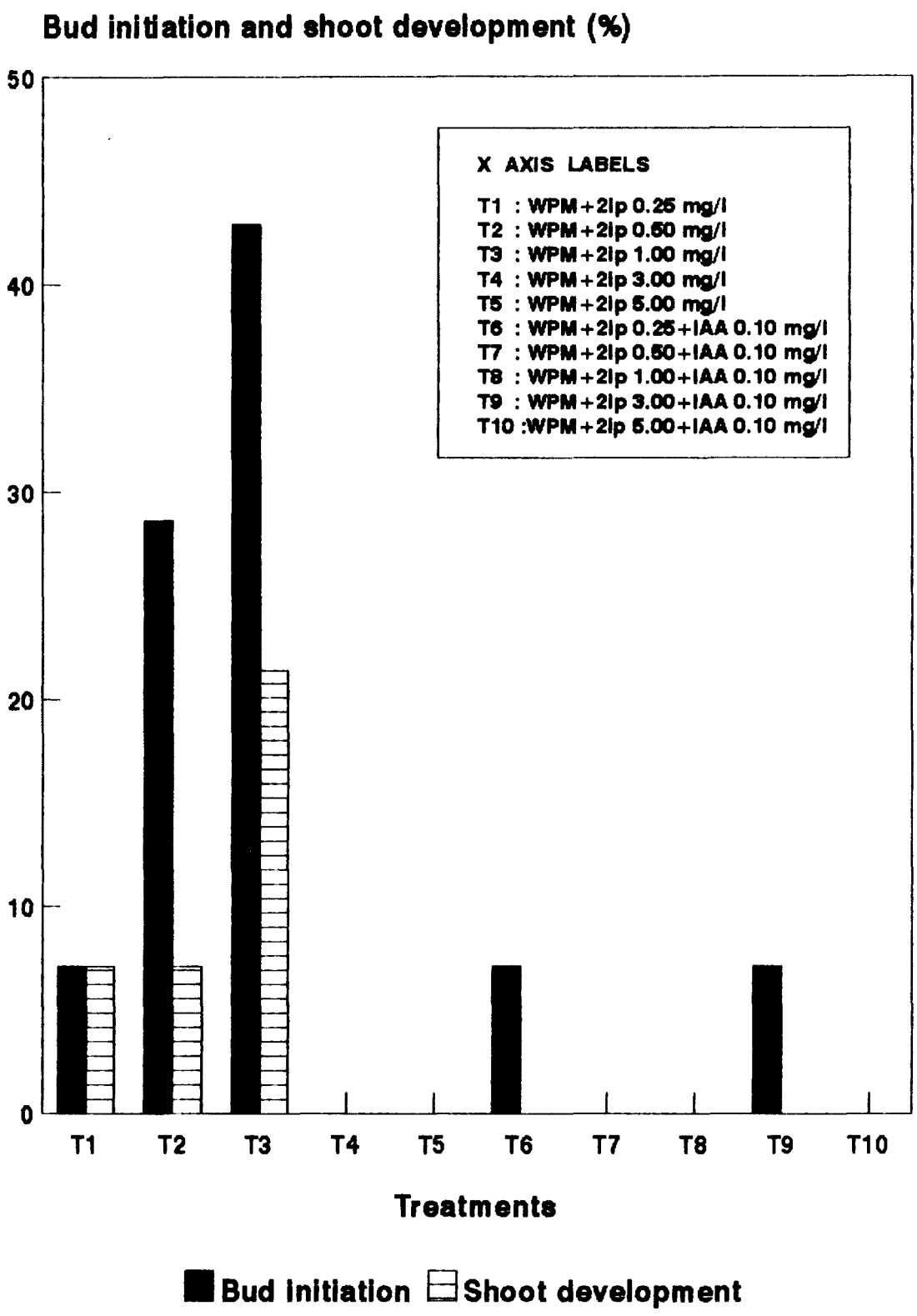


Fig.9. Effect of 2ip and IAA on bud initiation and shoot development in nodal explants from the plus tree of rosewood in WPM .

4.5.5.12. Effect of 2-ip and IAA on callus induction in WPM

Data on the influence of 2-ip and IAA on callus induction in nodal explants from the plus tree of *Dalbergia latifolia* in WPM are furnished in Table 20.

All the treatments induced callusing at the base of the explants. Percentage of callusing was 100 for the combinations of 2-ip 3.0 with IAA, 0.1 mg l^{-1} as well as 2-ip 5.0 with IAA 0.1 mg l^{-1}. Addition of auxin was found influencing the amount of callus produced. Lower concentrations of 2-ip without IAA gave pale yellow callus whereas all the other combinations produced light brown and compact callus.

4.5.6. Effect of other media supplements

The results obtained due to the addition of media supplements like adenine sulphate, casein hydrolysate, cycocel, phloroglucinol, L-glutamine, coconut water and activated charcoal in medium MS and WPM are furnished below:

4.5.6.1. Adenine sulphate

Among the various levels of AS tried in basal media MS and WPM, only one treatment viz., MS + AS 8 mg l^{-1} resulted in bud expansion and shoot proliferation in a limited number of cultures (7.1 per cent each). Mean shoot length was 1.0 cm with only one shoot per culture. All the other treatments

Table 20 Effect of 2-ip and IAA on callus induction in nodal explants from the plus tree of rosewood in WPM*

Treatment 2-ip (mg l^{-1})	IAA	% of cultures showing callusing at the base	Quantity of callus	Callus morphology
0.25	0	71.40	Light	Pale yellow compact
0.50	0	78.60	Light	Pale yellow compact
1.00	0	78.60	Light	Pale yellow compact
3.00	0	85.70	Light	Light brown compact
5.00	0	85.70	Medium	Light brown compact
0.25	0.10	78.60	Medium	Light brown compact
0.50	0.10	78.60	Medium	Light brown compact
1.00	0.10	82.10	Medium	Light brown compact
3.00	0.10	100.00	Medium	Light brown compact
5.00	0.10	100.00	Medium	Light brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

failed to induce bud break. However, all the 12 combinations were found successful in inducing callus. Medium MS with kinetin and IAA 1.0 and 0.1 mg l^{-1}, respectively, and supplemented with adenine sulphate at 4.0 or 8.0 mg l^{-1} produced dark brown and compact callus. The rest of the combinations gave rise to light brown and compact callus. Details are given in Table 21.

4.5.6.2. Casein hydrolysate

None of the media combinations of MS and WPM with CH could induce shoot proliferation in the axillary bud cultures of *D. latifolia*. However, as shown in Table 22, some of the treatments viz., MS + CH 100 or 500 mg l^{-1} and WPM + Kin 1.0 + IAA 0.1 + CH 100 or 500 mg l^{-1} supported bud initiation in few of the cultures (21.4 per cent in MS and 14.3 per cent in WPM). All the 12 combinations of CH with MS and WPM induced callusing in the explants. While in medium MS callusing was seen in 100 per cent of the cultures, around 75 per cent of the cultures showed callusing for the treatments with WPM.

4.5.6.3. Cycocel

Twelve different treatments were tried with cycocel. Data obtained are presented in Table 23 and Figure 10. The treatment with MS medium supplemented with CCC 1.0 mg l^{-1} only

Table 21 Effect of adenine sulphate on bud initiation, bud expansion, shoot proliferation and callus induction in nodal explants from the plus tree of rosewood*

Treatment (Supplements in mg l^{-1})	Percentage of cultures showing				Quantity of callus	Callus morphology
	Bud initiation	Bud expansion	Shoot proliferation	Callusing at the base		
MS+AS 1.0	0	0	0	28.6	Light	Light brown compact
MS+AS 4.0	14.3	0	0	28.6	Light	Light brown compact
MS+AS 8.0	14.3	7.1	7.1	42.9	Light	Light brown compact
MS+Kin 1.0+IAA 0.1+AS 1.0	0	0	0	28.6	Light	Light brown compact
MS+Kin 1.0+IAA 0.1+AS 4.0	0	0	0	57.1	Medium	Dark brown compact
MS+Kin 1.0+IAA 0.1+AS 8.0	0	0	0	71.4	Medium	Dark brown compact
WPM+AS 1.0	0	0	0	14.3	Light	Light brown compact
WPM+AS 4.0	0	0	0	14.3	Light	Light brown compact
WPM+AS 8.0	0	0	0	21.4	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+AS 1.0	0	0	0	14.3	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+AS 4.0	14.3	0	0	21.4	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+AS 8.0	14.3	0	0	21.4	Light	Light brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

Table 22 Effect of casein hydrolysate on bud initiation, bud expansion, shoot proliferation and callus induction in nodal explants from the plus tree of rosewood*

Treatment (Supplements in mg l^{-1})	Percentage of cultures showing				Quantity of callus	Callus morphology
	Bud initiation	Bud expansion	Shoot proliferation	Callusing at the base		
MS+CH 100.0	21.4	0	0	100.0	Light	Light brown compact
MS+CH 500.0	21.4	0	0	100.0	Light	Light brown compact
MS+CH 1000.0	0	0	0	100.0	Light	Light brown compact
MS+Kin 1.0+IAA 0.1+CH 100.0	0	0	0	100.0	Medium	Dark brown compact
MS+Kin 1.0+IAA 0.1+CH 500.0	21.4	0	0	100.0	Medium	Dark brown compact
MS+Kin 1.0+IAA 0.1+CH 1000.0	0	0	0	100.0	Medium	Dark brown compact
WPM+CH 100.0	14.3	0	0	71.4	Light	Light brown compact
WPM+CH 500.0	0	0	0	71.4	Light	Light brown compact
WPM+CH 1000.0	0	0	0	71.4	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+CH 100.0	14.3	0	0	75.0	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+CH 500.0	14.3	0	0	75.0	Medium	Light brown compact
WPM+Kin 1.0+IAA 0.1+CH 1000.0	0	0	0	78.6	Medium	Light brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

Table 23 Effect of cycocel on bud initiation, bud expansion, shoot proliferation and callus induction in nodal explants from the plus tree of rosewood*

Treatment (Supplements in mg l^{-1})	Percentage of cultures showing				Quantity of callus	Callus morphology
	Bud initiation	Bud expansion	Shoot proliferation	Callusing at the base		
MS+CCC 1.0	27.4	7.1	7.1	100.0	Light	Light brown compact
MS+CCC 3.0	50.0	0	0	100.0	Light	Light brown compact
MS+CCC 5.0	50.0	0	0	100.0	Light	Light brown compact
MS+Kin 1.0+IAA 0.1+CCC 1.0	28.6	0	0	100.0	Light	Light brown compact
MS+Kin 1.0+IAA 0.1+CCC 3.0	0	0	0	100.0	Light	Dark brown compact
MS+Kin 1.0+IAA 0.1+CCC 5.0	0	0	0	100.0	Light	Dark brown compact
WPM+CCC 1.0	14.3	0	0	64.3	Light	Light brown compact
WPM+CCC 3.0	7.1	0	0	64.3	Light	Light brown compact
WPM+CCC 5.0	7.1	0	0	71.4	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+CCC 1.0	14.3	0	0	71.4	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+CCC 3.0	7.1	0	0	71.4	Light	Light brown compact
WPM+Kin 1.0+IAA 0.1+CCC 5.0	7.1	0	0	78.6	Light	Light brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

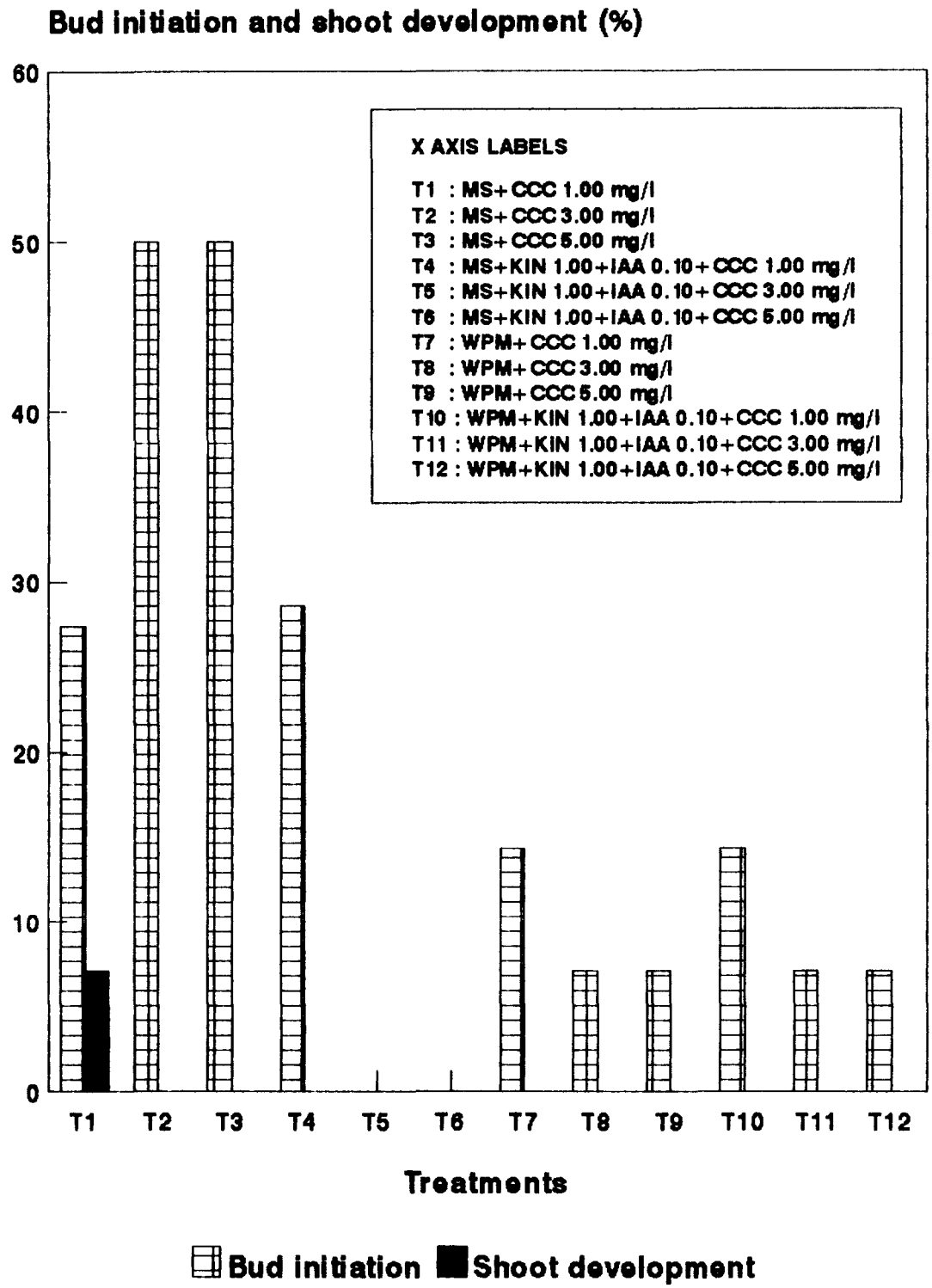


Fig.10. Effect of Cycocel on bud initiation and shoot development in nodal explants from the plus tree of rosewood.

could give rise to bud expansion and shoot growth. Percentage of bud expansion and shoot proliferation was just 7.1 with an average shoot length of 0.5 cm. Only one shoot per culture was formed. No leaf expansion was noticed.

