

***In vitro* PROPAGATION OF
Acacia auriculiformis
AND ITS HYBRID**

K.PADMA



Thesis submitted to the
University of Agricultural Sciences, Bangalore
in partial fulfillment of the requirements for the

**MASTER OF SCIENCE
in
CROP PHYSIOLOGY**

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This is to certify that the thesis entitled "*IN VITRO* PROPAGATION OF *ACACIA AURICULIFORMIS* AND ITS HYBRID" submitted by **Miss.K.Padma**, for the degree of **MASTER OF SCIENCE** in **CROP PHYSIOLOGY** of the University of Agricultural Sciences, Bangalore is a record of research work done by her during the period of her study in this University under my guidance and supervision, and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or other similar titles.

Bangalore
October , 1995


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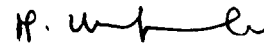
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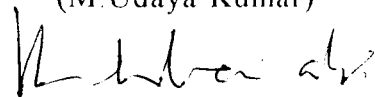

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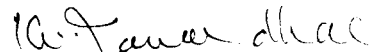
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*Bangalore
October , 1995*

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LIST OF ABBREVIATIONS

1. A = Absorbance
2. C = Celsius
3. Cft = Cubic feet
4. Chl.a= Chlorophyll a
5. Chl.b= Chlorophyll b
6. cm = Centimetres
7. DAI = Days after inoculation
8. DAT = Days after treatment
9. DDW= Double distilled water
10. g = Gram
11. gl^{-1} = Grams per litre
12. hrs = Hours
13. kg/cm^2 =Kilograms per square centimetre
14. M = Molar
15. mg = Milligrams
16. mgl^{-1} = Milligrams per litre
17. mM = Milli molar
18. mm = Milli metre
19. μm = Micro molar
20. μl = Micro litre
21. rpm = Rotations per minute
22. ppm = Parts per million
23. uv = Ultra violet
24. v = Volume
25. wt = Weight

LIST OF CHEMICALS AND REAGENTS USED

1. AC = Activated charcoal
2. AdSO₄ = Adenine sulphate
3. B₅ = Gamborg's B₅ medium
4. BA/BAP = Benzyl adenine/Banzyl amino purine
5. BSA = Bovine Serum Albumin
6. CBB = Coomassie Brilliant Blue
7. CaCl₂.2H₂O = Calcium chloride
8. Ca(NO₃)₂ = Calcium nitrate
9. CoCl₂.6H₂O = Cobaltous chloride
10. CuSO₄.5H₂O = Copper sulphate
11. 2,4-D = 2,4-Dichloro Phenyl Acetic Acid
12. FeSO₄.7H₂O = Ferrous sulphate
13. GA₃/GA = Gibberellic acid
14. H₃BO₃ = Boric acid
15. IAA = Indole Acetic Acid
16. IBA = Indole Butyric acid
17. KH₂PO₄ = Potassium Dihydrogen Phosphate
18. Kin = Kinetin
19. KNO₃ = Potassium nitrate
20. K₂SO₄ = Potassium sulphate
21. MgSO₄.7H₂O = Magnesium sulphate
22. MnSO₄.5H₂O = Manganous sulphate
23. MS = Murashige and Skoog's medium
24. NAA = Naphthalene Acetic Acid
25. Na₂MoO₄.2H₂O = Sodium molybdate
26. (NH₄)₂SO₄ = Ammonium sulphate
27. NH₄NO₃ = Ammonium nitrate
28. Na₂EDTA = Disodium Ethyl diamine Tetra Acetic Acid
29. NP = Polyvinyl polypyrrolidone
30. PMSF = Phenyl methyl sulphonyl fluoride
31. WPM = Woody plant medium
32. ZnSO₄.7H₂O = Zinc sulphate.

INTRODUCTION

I. INTRODUCTION

Forest biomass in future will not only be the main source of fuel and timber but also a major raw material for chemical industry. Several afforestation programmes like enrichment of the planting programme in degraded forests, waste land utilization for forestry, social forestry programme are all aimed at increasing the forest areas.

The objectives of the future afforestation programmes should not be for merely increasing the area but also should aim at increasing the biomass returns from forest ecosystems per unit land area.

Therefore one of the major objectives is to identify elite clones with intrinsic high growth rates by virtue of better adaptation to existing environmental factors. Such a clone will have in addition to high crop growth, tolerance to location specific biotic and abiotic stresses.

Therefore identification of elite plants and mass multiplication is a major component of future afforestation programme. However, multiplication of elite plants through conventional methods can not meet the requirement of the planting programme. In the recent years, effort has been made for mass multiplication by *in vitro* techniques. This opens up a new approach to develop a required planting material.

Micropropagation has an important role in the rapid multiplication of elite genotypes of tree species. This is particularly true in areas where many

species have only recently been domesticated, high growth rates are possible and the demand for wood is very high.

Research at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Division of Forestry, has concentrated on developing commercial micropropagation techniques for the rapid multiplication of *Acacia*, *Eucalyptus*, *Melaleuca* and some other Australian species. Such techniques need to be tested in other countries to exploit the unique advantages of micropropagation over alternate techniques.

To meet the energy need of rural masses one of the recent approaches is to introduce social forestry and agroforestry programmes. One of the major prerequisites is to identify the fast growing clone with good quality characteristics and tolerance to biotic and abiotic stresses.

Acacia auriculiformis A. Cunn. ex Benth is a leguminous, nitrogen fixing tree of the subfamily Mimosoideae. It is better known outside its natural range of distribution as a most adaptable species for all kinds of tree planting programmes in tropical humid and subhumid lowland regions. It is cultivated as an exotic tree in Asia, Africa and South America for more than half a century and is being increasingly used for reforestation in new areas.

Acacia auriculiformis has been placed in a new genus called *Racosperma* (Pedley 1986). Uses of *Acacia auriculiformis* are given below.

Fuel wood : The primary use of *Acacia auriculiformis* appears to be the fuel wood. It makes very good charcoal because it glows well with little smoke and doesn't spark.

Wood : The wood of *Acacia auriculiformis* makes very attractive furniture. It is also suited for construction work like framing, flooring, wood turning and carving.

Pulp : Plantation grown trees have been found to be very promising for the production of unbleached kraft pulp for bags, wrapping paper, liner board and high quality material sulphite semic hemial pulp for corrugating medium and higher grade products.

Green manure : This is made from phyllodes. It is also mixed planted with non nitrogen fixing tree species to maintain or to improve soil fertility. Phyllodes are very good for soil mulching.

Other forest products : The bark has sufficient tannins for possible commercial exploitation. A natural dye, used in a batik textile industry in Indonesia, is also extracted from the bark. Lac insects were found on the *Acacia auriculiformis* in India, and thus Lac culture using the species as host plants is possible. An edible mushroom *Tylopilus felleus*, is common under the plantation of *Acacia auriculiformis* in Thailand.

Agroforestry : Intercropping with peanut rice and kenaf has proved to be successful. It is used as nurse tree for tea plantations.

Erosion control : Its root system is superficial, and densely matted, making the species suitable for stabilising eroded land. It has been used widely in revegetation and rehabilitation of degraded land and green land in Indonesia and India.

Aesthetic value : It is a popular ornamental tree and its bright yellow flower make it attractive. Its dense foliage which remains through out the dry seasons also makes it an excellent shade tree.

Recently, a natural hybrid of *Acacia auriculiformis* identified at Shimoga area, has assumes greater importance. This has been shown to have high growth rate, fine branching, tendency for strong apical dominance which will eventually lead to a tree with single stem and a good length of clear bole. Thus production and propagation of the hybrid will be of considerable importance. Therefore there is a need to develop a rapid multiplication technique for the potential clones. Micropropagation techniques comes in handy for the rapid multiplication of this clone, thus resulting in genetically stable plantlets. So the objectives of the present investigation are:

1. Selection of suitable explant source.
2. To standardise the media for multiple shoot production.
3. To standardise the media for the induction of rooting of these multiple shoots.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The most important advances in tissue culture in India have been in the clonal micropropagation of wide range of species. This work was based mostly on the use of meristem tissue and not of callus tissue which often resulted in genetic variation. Whenever vegetative propagation is economically possible, it is generally preferred to asexual propagation because genetic characteristics are better maintained (Murashige 1974). He listed three ways for *in vitro* propagation.

En masse : They are

- a) enhanced release of axillary buds
- b) production of adventitious shoots either directly on organ culture or in callus and
- c) somatic embryogenesis

In the first route, shoot tips or nodal segments with axillary buds are cultured on suitable nutrient media which assures genetic uniformity of the progeny to a great extent (Rao and Lee, 1986). Now-a-days a number of horticultural and forestry tree species are rapidly multiplied commercially using the technique.

In the second route, somatic organogenesis is induced either directly or through callus. This has an advantage of rapid multiplication rate. One important disadvantage of this technique is that the generated material often shows genetic variability. This method is not recommended for clonal propagation.

Somatic embryogenesis, the third route is limited to a few species. It is one of the rapid methods of cloning plants *in vitro* (Evans et al., 1981).

In the recent past, a number of new methods of propagation processes are being developed for specific species using liquid culture via somatic embryogenesis, organogenesis or axillary-shoot multiplication. The advantage of this recent technology is the possibility of producing a much large number of cloned plants without increasing the laboratory area and other facilities in the lab. Sondahl (1993), gave a rough estimate of the advantages of liquid culture. He estimated that an embryogenic cell suspension charged to a one litre bioreactor vessel at a density of one million cells per ml and capable of 0.1% embryo regulation would yield approximately one million somatic embryos. Assuming efficiency for germination (50%), plantlet development (75%) and hardening (75%), the expected total number of plantlets would be 2,80,000 per one tube vessel.

The next common method of vegetative propagation is rooting of cuttings. However, rooting of cuttings is far from universally applicable because in some species, cuttings do not root even after treatment with growth regulators. Under such situations, micropropagation by tissue culture is the best solution for mass multiplication. Tissue culture technique is now extended to micropropagation of forest trees, medicinal plants, plantation crops, vegetable and field crops, fruit trees, and ornamentals for multiplication of elite clones or for rapid increase of scarce planting material.

It is wellknown that tissue culture techniques are used for micropropagation in crop plants and also in annual herbaceous plants (George and Sherington, 1984). Literature on the aseptic propagation of plants, in general and crops in particular, from root, leaf, embryo, stem, shoot explants and pollen and somatic embryogenesis is extensive. However, work pertaining to micro or in vitro propagation of forest species are comparatively limited. The first report of organogenesis in the tree tissue

culture was by Gauthret (1939) in which adventitious buds from cambial cultures were obtained, while light promoted their growth to leafy shoots, IAA inhibited.

I. Explant Source

Different sources of explants are used in micropropagation. Among them, axillary buds, shoot apices and stem segments, nodal segments, hypocotyls and cotyledons are extensively used.

a) Axillary bud

It is reported that stem cuttings with 2-4 axillary buds from clones of *Paulownia tomentosa*, *P. elongata*, *P. fortunei* and *P. coreana* and when cultured produced shoots which after being elongated in proliferation media were shifted for rooting (Bruneau, 1987).

Gassama and Duhoux (1987) tried with the nodes between two thorns bearing an axillary bud from stool shoots and root suckers of mature *Acacia albida* trees. Since root suckers had very little lignification, best results were obtained with nodes from root suckers.