With regard to callus induction, all the combinations showed positive response. Callus production was cent per cent for the cultures in MS medium. Light brown and compact callus (Plate 22) was produced in general.

4.5.6.4. Phloroglucinol

Addition of phloroglucinol at the rate of 200 and 500 mg l^{-1} in both media failed to induce bud break. However, cultures in all the combinations had a callusing tendency at the base (Table 24).

4.5.6.5. L-glutamine

No leaves were formed in any of the treatments tried. Leaf initials only were produced which again were found falling after a period of four to five days of emergence (Plates 23 and 24). Transferring the cultures into the medium containing BA 2.0 mg l^{-1} and L-glutamine 1000 or 1500 mg l^{-1} was found very effective in the retention of the leaf initials formed (Plate 25).

Table 24 Effect of phloroglucinol on bud initiation, bud expansion, shoot proliferation and callus induction in nodal explants from the plus tree of rosewood*

Treatment (Supplements in mg l^{-1})	Percentage of cultures showing				Quantity of callus	Callus morphology
	Bud initiation	Bud expansion	Shoot proliferation	Callusing at the base		
MS+PG 200.0	0	0	0	21.4	Very light	Black compact
MS+PG 500.0	0	0	0	14.3	Very light	Black compact
MS+Kin 1.0+IAA 0.1+PG 200.0	0	0	0	42.9	Very light	Black compact
MS+Kin 1.0+IAA 0.1+PG 500.0	0	0	0	42.9	Very light	Black compact
WPM+PG 200.0	0	0	0	14.3	Very light	Black compact
WPM+PG 500.0	0	0	0	7.1	Very light	Black compact
WPM+Kin 1.0+IAA 0.1+PG 200.0	0	0	0	21.4	Very light	Black compact
WPM+Kin 1.0+IAA 0.1+PG 500.0	0	0	0	28.6	Very light	Black compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

4.5.6.6. Coconut water

Effect of coconut water on bud initiation, bud expansion shoot proliferation and callus induction was studied with eight different treatments. Among the various combinations, coconut water at 10 per cent (v/v) added to MS + Kin 1.0 and IAA 0.1 mg l^{-1} only could produce bud break and shoot growth. This treatment had taken 14 days for bud initiation and 21 days for bud expansion. Average shoot length recorded was 1.0 cm with single shoot per culture. All the treatments were found effective in inducing callus. Data on the effect of coconut water on callus induction in nodal explants from the plus tree of rosewood are presented in Table 25.

4.5.6.7. Activated charcoal

None of the combinations in both media induced bud release or shoot proliferation. Percentage of cultures showing callusing at the base of the explants and the amount of callus was also low for all the combinations except for the treatment, MS + AC 0.25 per cent (Table 26).

4.6. Rooting *in vitro*

Rooting was attempted for the *in vitro* shoots. Addition of activated charcoal (1.0 per cent w/v) to the culture media (half strength MS) and pulse treatment to the excised shoot by

Table 25 Effect of coconut water on bud initiation, bud expansion, shoot proliferation and callus induction in nodal explants from the plus tree of roseood*

Treatment (Growth regulators in mg l^{-1})	Percentage of cultures showing				Quantity of callus	Callus morphology
	Bud initiation	Bud expansion	Shoot proliferation	Callusing at the base		
MS+CW 10%	0	0	0	71.4	Medium	Light brown compact
MS+CW 20%	21.4	0	0	71.4	Medium	Light brown compact
MS+Kin 1.0+IAA 0.1+CW 10%	14.3	14.3	14.3	100.0	Medium	Light brown compact
MS+Kin 1.0+IAA 0.1+CW 20%	14.3	0	0	100.0	Medium	Light brown compact
WPM+CW 10%	0	0	0	64.3	Medium	Light brown compact
WPM+CW 20%	0	0	0	71.4	Medium	Light brown compact
WPM+Kin 1.0+IAA 0.1+CW 10%	0	0	0	71.4	Medium	Light brown compact
WPM+Kin 1.0+IAA 0.1+CW 20%	0	0	0	78.6	Medium	Light brown compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

Table 26 Effect of activated charcoal on bud initiation, bud expansion, shoot proliferation and callus induction in nodal explants from the plus tree of rosewood*

Treatment (Growth regulators in mg l^{-1})	Percentage of cultures showing				Quantity of callus	Callus morphology
	Bud initiation	Bud expansion	Shoot proliferation	Callusing at the base		
MS+AC 0.25%	0	0	0	50.00	Very light	White compact
MS+AC 1.00%	0	0	0	7.10	Very light	White compact
MS+Kin 1.00+IAA 0.10+AC 0.25%	21.4	0	0	7.10	Very light	White compact
MS+Kin 1.00+IAA 0.01+AC1.00%	21.4	0	0	7.10	Very light	White compact
WPM+AC 0.25%	0	0	0	7.10	Very light	White compact
WPM+AC 1.00%	0	0	0	7.10	Very light	White compact
WPM+Kin 1.00+IAA 0.10+AC 0.25%	14.3	0	0	7.10	Very light	White compact
WPM+Kin 1.00+IAA 0.10+AC 1.00%	35.7	0	0	7.10	Very light	White compact

* All treatments replicated four times with seven cultures
Culture period - Six weeks

IBA (1000 mg l^{-1}) could not induce roots in these shoots. The shoots dried up without showing any symptom of growth in a period of three to four weeks of incubation.

4.7. Enhanced release of axillary buds of plus trees of *Dalbergia latifolia* - explants taken from the root suckers

4.7.1. Effect of fungicides in controlling the contamination rate

A prophylactic spraying of fungicides given to the root suckers was found effective in controlling the rate of contamination to the tune of 80 per cent of the cultures free of contamination during the month of May.

4.7.2. Treatment to overcome phenol problem

Nodal explants were immersed in a solution containing ascorbic acid and citric acid both at 150 mg l^{-1} each in sterile water for three hours before surface sterilisation to overcome the polyphenol interference. This treatment along with the addition of ascorbic acid, citric acid and PVP to the culture medium was found successful in avoiding browning of the medium.

4.7.3. Surface sterilization

An initial wiping of the explants with 70 per cent ethanol followed by immersion in 0.1 per cent mercuric chloride for a period of 12 minutes gave good result with only 20 per cent of the cultures showing contamination.

4.7.4. Effect of the media combinations

On MS medium supplemented with BA 1.0 mg l^{-1} and NAA 0.05 mg l^{-1} 92.9 per cent bud initiation was observed from seventh day onwards. Out of these only 42.9 per cent of the cultures recorded bud expansion and shoot proliferation when subcultured on MS medium modified with BA and kinetin 1.0 mg l^{-1} each. Average shoot length was 4.5 cm with single shoots per culture. Leaf initials only were formed without proper leaf expansion (Plate 26). Addition of L-glutamine (1000 or 1500 mg l^{-1}) to the medium was found effective in checking the abnormal falling of the initial leaves.

4.8. Rooting *in vitro*

The treatment done to induce rooting of the shoots produced *in vitro* failed to respond.

4.9. Indirect organogenesis

Callus cultures were successfully initiated from internodal segments from root suckers of plus trees of rosewood. A compact and white callus (Plate 27) was obtained on MS basal medium supplemented with 2,4-D 3.0 or 4.0 mg l^{-1}, BA 0.5 or 1.0 mg l^{-1}, NAA 1.0 or 2.0 mg l^{-1} and coconut water 10 or 15 per cent (v/v). For maintenance, callus was routinely subcultured on the same medium. The colour of the callus changed from white (Plate 28) to light brown (Plate 29) and finally to dark brown (Plate 30) after a period of four weeks of incubation. The callus index worked out was 253.6 for the first treatment and 257.1 for the second. Small pieces of these calli were cut and cultured on MS medium supplemented with BA 3.0 mg l^{-1} and NAA 0.5 mg l^{-1}. After a period of five weeks, shoot regeneration was noticed in 14.3 per cent of these callus cultures. However, leaf expansion was absent. Multiple shoot formation was also not noticed in any of these cultures (Plate 31).

Discussion

DISCUSSION

Forestry in India is indeed at the crossroads. The effective forest cover in the country is too low to offer long-range ecological security and supply goods and services to people and industry. Large scale plantation forestry and afforestation of degraded forest lands are the solutions to ensure future sufficiency. Availability of clonal progenies of superior genotypes from selected tree species will be a boon to such forestry programmes since this can ensure predictable returns. Conventional vegetative propagation methods, however, are often not possible in most of the tree species and where feasible, the number of seedlings that can be produced will be quite insufficient to meet the requirement of hundreds of thousands of planting materials. Under these circumstances, the role of tissue culture is of significance in enhancing the capability for the production of planting material of selected superior trees so as to boost production and productivity.

The genus *Dalbergia* is reputed throughout the world for a dozen or so species that yield the rosewood of commerce. Out of them four species recorded from Kerala, namely, *D. latifolia, D. sissoides, D. sissoo* and *D. melanoxylon* yield valuable timber. Among the four species, both *D. latifolia* and *D. sissoides* yield the highly priced rosewood of commerce (Nair, 1986). The present investigation was undertaken to establish an

in vitro rapid propagation method for Indian rosewood (*Dalbergia latifolia* Roxb.) using bud explants from selected plus trees. Explants from young rosewood trees were also used to study the difference in culture response between adult and juvenile plant materials. The results of this study are discussed below:

5.1. Culture contamination

Being perennial in nature and exposed to the vagaries of the environment, woody plants harbour a lot of micro-organisms. All tissue cultures are likely to end up contaminated if the inoculum or explant used is not obtained from properly disinfected plant material (Razdan, 1993). Doublin (1984) noticed that when the explants were collected from the field, the percentage of infection was over 90, regardless of the procedure used for explant sterilization. The results were similar in the present study with *Dalbergia* also. Irrespective of the explant sterilization protocol adopted, the culture contamination was 100 per cent when field explants were used, without fungicidal treatment. An initial treatment of the mother trees in the pre-culture stage (stage 0) with fungicidal spray was recommended by different authors (Legrand and Mississo, 1986; Mahato, 1992; Santhoshkumar, 1993; Divatar, 1994).

In our study, a prophylactic spraying with the combination of a systemic and a contact fungicide, Bavistin (Carbendazim) and Indofil M-45 (Mancozeb), respectively, both at 0.3 per cent given to the young trees as well as root suckers followed by immersing the explants initially wiped with 70 per cent ethanol, in mercuric chloride (0.1 per cent) for 12 minutes could control contamination of cultures to a considerable extent. In the case of plus trees, prophylactic spraying was not possible due to the inaccessibility of the branches and therefore immersing the exlants in the same fungicidal mixture for one hour followed by the surface sterilization procedure was found to be an equally effective method to reduce culture contamination. Presoaking the explants in fungicidal solution for reducing fungicidal contamination has been suggested by Broome and Zimmerman (1978) in black berry and Manzanera and Pardos (1990) in *Quercus suber* (Cork oak).

For surface sterilization, an initial wiping of the fungicide treated explants with 70 per cent ethyl alcohol followed by immersion in mercuric chloride (0.1 per cent) for 12 minutes was found very effective. Mercuric chloride as an effective sterilant for surface sterilization is well documented (George and Sherrington, 1984; Mahato, 1992; Santhoshkumar, 1993; Divatar, 1994). Cultures totally free of contamination were obtained from the explants of young trees during the months of

December and January. The percentage of contaminated cultures was reduced to as low as 20 when the explants treated both with the fungicides and the surface sterilant were used for culture in plus trees (March-April) as well as root suckers (May). Chlorine water treatment for 5 minutes was ineffective. Santhoshkumar (1993) reported that, in *Pterocarpus marsupium,* chlorine water treatment tried even upto 15 minutes duration could not control contamination effectively. However, chances of it being an effective sterilant at higher duration is not ruled out. The probable handicap of such a long duration treatment can be tissue damage and cell death.

Abnormal leaf development from explants in culture without shoot proliferation was noticed (Plate 4) when the young trees were sprayed everyday with the fungicides. This is probably due to the fungicidal toxicity on the explant. Reducing the frequency of fungicidal application to an interval of two days successfully eliminated this abnormality without any preceptible effect on culture contamination. Dodds and Roberts (1985) suggested to avoid the use of fungicides for sterilization since they or their degradation products may be metabolised by plant tissues with unpredictable results.

5.2. Polyphenol exudation

A serious problem generally faced in culturing adult tissues from woody species is the oxidation of phenolic substances leached out from the cut surface of the explant which turns the medium dark brown leading to the death of explant. Survival of explants was reduced to 20 per cent when polyphenol exudation was left unchecked (Razdan, 1993). Polyphenols can be oxidised either by peroxidases (Mayer and Harel, 1979; Vaughn and Duke, 1984) or by polyphenol oxidases (Mayer and Harel, 1979; Hu and Wang, 1983). The oxidised compounds are highly toxic, they form convalent bonds with the plant proteins thus inhibiting the enzyme activity (Hu and Wang, 1983) causing browning and death of the explant.

Exudation of polyphenols from explants taken from young trees of rosewood was practically nil. On the other hand browning of the medium was noticed in all the cultures when the explants taken from the plus trees (Plate 12) and root suckers were incubated without taking measures to control the phenolic exudation. Explant establishment of plus trees of rosewood, thus required special procedures to escape or avoid problems that are associated with polyphenol oxidation. Similar problem has been reported in other woody plant species like teak (Gupta *et al.*, 1980), eucalyptus (Gill and Gill, 1994) etc.

Among the pre-treatments tried for reducing the polyphenol interference, a combination of methods viz., soaking the explants in a solution of ascorbic acid and citric acid both at 150 mg l^{-1} for three hours and incorporation of 150 mg l^{-1} each of ascorbic acid and citric acid along with 0.7 per cent polyvinyl-pyrrolidone (PVP) in the culture medium was found to be very effective in preventing browning of the medium with only 20 per cent of the cultures showing browning. Reducing agents (anti-oxidants) which lower the redox potential of solutions are effective in preventing the blackening of isolated plant tissues or plant extracts, and it is often assumed that they prevent the oxidation of phenols. Polyvinyl-pyrrolidone can absorb the phenols through hydrogen bonding, preventing their oxidation (George and Sherrington, 1984). In the present study, browning intensity was found increasing with increasing concentration of BA in the medium. It has been reported that if the concentration of kinetin in the medium is more than 1.0 mg l^{-1}, the medium became darkly coloured in *in vitro* cultures of *Pelargonium* (George and Sherrington, 1984). This may be due to the fact that cytokinins at higher concentrations lead to the oxidation of polyphenols at a rapid rate.

5.3. Difference in response between genotypes

Difference in *in vitro* response between genotypes is not uncommon in tissue culture. In the present study, when the explants from the three selected plus trees were cultured on the same medium, they exhibited variation in response. The plus tree TR-1 was found to be superior (Table 8 and Figure 4). Response of *in vitro* culture vary between plant species and even within a species between varieties. There were large differences in the capacity of the explants from different selections of mature *Eucalyptus marginata* trees to survive in culture (McComb and Bennet, 1982). Genotype specific effects have been reported for *Sequoia sempervirens* also (Sul and Korban, 1994). The precise genetic regulation of endogenous growth regulating mechanisms can be one of the reasons for such variation in culture response between different genotypes.

5.4. Effect of media combinations

The most extensively used culture media for micropropagation in trees are MS (Murashige and Skoog, 1962) and WPM (Lloyd and McCown, 1980). Different combinations of growth regulators as well as growth supplements in MS and WPM have been tried to identify a suitable culture media for axillary bud cultures of plus trees of *Dalbergia latifolia*. For young trees, media combinations reported by Mahato (1992) were used (Table 3).

Axillary bud is a predetermined organ with the morphogenetic potential to give rise to a shoot in the absence of apical dominance. The basic phenomenon involved in the induction of bud release and subsequent plantlet production *in vitro* is reported to be through the action of plant hormones. Though little is known how a hormone evoke a particular response (Thorpe, 1980), the favourable effects on apical bud bursting and shoot production by cytokinins had been demonstrated by Murashige (1974). In addition, there are certain growth supplements of known and unknown chemical properties. These are also reported to be capable of influencing the morphogenesis of tissues in *in vitro* cultures (Skoog and Tsui, 1984; George and Sherrington, 1984; Gausman, 1986; Mahato, 1992; Divatar, 1994).

5.4.1. Growth regulators

Benzyl adenine when supplemented (0.25 or 2.0 mg l^{-1}) in both MS and WPM could induce bud initiation, bud expansion and shoot proliferation in young trees of rosewood (Table 6 and Figures 1,2 and 3). Increased concentration brought down the number of responding cultures and shoot length in both MS and WPM. However, increase in BA concentration led to an increased number of shoots per culture in MS medium. Eventhough all the treatments with BA resulted in giving cent per cent callusing at the base of the cultures, they were found inferior to those with

kinetin when axillary bud release was concerned. Among the different media combinations tried for the enhanced release of axillary buds of young trees of *Dalbergia latifolia*, the maximum frequency of bud initiation, bud expansion and shoot proliferation (100 per cent) was recorded in WPM with kinetin 1.0 or 2.0 mg l^{-1} along with IAA 0.1 mg l^{-1}. The combination WPM + Kin 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} was the best treatment with minimum number of days for bud initiation and bud expansion (5.1 and 7.1 days, respectively), maximum average shoot length (7.8 cm) and maximum mean number of leaves (4.5). The favourable effect of kinetin on bud break and shoot proliferation has been reported in other tree species like *Eucalyptus tereticornis* and *E. globulus* (Gupta and Mascarenhas, 1987), *Dalbergia latifolia* (Mahato, 1992) and *Pterocarpus marsupium* (Santhoshkumar, 1993).

The naturally occurring cytokinin, 2-ip has been reported to be more effective than kinetin or BA in a number of species like *Rhododendron* (Anderson, 1975), *Kalmia latifolia* (Lloyd and McCown, 1980) etc. Supplementing 2-ip at the levels, 0.5 or 1.0 mg l^{-1} to WPM were found to be the second best treatment when time taken for bud initiation and bud expansion, mean shoot length and mean number of leaves were concerned for axillary bud cultures of young trees (Table 6 and Figures 2 and 3). Both the combinations gave cent per cent callus induction with white compact calli. Multiple shoot formation was absent when WPM was supplemented with BA or 2-ip.

Among the various cytokinins used in plant tissue culture, BA is the cheapest and one of the most effective (Aboel-nil, 1987; Jang *et al.*, 1988; Scott *et al.*, 1988, Rai and Chandra, 1989; Vijayakumar *et al.*, 1990). In the present study also cytokinin BA was found to be effective in inducing organogenesis in plus trees of rosewood whereas kinetin was found superior to BA in cultures of young trees. Axillary bud explants directly taken from the plus tree of *D. latifolia* when cultured in MS medium containing BA and IAA at 2.0 and 0.1 mg l^{-1}, respectively, resulted in giving the highest value for bud initiation, bud expansion and shoot proliferation (46.4 per cent) and this was found statistically on par with MS + BA 2.0 mg l^{-1}. However, the average shoot length was only 1.0 cm when MS medium was amended with BA and IAA whereas BA at 2.0 mg l^{-1} alone produced an average shoot length of 4.0 cm (Plate 18). Mahato (1992) observed that auxins inhibited BA's capacity for inducing morphogenesis. Retarded shoot growth observed in the combination of MS amended with BA and IAA can be attributed to this inhibitory effect of auxin. In WPM, BA at 0.25 mg l^{-1} and BA along with IAA (2.0 and 0.1 mg l^{-1}, respectively) only succeeded in giving shoot proliferation with just 7.1 and 14.3 per cent of the cultures, respectively, showing shoot development. Higher levels of BA (3.0 and 5.0 mg l^{-1}) failed to show bud release in WPM (Table 11 and Figure 6).

Inhibitory effect of BA at higher concentrations has been reported in guava (Jaiswal and Amin, 1987) and Mangosteen (Goh *et al.*, 1988).

Kinetin is a common natural cytokinin widely used in plant tissue culture works (Sita *et al.*, 1980); Mascarenhas *et al.*, 1982). In our study kinetin was found superior to BA and 2-ip in axillary bud cultures of young trees of rosewood whereas BA was observed to be the most effective cytokinin in inducing organogenesis in nodal explants from plus trees. Kinetin was found inferior to both BA and 2-ip. Just two treatments with kinetin viz., MS + Kin 3.0 mg l^{-1} and WPM + Kin 2.0 mg l^{-1} + IAA 0.1 mg l^{-1} could give rise to shoot development whereas eight combinations with BA (Addition of 0.25 and 2.0 mg l^{-1} BA alone or 0.25, 0.50, 2.0 and 3.0 mg l^{-1} BA along with 0.1 mg l^{-1} IAA to MS medium and 0.25 mg l^{-1} BA alone or BA and IAA at 2.0 and 0.1 mg l^{-1}, respectively to WPM) and five combinations with 2-ip (supplementing 0.25, 0.50 or 1.00 mg l^{-1} 2-ip to WPM and 0.50 or 1.00 mg l^{-1} 2-ip to MS) resulted in shoot proliferation when axillary bud culture of rosewood plus tree was attempted (Tables 9, 11, 13, 15, 17 and 19 and Figures 5, 6, 7, 8 and 9). Slow growth, low propagation rate and weak *in vitro* performance of mature explants as compared to juvenile shoots has been discussed in different tree species like pear (Maarri *et al.*, 1986), *Corylus avellana* (Messeguer and Mele, 1987;

Rodriguez *et al.*, 1988), *Artocarpus heterophyllus* (Rajmohan and Kumaran, 1988). *Dalbergia latifolia* (Sita *et al.*, 1980 and Rao, 1986) etc.

Treatment combinations with 2-ip were found better than those with kinetin for the axillary bud culture of the plus tree of rosewood. However, only few cultures among the successful ones which showed bud initiation could give rise to shoot development (Tables 17, 19; Figures 8, 9 and Plate 21). The maximum mean shoot length was just 1.5 cm for the combinations with both kinetin and 2-ip.

Cent per cent callusing was noticed for all the treatment combinations with BA or kinetin in MS medium. Callusing percentage varied from 85.7 or 28.6 to 100 for the combinations with BA and kinetin, respectively, in WPM (Tables 10, 12, 14 and 16). Supplementing 5.0 mg l^{-1} 2-ip along with 0.1 mg l^{-1} IAA to MS or WPM and 3.0 and 0.1 mg l^{-1} 2-ip and IAA, respectively, to WPM resulted in 100 per cent callus induction (Tables 18 and 20). Addition of 3.0 or 5.0 mg l^{-1} of 2-ip alone to MS or WPM could result only a callusing percentage of 85.7. The interaction of cytokinin and auxin is complex. Auxin added to the medium has already been reported to nullify the suppressive effect of high cytokinin content (Lundergan and Janick, 1980). In the present investigation, MS medium was found better than WPM in general

when callus induction of nodal explants from plus trees was concerned. Compact calli having a range of colours from white, pale yellow, light brown or dark brown only were formed.

Evidences of different kinds of chemicals of the same class of hormones, evoking different response is well documented. For example, Vieitez (1980) observed that axillary bud proliferation could be induced by BA in chestnut while kinetin was without any effect. In the present investigation kinetin was found to be the best for young trees whereas it had shown little effect with the plus trees. Among the three cytokinins tried, BA proved to be the best for the enhanced release of axillary buds of plus trees.

None of the combinations involving cytokinins and auxins could improve the problem of lack of multiple shoot formation or leaf expansion in culture (Plates 18, 20 and 21).

On MS medium supplemented with BA 1.0 mg l^{-1} and NAA 0.05 mg l^{-1} 92.9 per cent bud initiation was observed in case of explants from root suckers. Bud expansion and shoot proliferation percentage was 42.9 per cent with an average shoot length of 4.5 cm. Here also no multiple shoots or leaves were formed (Plate 26).

5.4.2. Other growth supplements

5.4.2.1. Adenine sulphate

The possible growth regulatory effect of adenine and its more soluble form adenine sulphate has been exploited in tissue culture of *Pterocarpus santalinus* (Patri *et al.*, 1988), young trees of *Dalbergia latifolia* (Mahato, 1992) etc. The beneficial effects of adenine may be due to its cytokinin like activity (Skoog and Tsui, 1948). Among the various levels of AS tried in basal media MS and WPM, only one treatment viz., MS + AS 8 mg l^{-1} resulted in bud expansion and shoot proliferation in a limited number of cultures (7.1 per cent). All the other treatments failed to induce bud break (Table 21). In this connection, Jarret *et al.* (1980) observed the inhibitory role of adenine sulphate in shoot formation.

5.4.2.2. Casein hydrolysate

Amino acids are routinely added to the media as they provide an immediately available source of nitrogen and their uptake can be much more rapid than that of inorganic nitrogen in the same medium (Simpkins *et al.*, 1970). Casein hydrolysate is a complex mixture of several amino acids. Enhancement in multiple shoot production by adding CH has been reported by Mascarenhas *et al.* (1982) in *Hevea brasiliensis* and Mahato (1992) in *Dalbergia latifolia.* However, in our investigation none of the combinations of MS and WPM with CH could induce shoot

proliferation (Table 22). Similar observations of no favourable effect of casein hydrolysate have been reported by George and Sherrington (1984) in several plant species both in monocots and dicots and Divatar (1994) in *Vateria indica*. Callusing was observed in all the treatments. Medium MS was found superior to WPM when callus induction supported by CH was concerned.

5.4.2.3. Cycocel

Cycocel (Chloromequat chloride/CCC) is a choline derivative containing substituted chlorine. This compound has been reported to influence leaf morphogenesis and leaf anatomy including palisade cell length, spongy parenchyma layer thickness and mesophyll air space (Gausman, 1986). These observations together with the generally observed increase in chlorophyll concentration (Davis and Curry, 1991) had possible implication of CCC in culture. In addition to these, CCC may sometimes prolong the leaf retention by delaying leaf senescence. Among the twelve different treatment combinations tried with CCC, supplementing MS medium with 1.0 mg l^{-1} CCC only could give rise to bud expansion and shoot growth. Performance was found to be poor with a shoot proliferation percentage of just 7.1 (Table 23 and Figure 10). Leaf expansion was absent. Results of the investigations carried out to study the effect of this growth inhibitor by Santhoshkumar (1993) showed that it negatively influenced bud break from axillary buds of bijasal.

5.4.2.4. Phloroglucinol

Polyphenolic compounds like phloroglucinol in the medium has been found to have a beneficial role in organogenesis and growth (Hunter, 1979; Mallika *et al.*, 1992). Addition of this compound in MS or WPM during the present investigation, however, did not give any favourable response to the axillary buds of plus trees of *Dalbergia latifolia* except for callus induction (Table 24). A large number of workers who have compared the responses of rosaceous fruit trees in media with or without phloroglucinol found this compound to be without advantage or even inhibitory (Quoirin *et al.*, 1977; Singha, 1980; Zimmerman and Broome, 1981).

5.4.2.5. L-glutamine

Addition of L-glutamine at 1000 or 1500 mg l^{-1} to the culture medium was found very effective in preventing precocious drop of leaf initials (Plates 23, 24 and 25). L-glutamine at higher concentrations reduced precocious leaf drop and the growth of the shoots was normal (Sita and Swamy, 1992).

Glutamine is a key compound in nitrogen metabolism in both procaryotic and eucaryotic organisms. It plays different metabolic roles in different types of cells. Not only is it a component of many proteins but it also is a precursor of a large number of important biochemical compounds. In addition, it participates in an essentially irreversible ATP dependent

synthesis of glutamic acid from ÿ-ketoglutaric acid and thus in turn serves as a donor of NH_2 groups in the biosynthesis of a large number of amino acids by transamination (Conn and stumpf, 1976). Thus the favourable effect of L-glutamine in rosewood cultures can tentatively be ascribed to its key role in many of the vital metabolic activities.