In vitro mass propagation of *Lagerstroemia indica* f. *alba pender* was also carried out with stem explants containing axillary buds and cultured on medium supplemented with various concentrations of BAP, NAA and IBA. Shoots were formed in all the buds and then shifted for rooting (Lee et al., 1987).

Youn et al., (1988) collected buds from matured trees of *Tilia chordata* and cultured on three different media, with different concentrations of BA. Best shoot development was observed in Woody Plant Medium.

Kim and Lee (1988) achieved micropropagation of *Jujuba* cultivars through axillary bud culture. Axillary buds of *Ziziphus cv. Geumsung* showed the best shoot and root growth after eight weeks.

In vitro development of plantlets from axillary buds of *Acacia auriculiformis* was achieved. Multiple shoots have developed from axillary buds excised from *in vitro* grown seedlings of *Acacia auriculiformis* (Aradhana et al., 1989).

b) Shoot apices and stem segments

It is reported that in Guava 3-6 shoots developed on explant collected from field grown plants and 5-10 shoots developed on explants developed via **in vitro** proliferated shoots within twelve weeks of culture. A prior transfer of shoot clumps to a medium containing a lower concentration of BA before the harvesting of cuttings for rooting allowed rapid extension growth and increased the number of usable shoots for culture (Mohammed et al., 1987).

Shoot tip explants of *Melia azadarch* var. *gigantea* were established in media with different hormones. Multiplication was observed in this cultured explant (Domecq, 1988).

Rajmohan *et al.*, (1988) reported the influence of explant source on the *in vitro* propagation of Jack. Shoot apices from different aged trees were collected (two or six month old, 5-10 and 30 years old) and cultured on establishment media where the shoots developed. Then the developed shoots were transferred to proliferation media and elongation media. All the explants responded but highest response was on shoot apices from two or six months old seedlings.

Micropropagation of valley Oak shoots was done by making use of stem segments 5 cms long from one year old seedlings. Twenty eight days after their culture on medium, a great proportion of segments had developed buds in one medium (BTM=Broad tree medium) and more segments on Gresshoff-Doy medium showed shoot growth (Johnson and Walker, 1990).

Micropropagation of *Ficus benjamina* clones was also initiated from shoot tips. The shoot formation and growth *in vitro* were followed during subculturing, whereafter the developed shoot clusters were rooted (Kristiansen, 1992).

Feng H. Huang *et al.*, (1994) have tried shoot tips of 5 mm long from three week old seedlings of *Acacia mearensii* germinated *in vitro*. These shoot tips were cultured on medium with different growth regulators at various concentrations. Multiple shoot were promoted in these explants.

c) Hypocotyls

Vegetative propagation of sandal wood plants was carried out using hypocotyl segments as the explants. Excised Hypocotyl segments from the seedlings of

Santalum album grown *in vitro* were cultured aseptically on various nutrient media. Regeneration of shoot buds on explant was observed on basal media supplemented with auxins like IAA, IBA, NAA or NOA (Rao and Bapat, 1977).

Tomar and Gupta (1988) used excised segments of one cm long hypocotyl from 10-12 day old seedlings of *Albizia amara*, *Albizia lucida* and *Albizia richardiana* (all leguminous trees) and cultured on a medium with different concentrations of growth regulators like IAA, IBA, NAA, 2,4-D, BA or Kinetin. Maximum shoots per explant were developed on medium with lower concentration of 2,4-D and higher concentration of BA.

In general, shoot apex or stem segments with axillary buds or lateral buds were used to induce multiple shoot production. Shoot apex in many species produced large number of multiple shoots. Many scientists were also successful in getting multiple shoots from lateral buds.

II. Growth Media

Different growth media were standardised for micropropagation. The media used and the concentrations of the important components in the media also differs from one species to another.

Gassama and Duhoux (1987) attempted micropropagation of adult trees of *Acacia albida* on three media viz. Lin and Staba, MS and MS media supplemented with K, Mg and PO_4 apart from growth regulators (0.1 mg/l⁻¹ NAA and 0-0.5 mg/l⁻¹ BA). Best results were obtained in MS media supplemented (with 0.5 mg/l⁻¹ BA and 0.01 mg/l⁻¹ NAA).

John F. Frett (1987) arrived at the conclusion that full or half MS salts were superior to 1/4 MS salts based on the number of shoots produced in *Salvia greggi*. Multiple shoot production was reported on MS media supplemented with BAP, Adenine sulphate and Sodium dihydrogen phosphate in the juvenile tissues of *in vitro* germinated seedlings of *Eucalyptus camaldulensis* (Kumar and Ayyappan, 1987).

Excised cotyledons from germinated seeds of *Dalbergia sissoo* were successfully responded on MS medium (with casein hydrolysate and BA) in the production of multiple shoots. (Bhandary, 1988).

Different explants (cotyledon, hypocotyl and root explant) from *Lacandula latifolia* and *L.stocches* sub. sp *stocchis* were tested for their morphogenetic response on MS basal medium and MS with different combinations of auxins and cytokinins. Differential morphogenetic responses were mainly observed on shoot bud differentiation in MS medium with auxins (Calvo and Segura, 1988).

Modified MS media with supplements (BA, IBA and GA3) was found suitable for shoot tip explants of *Melia azadarach* var. *gigantea*. Bud multiplication was favoured by transferring to the same medium but without auxin (IBA) (Domecq, 1988).

Rajmohan and Mohankumar (1988) standardised the culture medium and culture conditions for Jack (*Artocarpus heterophyllus*). Initially the explants from *in vitro* grown and field grown were cultured on an establishment medium which is MS with some supplements (MS + 1 mg^l⁻¹ + 1% AC) in darkness for four weeks with

repeated subculturing. The cultures were exposed to light for 2 weeks and again inoculated on a proliferation media which was again MS (with 0.2 NAA at different BA concentrations 5.0, 7.5, 10.0, 12.5, 20 or 40). Then the shoots were transferred to elongation medium i.e., MS (with 2.0 mg l⁻¹ BA + 0.2 mg l⁻¹ NAA + 500 mg l⁻¹ insoluble PVP) and later on transferred to rooting medium.

Rapid multiplication of axillary meristems and direct shoot development was achieved from the nodal explants of mature *Eucalyptus tereticornis* Sm. cultured on MS medium (with 5.3 µM NAA, 1.1 µM IAA and 4.4 µM BA). Subculturing in the same media with different concentrations of hormones (0.44-0.88 µM BA + 0.1 µM NAA) yielded large number of axillary microshoots (Rao, 1988).

Explants from juvenile tissues and matured tissues of *Corylus avellana* L. when cultured on solid MS medium with combinations of BA, IAA and GA₃, resulted in the production of single shoot or clusters of shoot (Rodriguez et al., 1988).

Development of axillary shoots was induced in explants (embryonic axes) of *Shorea roxburghii* G. Don. cultured on a modified MS medium containing BA at 5 mg l⁻¹ (Scott et al., 1988).

Tomar and Gupta (1988) attempted *in vitro* plant regeneration of leguminous trees (*Albizia* sp) On B₅ medium. Explants from *in vitro* grown seedlings of *Albizia amara*, *Albizia lucida*, and *A. richardiana* were cultured on B₅ basal medium with different concentrations of auxins and cytokinins. Maximum shoots were produced at an optimum combination of auxins and cytokinin in B₅ medium.

Multiple shoot production in *Acacia auriculiformis* in B₆ basal medium supplemented with some adjuvants (coconut milk 5-10%) and growth regulator (BA=10⁻⁶ M) (Aradhana et al., 1989).

Micropropagation techniques for explants of *Acacia mangium* which were aseptically germinated were standardised. MS medium supplemented with cytokinins (6 BAP + Kin) at different concentration levels was found to be best for higher shoot multiplication (Darus Haji Ahmad, 1989).

Multiple shoots were induced on explants from *in vitro* germinated seedlings of Indian Rose wood (*Dalbergia latifolia*). This was achieved on MS medium supplemented with cytokinins and auxins (Ravishankar et al., 1989).

In vitro propagation of *Datura insignis* was carried out by culturing the explants from seedlings which were aseptically germinated on Ms medium. This medium was incorporated with a combination of cytokinin and auxin or cytokinin alone. Even for elongation medium, same medium was used with different concentration levels of growth regulators. (Maria and Dorsanto, 1990).

An *in vitro* technique for micropropagation of the clove tree, *Syzygium aromaticum* was standardised for the multiple shoot production. Explants were tried in two different media, MS with half strength salts and **Gamborg's** media supplemented with a combination of cytokinin and auxin. The explants were tried in both the media indicating that there is no significant differences between the two media used (Mathew and Hariharan, 1990). For the plant regeneration of a tree legume, *Tamarindus indica* (L), maximum shoot bud differentiation occurred when

the adaxial surface of the entire cotyledon which was the explant source was in contact with MS medium with a cytokinin incorporated (5×10^{-6} M BAP) (Jaiwal and Gulati, 1991).

Two cold tolerant species (*Eucalyptus macarthurii*) and a cold tolerant hybrid (*E. macarthurii* x *E. grandis*) were propagated *in vitro* from explants collected from field grown seedlings and from clonal hedges. Shoot growth was initiated on modified MS medium and later on a modified MS medium with 0.2 mg l^{-1} BA and 10.0 mg l^{-1} NAA. The modified medium was most effective in promoting shoot proliferation (Le Roux *et al.*, 1991).

Hypocotyl explants of *Acacia auriculiformis* showed the greatest morphogenetic response when cultured on modified MS medium with Kinetin, BAP and NAA singly or in combination (Rao *et al.*, 1991).

Darus Haji Ahmed (1989) attempted micropropagation technique for the hybrid i.e., *Acacia mangium* x *A. auriculiformis*. The explants from *in vitro* grown seedlings were cultured in full strength MS medium with 3% sucrose and different concentrations of BAP. Subculturing in fresh MS medium resulted in shoot multiplication and elongation.

Explants from a 12 yr old mulberry tree, *Morus nigra* L. (Yadav *et al.*, 1991) were cultured on MS medium which was supplemented with BA and Kinetin. Shoot proliferation was maximum in this medium at BA 1.0 mg l^{-1} .

D'Silva and D'Souza (1992) reported multiple bud induction from the explants of cashew seedlings, *Anacardium occidentale*, cultured in MS medium. When MS medium was supplemented with higher sucrose (117 mM sucrose), maltose (14.6 mM) and BA (22.2 μ M), maximum number of buds were initiated. The buds were again subcultured in MS medium and were harvested after being elongated in the same medium.

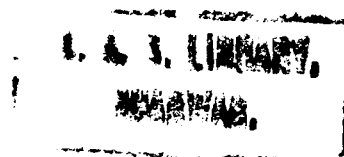
In vitro culture of eight *Ficus benjamina* clones were initiated from the explants when cultured in MS medium. Shoot formation and growth was obtained during subculture on MS medium with added NaFeEDTA, Thiamine HCl, Myoinositol and BA (Kristiansen, 1992).

Adenenthera pavonina L. explants were cultured *in vitro* in MS medium. Best shoot multiplication rate was obtained in this medium with BA and Kinetin (Bejoy and Hariharan, 1993).

Multiple shoots were successfully induced in the explants of *Chloroxylon switenia* (DC) (Satin Wood) on MS medium supplemented with various concentrations and combinations of auxins and Cytokinins (Tomar and Gupta, 1988).