5.4.2.6. Coconut water

Among the different combinations, coconut water at 10 per cent (v/v) added to MS + Kin 1.0 + IAA 0.1 mg l^{-1} only could produce bud break and shoot growth (Table 25). However, all the treatments were found effective in inducing callus. According to Straus and Rodney (1960), the favourable effect of coconut water 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. Addition of coconut water in medium has been reported to enhance both shoot growth and multiple shoot formation in a number of tree species (Mittal *et al.*, 1989; Rai and Chandra, 1989).

Coconut water contains myo-inositol which lead to the inclusion of this chemical in many media (Pollard *et al.*, 1965). Lack of beneficial effect of coconut water on shoot formation and leaf production has been noticed in *Dalbergia latifolia* (Mahato, 1992) and *Vateria indica* (Divatar, 1994).

5.4.2.7. Activated charcoal

Addition of activated charcoal in tissue culture media may have beneficial or harmful effects. Activated charcoal is not a growth regulator in the strict sense, but its capability to adsorb a wide range of compounds has made it a valuable medium supplement. In tree micropropagation, AC is of great use as it can adsorb inhibitory compounds secreted from explants. Growth, rooting, organogenesis and embryogenesis are reported to be stimulated in a wide variety of species and tissues (Wang and Huang, 1976) and inhibited in certain others on the other hand (Fridborg and Eriksson, 1975). In the present study, none of the combinations of AC in both MS and WPM induced bud release or shoot proliferation in rosewood. All the levels of this supplement except MS + AC 0.25 per cent, exhibited inhibitory effect on callusing also. The percentage of cultures showing callus as well as the amount of callus was low for all these combinations (Table 26). Inhibitory effect of AC may be due to its binding with plant growth regulators and other metabolites (Weatherhead *et al.*, 1978). Scott *et al.* (1990) has reported that activated charcoal added to liquid MS reduced IAA and IBA concentrations by over 97 per cent. Activated charcoal exhibiting a negative influence on bud break was reported in *Dalbergia latifolia* (Mahato, 1992) and *Pterocarpus marsupium* (Santhoshkumar, 1993). No browning was noticed in any of the cultures with AC. This is probably due to the capability of AC

to absorb or bind to the phenolic substances secreted by the explants, which otherwise get oxidised to quinols which impart brown colouration to the medium and toxic effect to the explant. Addition of AC may also prevent browning of tissues (Tisserat, 1979).

5.5. Rooting

Generally, auxins favour root formation. Most of the researchers have reported that *in vitro* rooting can successfully be achieved by reducing salt concentration in the media, particularly in MS, B_5 and WPM, which contain high salt concentrations. Lane (1979) observed abundant rooting when the salt concentration was reduced to one half or one third in the medium. But in such cases, sometimes poor top growth was resulted (Gupta *et al.*, 1981). High concentrations of auxin inhibited root elongation (Thimman, 1977).

In the present study, addition of activated charcoal (one per cent) to half strength MS medium and pulse treatment to excised shoot by IBA (1000 mg l^{-1}) registered 95 per cent rooting of shoots from young source trees. Rooting trial was a failure with the excised *in vitro* grown shoots of the plus trees as well as root suckers. The loss of rooting ability with age is common among woody perennials. Shoots taken from older trees or from

higher up the stem show a decline in rooting ability (Hackett, 1988). Mahato (1992) reported that rooting in *in vitro* shoots of saplings or young trees of rosewood could readily be achieved by a root inductive auxin treatment (IBA 1000 mg l^{-1}) followed by culturing in an auxin free medium. Reports on *in vitro* propagation of forest trees show that, explants that have shown positive response in culture for regeneration are largely restricted to juvenile material. Amin and Jaiswal (1993) reported that microcuttings taken from primary cultures initiated from bud explants of mature field grown trees of *Artocarpus heterophyllus* were very difficult to root. Regardless of the media composition, growth regulator concentration and culture environment, only about five per cent of cuttings from newly established cultures produced roots. Contrary to that, shoots originated from the jackfruit seedling explants could be rooted easily. In *Eucalyptus citriodora*, shoots have been regenerated from adult material but *in vitro* multiplied shoots developed roots only after passing them through a series of subcultures (Razdan, 1993). Weak *in vitro* performance of mature explants as compared to juvenile shoots has been discussed by different workers (Maarri *et al.*, 1986 in pear; Messeguer and Mele, 1987 in *Coryluys avellana;* Rajmohan and Kumaran, 1988 in *Artocarpus heterophyllus* etc.).

Rooting under *in vitro* is one of the most labour intensive parts of micropropagation (Debergh and Maene, 1981). *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). Such *ex vitro* rooting provides facilities for simultaneous rooting and hardening of the shoots so that the mortality of plantlets while planting out from the rooting medium as well as the time required for final planting out can be reduced to a great extent. In our study, *in vitro* produced shoots of young trees of rosewood when transferred to sterilized sand in cups after IBA (1000 mg l^{-1}) treatment failed to produce roots. Sand alone as a medium for *ex vitro* rooting appears to be unsuitable.

Direct rooting from the explants in primary cultures was noticed (14.3 per cent) when the nodal explants from young trees were cultured on WPM supplemented with kinetin 1.0 or 2.0 mg l^{-1} along with 0.1 mg l^{-1} IAA (Plates 8 and 9). This probably was just an accidental finding.

5.6. Planting out and acclimatization

Acclimatization is critical to any micro-propagation scheme since shoots and plantlets produced *in vitro* must be readapted to the less humid environmental conditions outside the culture vessels. A period of humidity acclimatization is 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 investigation, high relative humidity was maintained during the initial period of planting out with the help of polythene bags (Plate 10) and intermittent cold water sprays. After two weeks, the bags were lifted partially for short intervals to make the plantlets hardened with respect to lower relative humidity. Gradually within two weeks, the cover could be completely removed and the plantlets exposed to ambient conditions of temperature and humidity (Plate 11). Similar method 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 was successfully adopted by Murashige (1977). Plants believed to develop their stomatal control mechanism during the period under high humidity, which helps them to reduce excessive water loss when planted out. Application of 5 to 10 ml nutrient solution containing MS organic salts at half concentration on alternate days enhanced the survival and promoted normal growth of the plantlets once they were planted out from the rooting medium. Rajmohan (1985) and Mahato (1992) also reported the use of a nutrient solution similar to the above for the successful nurture of *in vitro* raised jack and rosewood

plantlets, respectively. In our study 65 per cent of the plantlets survived after the acclimatization process. It was observed that plantlets which showed direct rooting failed to survive when planted out. This may be due to the improper vascular connection between roots and shoots.

5.7. Indirect organogenesis

Indirect organogenesis is the fastest method of shoot multiplication and been suggested as a potential method of cloning plant species (Murashige, 1974). callus, which shows stable characteristics under specific conditions after subculture through many successive passages, is a suitable material for cytodifferentiation. The advantage of using such callus is that it is composed of a fairly homogeneous mass of cells and can be proliferated in large amounts under known culture conditions (Razdan, 1993). The most serious drawback in the use of callus culture is the possible genetic instability of the cells. However, successful micropropagation technique following this route have been reported in several species including *Sesbania bispinosa* (Sinha and Mallick, 1991), *Bauhinia purpurea* (Kumar, 1992), *Cephalotaxus harringtonia* (Wickremesinhe and Arteca, 1993), *Populus trichocarpa* x *deltoides* cv. Hunnegem (Jehan *et al.*, 1994) etc. Callus cultures were successfully initiated from

internodal segments from root suckers of plus trees of rosewood (Plates 27, 28, 29 and 30). In one of these cultures shoot regeneration was noticed (Plate 31). Since leaf formation and further growth of these shoots were absent more studies could not be taken up.

The present investigation clearly depicted the difference in culture response between juvenile and adult explants from *Dalbergia latifolia*. While *in vitro* propagation using explants from young trees of rosewood could be achieved and plantlets could be regenerated with cent per cent repeatability, micro-propagation of the selected plus trees of rosewood faced with many obstacles. Though bud break and shoot morphogenesis was noticed in some of the media combinations tried, leaf expansion as well as further rooting of the shoots could not be obtained. Lack of multiple shoot production was another handicap. It is observed that culture systems that work for one species or explant are often not readily adaptable to others (Hulse, 1992).

A major constraint to orderly progress in *in vitro* culture is inadequacy of reliable quantitative physiological, biochemical and cell biological data relative to the species to be cultured; together with an understanding of the critical functions of phytohormones, plant growth regulators and other critical media constituents. Little is known about the

physiological and genetic mechanisms that control the differences between the juvenile and mature physiological state of cells, tissues or the plant. Consequently plant cell and tissue culture of forest trees relies more upon empirical experience than fundamental principles. Micropropagation of mature trees can be achieved only through a succession of experiments. Abnormalities in the beginning can be eliminated in progressive steps by adjusting the culture conditions. We may have to depend on certain markers to design better culture conditions. For example, the appearance of leaf specific mRNA or proteins could possibly be used as markers to indicate the progress. Dong and Dunstan (1994) reported that DNA content and the content of intracellular proteins can be used as indicators of embryo yield in suspension cultures of *Picea glauca*.

Further studies are certainly needed to formulate a protocol for the *in vitro* propagation of the superior genotypes of Indian rosewood, taking the explants directly from the field grown trees. More basic research into the biochemistry of organogenesis and the physiology of root and leaf initiation in this species is warranted with increased co-operation between scientists concerned with fundamental and applied research.

Summary

SUMMARY

A research programme entitled "**Clonal propagation of selected plus trees of Indian rosewood (*Dalbergia latifolia* Roxb.) through tissue culture**" was undertaken at College of Forestry, Vellanikkara during the period 1993-95. Three selected plus trees as well as root suckers from one of these trees were used as the explant sources. Explants from young (eight to ten year old) rosewood trees were also used in the present investigation, to know the difference, if any, in culture response between juvenile and adult plant materials. The experiment was laid out in a Completely Randomized Design with 121 treatments. The highlights of the study can be summarized as follows:

1. A prophylactic spray with the mixture of a contact and a systemic fungicide, Indofil M-45 (Mancozeb) and Bavistin (Carbendazim), respectively, both at 0.3 per cent given to the young trees and root suckers of rosewood or immersing the explants from the plus trees in the same fungicidal mixture for one hour duration remarkably reduced the culture contamination.

2. Abnormal leaf development from explant in culture without shoot proliferation was noticed when the young trees were sprayed everyday with the fungicides. Reducing the

frequency of fungicidal application at an interval of two days successfully eliminated this abnormality without any perceptible effect on culture contamination.

3. An initial wiping of the fungicide treated explants with 70 per cent ethanol, followed by immersion in 0.1 per cent mercuric chloride for 12 minutes was found to be a very effective surface sterilization method.

4. Chlorine water was found to be totally ineffective for controlling culture contamination.

5. Phenol problem was practically nil when the explants from young trees were used for culture.

6. Browning of the medium due to polyphenol interference was very severe when the explants from plus trees as well as root suckers were cultured without pre-treatment.

7. No individual treatment was found successful in controlling the polyphenol interference. However, a treatment combination involving soaking the explants in a solution of ascorbic acid and citric acid both at 150 mg l^{-1} for three hours and incorporation of 150 mg l^{-1} each of ascorbic acid and citric acid and 0.7 per cent polyvinylpyrrolidone (PVP)

in the culture medium was found to be very effective in preventing browning of the medium.

8. The treatment, WPM + Kin 1.0 + IAA 0.1 mg l^{-1} was found to be the best treatment for the enhanced release of axillary buds of young rosewood trees with 100 per cent shoot proliferation, maximum average shoot length (7.8 cm) and mean number of leaves (4.5). This treatment took minimum number of days for bud initiation (5.1) and bud expansion (7.1).

9. The highest number of shoots per culture (3.5) was obtained by the addition of 2.0 mg l^{-1} BA to MS medium for young trees.

10. Explants from young rosewood trees stored in refrigerator for one or two days failed to show any response in culture.

11. All the eight treatment combinations tried for young trees resulted in inducing callus at the base of the explants for all the cultures. Addition of silver nitrate at 5.0 mg l^{-1} level did not exhibit any influence on inhibiting callus induction. However, callus growth was not found to inhibit the growth of the cultures.

12. For *in vitro* rooting of shoots, pulse treatment with IBA (1000 mg l^{-1}) to individual shoot and then transfer to half strength MS medium containing 1.0 per cent activated charcoal was found very effective in case of young trees.

13. *In vitro* produced shoots from young rosewood trees when transferred to sterilized sand in cups after IBA (1000 mg l^{-1}) treatment failed to produce roots.

14. Transparent plastic covers were found suitable as humidity maintenance devices, maintaining high relative humidity supporting 65 per cent of plantlet survival for young trees.

15. Direct shoot and root formation was noticed when the nodal explants from young trees were cultured on WPM supplemented with kinetin 1.0 or 2.0 mg l^{-1} along with 0.1 mg l^{-1} IAA. However, these plantlets failed to survive when planted out.

16. Difference in culture response between genotypes was observed when explants from the three selected plus trees were cultured on the same medium.

17. Among the three cytokinins (BA, Kinetin and 2-ip) tried, BA proved its superiority over the other two in inducing organogenesis in plus trees of rosewood whereas kinetin was found superior to BA in cultures of young trees.

18. The combination of MS + BA 2.0 mg l^{-1} was identified as the best treatment for getting a maximum shoot length of 4.0 cm with the nodal explants from the plus trees of rosewood.

19. Addition of the auxin IAA at 0.1 mg l^{-1} to the medium inhibited BA's capacity for inducing morphogenesis, with retarded shoot growth.

20. Kinetin was found inferior to BA and 2-ip in axillary bud cultures of plus trees.

21. Cent per cent callusing was noticed for all the treatment combinations with BA or kinetin in MS medium

22. None of the combinations of activated charcoal casein hydrolysate and phloroglucinol in both MS and WPM induced shoot proliferation in plus trees.

23. Adenine sulphate, cycocel and coconut water added to the media showed a little effect on shoot development of the selected plus trees of rosewood.

24. On MS medium supplemented with BA 1.0 mg l^{-1} and NAA 0.05 mg l^{-1} 92.9 per cent bud initiation was observed in case of explants from root suckers. Bud expansion and shoot proliferation percentage were 42.9 per cent each with an average shoot length of 4.5 cm.

25. None of the combinations involving cytokinins, auxins and the other supplements could improve the problem of lack of multiple shoot formation or leaf expansion in plus trees as well as root suckers.

26. Addition of L-glutamine at 1000 or 1500 mg l^{-1} to the culture medium was found very effective in preventing precocious drop of leaf initials formed.