Explants of *Acacia mearnsii* which were obtained from seedlings raised in hormone free MS Medium were cultured on 3/4 Strength MS medium with combinations of auxins and cyctokinins. This resulted in the production of multiple shoots (Feng H.Huang, 1994).

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A number of standardised culture media were used to induce multiple shoot production in tree species. Modified MS media was often used to induce multiple shoot production. However a few scientists attempted the use of B₅ medium, WPM, BTM and GD. In one reference with *Acacia auriculiformis*, B₅ medium was found effective in inducing multiple shoot production. However not much work is available on comparative effect of different median induction of multiple shoots.

III. Hormones

a) Shoot multiplication

Hormones play an important role initiating morphogenetic response. It is the proportion of hormones which counts for morphogenetic response.

It has been reported that for the *in vitro* mass propagation of *Lagerstroemia indica* f. *alba*, various concentrations of BAP, NAA and IBA were incorporated to the medium. A combination of 0.05 mg l⁻¹ of BA and 0.01 mg l⁻¹ NAA resulted in shoot formation on the buds cultured. Multiple shoot production was greatest with 2.0 mg l⁻¹ BAP and 0.01 mg l⁻¹ NAA (Lee et al., 1987).

Mohammed *et al.* (1987) reported that BA (4.5 μM) alone, without any auxin and gibberellin was found to give best shoot multiplication rate during the clonal propagation of Guava. Five to ten shoots developed on explants taken from *in vitro* proliferated shoots within 12 wks of culture. A prior transfer of shoot clumps to a low BAP medium (0.5 μM) before harvesting of cuttings allowed rapid extension growth and increased the number of usable shoots per culture.

Medium containing a combination of BA and NAA responded in multiple shoot production of *Paulownia* of 1-2 cms long and attained a length of 3-4 cms long when transferred to a hormone free medium and then used for rooting (Bruneau, 1988).

Young shoots of *Quercus suber* c. seedlings after being multiplied in Duryan medium, were separated and subcultured in the same medium with BAP at 2.25 mg l⁻¹. After three subcultures, at 25 days interval, young shoots were transferred to a low BAP (0.5 mg l⁻¹) medium for growth and transferred for rooting (Deidda et al., 1988).

Rajmohan and Mohankumaran (1988) reported *in vitro* propagation of Jack (*Artocarpus heterophyllus*). Explants from in vitro grown seedlings were cultured in an establishment medium which was incorporated with 1.0 mg l⁻¹ GA₃. Again the cultures were inoculated to a proliferation medium containing 0.2 mg l⁻¹ NAA, containing BA at 5.0, 7.5, 10.0, 12.5, 20.0, or 40.0 mg l⁻¹. Shoots from here were transferred to elongation medium containing 2.0 mg l⁻¹ BA + 0.2 mg l⁻¹ NAA and used for rooting.

Explants from matured *Eucalyptus tereticornis* Sm. when inoculated in medium with 5.3 μM NAA 1.1 μM IAA and 4.4 μM BA resulted in the rapid multiplication of axillary meristems and direct shoot development. Repeated subcultures of the second generation of shoots on media containing 0.44 - 0.88 μM BA + 0.1 μM NAA yielded large number of axillary microshoots. Then transferred for rooting (Rao, 1988).

Explants from *in vitro* grown seedlings of *Albizia amara*, *A. lucida* and *A. richardiana* (all leguminous trees) were cultured on medium with different concentrations of IAA, IBA and NAA, 2,4-D, BA or Kinetin. Maximum shoots were developed on medium with 0.1 μ M BA for *A. lucida* and *A. richardiana* (Tomar *et al.*, 1988).

Media containing 6-Benzyl amino purine at an optimum concentration of 5.0 mg l^{-1} resulted in the development of axillary shoots from the explants of *Shorea roxburghii* G. Don. Excised axillary shoots were used in multiplication (Scott *et al.*, 1988).

Multiple shoots were developed from the explants excised from *in vitro* grown seedlings of *Acacia auriculiformis* on media incorporated with coconut milk (5-10%) and BA 10^{-2} M. (Aradhana *et al.*, 1989).

Ravishankar *et al.*, (1989) have reported that multiple shoots were induced on excised explants of *in vitro* germinated seedlings of Indian Rose wood (*Dalbergia latifolia*) on medium supplemented with cytokinins and auxins.

Explants of *Datura insignis* were cultured on medium supplemented either with BA alone or in combination with 2,4-D or IAA. Best shoot multiplication and elongation was obtained in medium with 1.0 mg l^{-1} of BA among various growth regulator concentrations (Maria cristina and Dornanto, 1990).

Mathew *et al.*, (1990) reported best multiple shoot induction from explants of *Syzygium aromaticum* seedlings in two media supplemented with 3.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA among all other combinations, in both the media.

Darus Haji Ahmad (1991) have reported maximum number of shoots per explant for a hybrid of *Acacia auriculiformis* x *Acacia mangium* containing 0.5 mg l⁻¹ of BA among 0.5, 1.0, 2.0 and 4.0 mg l⁻¹ of BAP. Explants were excised from the developed shoots of the previous cultures and subcultured into fresh medium with same concentration of BAP. The process was carried out for seven passages of two months each. In the first culture, the average number of shoots per explant was 21.6 with an average length of 0.61 cm. In the seventh recycle culture, there was an average of 31.0 shoots per explant and an average shoot length of 1.3 cms. Shoots longer than 0.5 cms were transferred to rooting media.

MS medium containing 5 x 10⁻⁶ M. BA resulted in the maximum shoot bud differentiation (100%) when the adaxial surface of the cotyledons of a tree legume i.e., *Tamarindus indica* (L) was in contact with medium (Jaiwal and Gulati, 1991).

Explants from a cold tolerant species (*Eucalyptus macarthurii*) and a cold tolerant hybrid (*Eucalyptus macarthurii* x *E. grandis*) which were propagated *in vitro* responded better on the medium supplemented with 0.1 mg l⁻¹ BA and NAA 10.0 mg l⁻¹. The shoot growth promotion was most effective in promoting shoot proliferation (Le Roux *et al.*, 1991).

Axillary shoot proliferation was achieved in explants of leguminous trees, *Bauhinia variegata* and *Parkinsonia aciculata* (Jaideep *et al.*, 1992) in a medium supplemented with 2.2 - 31.1 μM of BA.

Multiple shoots were successfully induced from explants of *Chloroxylon switenia* BC. (Satin wood) in a medium supplemented with various concentrations and combinations of auxins and cytokinins. The highest number of shoots were obtained with in medium with 0.5 mg l^{-1} BAP and also 2.0 mg l^{-1} of NAA + BAP (Umer *et al.*, 1993).

Explants of *Acacia mearensii* cultured on medium with BAP at 2.0 mg l^{-1} and higher, combined with or without 0.01 mg l^{-1} IBA resulted in multiple shoot formation. Shoot multiplication was achieved in media with 2.0 mg l^{-1} BAP and 0.01 mg l^{-1} IBA. Higher levels of BAP promoted more multiple shoot formation and shoot elongation (Feng *et al.*, 1994).

As shown by number of earlier works, cytokinins were highly effective in inducing multiple shoot production in tree species. In general, the medium supplemented with high cytokinin to auxin ratio was found more effective. Benzyl adenine is often used as a source of cytokinin and more effective in the induction of multiple shoots.

b) Rooting

Successful rooting of shoots of *Albizia lebback* L. were achieved with 2.0 mg l^{-1} IAA and could successfully transferred into soil (Paramjit *et al.*, 1982).

Nadgir *et al.*, (1984) reported successful rooting and shoots of *Dendrocalamus strictus* on half strength medium with activated charcoal after treatment with IBA for 96 hrs in dark.

In *Leucama leucocephala*, rooting was achieved at 5.0 μ M IAA (Hisajima *et al.*, 1987).

Basal MS medium initiated rooting of shoots of *Eucalyptus camaldulensis* (Kumar and Ayyappan, 1987). In case of Guava *in vitro* shoots, rooting was achieved in half strength MS medium with 1.0 g l^{-1} of IBA NAA and activated charcoal (Muhammad *et al.*, 1987).

Rooting of *Tilia chordata* shoots was obtained in half strength Ide and Santo medium with 3.0 mg l^{-1} IBA (Youn *et al.*, 1988).

Rooting experiments in young shoots of *Quercus suber* L. seedlings was found to be best in 1/2 strength Durzan medium with 1.0 mg l^{-1} IBA and 0.1 mg l^{-1} NAA. It yielded 70% rooting (Deidda *et al.*, 1988).

Rooting of *Eucalyptus tereticornis* Sm. shoots was in half strength MS medium with 4.9 μ M IBA, 5.5 μ M IAA and 5.3 μ M NAA. half strength semisolid hormone free MS medium with charcoal and MS liquid medium without charcoal or hormones induced rooting of shoots in the dark (Rao, 1988).

Treatment with IBA at 1.0 mg/ml for 10 secs. initiated rooting of shoots of *Corylus avellana* L.. (Rodriguez *et al.*, 1988).

Vigorous root development was observed in the shoots of *Shorea roxburghii* G.DM in liquid medium with NAA and IBA 0.1 mg l⁻¹ each (Scott *et al.*, 1988).

Rooting of *Acacia auriculiformis* shoots was in B₅ medium augmented with IAA at 10⁻⁷ M. or NAA at 10⁻⁶ M. or 10⁻⁷ M. (Aradhana *et al.*, 1989).

Shoots of Indian Rose wood (*Dalbergia latifolia*) treated with NAA, IAA and IBA (1.0 mg l⁻¹ each) and subsequently transferred to hormone free half strength MS medium induced rooting (Ravishankar *et al.*, 1989).

Growth regulator free medium initiated the rooting of *Datura insignis* Brab. shoots (Maria and Domanto, 1990).

Ramesh *et al.*, (1990) transferred shoots grown on medium containing IAA at 6.0 μM. for 2 wks and then transferred to basal media, resulted in tap root production.

Rooting of *Camillia sinensis* CV T-78 was achieved by placing the microshoots on Inductive medium for 10 days and transferred shoots to hormone free MS medium by treating the microshoots with IBA at 500 mg l⁻¹ for 30-40 min. (Timirbaran *et al.*, 1992).

Shoots of *Adenanthera pavonina* L. respond to rhizogenesis when cultured onto half strength MS medium with 2.0 mg l⁻¹ IBA and 0.5 mg l⁻¹ NAA (Bejoy *et al.*, 1993).

Perez *et al.* (1994) reported rooting of *Frazinus angustifolia* on Woody Plant medium (WPM) supplemented with 0.98- 4.9 μ M. IBA. There was 85% survival in the rooted plantlets when transferred to soil and acclimatised.

Multiple shoots transferred to high auxin medium to induce rooting. The three main sources of auxins used for rooting in many experiments were NAA, IAA incorporated in half MS medium.

IV. Physiological age

In micropropagation techniques, it has been observed that even the age of the explant will count a lot. This has been showed evidently by number of workers.

In the micropropagation of sandal wood plants two types of explants were used (Rao *et al.*, 1977).

1. Hypocotyl segments excised from seedlings of *Santalum album* grown *in vitro* and
2. Stem segments from adult plants.

Among these two explants, it was the hypocotyl segments which responded best for hormonal treatment interms of shoot bud regeneration.

In the clonal propagation of guava, the explants taken were from field grown plants and those from *in vitro* proliferated shoots. However, the number of shoots developed was 5-10 in shoots developed from *in vitro* proliferated shoots as compared to 3-6 shoots in explants of from field grown plants (Mohammed *et al.*, 1987).

In Jack (*Artocarpus heterophyllus*), shoot apices about one cm long from two month old seedling or six month old grafted plants and fresh stem shoots of 5-10 and 30 yrs old trees were used as explants and cultured. Seedling apices showed a 17.4 fold multiplication rate in 5 wks with 100 per cent rooting. For stem shoot apices from 5-10 and 30 year old trees, the multiplication rate was 4.5, 2.8 and 2.09 fold respectively in 5 wks with 70 per cent rooting (Rajmohan and Mohankumaran, 1988). Explants from embryogenic material (45 days old) and adult material (2 yr old) of *Corylus avellana* L. (Rodriguez *et al.*, 1988) were cultured on solid medium with several concentrations and combinations of different hormones. Embryogenic explants gave single shoot or clusters of shoots after 30 days in all combinations assayed. Adult explants needed a longer culture period and more specific combinations of plant growth regulators (Rodriguez *et al.*, 1988).

Cotyledonary nodes from germinated seedlings of *Camillia sinensis* cv T-78 and nodal explants of field grown plants were cultured. Cotyledonary nodes produced the highest mean number of microshoots per explants (Timirbaran et al, 1992).

But in *Frazinua angustifolia* shoot tips and nodal explants from both mature and juvenile plants have responded almost same to the treatment. The number of multiple shoots produced in new explants, from matured explants was 5.3 and new explants from juvenile explant was 5.6 (Perez *et al.*, 1994).

MATERIAL AND METHODS

III. MATERIALS AND METHODS

In the first part of the investigation, the morphological and physiological differences of the suspected parents i.e., *Acacia auriculiformis*, *Acacia mangium* and their hybrid i.e., the hybrid of *Acacia auriculiformis* and *Acacia mangium* were studied.

The morphological and physiological differences included the parameters like size, total protein content, chlorophyll content and the initial growth rate of the trees.

In the second part of the study, methods were standardised for the micropropagation of *Acacia auriculiformis* and its hybrid. The micropropagation technique involves standardising optimum concentrations of growth regulator for the production of multiple shoots and efficient rooting in an optimum concentration of any growth regulator which induces rooting.

Morphological differences

The morphological differences of *Acacia auriculiformis*, *Acacia mangium* and the hybrid included the parameters like, size of the three tree species, leaf morphology and size and also the seed shape and colour.

The size of the hybrid has the maximum size with high branching followed by *Acacia auriculiformis* and *Acacia mangium* (Plate 1).

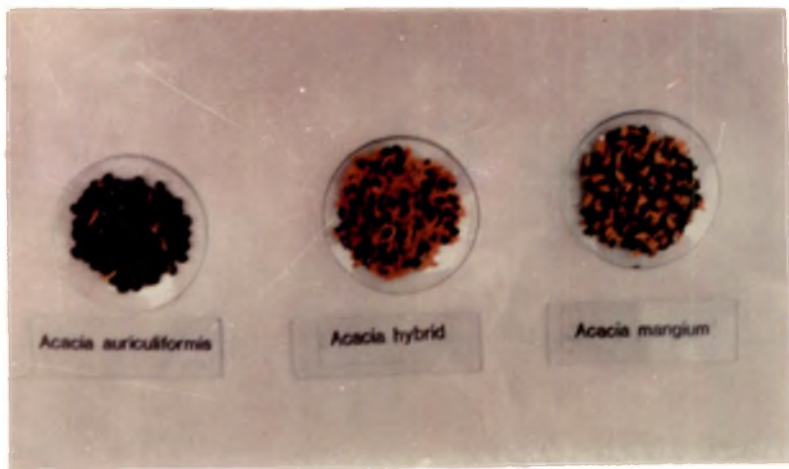
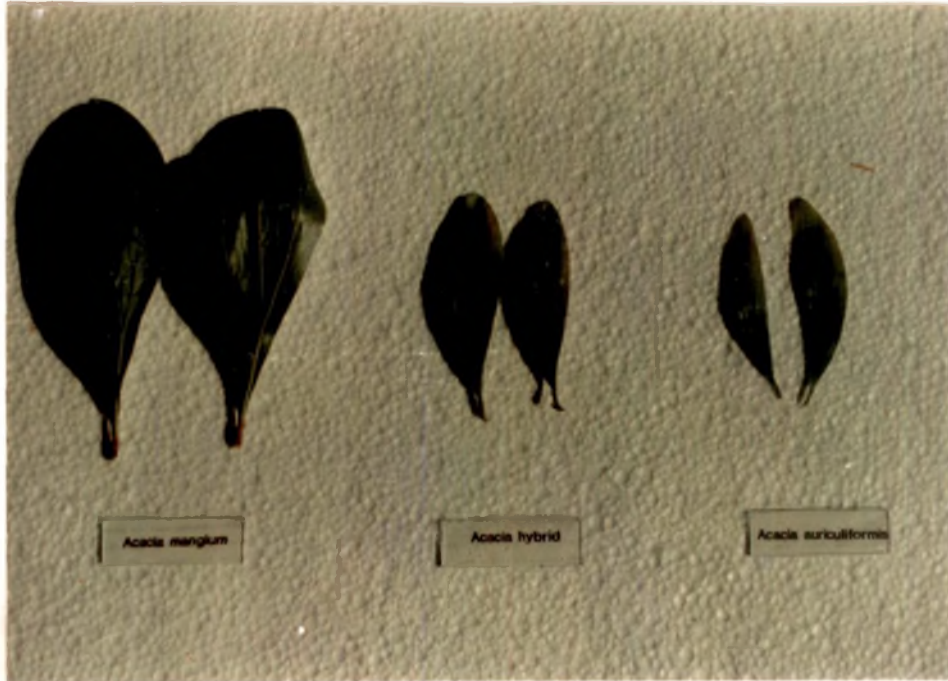
Acacia mangium has biggest leaves and *Acacia auriculiformis* has the smallest leaves and hybrid is intermediate between them (plate 2a).

Plate 1: Plate showing the growth of one and a half year old *Acacia auriculiformis*, *Acacia mangium* and their putative hybrid.

- a: *Acacia auriculiformis*
- b: *Acacia mangium*
- c: *Acacia* hybrid



Plate 2a & b: Morphology of the leaves and seeds of *Acacia auriculiformis*,
Acacia mangium and their putative hybrid.



Largest seeds were found in *Acacia auriculiformis* with yellow coloured arils. Whereas mangium has the smallest seeds with orange tinged yellow coloured arils. Hybrid has seed size in between the parents with orange coloured arils (Plate 2b).

Physiological differences

Three preliminary experiments were conducted to compare to the chlorophyll content, total protein content and the three volumes of *Acacia auriculiformis*, *Acacia mangium* and their hybrid.

EXPT. 3.1. ESTIMATION OF TOTAL CHLOROPHYLL

Fully expanded young leaf of half year old tree weighing about 1000 mg was extracted in 80% Acetone. Known weight of leaf material was homogenised in clean pestle and mortar and centrifuged at 5000-6000 rpm for 5-10 minutes. Supernatant was collected and residue was washed with acetone until the residue becomes clear (Witham *et al.*, 1971). Volume was made upto 25 ml. Absorbance was recorded at 645 nm and 663 nm and chl a, chl b and total chlorophyll was calculated using the formula given as,

$$\text{mg chl a/gm tissue} = [12.7(A_{663}) - 2.69(A_{645})] V/1000 \times Wt.$$

$$\text{mg chl b/gm tissue} = [22.9(A_{645}) - 4.68(A_{663})] V/1000 \times Wt.$$

$$\text{Total chlorophyll} = [20.2(A_{645}) + 8.02(A_{663})] V/1000 \times Wt.$$

where A = Absorbance at specific wavelength

V = Volume of chlorophyll extract in 80% Acetone

W = Fresh weight of leaf taken for extraction

EXPT. 3.2. EXTRACTION AND QUANTIFICATION OF SOLUBLE PROTEINS

Leaves from tender/young branches were homogenised at 4°C. in two volumes of Tris Hcl buffer pH 8.5 containing 0.15 M. Tris, 0.15 M. Sodium dithionate, 0.15 M. Ascorbic acid, 1 mM PMSF, and 5 mM Mercaptoethanol using pestle and mortar. The cell debris were precipitated by centrifugation at 12,000 rpm for 15 minutes at 4°C. and the total protein was quantified in the supernatant using a simple proteindye binding technique, according to Bradford (1976), using BSA as the standard. The method is based on the principle that the Coumassic Brilliant Blue (CBB) G-250 binds to proteins and gives a consistent blue colour. The protein dye complex has a higher extinction coefficient, thus leading to a great sensitivity in measurement of protein. This binding of dye to protein is a very rapid process (approximately two minutes). The method is devoid of interferences by other soluble components.

EXPT. 3.3. ESTIMATION OF TREE VOLUME OF *ACACIA AURICULIFORMIS*, *ACACIA MANGIUM* AND THEIR HYBRID

The three species under consideration were *Acacia auriculiformis*, *Acacia mangium* and *Acacia hybrid*. For the estimation of volume, the measurements taken were, tree height and diameter.

Tree heights were measured using Multimeter. This instrument is widely used to measure the tree heights of most of the forestry species including the measurement of the altitude of the forest. It works on the principles of Trigonometry. This has five scales, A,B,C,D and E.

1. The scale A measures tree height in metres above the eye level when the distance between the observer and the tree is 20 mts.

2. Scale B measures the tree height when the distance is 30 mts.

3. Scale C reads the complement of the angle between the tree tip and observer's eye level.

4. Scale D reads the slope i.e., whenever the trees are on the sloping land, it gives the % slope which will be useful in converting it into horizontal distance.

5. Scale E reads tree height in percentage and can be taken at any distance from the tree.

Since it is very difficult to go for a fixed distance which is to be used in Scale A and B, Scale E was made use. Standing a few metres away from the tree, tree height was measured using Scale E of the instrument.

The reading of the Scale E was noted down and without changing the position, the horizontal distance between the observer and the tree was measured.

Since the trees were in the horizontal ground as it is a plantation, only the horizontal distance was taken. Using these parameters (Scale E reading and the horizontal distance), tree height was calculated using the formula given below.

$$\text{Total height} = \frac{\text{Scale E reading} \times \text{horizontal distance} + \text{Observer's eye height}}{100}$$

For the same tree, diameter was measured at breast height (DBH=1.37 mts from the ground level). Whenever the abnormalities are found at DBH, the measurement was taken either just above or below the DBH. Two measurements were made at right angles to each other to arrive at the exact diameter of the tree as most of the trees are not cylindrical. Average diameter was taken for calculating the basal area or the circumference. Whenever the trees exhibit branching below DBH, each branch was considered as a individual tree, diameters were taken for all the branches. The height of the respective branches are also taken for calculating the volume. The basal area was estimated or calculated using the formula,

$$\text{Basal area} = \frac{\pi d^2}{4}$$

Since the trees are not exactly cylindrical in shape, the formula corresponding to the cylinder can not be applied for the calculation of volume. Always the tree is tapering towards the tip. So, in order to get the correct volume of trees, a factor needed to be included and that factor is called as Form factor (FF) which is always less than one. This is calculated by measuring the diameter or girth at different heights of the tree for large number of trees of the same species. Since the plantation area was small with only few trees, form factor was calculated by measuring the girths of ten trees. The form factors for different species are:

Acacia auriculiformis	-	0.65
Acacia mangium	-	0.65
Acacia hybrid	-	0.70

Finally the volume was calculated for ten trees in each species parameters viz tree height, basal area and form factor.

$$\text{Volume} = \text{Tree height} \times \text{basal area} \times \text{FF}$$

The volume was expressed in cft (Cubic feet).