27. Rooting trial was a failure with the excised *in vitro* grown shoots of the plus trees as well as root suckers.

28. Callus cultures were successfully initiated from internodal segments from root suckers of the plus tree of rosewood. However, no further morphogenesis could be obtained.

References

REFERENCES

Aboel-nil, M.M. 1987. Micropropagation of *Casuarina equisetifolia*. *Cell and Tissue Culture in Forestry. Vol.III.* (Eds. Bonga, J.M. and Durzan, D.J.) Martinus Nijhoff, Boston. p.400-409.

Ahmad, D.H. 1990. Micropropagation of *Acacia mangium* from aseptically germinated seedlings. *J. Trop. For. Sci.* **3**: 204-208.

Amatya, N. and Rajbhandary, S.B. 1990. *In vitro* Propagation of *Ficus lacor* Buch-Ham. *Banko Janakari.* **3**(1): 30-35.

Amin, M.N. and Jaiswal, V.S.1988. Micropropagation as an aid to rapid cloning of a guava cultivar. *Scientia Horticulturae* **36**(1-2): 89-95.

Amin, M.N. and Jaiswal, V.S. 1993. *In vitro* response of apical bud explants from mature trees of jackfruit (*Artocarpus heterophyllus*). *Pl. Cell Tissue Organ Cult.* **33**: 59-65.

Ancora, G., Belidonna, M.L. and Cuzzo, L.L. 1981. Globe artichoke plants obtained from shoot apices through rapid *in vitro* micropropagation. *Sci. Hort.* **14**: 207-213.

*Anderson, W.C. 1975. Propagation of rhododendrons by tissue culture: Part I, development of a culture medium for multiplication of shoots. *Comb. Proc. Int. Pl. Prop. Soc.* **25**: 129-135.

Arnold, M.A., Lineberger, R.D. and Struve, D.K. 1994. Copper compounds influence *in vitro* rooting of birch microcuttings. *J. Am. Soc. Hortic. Sci.***119**(1): 74-79.

Arnold, S.V. and Erikson, T. 1986. Norway spruce (*Picea abies* L.). *Biotechnology in Agriculture and Forestry Vol. I.* (Ed. Bajaj. Y.P.S). Springer Verlag, New York. p. 275-290.

Ashok, A. 1985. *In vitro* shoot differentiation in *Eucalyptus citriodora* : Effect of activated charcoal. *Indian J. For.* **8**(4): 304-341.

Bejoy, M. and Hariharan, M. 1993. *In vitro* propagation of *Adenanthera pavonia* L. *J. Tree Sci.* **12** (2): 69-72.

Bennett, I.J., Mccomb, J.A., Tonkin, C.M. and Mcdavid, D.A. 1994. Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. *Ann. Bot.* **74**(1): 53-58.

Bhatnagar, S.P. and Singh, M.N. 1984. Organogenesis in the cultured female gametophyte of *Euphedra foliata. J. Exp. Bot.* **35**: 268-278.

Bhatnagar, S.P., Singh, M.N. and Kapur, N. 1983. Preliminary investigations on organ differentiation in tissue culture of *Cedrus deodara* and *Pinus roxburghii. Indian J. Exp. Biol.* **21**: 524-526.

Biddington, N.L. 1992. The influence of ethylene in plant tissue culture. *Pl. Growth Regul.* **11**: 173-187.

Bonga, J.M. 1982. Vegetative propagation in relation to juvenility, maturity and rejuvenation. *Tissue Culture in Forestry.* (Eds. Bonga, J.M. and Durzan, D.J.). Martinus Nijhoff, Boston. p.78-90.

Broome, C. and Zimmerman, R.H. 1978. *In vitro* propagation of blackberry. *Hortscience* **13**(2): 151-153.

Brown, C.L. and Sommer, H.E. 1982. Vegetative propagation of dicotyledenous trees. *Tissue Culture in Forestry.* (Eds. Bonga, J.M. and Durzan, D.J.). Martinus Nijhoff, Boston. p.109-149.

Chauhan, V.A., Josekutty, P.C. and Prathapan, G. 1994. Rapid micropropagation of *Dalbergia sissoo* Roxbo. *In Vitro.* **30A**, **3**(2): 81.

Chimmala, A.R. 1994. Micropropagation of forest tree species using tissue culture. *In Vitro Pl.* **30**(4): 232.

Chu, C.C. 1978. The N_6 medium and its application to anther culture of cereal crops. *Proceedings of Symposium on Plant Tissue Culture* Science press, peking. p.43-50.

Colleman, G.D. and Ernst, S.G. 1990. Axillary shoot proliferation and growth of *Populus deltoides* shoot cultures. *Pl. Cell. Rep.* **9**: 165-167.

Conn, E.E. and Stumpf, P.K. 1976. *Outlines of Biochemistry* (4th Edn.). Wiley Eastern Limited, New Delhi. p.565.

C.S.I.R. 1952. *The Wealth of India, Raw Materials. Vol.III D-E.* Council of Scientific and Industrial Research, New Delhi. p.4-7.

*Datta, K. and Datta, S.K. 1985. Auxin + KNO_3 induced regeneration in apical budsod trees through tissue culture. *Curr. Sci.* **54**: 248-249.

Doublin, P. 1984. Extractable production section XI. *Hand Book of Plant Cell Culture.* (Eds. Evans, P.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y.). McMillan Publishing Co., New York. p.541-564.

Fridborg, G. and Eriksson, T. 1975. The effect of activated charcoal on tissue culture : absorption of metabolites inhibiting morphogenesis. *Physiol. Pl.* **40**: 104-106.

Gamborg, O.L., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soyabean root cells. *Exp. Res.* **50**: 151-158.

Gamborg, O.L., Davis, B.D. and Stahlhut, R.W. 1983. Somatic embryogenesis in cell cultures of *Glycine* species. *Pl. Cell Rep.* **2**: 209-212.

Gamborg, O.L. and Shyluk, J.P. 1981. Nutrition, media and characteristics of plant cell and tissue cultures. *Plant Tissue Culture : Methods and Applications in Agriculture.* (Ed. Thorpe, T.A.). Academic Press, New York. p.21-44.

Garland, P. and Stoltz, L.P. 1981. Micropropagation of Pissardiplum. *Ann. Bot.* **48**: 357-389.

*Gausman, H.W. 1986. *Onium Bioregulators.* West printing, Lubbock. Tenas. p.80-86.

*Gautheret, R.J. 1942. Manual technique de culture de tissue vegetaux paris: Masson.

George, E.F. and Sherrington, P.D. 1984. *Plant Propagation by Tissue Culture.* Exegetics Limited, England. pp.690.

David, A., David, H. and Mateille, T. 1982. *In vitro* adventitious budding on *Pinus pinaster* cotyledons and needles. *Physiol. Pl.* **5**: 102-107.

Davis, J.D. and Curry, F.A. 1991. Chemical regulation of vegetative growth. *Critical Rev. Pl. Sci.* **10**: 151-188.

Debergh, P.C. and Maene, L.G. 1981. A scheme for commerical propagation of ornamental plants by tissue culture. *Sci. Hort.* **14**: 334-335.

Deshpande, S.R., Josekutty, P.C. and Prathapan, G. 1994. Rapid micropropagation of *Ficus religiosa* L. *In Vitro.* **30A**, **3**(2): 81-82.

Devi, S.A.M. and Nataraja, K. 1987. *In vitro* regeneration and establishment of plantlets in stem cultures of *Dalbergia latifolia* Roxb. *Indian For.* **113**(7): 501-506.

Dhingra, M., Sujatha, M. and Ranganath, A.R.G. 1991. Rapid multiplication of *Melia azedarach* L. through tissue culture. *J. Oilseed Res.* **8**(2): 215-219.

Divatar, A.B. 1994. *In vitro* propagation of Malabar White Pine (*Vateria indica* L.) through Tissue Culture. M.Sc. Thesis, Kerala Agric. Univ., Vellanikkara, Thrissur. pp.118.

Dodds, J.H. and Roberts, L.W. 1985. *Experiments in Plant Tissue Culture.* Cambridge University Press, London. pp.28.

Dong, J.Z. and Dunstan, D.I. 1994. Growth parameters, protein and DNA synthesis of an embryogenic suspension culture of White spruce(*Picea glauca*). *J. Pl. Physiol.***144**(2): 201-208.

Gill, R.I.S. and Gill, S.S. 1994. *In vitro* exudation of phenols in *Eucalyptus. Indian For.* **120**(6): 504-509.

Gillis, M.R. and Kane, M.E. 1994. *In vitro* multiplication of *Stewartia malacodendron* L., an endangered woody species *Hortscience.* **29**(5): 516.

Goh, H.K.L., Rao, A.N. and Loh, C.S. 1988. *In vitro* plantlet formation in mangosteen (*Garcinia mangostana* L.). *Ann. Bot.* **62**: 87-93.

Gomez, M.P. and Segura, J. 1994. Factors controlling adventitious bud induction and plant regeneration in mature *Juniperus oxycedrus* leaves cultured *in vitro. In Vitro Pl.* **30**(4): 210-218.

Goyal, Y., Binham, R.L. and Felker, P. 1985. Propagation of the tropical tree *Leucaena leucocephala* K67 by *in vitro* bud culture. *Pl. Cell. Tissue Organ Cult.* **4**: 3-10.

Gupta, P.K. and Durzan, D.Z. 1985. Shoot multi-plication from mature trees of Douglas fir (*Pseudotsuga menziesii)* and sugar pine *(Pinus lambertiana). Pl. Cell Rep.* **4**: 177-179.

Gupta, P.K. and Mascarenhas, A. 1987. *Eucalyptus. Cell and Tissue Culture in Forestry.* (Eds. Bonga, J.M. and Durzan, D.J.). Martinus Nijhoff, Boston. p.316-325.

Gupta, P.K., Mascarenhas, A.F. and Jagannathan, V. 1981. Tissue culture of forest trees - clonal propagation of mature trees of *Eucalyptus citriodora* Hook by tissue culture. *Pl. Sci. Lett.* **20**: 195-201.

Gupta, P.K., Nadgir, A.L., Mascarenhas, A.F. and Jagannathan, V. 1980. Tissue culture of forest trees: colonal multiplication of *Tectona grandis* L. (Teak) by tissue culture. *Pl. Sci. Lett.* **17**: 259-268.

Hackett, W.P. 1988. Donor plant maturation and adventitious root formation. *Adventitious Root Formation in Cuttings*. (Eds. Davis, T.D., Haissiq, B.E. and Sankhla, N.). Dioscorides Press, Portland. p.11-28.

Hammatt, N. and Ridout, M.S. 1992. Micropropagation of common ash (*Fraxinus excelsior). Pl. Cell. Tissue Organ Cult.* **13**: 67-74.

Hanus, D. and Rohr, R. 1987. *In vitro* plantlet regeneration from juvenile and mature sycamore maple. *Acta Horticulturae* **212**: 77-82.

Hasnian, S. and Cheliak, W. 1986. Tissue culture in forestry : economic and genetic potential. *For. Chron.* **62**(4): 219-255.

*Heller, R. 1953. Recherches sur la nutrition minerale de tissus vegetaux cultives *in vitro. Ann. Sci. Nat. Bot. Biol. Veg.* **14**: 1-223.

*Hildebrandt, A.C., Riker, A.J. and Duggar, B.M. 1946. The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue cultures. *Am. J. Bot.* **33**: 591-597.

*Hoque, M.I., Ara, A. and Sarker, R.H. 1992. Morphogenesis in some fast growing trees. *Conf. Abstr.* (Univ. Dhaka, Bangladesh). **2**: 37.

Horgan, K. 1987. *Pinus radiata. Cell and Tissue Culture in Forestry. Vol III.* (Eds. Bonga, J.M. Durzan, D.J.). Martinus Nijhoff, Boston. p. 416.

Hu, C.Y. and Wang, P.J. 1983. Meristem, shoot tip and bud cultures. *Handbook of Plant Cell Culture. Vol.I. Techniques for Propagation and Breeding.* (Eds. Evans, D.A., Sharp, W.R. Ammirato, P.V. and Yamada, Y.). McMillan Pub. Co., New York. p. 177-227.

Huetteman, C.A. and Preece, J.E. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Pl. Cell Tissue Organ Cult.* **33**: 105-119.

Hulse, J.H. 1992. Plant cell and tissue culture : Progress and prospects. *Rapid propagation of Fast-growing Woody Species* (Ed. Baker, F.W.G.). CAB International, U.K. p.1-6.

Hunter, C.S. 1979. *In vitro* culture of *Cinchona ledgeriana. J. Hort. Sci.* **54**: 111-174.

Hussey, G. 1978. The application of tissue culture to the vegetative propagation of plants. *Sci. Prog.* **65**: 185-208.

Hussey, G. 1983. *In vitro* propagation of horticultural and agricultural crops. *Plant Biotechnology.* (Eds. Mantell, S.H. and Smith, H.). Cambridge University Press, Cambridge. p.11-138.

*Jaiswal, P.K. and Gulati. 1991. *In vitro* high frequency plant regeneration from a tree legume, *Tamarindus indicus* L. *Pl. Cell Rep.* **10**: 569-573.

Jaiswal, V.S. and Amin, M.N. 1987. *In vitro* propagation of guava from shoot cultures of mature trees. *J. Pl. Physiol.* **130**(1): 7-12.

*Jang, S.S., Lee, J.S., Lee, S.K. and Shim, S.Y. 1988. Plant regeneration from cell cultures of *Populus glandulosa* Suwon. *Korea Repub. Res. Rep. Inst. For. Genet.***24**: 107-113.

Jarret, R.L., Hasegawa, P.M. and Ericksson, H.T. 1980. Effects of medium components on shoot formation from cultured tuber discs of potato. *J. Am. Hort. Sci.* **102**: 177-184.

Jehan, H., Brown, S., Marie, D., Noin, M., Prouteau, M. and Chiriqui, D. 1994. Ontogenesis and ploidy level of plantlets regenerated from *Populus trichocarpa x deltoides* cv. Hunnegem root, leaf and stem explants. *J. Pl. Physiol.* **144**(4-5): 576-585.

Jones, T.C., Batchelor, C.A. and Harris, P.J.C. 1990. *In vitro* culture and propagation of *Acacia* species (*A. bivenosa, A. holosericea, A. Salicina, A. Saligna* and *A. Sclerosperma). Int. Tree Crops J.* **6**: 183-192.

Joshi, R., Shekhawat, N.S. and Rathore, T.S. 1991. Micropropagation of *Anogeissus Pendula* - An arid forest tree. *Indian J. Exp. Biol.* **29**: 615-618.

Kackar, N.L., Solanki, K.R., Singh, M. and Vyas, S.C. 1991. Micropropagation of *Prosopis cineraria. Indian J. Exp. Biol.* **29**: 65-67.

Kim, D.S. and Lee, S.P. 1988. Study on micro-propagation of jujuba cultivar through axillary bud culture. *J. Korean For. Sci.* **77**(4): 445-452.

Kim, Y.W., Lee, B.C., Lee, S.K. and Jang, S.S. 1994. Somatic embryogenesis and plant regeneration in *Quercus acutissima*. *Pl. Cell. Rep.* **13**(6): 315-318.

Kleinschmit, J. 1974. A programme for large scale cutting propagation of Norway spruce. *N.Z. J. For Sci.* **4**: 359-366.

*Knudson, C. 1946. A new nutrient solution for the germination of orchid seeds. Bull. Am. Orchid. Soc. **15**: 214-217.

*Kogl, F., Hagen-Smith, A.J. and Erxleben, M. 1934. Uber ein neus auxin (hetero auxin) aus harn XI. Mitteilung. *Z. Physiol. Chem.* **228**: 90-103.

Kolveska-pletikapis, B., Jelasba, S., Berljak and Vidakovic. 1983. Bud and shoot formation in juvenile tissue cultures of *Pinus nigra. Silvae Genetica.* **32**: 115-119.

Kopp, M.S. and Nataraja, K. 1990. *In vitro* plantlet regeneration from shoot tip cultures of *Tamarindus indicus* L. *Indian J. For.* **13**(1): 30-33.

Krikorian, A.D. 1982. Cloning higher plants from asceptically cultured tissue and organs *Biol. Rev.* **57**: 151-218.

Kumar, A. 1992. Micropropagation of mature leguminous tree; *Bauhinia purpurea. Pl. Cell Tissue Organ Cult.* **31**: 257-259.

Kumar, A., Tandon, P. and Sharma, A. 1991. Morphogenetic responses of cultured cells of cambial origin of a mature tree. *Dalbergia sissoo. Pl. Cell. Rep.* **9**: 703-706.

Kumar, R.R. and Ayyappan, P. 1987. Rapid clonal multiplication of *Eucalyptus camaldulensis* : *In vitro* culture. *Planters chron.* **8**(7): 225-227.

Lane, W.D. 1979. The influence of growth regulators on root and shoot initiation from flax meristem tips and hypocotyls *in vitro*. *Physiol. Pl.* **45**: 260-264.

*Lee, S.K. and Rao, A.N. 1980. Tissue culture of certain tropical trees. *Plant Cell Cultures: Results and Perspectives.* (Eds. Sala, F., Parisi, B., Cella, R. and Cifferi, P.). Elsevier, Amsterdam. p. 305-311.

*Legrand, B. and Mississo, E. 1986. Effect of size of the explants and growth regulators on the development of tissues of *Theobroma cacao* L. var. Amelonado cultivated *in vitro*. *Cafe Cacao The.* **30**(4): 239-246.

Leifert, C., Waites, B., Keetley, J.W., Wright, S.M., Nicholas, J.R. and Waites, W.M. 1994. Effect of medium acidification on filamentous fungi, yeasts and bacterial contaminants in *Delphinium* tissue culture. *Pl. Cell Tissue Organ Cult.* **36**(2): 149-155.

Lelu, M.A., Bastien, C., Klimaszewska, K., Ward, C. and Charest, P.J. 1994. An improved method for somatic plantlet production in hybrid larch (Larch x leptoeuropaea) Part I. Somatic embryo maturation. *Pl. Cell Tissue Organ Cult.* **36**(1): 107-115.

Levy, L.W. 1981. A large scale application of tissue culture. The mass propagation of pyrethrum clones in Ecuador. *Environ. Exp. Bot.* **21**: 389-395.

Linsmaier, M.R. and Skoog, F. 1965. Organic growth factor reequirement of tobacco cultures. *Physiol. Pl.* **18**: 100-127.

Lloyd, B. and McCown, B. 1980. Commercially feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot tip culture. *Comb. Proc. Int. Pl. Prop. Soc.* **30**: 421-427.

Lundergan, C. and Janick, J. 1980. Regulation of apple shoot proliferation and growth *in vitro*. *Hort. Res.* **20**: 19-24.

*Maarri, K.A.L., Duron, M., Arnaud, Y. and Miginaic, E. 1986. Comparative study on the response to micropropagation of adult Passe Crassane pears and seedlings in meristem culture. *Comptes Rendus Academic Agriculture France* **72**(5): 413-421.

Macek, T., Kral, J., Vanek, T., Blazek, J. and Mackova, M. 1994. Chemical sterilization of nutrient media for plant cell cultures using diethylpyrocarbonate. *Biotechnol. Tech.* **8**(12): 885-888.

Mahato, K.C. 1992. *In vitro* Propagation of *Dalbergia latifolia* Roxb. through Tissue Culture. M.Sc. Thesis. Kerala Agric. Univ., Vellanikkara, Thrissur. pp. 111.

Mallika, V.K., Sankar, A.M., Sindhu, K., Rekha, C., Vijayakumar, N.K. and Nair, R.V. 1992. Plantlet regeneration *in vitro* from nodal segments of cocoa (*Theobroma cacao* L.) and field planting. *J. plantn. Crops* **20**: 114-122.

Manzanera, J.A. and Pardos, J.A. 1990. Micro-propagation of juvenile and adult *Quercus suber* L. *Pl. Cell Tissue Organ Cult.* **21**: 1-8.

Marino, G., Bertazza, G., Magnanini, E. and Altan, A.D. 1993. Comparative effects of sorbitol and sucrose as main carbon energy sources in micropropagation of apricot. *Pl. Cell Tissue Organ Cult.* **34**: 235-244.

Maroti, M. and Levy, E. 1977. Hormonal regulation of the organisation from meristem cultures. *Use of Tissue Culture in Plant Breeding.* (Ed. Novak, F.J.). Czech Acad. Sci., Prague. pp.337.

Mascarenhas, A.F., Hazara, Z., Potdar, V., Kulkarni, D.K. and Gupta, P.K. 1982. Rapid clonal multiplication of mature forerst trees through tissue culture. *Plant Tissue Culture.* (Ed. Fujiwara, A.). Proc. 5th Int. Cong. Pl. Tissue Cell Cult., Tokyo.

Mascarenhas, A.F., Kendurkar, S.V., Gupta, P.K., Khurpe, S.S. and Agarwal, D.C. 1987. Teak. *Cell and Tissue Culture in Forestry. Vol. III.* (Eds. Bonga, J.M. and Durzan, D.J.). Martinus Nijhoff, Boston. p. 300-315.

Mathur, J. and Mukunthakumar, S. 1992. Micro-propagation of *Bauhinea variegata* and *Parkinsonia aculeata* from nodal explants of mature tree. *Pl. Cell Tissue Organ Cult.* **28**: 119-121.

Mathuri, I. and Chandra, N. 1983. Induced regeneration in stem explants of *Acacia nilotica. Curr. Sci.* **52**: 882-883.

Mayer, A.M. and Harel, E. 1979. Polyphenol oxidases in plants. *Phytochem.* **18**: 193-215.

McComb, J.M. and Bennet, I.S. 1982. Vegetative propagation of Eucalyptus using tissue culture and its application to forest improvement in Western Australia. *Plant Tissue Culture* (Ed. Fujiwara, A.). Jap. Ass. Pl. Tissue. Cult., Tokyo. p. 721-722.

Messeguer, J. and Mele, E. 1987. *In vitro* propagation of adult material and seedlings of *Corylus avesllana. Acta Horticulturae* **212**: 499-501.

Mittal, A., Agarwala, R. and Gupta, S.C. 1989. *In vitro* development of plant cells from axillary buds of *Acacia auriculiformis* : a leguminous tree. *Pl. Cell Tissue Organ Cult.* **19**: 65-70.

Montoro, P., Etienne, H. and Carron, M.P. 1995. Effect of calcium on callus friability and somatic embryogenesis in *Hevea brasiliensis* Mull. Arg. : relations with callus mineral nutrition, nitrogen metabolism and water parameters. *J. Exp. Bot.* **46**(283): 255-261.

More, T.C. 1979. *Biochemistry and Physiology of Plant Hormones.* Springer Verlag, New York.p.1-29.

Murashige, T. 1974. Plant propagation by tissue culture. *Ann. Rev. Pl. Physiol.* **25**: 135-166.

Murashige, T. 1977. Clonal propagation of horticultural crops through tissue culture. *Plant Tissue Culture and its Biotechnological Applications.* (Eds. Barz, W., Reinhard, E. and Zenk, M.H.). Springer Verlag, New York. p. 392-403.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Pl.* **15**: 473-497.

Nadgir, A.L., Phadke, C.H., Gupta, P.K., Parshamani, V.A. and Mascarenhas, A.F. 1984. Rapid multiplication of bamboo by tissue culture. *Silvae Genetica.* **33**: 216-223.

Nair, K.K.N. 1986. *Preservation of Dalbergia* Lf *in Kerala by Establishment of a Germplasm Bank.* Kerala For. Res. Inst., Peechi, India. p. 17-20.

Nandwani, D. and Ramawat, K.G. 1991. Callus culture and plantlets formation from nodal explants of *Prosopis juliflora. Indian J. Exp. Biol.* **29**: 523-527.

Nandwani, D. and Ramawat, K.G. 1992. High frequency plantlets regeneration from seedling explants of *Prosopis tamarugo. Pl. Cell Tissue Organ Cult.* **29**: 173-178.

*Nitsch, J.P. 1951. Growth and development *in vitro* of excised ovules. *Am. J. Bot.* **38**: 566-577.

Nitsch, J.P. and Nitsch, G. 1969. Haploid plants from pollen grains. *Science.* **163**: 85-87.

Norton, M.E. and Norton, C.R. 1986. *In vitro* shoot proliferation of *Prunus* and *Spirea* in relation to explant type. *Pl. Propagator.* 32(3): 5.

Parfitt, D.E. and Almehdi, A.A. 1994. Use of high CO_2 atmosphere and medium modification for the successful micropropagation of pistachio. *Sci. Hortic.* **56**(4): 321-329.

Patri, S., Bhatnagar, S.P. and Bhojwani, S.S. 1988. Preliminary investigations on the micropropagation of a leguminous timber tree: *Pterocarpus santalinus*. *Phytomorph.* **38** (1): 41-45.

Paul, H., Belaizi, M. and Sangwan - Norreel, B.S. 1994. Somatic embryogenesis in apple - propagation from cotyledon culture. *J. Pl. Physiol.* **143**(1): 78-86.

Perez-parron, M.A., Gonzalez-benito, M.E. and Perez, C. 1994. Micropropagation of *Fraxinus angustifolia* from mature and juvenile plant material. *Pl. Cell Tissue Organ Cult.* **37**(3): 297-302.

Pierik, R.L.M. 1989. *In Vitro Culture of Higher Plants.* Martinus Nijhoff, Boston. pp. 344.

Pollard, J.K., Shantz, E.M. and Steward, F.C. 1965. Hexitols in coconut milk: their role in nature of dividing cells. *Pl. Physiol.* **30**: 492.

Prutpongse, P. and Gavinlertvatana, P. 1992. *In vitro* micropropagation of 54 species from 15 genera of bamboo. *Hortscience.* **27**(5): 453-454.

Pua, E.C. and Chong, C. 1984. Requirement for sorbitol (D-glucitol) as carbon source for *in vitro* propagation of *Malus robusta*. *Can. J. Bot.* **62**(5): 1545-1549.

*Quoirin, M., Lepoivre, P. and Boxus, P. 1977. Un premier bilan de 10 annees de recherches sur les cultures de meristemes et la multiplication *"in vitro"* de fruitiers ligneux. C.R. Rech. 1976-1977 et Rapports de Synthese. *Stat. Cult. Fruit. et Marich. Gembloux.* p.93-117.

Rai, R.V. and Chandra, K.S.J. 1989. Micro-propagation of Indian rosewood by tissue culture. *Ann. Bot.* **64**: 43-46.

Rajmohan, K. 1985. Standardisation of Tissue/Meristem Culture Techniques in Important Horticultural Crops. Ph.D. thesis. Kerala Agric. Univ., Vellanikkara, Thrissur.

Rajmohan, K. and Kumaran, M.N. 1988. Influence of explant source on the *in vitro* propagation of jack (*Artocarpus heterophyllus* Lam). *Agric. Res. J. Kerala* **26**(2): 169-174.

Ramesh, K. and Padhya, M.A. 1990. *In vitro* propagation of neem, *Azadirachta indica* (A. Juss), from leaf discs. *Indian J. Exp. Biol.* **28**(10): 932-935.

Rao, K.S. 1986. Plantlets from the somatic callus tissues of the east Indian red wood (*Dalbergia lalifolia* Roxb). *Pl. Cell Rep.* **3**: 199-201.

*Rao, V.R. and Rao, I.U. 1988. Tissue culture approaches to the mass propagation and genetic improvement of bamboos. Proceedings of the International Bamboo Workshop.p.151-158.

Razdan, M.K. 1993. *An Introduction to plant Tissue Culture.* Oxford and IBH Publishing Co., New Delhi. p. 276-283.

*Reinert, J. and White, P.R. 1956. The cultivation *in vitro* of tumor tissues and normal tissues of *Picea glauca. Physiol. Plantarum.* **9**: 177-189.

Roberts, D.R., Webster, F.B., Grossnickle, S.C. and Sutton, B.C.S. 1992. Application of somatic embryogenesis to clonal propagation of spruce. *Biotechnol. Agric.* p.1.

Rodriguez, R., Diaz-sala, C. and Ancora, G. 1988. Sequential cultures of explants taken from adult *Corylus avellana* L. *Acta Horticulturae* **227**:45-49.

Rohman, S.M., Hossain, M., Biswas, B.K., Joarder, O.I. and Islam, R. 1993. Micropropagation of *Caesalpinia pulcherrima* through nodal bud culture of mature tree. *Pl. Cell Tissue Organ Cult.* **32**(3): 363-365.

Rout, G.R. and Das, P. 1994. Somatic embryogenesis and *in vitro* flowering of three species of bamboo. *Pl. Cell. Rep.* **13**(12): 683-686.

Rucker, W. 1982. Callus and organ formation of *Digitalis* leaf cuttings. *Plant Tissue Culture.* (Ed. Fujiwara, A.). Jap. Ass. Pl. Tissue Cult., Tokyo. p. 195-196.