II. MICROPROPAGATION TECHNIQUES

The following aspects were considered during the standardisation of Micropropagation technique.

Plant material

The plant materials which were used for the present study were *Acacia auriculiformis* and its hybrid.

a) *Acacia auriculiformis* A. Cunn. ex Benth

This is a small to medium sized tree, 8-20 mts. tall, heavily branched with a short, crooked stem. Young trees have smooth bark, grey in colour but becomes rough and dark brown with age. Newly germinated seedlings produce bipinnate leaves but soon develop phyllodes, at leaf node number 3 or 4. Phyllodes are 1.5-2.5 cm wide and 4-9 times as long as wide. Flower are in spikes to 8 cms long in pairs. Pods are flat, woody, glaucous, transversely veined. Seeds are held transversely in the pod 4-6 mm. long and 3-4 mm. wide and each is encircled by a long yellow funicle (arils).

b) Acacia hybrid

It is opined that there is a natural hybrid of *Acacia auriculiformis* and *Acacia mangium*. The morphological studies of this hybrid are in comparison with *Acacia auriculiformis*. This is a tall tree and there is fine branching. Bipinnate leaves are produced first but phyllode development is bit late compared to *Acacia auriculiformis*. Phyllodes are 4-5 cms. wide and proportionately long. Seeds are small. Seeds are encircled by a long orange coloured funicle (arils).

CHEMICALS

The chemicals which were used for the preparation of culture media and solutions were Analytical Reagents (AR grade purity) supplied by Merck , Glaxo and Sigma company.

GLASSWARES

All the glasswares like test tubes, beakers and pipettes used for tissue culture were of Borosil supplied by Borosil Company. The test tubes which used for the dispersion of media were of the size 150 x 25 mm diameter.

The plastic caps which were found comfortable than other coverings were supplied by Laxbro Company. They were of the diameter 6.25 cm.

PREPARATION OF GLASSWARES

The glasswares were dipped in 5% Chromic acid over night. The next day they were cleaned in Teepol followed by thorough rinsing with tap water and double distilled water (DDW). Then they were dried in hot air oven.

Stock Solutions

Any media will have different groups of stock solutions. Stock solutions of all these groups are prepared for 10 lts. Different salts of each group were weighed separately, dissolved in DDW one by one. These solutions were mixed thoroughly in volumetric flasks and the volume was made up to obtain stock solutions with known concentrations of each constituent of the group. Stock solutions were stored in refrigerator at 4°C.

Growth Regulators

A known quantity of Naphthalene Acetic Acid (NAA), Indole Butyric Acid (IBA) and 2,4 Dichloro phenoxy Acetic Acid (2,4-D) were weighed, dissolved separately in a minimal quantity of redistilled ethyl alcohol and the volume was made up with DDW to a known volume.

Benzyl Amino Purine (BAP), Kinetin, Zeatin and Adenosine Sulphate were weighed, dissolved separately in a minimal quantity of 0.1 N Hydrochloric acid and volume was made up with DDW to a known volume.

All the growth regulators were stored in refrigerator at 4°C.

Preparation of Media

Required quantity of Sucrose was weighed and dissolved in DDW. The required volumes of each stock solution as detailed in Table 1 to 3 were sequentially pipetted out into it. All these solutions were mixed thoroughly in the volumetric flask and the volume was made up to a known volume with DDW.

MEDIA

The media which were used in the present study are

1. Murashige and Skoog's medium (Murashige and Skoog, 1962) (Table 3.1).
2. B5 Medium (Gamborg et al, 1968) (Table 3.2).
3. Woody Plant Medium (McCown and Lloyd, 1981) (Table 3.3).

Adjustment of pH

The pH of all media were maintained between 5.5 and 6.0. The pH of the media was adjusted using a pH meter with 1 N. HCl and or 1 N. NaOH. Known quantity of phytoigel was added and then heated for dissolving phytoigel. The media was dispensed into test tubes at the rate of 15 ml. and plugged with non-absorbant cotton plugs or aluminium foils or Laxbro caps.

Autoclaving

Vertical type of autoclave was used. The testtubes with medium were autoclaved at 1.06 Kg/cm² (121°C) for 15 min. The test tubes were then taken out

Table 3.1. Composition of Murashige media (1962)

Ingredients	Salt concentration in stock solution (g l ⁻¹)	Aliquot for one liter of medium	Final concentration of salt in one liter (mg)	Groups
NH ₄ NO ₃	66.00		1650	I
KNO ₃	76.00		1900	
MgSO ₄ ·7H ₂ O	14.80	25	370	
KH ₂ PO ₄	6.80		70	
CaCl ₂ ·2H ₂ O	4.4	10	440	II
Na EDTA	7.45	5	37.20	III
FeSO ₂ ·7H ₂ O	5.57		27.20	
H ₂ BO ₃	0.62		6.20	
ZnSO ₄ ·4H ₂ O	0.86		8.60	
MnSO ₄ ·4H ₂ O	2.23	10	22.30	IV
KI	0.083		0.83	
Na ₂ MoO ₄ ·2H ₂ O	0.025		0.25	
CuSO ₄ ·5H ₂ O	0.025		0.25	
CoCl ₂ ·6H ₂ O	0.025		0.25	
Glycine	0.2		2.0	
Myoinositol	10.0		100.0	
Thiamine HCl	0.01		0.1	
Pyridoxine HCl	0.05	10	0.5	V
Nicotinic acid	0.05		0.5	
Sucrose	2%	20.0 g		
Phytogel	0.25%	2.5 g		
pH	5.8			

Table 3.2.

B₅ medium, Gamborg et al (1968)

35

Groups	Compounds	Concentration Reqd (mg l ⁻¹)	Amt of the compound taken (g)	Vol. made up ml	Aliquat taken
GROUP I	MgSO ₄ ·7H ₂ O	250	2.5	100	10
	NaH ₂ PO ₄	150	1.5		
	(NH ₄) ₂ SO ₄	134	1.34		
GROUP II	KNO ₃	2500	25.0	100	10
GROUP III	H ₃ BO ₃	3	0.03	100	10
	MnSO ₄ ·H ₂ O	10	0.1		
	ZnSO ₄ ·7H ₂ O	2	0.02		
	Na ₂ MoO ₄ ·2H ₂ O	0.25	0.0025		
	CuSO ₄ ·5H ₂ O	0.025	0.00025		
	CoCl ₂ ·6H ₂ O	0.025	0.00025		
GROUP IV	CaCl ₂ ·2H ₂ O	100	1.0	100	10
GROUP V	Na ₂ FeEDTA	43	0.43	100	10
GROUP VI	Thiamine HCl	10	0.1	100	10
	Pyridoxine HCl	1	0.01		
	Nicotinic acid	1	0.01		
	Myoinositol	100	1.0		
Sucrose	2%	20.0 g			
Phytogel	0.25%	2.5 g			
pH	5.5				

Table 3.3. Woody plant medium (WPM)
(McCown and Lloyd, 1981)

Groups	Compounds	Concentration Reqd (mg l ⁻¹)	Amt. of the compound taken (g)	Vol. made up ml	Aliquat taken
GROUP A	NH ₄ NO ₃	400.0	4.0	100	10
	Ca(NO ₃) ₂	556.0	5.56		
GROUP B	K ₂ SO ₄	990.0	9.90	100	10
	CaCl ₂ ·2H ₂ O	96.0	0.96		
GROUP C	KH ₂ PO ₄	170.0	1.7	50	5
	H ₃ BO ₃	6.2	0.062		
	Na ₂ MoO ₄	0.25	0.0025		
GROUP D	MgSO ₄ ·7H ₂ O	370.0	18.5	250	5
	MnSO ₄	22.3	1.115		
	ZnSO ₄ ·7H ₂ O	8.6	0.43		
	CuSO ₄ ·5H ₂ O	0.25	0.012		
GROUP E	FeSO ₄ ·7H ₂ O	27.8	0.556	100	5
	Na ₂ EDTA	37.3	0.746		
GROUP F	Thiamine HCl	1.0	0.02	100	5
	Nicotinic acid	0.5	0.01		
	Pyridoxine HCl	0.5	0.01		
	Glycine	2.0	0.04		
GROUP G	Myoinositol	100.0	2.0	100	5
Sucrose	2%	20.0 g			
Phytogel	0.25%	2.5 g			
pH	5.8				

and were allowed to cool. They were kept in the culture room at $25\pm 2^{\circ}\text{C}$. Inoculation of seeds and explants were done after 2-3 days ensuring that the tubes were free from contamination and conditioned.

***IN VITRO* SEED CULTURE**

Pretreatment of seeds

Healthy *Acacia auriculiformis* seeds were selected. They were treated with conc. H_2SO_4 for 2-3 minutes. Then washed 3-4 times under tap water. Then they were washed with detergent for 4-5 minutes. Again they were washed under tap water for 4-5 times. Later they were treated with 70% ethyl alcohol for 2-3 minutes. Again washed under tap water for 4-5 times. Then the seeds were dipped in 10 ppm of BA solution for 48 hrs. These seeds were used for inoculation under uv fitted laminar flow chamber.

Inoculation of Seeds and Incubation

The seeds which were dipped in BA solution was, now inoculated in flasks containing media under aseptic conditions. The imbibed seeds in BA were treated with 0.1% HgCl_2 and a drop of Tween, for 5-6 minutes. The seeds were shaken well. Then they were washed thoroughly with autoclaved (sterilised) DDW until the complete removal of lather (5-6 times). The seeds were then inoculated in 1/2 strength MS medium provided with 0.1 mg l^{-1} BAP and 0.1 % Activated charcoal.

The the flasks were incubated at $25\pm 2^{\circ}\text{C}$ under fluorescent light of 2000 lux. Sixteen hours of light and eight hours of dark were maintained. Ten days old seedlings were used for further inoculation of the explant.

TISSUE CULTURE OR MICROPROPAGATION

Explants

The explants used for tissue culture were

1. Mesocotyls with shoot tips and axillary buds from *in vitro* grown seedlings.
2. Axillary buds from matured *Acacia auriculiformis* trees.

The explants were inoculated onto the medium under aseptic conditions.

Sterilization of explants

The *in vitro* grown explants were treated with 0.1% HgCl_2 and Tween for 2-3 minutes and then washed with sterilized DDW until the complete removal of lather. The axillary buds brought from field were kept under running water for 2-3 hours. Then they were dipped in 0.1% Bavestin. Again washed under tap water for 2-3 times. Then the branches with axillary buds were swabbed with cotton dipped in Ethyl alcohol. Again these axillary buds were treated with 0.1% HgCl_2 and Tween for 9-10 min. Washing was done as mentioned above and implanted in the medium and covered with cotton pluggs.

Inoculation

A known weight of sterilized explants (both in vitro and field grown) were implanted in the media in the inoculation hood aseptically. Here the media was incorporated with different growth regulator's at different concentrations.

Incubation

Incubation was done at $25\pm 2^{\circ}\text{C}$ under fluroscent light of 2000 lux. Sixteen hours of light and eight hours of dark are maintained.

Recording of Data

Data was recorded on parameters like the difference in morphological behaviour, production of multiple shoots and its number, after a known number of days. The number of roots per shoot was also recorded.

For the production of multiple shoots, some micropropagation experiments were designed.

EXPERIMENTAL DETAILS OF MICROPROPAGATION TECHNIQUE**EXPT. 3.4. PRODUCTION OF ACCESSORY BUDS ON MATURED TREE SPECIES**

The main objective of this experiment was to get more bud sprouts and to see their response in *in vitro* experiment, when used as an explant source.

In this experiment, different combinations of NAA, GA and BA as the hormonal source were treated to buds selected on matured trees. The young branches selected were detopped to remove apical dominance and then the growth regulators were applied to the dormant buds in the axils of the leaves which cover eight buds in the apical region of the cut branches.

EXPT. 3.5. SELECTION OF EXPLANT SOURCE AND STANDARDISATION OF MEDIA

It is a well known fact that different plant species respond differently to the media composition and to the explant source for multiple shoot production through *in vitro* technology. So this experiment was designed with an aim to select the correct explant source and also the appropriate composition of the medium for obtaining the maximum response.

a) Axillary buds from matured trees

Axillary buds measuring 0.5 to 1.0 cm length were selected from branches of a matured tree. Buds after being sterilized, were inoculated to B₅, MS (Murashige and Skoog) and WPM (Woody Plant Medium). Observations were made on the sprouting of buds at different intervals.

b) Explants from *in vitro* grown seedlings

In this experiment, healthy seeds were selected, surface sterilized and germinated under sterile condition. After obtaining uniform size of the seedlings, different explants from the seedlings were selected and inoculated on B₅, MS and

WPM aseptically. Periodical observations were made. The medium whichever was found responsive on the growth of the explant was considered as the best medium.

EXPT. 3.6. RESPONSE OF MESOCOTYL AS EXPLANT SOURCE IN THE PRODUCTION OF MULTIPLE SHOOTS

The present experiment was designed with the aim to select the best combination of growth regulators for obtaining the maximum production of multiple shoots from mesocotyl explants. Best media selected i.e., B₅, was supplemented with different combinations of BA ranging from 0-10 mg l⁻¹ keeping NAA at 0.1 mg l⁻¹. The mesocotyl explants were inoculated and incubated. Observations were recorded periodically on the production of multiple shoots and also the morphological differences.

EXPT. 3.7. INFLUENCE OF INCREASED NAA LEVEL ON MULTIPLE SHOOT PRODUCTION FROM MESOCOTYL EXPLANTS

This experiment was conducted to observe any difference in multiple shoot production for increased concentration or level of NAA from 0.1 to 0.25 mg l⁻¹. In order to enhance the number of multiple shoots, higher level of NAA at 0.25 mg l⁻¹ was tried with varying concentrations of BA. Observations were recorded on the parameters like morphological behaviour and multiple shoot production.

EXPT. 3.8. INFLUENCE OF VARYING CONCENTRATION OF AUXIN AND CYTOKININ IN THE MEDIUM ON MULTIPLE SHOOT PRODUCTION DERIVED FROM EXPLANTS MATURED TREE SPECIES

The experiment was designed to select the explant source directly from the matured trees for *in vitro* experimentation. Young axillary buds of uniform size from matured tree of *Acacia auriculiformis* were selected as explant source. The explants

were treated with Absolute alcohol followed by 0.1% Bavistin and 0.1% Mercuric chloride. The B₅ medium was supplemented with different concentrations of BA ranging from 1,2,3,4,5,6,8 and 10 mg l⁻¹ in combination with 0.1 mg l⁻¹ NAA. The inoculated tubes were incubated. Observations taken at 15 days interval upto 60th day after inoculation.

EXPT. 3.9. RESPONSE OF *IN VITRO* GROWN AXILLARY BUDS ON MS MEDIUM SUPPLEMENTED WITH DIFFERENT COMBINATIONS OF GROWTH REGULATORS

This experiment was conducted to see the response of *in vitro* grown axillary buds derived from the *in vitro* grown elongated mesocotyl and its branches. They were inoculated on MS medium supplemented with different concentrations of BA along with NAA and 2,4-D. Observations were recorded 20 days after inoculation on multiple shoots produced per inoculum and their length.

EXPT. 3.10. RESPONSE OF MESOCOTYL EXPLANT ON MS MEDIA SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF AUXINS AND CYTOKININS

The main aim of the experiment was to see the effect of high inorganic salt medium on the production of multiple shoot per explant.

In this experiment, a combination of BA and NAA, and BA alone are incorporated in MS media to which mesocotyl explants were inoculated. The concentrations of BA was varied keeping NAA at 0.1 mg l⁻¹ and in another set, only BA was incorporated without NAA.

EXPT. 3.11. EFFECT OF DIFFERENT SOURCES OF CYTOKININS IN MS MEDIA ON MULTIPLE SHOOT PRODUCTION IN EXPLANTS FROM *IN VITRO* DEVELOPED SHOOTLETS

The present experiment was conducted to select the potent cytokinin for maximum number of multiple shoots on MS medium. The MS medium was supplemented with different sources of cytokinins i.e., BA, Kinetin and Adenine sulphate with different concentrations.

Two types of explant sources i.e., axillary bud and internodal segments were selected from the *in vitro* grown multiple shoots and inoculated. Periodical observations at intervals of 15 days.

EXPT. 3.12. *IN VITRO* MAINTENANCE OF EXPLANT SOURCE FOR FUTURE GENERATION OF STANDARDISED MEDIUM

This experiment was conducted in order to confirm and to develop a protocol for *in vitro* maintenance of the explant source. For this, the standardised medium i.e., B₅, was supplemented with 3.0 mg l⁻¹ BA with 0.1 mg l⁻¹ NAA on the development of multiple shoot from explants of different generations. Explants selected from the *in vitro* grown seedlings formed the I generation and explants from the I generation formed the II generation. Observations were taken periodically after the explants being inoculated.

EXPT. 3.13. INDUCTION OF ROOTING ON *IN VITRO* GROWN SHOOTLETS

The experiment was conducted to induce rooting for the shootlets that were developed. Half strength MS medium was supplemented with two sources of root

inducing hormones viz. IAA and NAA at two concentrations, 0.1 and 0.25 mg/l. Uniform length of shootlets were inoculated on the root inducing medium. Observations were made for every 15 days.

EXPT. 3.14. STATISTICAL ANALYSIS

Statistical analysis was carried out by taking per cent success for some experiment. A Factorial ANOVA was designed for another few experiments. A few of the experiments were interpreted on the morphological differences.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Several preliminary experiments were conducted to make a comparison between the parents *Acacia auriculiformis*, *Acacia mangium* and their putative hybrid. The first three experiments were conducted with the above mentioned aim. Rest of the experiments were designed to standardise the explant source, media, the best growth regulator combination for multiple shoot production and root induction. The results of the experiments are given below.

4.1. EXTRACTION AND ESTIMATION OF TOTAL CHLOROPHYLL IN LEAVES

The results indicated that Chl a and Total Chlorophyll were more in *Acacia auriculiformis*, followed by the hybrid. *Acacia mangium* had maximum amount of Chl b, followed by the hybrid (Table 4.1).

4.2. EXTRACTION AND QUANTIFICATION OF SOLUBLE PROTEINS IN LEAVES

The results generated in Table 4.2 indicated total soluble protein content was more in *Acacia auriculiformis*, followed by hybrid. *Acacia mangium* contained less total soluble proteins.

4.3. CALCULATION OF TOTAL TREE VOLUMES OF ACACIA AURICULIFORMIS, ACACIA MANGIUM AND THEIR HYBRID

The data on total volume of the trees of two parents i.e., *Acacia auriculiformis*, *Acacia mangium* and their hybrid are presented. The aim of

Table 4.1: Chlorophyll content in the leaves of Acacia auriculiformis
Acacia Mangium and their putative hybrid.

Species	Chl.a (mg/gm fr.wt)	Chl.b (mg/gm fr.wt)	Total Chlorophyll
<u>Acacia auriculiformis</u>	3.852	1.84	5.692
Acacia hybrid	2.496	1.03	3.526
<u>Acacia mangium</u>	2.221	1.04	3.261

Table 4.2. Protein content in the leaves of *Acacia auriculiformis*, *Acacia mangium* and their putative hybrid

Species	Protein content (mg/gm) fresh weight
<i>Acacia auriculiformis</i>	18.22
<i>Acacia</i> hybrid	13.54
<i>Acacia mangium</i>	9.47

calculating the total volume of these species was to know the growth rates. Results are interpreted in Table 4.3. The total volume was more in the hybrid followed by *Acacia auriculiformis* and then *Acacia mangium*.

4.4. PRODUCTION OF ACCESSORY BUDS ON MATURED TREE SPECIES

For micropropagation experiments, the age of the tissue used as an explant plays an important role. In order to achieve the desired results in *in vitro* experiments the explant source is important. Preliminary experiments were conducted to induce growth of axillary buds on matured trees through hormonal treatment. The main objective was to obtain better and more bud sprout and to see their response *in vitro* experiments. The selected young branches were detopped to remove apical dominance and growth regulators were applied to the dominant buds in the axils of the leaves covering 8 buds in the apical region of the cut branches. Periodical observations were made on the growth of the buds (Table 4.4).

The observations revealed that the growth regulator treatment in general reduced the number of buds sprouted (Table 4.4). The method of application was that the selected young branches were detopped to remove apical dominance and growth regulators were applied to the dormant buds in the axils of the leaves covering eight buds in the apical region of the cut branches. The reduction in bud sprouting was maximum in treatment with 10 mg l⁻¹ NAA, 50 mg l⁻¹ BA and 0.2 mg l⁻¹ GA. In treatment with 10 mg l⁻¹ NAA, 10 mg l⁻¹ BA and 0.2 mg l⁻¹ GA, about 10% of the treated buds showed elongation in 20 days after treatment. However, a few of the buds senesced after elongation.

Table 4.3. Comparative tree volumes of one and a half year old trees of *Acacia auriculiformis*, *Acacia mangium* and their putative hybrid

Species	Volume (cft)
<i>Acacia auriculiformis</i>	0.064
<i>Acacia</i> hybrid	0.207
<i>Acacia mangium</i>	0.035

CD (5%) : 0.01
F test : *

Table 4.4. Influence of application of growth regulator mixtures to the branch on the growth of axillary buds under in *in vivo* conditions

Sl.No.	Treatment	Per cent of buds sprouted (after 1wk)	Per cent of buds elongated (after 2 wk)	% of buds dried (after 3 wk)	% of buds not sprouted (after 3 wk)	Avg. No. of buds sprouted per branch
1	T ₁	96	-	-	2.3	7.8
2	T ₂	65	-	-	34.5	5.6
3	T ₃	56	10	2.6	7.5	6.7
4	T ₄	86	-	-	13.7	6.7
5	T ₅	75	-	-	25.0	6.0
6	T ₆	86	-	-	13.7	6.7
		NAA (mg l ⁻¹)	- GA (mg l ⁻¹)	+ BA (mg l ⁻¹)		
	T ₁	0	0	0		
	T ₂	10	50	0.2		
	T ₃	10	10	0.2		
	T ₄	2	50	0.2		
	T ₅	2	10	0.2		
	T ₆	0.2	10	0.2		

4.5. SELECTION OF EXPLANT SOURCE AND STANDARDISATION OF MEDIA

The experiment is designed to select the explant source both from the matured trees and also from young seedlings. The objective was to study the differential potential response of these explants to growth and multiplication in *in vitro* conditions.

a. Axillary buds from matured trees.