Ruredzo, T.J. and Hanson, J. 1993. Plant recovery from seedling-derived shoot tips of *Faidherbia albida* growing *in vitro*. *Agroforestry Systems.* **22**(1): 59-65.

Sankhla, D., Davis, T.D. and Sankhla, N. 1993. Effect of gibberellin biosynthesis inhibitors on shoot regeneration from hypocotyl explants of *Albizzia julibrissin*. *Pl.Cell Rep.***13**(2): 115-118.

Sankhla, D., Davis, T.D. and Sankhla, N. 1994. Thidiazuron induced *in vitro* shoot fromation from roots of intact seedlings of *Albizia julibrissin*. *Pl. Growth Regul.* **14**(3): 267-272.

Santhoshkumar, A.V. 1993. *In vitro* Propagation of Bijasal (*Pterocarpus marsupium* Roxb.) through Tissue Culture. M.Sc. Thesis. Kerala Agric. Univ., Vellanikkara, Thrissur. pp. 101.

Schackel, K.A., Novello, O. and Sutter, E.G. 1990. Stomatal function and cuticular conductance in whole feed apple. *J. Am. Soc. Hort. Sci.* **115**: 468-472.

Schenk, R.V. and Hildebrandt, A.C. 1972. Medium and Techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* **50**: 199-204.

Schwarz, O.J., Schlarbaum, S.E. and Beetry, R.M. 1988. Plantlet regeneration from mature zygotic embryos of Eastern White Pine (*Pinus strobus* L). *Pl. Cell Rep.* **7**: 174-177.

Scott, E.S., Rao, A.N. and Loh, C.S. 1988. Production of plantlets of *Shorea roxburghii* G. Don. from embryogenic callus cultured *in vitro*. *Ann. Bot.* **61**(2): 233-236.

Scott, E.S., Rao, A.N. and Loh, C.S. 1990. Tissue culture of Dipterocarpus. Bull. Nat. Univ. Singapore. **20**: 175-179.

Sen,S., Magallanes-cedeno, M.E. and Kamps, R.H. 1994. *In vitro* micropropagation of Afghan pine for Christmas tree production. *Can. J. For. Res.* **24**(6): 1248-1252.

Shankar, S. and Ram, H.Y.M. 1990. Plantlet regeneration from tissue cultures of *Sesbania grandiflora*. *Curr. Sci.* **59**: 39-43.

Shields, R., Robinson, S.J. and Anslow, P.A. 1984. Use of fungicides in plant tissue culture. *Pl. Cell. Rep.* **3**: 33-36.

Skirvin, R.M. 1980. Fruit crops. *Cloning Agricultural Plants via in vitro Technique.* (Ed. Conger, B.V.). C.R.S. Press, Boca, Raton, Florida. p. 51-139.

Skirvin, R.M. and Chu, M.C. 1979. *In vitro* propagation of 'Forever Yours' rose. *Hortscience.* **14**: 608-610.

*Skoog, F. and Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tissue cultured *in vitro*. *Symp. Soc. Exp. Biol.* **11**: 118-131.

*Skoog, F. and Tsui, C. 1948. Chemical control of growth and formation in tobacco stem segments and callus cultured *in vitro*. *Ann. Bot.* **35**: 782.

Simpkins, I., Collins, H.A. and Street, H.E. 1970. The growth of *Acer pseudoplatanus* L. cells grown in suspension cultures. *Physiol. Pl.* **23**: 385-396.

Singha, S. 1980. *In vitro* propagation of 'Seckel' pear. *Proceedings of the Conference on Nursery Production of Fruit Plants through Tissue Culture - Applications and Feasibility.* Aagric. Res. Sci. Educ. Adm., U.S.D.A., Beltsville. p. 59-68.

Sinha, R.K. and Mallick, R. 1991. Plantlets from somatic callus tissue of the woody legume *Sesbania bispinosa*. *Pl. Cell Rep.* **10**: 247-250.

Sinha, R.K. and Mallick, R. 1993. Regeneration and multiplication of shoots in *Albizzia falcataria*. *Pl. Cell. Tissue Organ Cult.* **23**(2): 259-261.

Sita, G.L., Chattopadhyay, S. and Tejavathi, D.H. 1986. Plant regeneration from shoot callus of rosewood. *Pl. Cell Rep.* **5**: 266-268.

Sita, G.L., Ram, R.N.V. and Vaidyanathan, C.S. 1980. Triploid plants from endosperm cultures of sandalwood by experimental embryogenesis. *Pl. Sci Lett.* **20**: 63-69.

Sita, G.L., Sreenath, K.S. and Sujata, S. 1992. Plantlet production from shoot tip cultures of red sandalwood (*Pterocarpus santalinus* L.). *Curr. Sci.* **62**(7): 532-535.

Sita, G.L. and Swamy, B.V.R. 1992. Application of cell and tissue culture technology for mass propagation of elite trees with special reference to rosewood (*Dalbergia lalifolia* Roxb.). *Indian For.* **118**(1): 36-47.

Sita, G.L. and Swamy, B.V.R. 1993. Regeneration of plantlets from leaf disc-cultures of rosewood; control of leaf abscission and shoot tip necrosis. *Pl. Sci.* **88**(1): 107-112.

Smith, D.R. 1986. *Radata pine (Pinus radiata). Biotechnology in Agriculture and Forestry. Vol. I.* (Ed. Bajaj. Y.P.S.). Springer Verlag, New York. p. 275-290.

Snedecor, G.W. and Cochran, W.G. 1967. *Statistical methods* (6th ed.). Oxford and IBH Publishing Co., Calcutta, pp. 593.

Sohal, H.S. and Srivastava, A.K. 1994. *Environment and Biotechnology.* Ashish Publishing House, New Delhi. p.181-198.

Son, S.H. and Hall, R.B. 1990. Multiple shoot regeneration from root organ cultures of *Populus alba* x *P. grandidentada. Pl. Cell Tissue Organ Cult.* **22**: 53-57.

Straus, J. and Rodney, R.E. 1960. Response of *Cupressus funebris* tissue culture to gibberellins. *Science.* **131**: 1036-1087.

Sul, I.W. and Korban, S.S. 1994. Effect of different cytokinins on axillary shoot proliferation and elongation of several genotypes of *Sequoia sempervirens. In Vitro Pl.* **30**(3): 131-135.

Sutter, E.G., Fabbri, A. and Dunston, S. 1985. Morphological adaptation of leaves of strawberry plants grown *in vitro* after removal from culture. *Tissue Culture in Forestry and Agriculture. (*Eds. Hanke, R.R., Hughes, K.W., constantin, M.J. and Hollaender, A.). Plenum Press, New York. p.358-359.

Tabrett, A.M. and Hammatt, N. 1992. Regeneration of shoots from embryo hypocotyls of common ash (*Fraxinus excelsior). Pl. Cell Rep.* **11**: 514-518.

Tewary, P.K. and Rao, S.G. 1990. Multiple shoot formation through shoot apex culture of mulberry. *Indian J. For.* **13**(2): 109-111.

Thimman, K.U. 1977. *Hormone Action in the Whole Life of Plant.* Univ. Massachusetts Press. Amherst. p.12-26.

Thorpe, T.E. 1980. *Frontiers of Plant Tissue Culture.* Univ. Calgary Press, Canada. p. 49-58.

Tisserat, B. 1979. Propagation of date palm (*Phoenix dactylifera* L.) *in vitro. J. Exp. Bot.* **30**: 1275-1283.

Troup, R.S. 1921. *The Silviculture of Indian Trees. Vol.I.* International Book Distributors, Dehra Dun. p.244-331.

Vacin, E. and Went, F. 1949. Use of tomato juice in the asymbiotic germination of orchid seeds. *Bot.Gaz.* **110**:605-613.

*Vasil, I.K. and Vasil, V. 1980. Clonal propagation. *Int. Rev. Cytol. Suppl.* **11**(A): 145-173.

Vaughn, K.C. and Duke, S.O. 1984. Function of polyphenol oxidase in higher plants. *Physiologia Plantarum* **60**: 106-112.

Vieitez, A.M., Ferro, E.M. and Ballester, A. 1993. Micropropagation of *Fagus sylvatica* L. *In Vitro Pl.* **29**(4): 183-188.

Vieitez, A.M. and Vieitez, E. 1980. Plantlet formation from embryonic tissue of chestnut grown *in vitro. Physiol. Pl.* **50**: 127-130.

Vijayakumar, N.K., Ferret, P.P. and Sharik, T.L. 1990. *In vitro* propagation of the endanged Virginia round leaf birch (*Betula uber* (*Ashe*) *Fervi.*) using dormant buds. *For. Sci.* **36**(3): 842-846.

Wang, S.J. and Huang, L.C. 1976. Beneficial effects of activated charcoal on plant tissue and organ cultures. *In vitro.* **12**: 260.

Weaatherhead, M.A., Burdon, J. and Henshaw, G.G. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. *Z. Pflanzen Physiol.* **89**: 141.

Welander, M., Welander, N.T. and Brackman, A.S. 1989. Regulation of *in vitro* shoot multiplication in *Syringa, Alnus* and *Malus* by different carbon sources.*J. Hort. Sci.***64**:361-366.

*Went, F.W. 1926. On growth accelerating substances in the coleoptiles of *Avena sativa* . *Proc. Kon. Acad.* Wentensch Amst. **30**: 10-19.

*White, P.R. 1943. Nutrient deficiency studies and an improved inorganic nutrient for cultivation of excised tomato roots. *Growth.* **7**: 53-65.

Wickremesinhe, E.R. and Arteca, R.N. 1993. Establish-ment of fast-growing callus and root cultures of *Cephalotaxus harringtonia. Pl. Cell Rep.* **12**(2): 80-83.

Wilson, Z.A. and Power, J.B. 1989. Elimination of systemic contamination in explant and protoplast cultures of rubber (*Hevea brasiliensis* mull. Arg.). *Pl.Cell Rep.***7**(8): 622-625.

Yadav, V., Lal, M. and Sjaiswl, V.S. 1990. *In vitro* micropropagation of the tropical fruit tree *Syzygium cumini. Pl. Cell Tissue Organ Cult.* **22**: 87-90.

Yasseen, M.Y. 1994. Shoot proliferation and plant formation from neem (*Azadirachta indica* Juss.) with thidiazuron. *Hortscience.* **29**(5): 515.

Yeoman, M.M. 1986. *Plant Cell Culture Technology.* Blackwell Scientific Publication, Melbourne. pp.33.

Yu, Y.B. 1991. Study on some factors in tissue culture of lychee (*Litchi chinensis*). *Fujian agric. Sci. Tech.* (5): 17-18.

Zimmerman, R.H. and Broome, O.C. 1981. Phloroglucinol and *in vitro* rooting of apple cultivar cuttings. *J. Am. Soc. Hortic. Sci.* **106**: 648-652.

Ziv, M. 1986. *In vitro* hardening and acclimatization of tissue culture plants. *Plant Tissue Culture and its Agricultural Application.* (Eds. Winther, L.A. and Alderson, P.G.). Butterworths, London. p. 187-196.

* Original not seen

Plates

Plate 1 Selected plus tree of rosewood (TR-1)

Plate 2 Selected plus tree of rosewood (TR-2)

Plate 3 Selected plus tree of rosewood (TR-3)

Plate 4 Abnormal leaf development from explants without shoot proliferation due to the fungicidal spray given daily to the young rosewood trees.
Medium: WPM+Kin 2.0+IAA 0.1 mg l^{-1}

Plate 5 Bud expansion and further morphogenesis in nodal explants from young trees of rosewood.
Medium: WPM+Kin 1.0+IAA 0.1 mg l^{-1}

Plate 6 Multiple shoot formation in nodal explants from young trees of rosewood.
Medium: MS+BA 2.0 mg l^{-1}

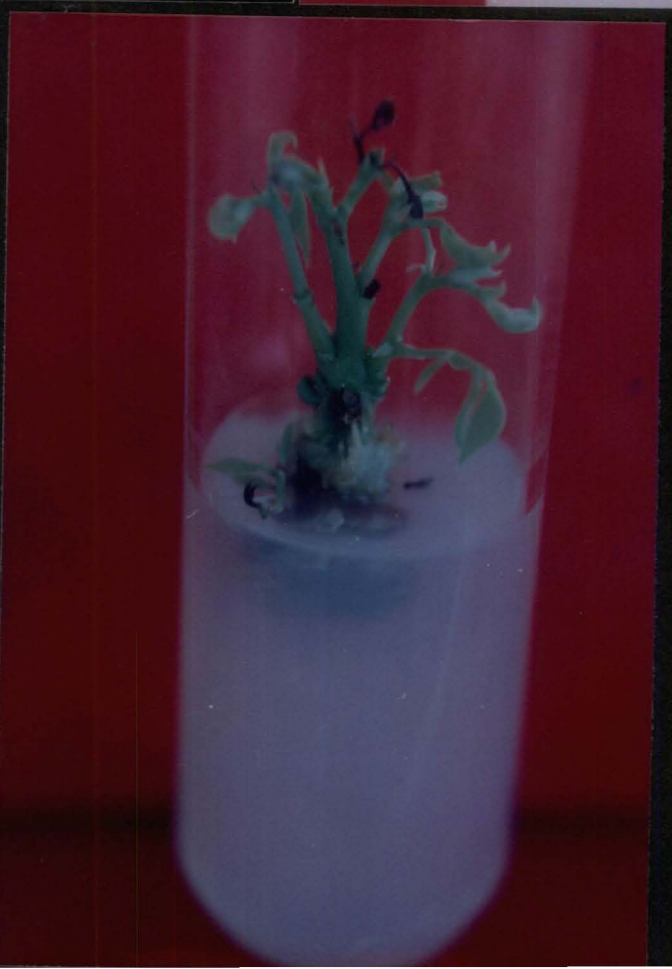


Plate 7 *In vitro* rooting of shoots of young rosewood trees in ½ MS medium supplemented with AC one per cent after pulse treatment with IBA 1000 mg l^{-1}

Plate 8 Direct rooting of primary cultures of young trees of rosewood.
Medium: WPM+Kin 1.0+IAA 0.1 mg l^{-1}

Plate 9 Direct rooting of primary cultures of young trees rosewood.
Medium: WPM+Kin 2.0+IAA 0.1 mg l^{-1}

Plate 10 Acclimatization of the plantlet regenerated from nodal explants of young rosewood trees.