Axillary buds measuring 0.5-1.0 cm length were selected from branches of matured tree. These buds were surface sterilised and inoculated on the basal Gamborg's B₅ (B₅), Murashige and Skoog (MS), and Woody plant media (WPM). Inoculated buds did not show any type of response even thirty days after inoculation. These results indicated that axillary buds from the branches of a matured tree do not normally respond in terms of multiple shoot production.

b. Explants selected from young seedlings grown *in vitro*

Different plant species respond differently to the media composition and to the explant source for multiple shoot production in *in vitro* technology. Hence, the primary objective is to select the right explant source and also the appropriate composition of the medium for obtaining maximum response. In this experiment, the healthy seeds were selected, surface sterilised and germinated under sterile conditions. Uniform size seedlings were obtained and the explants, mesocotyl with shoot tip, shoot tip, cotyledon and internodal

regions were aseptically separated and inoculated on three different standard media- Murashige and Skoog, Gamborg's B₅ and Woody plant media. These media were uniformly supplemented with 3 mg l⁻¹ BAP and 2% Sucrose. The media was dispensed in culture tubes and the tubes with inoculum were incubated. The observations were made periodically and the results recorded after 25 days of incubation.

The results presented in Table 4.5 indicate that the explant mesocotyl along with shoot bud gave better response in terms of the elongation of the shoot bud, maintenance of chlorophyll content and production of axillary buds as compared to the other explant source. The next best explant source was shoot bud alone. The other explant did not show any type of growth response. This has clearly given an indication that the mesocotyl along with the shoot bud is the best explant for *in vitro* micropropagation.

The results revealed that B₅ and MS were the best media. WPM medium did not have any response (Plate 3a). Among B₅ and MS media, B₅ medium responded better in producing more number of axillary buds than MS medium (Plate 3b) (Table 4.6).

Among the media chosen, both MS and B₅ media were found to be suitable for vigorous growth of the explant than that of WPM. In the ongoing experiments both B₅ and MS media were used to establish further superiority among the selected media for micropropagation of *Acacia auriculiformis*.

Table 4.5. Standardisation of culture media and explant source from young seedlings (per cent of explants which showed growth* after being inoculated on the media)

MEDIA	Explant sources				
	MC+ST	ST	MC	IN	C
MS	66	66	0	0	0
B _s	100	100	0	0	0
WPM	33	33	0	0	0

where

MC = Mesocotyl

ST = Shoot tip with an apical bud

IN = Internode

C = Cotyledon

* Elongation growth as well as maintenance of chlorophyll

These values indicate the percent of explant which showed continuous growth in the medium (elongation growth as well as maintenance of chlorophyll content)

Plate 3a: Response of mesocotyl explants of *Acacia auriculiformis* on different media.

- A: MS medium + 3.0 mg^l⁻¹ BAP**
- B: B₅ medium + 3.0 mg^l⁻¹ BAP**
- C: WPM medium + 3.0 mg^l⁻¹ BAP**



Table 4.6. Comparison of MS and B₅ media supplemented with BA on axillary bud production

Treatments	No. of explants inoculated	Total number of buds produced	Buds/explant
MS + 3 BAP	12	6	0.5
B ₅ + 3 BAP	12	24	2.0
CD (5%) : 0.42			
F test : *			

Plate 3b: Comparison of MS and B₅ media supplemented with BA on axillary bud production.



Morphological behaviour

of mesocotyl on different media

4.6. RESPONSE OF MESOCOTYL AS EXPLANT SOURCE IN THE PRODUCTION OF MULTIPLE SHOOTS.

The results of the previous experiments clearly suggested that the mesocotyl portion from the *in vitro* grown seedlings responded well in the production of axillary buds over B₅ medium. The present investigation was designed to select the best combination of growth regulators for obtaining maximum production of multiple shoots from mesocotyl explants.

The B₅ media was supplemented with different combinations of BA keeping NAA at 0.1 mg l⁻¹. The mesocotyl explants were inoculated and incubated. Observations were recorded both on morphological changes of the explant (Table 4.7), in the number of axillary buds produced (Table 4.8) and multiple shoot production (Table 4.9) at 10 days and 30 days after incubation.

At 10 days after incubation it was noticed that there were differences in the lengths of the main stem and also in the number of multiple shoots produced. The results obtained suggested that BA at 3 mg l⁻¹ along with 0.1 mg l⁻¹ NAA resulted in maximum response, both in terms of the response of number of explants, elongation of the explant and thickness of the elongated stem.

Elongation of the stem increased with increase in BA concentration upto 2 mg l⁻¹ and further response remained constant upto 6 mg l⁻¹. A further increase in BA concentration decreased elongation of the stem tips.

Table 4.7. Influence of different concentrations of Benzyl Adenine and NAA on the growth of apical bud

Morphological observations at ten days after incubation

MC+SB in B ₅ media with BA + NAA mg l ⁻¹	Elongation of stem tip	Score %	Stem thick- ness	No. of ax. buds	Opening of leaflets	Relative Length of side branch
1) Control	+	80	+	1	partial	-
2) 1 + 0.1	++	75	+	2	Full	+
3) 2 + 0.1	+++	100	++	2	Full	+
4) 3 + 0.1	+++	100	+++	3	Full	+
5) 4 + 0.1	+++	100	++	1	Partial	+
6) 5 + 0.1	+++	100	++	1	Partial	+
7) 8 + 0.1	+++	100	+	1	Partial	+
8) 10 + 0.1	++	75	+	1	Partial	+

where MC = Mesocotyl

SB = Shoot bud with apical bud

Elongation : + = 10.0 mm

++ = 20.0 mm

+++ = 30.0 mm

Stem thickness : + = 1.0 mm

++ = 1.5 mm

+++ = 2.0 mm

Table 4.9. Influence of varying concentrations of BA and NAA on the production of axillary buds on the main axis of mesocotyl explant

(10 days after treatment)

Treatments BA+NAA mg l ⁻¹	Explants inoculated	Total No. of buds produced	Buds/explant
1) 0	12	12	1
2) 1 + 0.1	12	24	2
3) 2 + 0.1	12	24	2
4) 3 + 0.1	12	36	3
5) 4 + 0.1	12	12	1
6) 5 + 0.1	12	12	1
7) 8 + 0.1	12	12	1
8) 10 + 0.1	12	12	1

CD (5%) : 0.74
F test : *

Plate4a: Response of varying concentrations of BA on the production of axillary buds on the main axis of mesocotyl explants of *Acacia auriculiformis*.

C=B₅

1=B₅ + 1 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

2=B₅ + 2 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

3=B₅ + 3 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

4=B₅ + 4 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

5=B₅ + 5 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

6=B₅ + 8 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

7=B₅ + 10 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

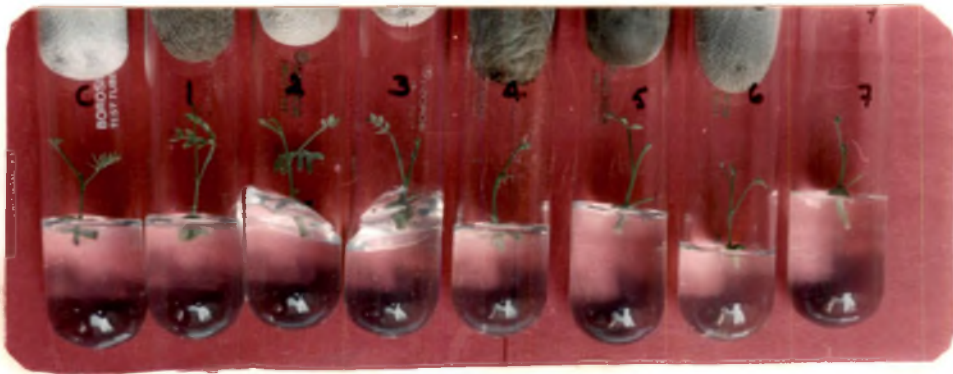


Table 4.9. Influence of varying concentrations of BA and NAA on the production of multiple shoots from mesocotyl explant (30 days after treatment)

	Treatments BA+NAA mg/l	No. of explants inoculated	No. of multiple shoots	Shoots/explant
1)	0	12	-	0
2)	1 + 0.1	12	24	2
3)	2 + 0.1	12	24	2
4)	3 + 0.1	12	60	5
5)	4 + 0.1	12	48	4
6)	5 + 0.1	12	48	4
7)	8 + 0.1	12	36	3
8)	10 + 0.1	12	24	2

CD (5%) : 1.147

F test : **

Plate 4c: Effect of 3 mg^l⁻¹ BA with 0.1 mg^l⁻¹ NAA on multiple shoot production.

3=B₅ + 3 mg^l⁻¹ BA + 0.1 mg^l⁻¹ NAA



Plate 4b: Response of varying concentrations of BA on the production of multiple shoots from the mesocotyl of *Acacia auriculiformis*.

C=B₅

1=B₅ + 1 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

2=B₅ + 2 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

3=B₅ + 3 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

4=B₅ + 4 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

5=B₅ + 5 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

6=B₅ + 8 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA

7=B₅ + 10 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA



The stem thickness, increased upto 5 mg l⁻¹ of BA and the concentration of BA and showed maximum reponse of 3 mg l⁻¹ with 0.1 mg l⁻¹ NAA. The number of axillary buds produced on the main axis of the growing apical bud increased with increase in concentration of BA in the medium upto 3 mg l⁻¹. With further increase in BA, the number of axillary buds produced was only one per explant. On an average treatment combination of 3 mg l⁻¹ BA with 0.1 mg l⁻¹ NAA resulted in 3 axillary buds on the main axis.

The number of multiple shoots produced in relation to different combinations of BA in the medium was recorded 30 days after inoculation of the explant. Maximum number of multiple shoots observed were five when the medium was supplemented with 3 mg l⁻¹ of BA with 0.1 mg l⁻¹ NAA. The average number of shoots per explant was 4 in the treatments supplemented with 4 mg l⁻¹ BA with 0.1 mg l⁻¹ NAA, and 5 mg l⁻¹ BA with 0.1 mg l⁻¹ NAA (Table 4.9).

4.7. INFLUENCE OF DIFFERENT NAA LEVELS ON MULTIPLE SHOOT PRODUCTION FROM MESOCOTYL EXPLANTS

In the previous experiment, it was noticed that the mesocotyl shoot showed good response in terms of multiple shoot production. When cultured in the medium containing 3 mg l⁻¹ BAP with 0.1 ppm NAA as the hormonal source, it resulted in an average of 5 multiple shoots/explant. In order to enhance the number of multiple shoots, higher dosage of NAA at 0.25 mg l⁻¹ was tried in this experiment with different levels of BA. Similar observations both in terms of the morphological behaviour of the explant at

10 days after culture and the number of multiple shoots produced at 30 days after culture were recorded and are presented in (Table 4.10 and 4.11).