Plate 11 *In vitro* produced plantlet, regenerated from nodal explants of young rosewood tree, ready for planting out

Plate 12 A dead culture from the plus tree of rosewood due to polyphenol interference.
Medium: MS+BA 2.0 mg l^{-1}

Plate 13 Nodal explants from the plus tree of rosewood remaining without response in MS basal medium.
Culture period: Four weeks

Plate 14 Nodal explants from the plus tree of rosewood remaining without response in basal WPM.
Culture Period: Four weeks

Plate 15 Nodal explants from the rosewood plus tree showing bud initiation in MS medium containing BA and IAA at 2.0 and 0.2 mg l^{-1}, respectively.

Plate 16 Nodal explants from the plus tree of rosewood showing bud expansion.
Medium: MS+BA 2.0+IAA 0.1 mg l^{-1}

Plate 17 Nodal explants from the plus tree of rosewood showing shoot proliferation.
Medium: MS+BA 2.0+IAA 0.1 mg l^{-1}

Plate 18 Shoot formation in nodal explants from the plus tree of rosewood without multiple shoots or leaf expansion (Leaf initials are seen).
Medium: MS+BA 2.0 mg l^{-1}

Plate 19 Dark brown and compact callus formed at the base of the nodal explant from the plus tree of rosewood. Medium: MS+BA 5.0 mg l^{-1}

Plate 20 Shoot formation in nodal explants from the plus tree of rosewood without leaf expansion. Medium: WPM+BA 2.0+IAA 0.1 mg l^{-1}

Plate 21 Shoot proliferation in nodal explant from the plus tree of rosewood without leaf formation.
Medium: MS+2-ip 1.0 mg l^{-1}

Plate 22 Light brown and compact callus formed at the base of the nodal explant from the plus tree of rosewood.
Medium:MS+Kin 1.0+IAA 0.1+CCC 1.0 mg l^{-1}

Plate 23 Precocious fall of leaf initials formed in nodal explant from the plus tree of rosewood.
Medium: WPM+BA 2.0+IAA 0.1 mg l^{-1}

Plate 24 Precocious fall of leaf initials formed in nodal explant from the plus tree of rosewood.
Medium: MS+BA 2.0 mg l^{-1}

Plate 25 Effect of L-glutamine in the retention of leaf initials formed in the nodal explant from the plus tree of rosewood.
Medium:MS+BA 2.0 L-glutamine 1500 mg l^{-1}

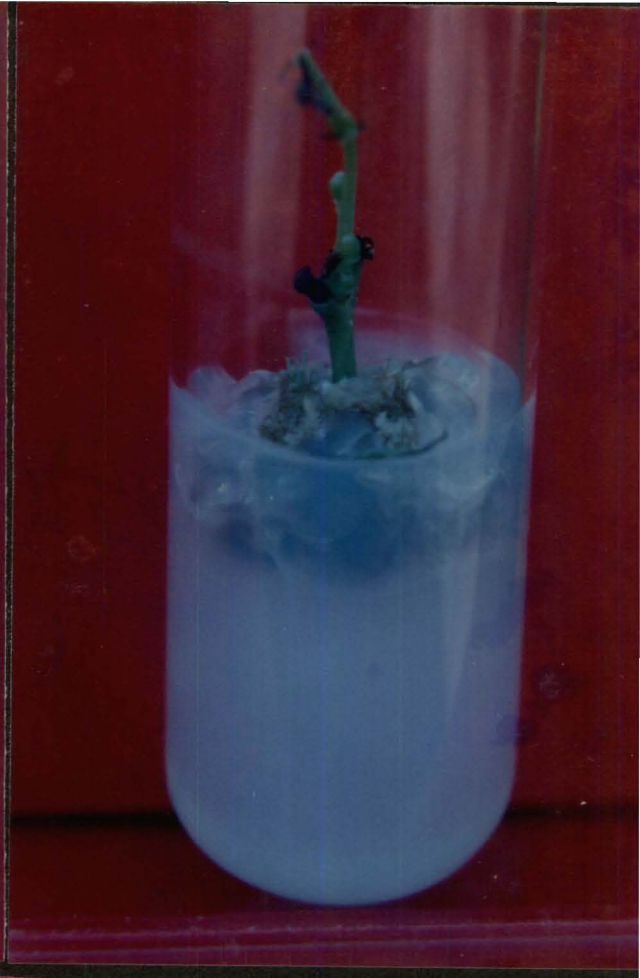


Plate 26 Shoot development in nodal explant from the root sucker of the plus tree of rosewood without leaf expansion.
Medium: MS+BA 1.0+Kin 1.0 mg l^{-1}

Plate 27 Callus induction in internodal explant from the root sucker of the plus tree of rosewood.
Medium: MS + BA 0.5 + NAA 1.0+2,4-D 3.0 mg l^{-1} + CW 10%

Plate 28 Sub culturing of the white compact callus formed in the medium MS+BA 0.5+NAA 1.0+2,4-D 3.0 mg l^{-1} + CW 10%

Plate 29 Change in colour of the callus from white to light brown after a period of two weeks of incubation. Medium: MS + BA 0.5 + NAA 1.0+2,4-D 3.0 mg l^{-1} + CW 10%

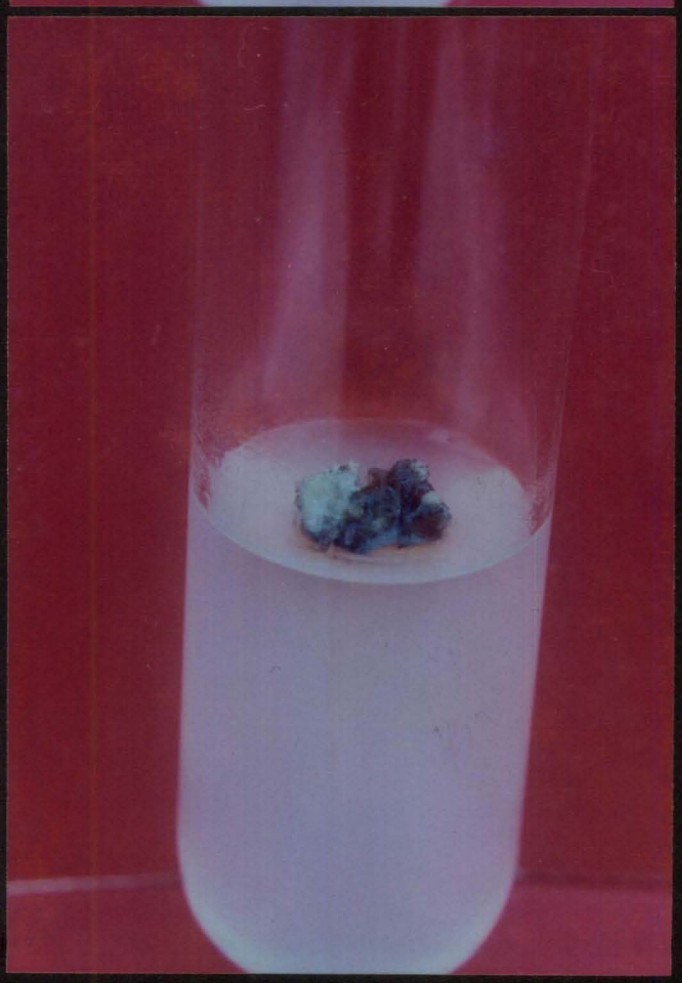
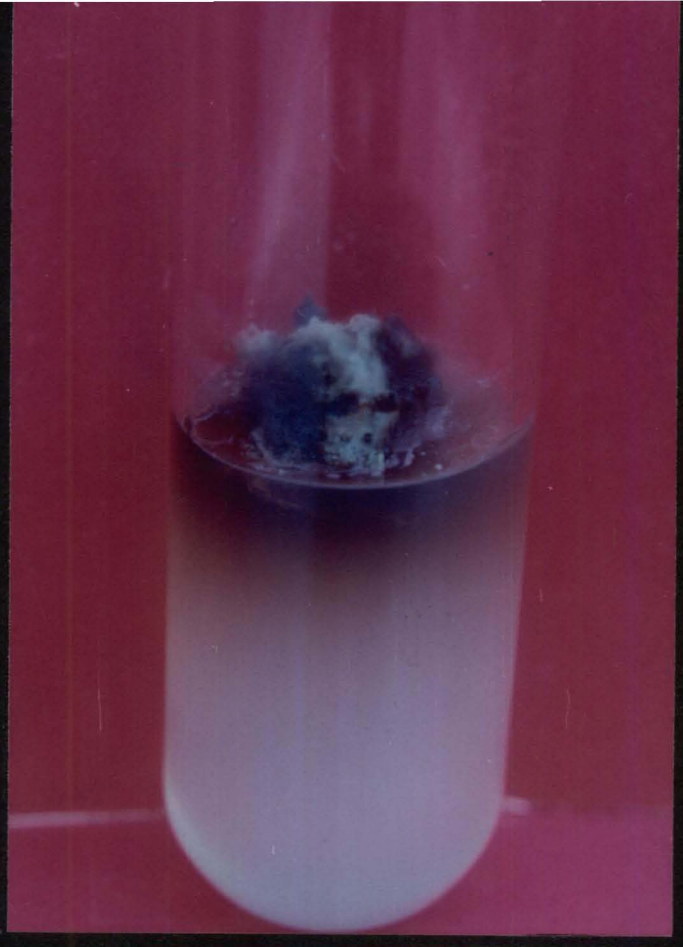


Plate 30 Change in colour of the callus from white to dark brown after a period of four weeks of incubation.
Medium: MS + BA 0.5 + NAA 1.0+2,4-D 3.0 mg l^{-1} + CW 10%

Plate 31 Shoot regeneration from callus cultures of root suckers of the rosewood plus tree after five weeks culture.
Medium: MS + BA 0.5 + NAA 1.0+2,4-D 3.0 mg l^{-1} + CW 10%



Annexure

ANNEXURE I

ABSTRACT OF ANALYSIS OF VARIANCE

1. Experiment with young trees*

	Treatment mean square	Error mean square	F-value
Degrees of freedom	7	24	
Characters:			
a. Time taken for bud initiation	1.622	0.054	29.947
b. Time taken for bud expansion	6.044	0.028	218.140
c. Percentage of bud initiation	970.908	2.038	476.519
d. Percentage of bud expansion and shoot proliferation	1379.114	2.788	494.749
e. Mean number of shoots	3.429	0.167	20.571
f. Mean shoot length	14.196	0.438	32.449
g. Mean number of leaves	3.411	0.375	9.095

Contd.....

2. Experiments with plus trees*

2.1. Difference in culture response between the selected plus trees

	Treatment mean square	Error mean square	F-value
Degrees of freedom	2	9	
Characters:			
a. Percentage of bud initiation, bud expansion and shoot proliferation	290.973	3.193	91.119
b. Mean number of shoots	4.333	0.167	26.000

2.2. Effect of various media combinations

2.2.1. BA and IAA in MS medium:

Degrees of freedom	5	18	
Characters			
Percentage of bud initiation, bud expansion and shoot proliferation	605.495	3.370	179.672

2.2.2. Kinetin and IAA in WPM:

Degrees of freedom	4	15	
Characters:			
a. Time taken for bud initiation	45.200	0.800	56.500
b. Percentage of bud initiation	587.708	6.533	89.955

Contd.....

	Treatment mean square	Error mean square	F-value
2.2.3. 2-ip and IAA in MS medium:			
Degrees of freedom	3	12	
Character:			
Percentage of bud initiation	499.317	7.448	67.041
2.2.4. 2-ip and IAA in WPM:			
Degrees of freedom	4	15	
Character:			
Percentage of bud initiation	569.135	4.234	134.436
2.2.5. Effect of CCC:			
Degrees of freedom	9	30	
Character:			
Percentage of bud initiation	561.298	3.873	144.913

* All the treatments were found significantly different at the level of significance 0.0001

CLONAL PROPAGATION OF SELECTED PLUS TREES OF INDIAN ROSEWOOD (*DALBERGIA LATIFOLIA* ROXB.) THROUGH TISSUE CULTURE

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ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Science in Forestry

KERALA AGRICULTURAL UNIVERSITY

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ABSTRACT

An investigation was carried out at College of Forestry, Vellanikkara during 1993-1995 on micropropagation of selected plus trees of Indian rosewood (*Dalbergia latifolia* Roxb.) through tissue culture. Nodal or internodal explants from the three selected plus trees as well as root suckers of one among them were used for the study. Explants from young (eight to ten year old) rosewood trees were also taken, inorder to know the difference in culture response if any, between adult and juvenile plant materials.

Prophylactic spraying with the mixture of Bavistin and Indofil M-45 given to young trees as well as root suckers or immersing the explants from the plus trees for one hour in the same fungicidal mixture coupled with surface sterilization of explants with 0.1 per cent mercuric chloride for 12 minutes could control culture contamination very effectively. Phenol problem was nil for young trees whereas treatments with ascorbic acid, citric acid and polyvinylpyrrolidone were essential to check browning of the cultures of explants from plus trees as well as root suckers. Woody plant medium supplemented with 1.0 mg l^{-1} kinetin alongwith 0.1 mg l^{-1} IAA was the best combination for young trees whereas addition of 2.0 mg l^{-1} BA to MS medium proved to be better than the others, in case of plus trees.

While *in vitro* propagation using explants from young trees of rosewood could be achieved and plantlets could be regenerated with cent per cent repeatability, micropropagation of the selected plus trees of rosewood faced with many obstacles. Though bud break and shoot morphogenesis was noticed in some of the media combinations tried with BA, kinetin and 2-ip alone or along with IAA or NAA, none of them could improve the problem of lack of multiple shoot formation or leaf expansion. Adenine sulphate, casein hydrolysate, cycocel, phloroglucinol, coconut water and activated charcoal were found to have no significant beneficial effect on culture of plus trees of rosewood. However, L-glutamine added to the medium at higher concentrations was found preventing precocious drop of leaf initials.

In vitro rooting was achieved by resorting to a pulse treatment of the shoots with IBA (1000 mg l^{-1}) and culturing them in half strength MS medium containing 1.0 per cent activated charcoal and hardening and planting out of the plantlets was also carried out for young trees. Rooting trial was a failure with plus trees as well as root suckers. Callus cultures were successfully initiated from internodal segments from root suckers. Shoot regeneration was noticed in 14.3 per cent of these cultures. However, leaf expansion or multiple shoot formation could not be obtained. The study clearly portrays the difference in culture response between juvenile and adult explants from *Dalbergia latifolia*.