Observations recorded 10 days after incubation indicated that the elongation of the main axis was more in treatments with 1,2,5,7 and 8 mg l⁻¹ BA with 0.25 mg l⁻¹ NAA compared to basal medium. However, the elongation of the main axis was relatively less compared to the previous experiment 4.3 in which 0.1 mg l⁻¹ NAA was used.

Interestingly when the concentration of NAA was 0.25 mg l⁻¹ in the medium, the length of the side branches from the main axis was relatively more compared to the length of the side branches when NAA was 0.1 mg l⁻¹.

In presence of 0.25 mg l⁻¹ NAA the stem thickness, number of axillary buds per explant also did not vary significantly. The number of axillary buds produced on the main axis increased in the treatments with 1,0 mg l⁻¹ BA and 0.1 mg l⁻¹ NAA compared to the basal media. Further increase in BA concentration in presence of 0.25 mg l⁻¹ NAA was not effective in increasing the number of axillary buds produced on the main axis. On an average the number of axillary buds produced per explant was one when BA present was from 1-8 mg l⁻¹.

4.8. INFLUENCE OF VARYING CONCENTRATIONS OF AUXIN AND CYTOKININ IN THE MEDIUM ON MULTIPLE SHOOT PRODUCTION FROM EXPLANTS DERIVED FROM MATURE TREES

It is a well established fact that the tree species are highly heterozygous in nature. In order to maintain the genetic purity of the species and also to

Table 4.10. Effect of different concentrations of BA alongwith increased NAA on the growth of apical bud
(Morphological observation after 10 days of growth in the medium)

MC+SB (BA + NAA) mg l ⁻¹	Elongation of stem tip	Score %	Stem thickness	No. of axillary buds	Opening of leaf- lets	Length of side branch
1) 0	+	80	+	1	Partial	++
2) 1 + 0.25	++	90	+	1	“	+++
3) 2 + 0.25	++	100	+	1	“	+++
4) 3 + 0.25	+	100	+	1	“	++
5) 4 + 0.25	+	80	+	1	“	++
6) 5 + 0.25	++	100	+	1	“	+++
7) 6 + 0.25	+	100	+	1	“	++
8) 7 + 0.25	++	100	+	1	“	+++
9) 8 + 0.25	++	100	+	1	“	+++

Elongation : + = 10.0 mm
 ++ = 20.0 mm
 +++ = 30.0 mm

Stem thickness : + = 1.0 mm
 ++ = 1.5 mm
 +++ = 2.0 mm

Table 4.11 Effect of different concentrations of BA alongwith increased NAA on multiple shoot production and axillary bud production

Treatments (B with BA + NAA)	Explants inoculated	No. of axillary buds produced	Buds/ explant	No. of multiple shoots produced
1) 0	12	6	0.5	0
2) 1 + 0.25	12	12	1	0
3) 2 + 0.25	12	12	1	0
4) 3 + 0.25	12	12	1	0
5) 4 + 0.25	12	12	1	0
6) 5 + 0.25	12	12	1	0
7) 6 + 0.25	12	12	1	0
8) 7 + 0.25	12	12	1	0
9) 8 + 0.25	12	12	1	0

CD (5%) : 0.7541

F.Test : NS

Plate 5: Effect of higher NAA level on the multiple shoot production from mesocotyl explants.

C=B₅

1=B₅ + 1 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA

2=B₅ + 2 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA

3=B₅ + 3 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA

4=B₅ + 4 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA

5=B₅ + 5 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA

6=B₅ + 6 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA

7=B₅ + 7 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA

8=B₅ + 8 mg l⁻¹ BA + 0.25 mg l⁻¹ NAA



avoid segregation it was designed to select the explant source directly from the matured trees for *in vitro* experimentation. Young axillary buds of uniform size from matured tree of *Acacia auriculiformis* were selected as explant source. The explants were treated with Absolute alcohol followed by 0.1% Bavestin and 0.1% Mercuric chloride. The B₅ medium was supplemented with different concentrations of BA ranging from 1,2,3,4,5,8 and 10 mg l⁻¹ in combination with 0.1 mg l⁻¹ NAA. The inoculated tubes were incubated.

Periodical observations were made at 15 days interval upto 60th day after incubation. The results indicated that none of the treatment combinations were effective in inducing multiple shoot production in axillary buds selected from matured trees.

Since the axillary buds from the trees did not respond for multiple shoot production, the buds were scouped out from the trees directly and inoculated in the medium with different combinations of growth regulators as given in materia-ls and methods.

Observations recorded at 15 days interval upto 60 th day after incubation indicated that even maintaining the bud in close contact with the inoculation media also is not effective in inducing growth and multiple shoot production of the buds. This further confirmed that explants from mature trees generally do not respond to multiple shoot production.

4.9. RESPONSE OF *IN VITRO* GROWN AXILLARY BUDS ON MS MEDIA SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF GROWTH REGULATORS

The results obtained in the experiment 4.3 indicated that B₅ medium when supplemented with 3 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA gave good response when the explant source was selected from the *in vitro* grown seedlings. In order to see the response of *in vitro* grown axillary buds selected from elongated mesocotyl and its branches, they were inoculated on MS medium containing high levels of inorganic salts and supplemented with different concentrations of BA along with NAA and 2,4-D. Observations were recorded 20 days after inoculation, on multi-ple shoots produced/inoculum and their length.

All the treatments were effective in inducing multiple shoot production from the axillary buds except on basal medium (control). Amongst the auxin sources used, NAA was more effective in inducing more number of multiple shoots compared to 2,4-D. Amongst the growth regulator combinations, maximum number of multiple shoots of 10/explant was observed in the treatment with 1.0 mg l⁻¹ BA + 0.1 mg l⁻¹ NAA (Table 4.12; Plate 6).

The maximum length of the multiple shoot was recorded at 40 days after inoculation. The data indicated that in presence of 1.0 mg l⁻¹ BA with 0.1 mg l⁻¹ NAA the average length was approximately 5.2 cm.

Table 4.12. Response of *in vitro* grown axillary buds on MS medium obtained after 40 days

	Treatments mg l ⁻¹	No. of explant inoculated	No. of Multiple shoots produced	Multiple Shoot/ explant	Shoot Length
1.	A=Control	10	0	0	0
2.	B=1 BA + 0.1 NAA	10	100	10	5.2
3.	C=2 BA + 0.1 NAA	10	80	8	4.3
4.	D=1 BA + 0.1 2,4-D	10	60	6	2.1
5.	E=2 BA + 0.1 2,4-D	10	70	7	3.5
For No. of multiple shoots			For shoot Length		
CD (5%) = 1.16			CD (5%) = 1.12		
F.test = **			F test = **		

Plate 6: Response of in vitro grown axillary buds on MS media supplemented with different concentrations of auxins and cytokinins.

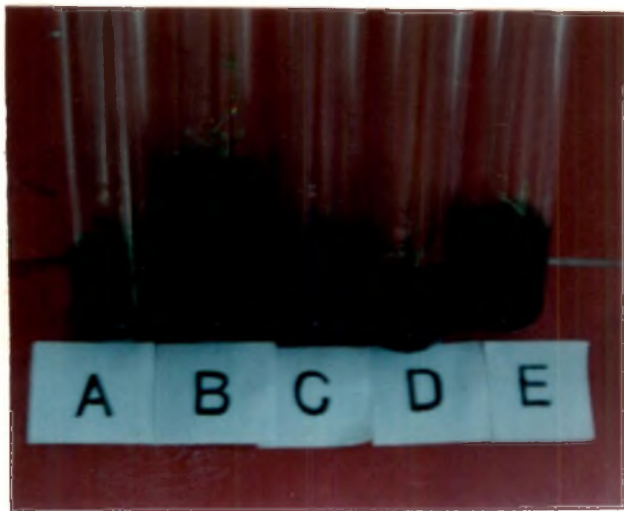
A= CONTROL

B= 1 mg l^{-1} BA + 0.1 mg l^{-1} NAA

C= 2 mg l^{-1} BA + 0.1 mg l^{-1} NAA

D= 1 mg l^{-1} BA + 0.1 mg l^{-1} 2,4-D

E= 2 mg l^{-1} BA + 0.1 mg l^{-1} 2,4-D



4.10. RESPONSE OF MESOCOTYL EXPLANT ON MS MEDIA SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF AUXIN AND CYTOKININ

The results obtained in the the earlier experiment, indicated that B₅ medium when supplemented with 3.0 mg l⁻¹ BA and 0.1 mg l⁻¹ NAA gave maximum number of multiple shoots when mesocotyl was used as the explant. The number of multiple shoots obtained on B₅ medium was 5 per explant. The experiment is designed to see the effect of high inorganic salt medium on the production of multiple shoot per explant. MS medium with high inorganic salt source was supplemented with different concentrations of BA alone and BA along with 0.1 mg l⁻¹ NAA and the mesocotyl explants were inoculated.

The results presented in Table 4.13, clearly indicated that though there was production of multiple shoots on the medium containing BA alone than that of the medium containing a combination of BA and NAA, however, the multiple shoots were not as healthy as those produced on B₅ medium (Plate 7).

4.11. EFFECT OF DIFFERENT SOURCES OF CYTOKININS IN MS MEDIUM ON MULTIPLE SHOOT PRODUCTION IN EXPLANTS FROM *IN VITRO* GROWN SHOOTLETS

In the earlier experiment 4.6, it was observed that the explant on MS medium showed comparatively better result than that of B₅ medium in the production of multiple shoots from the *in vitro* grown axillary buds. The present experiment is designed to select the potent cytokinin for maximum number of multiple shoots on MS medium. The MS medium was

Table 4.13. Response of mesocotyl explants on MS medium supplemented with different concentrations of auxin and cytokinin

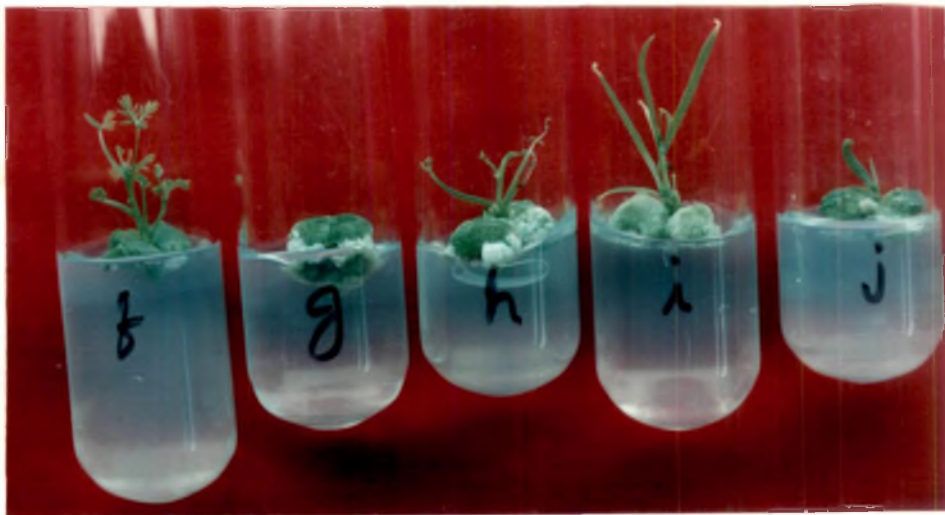
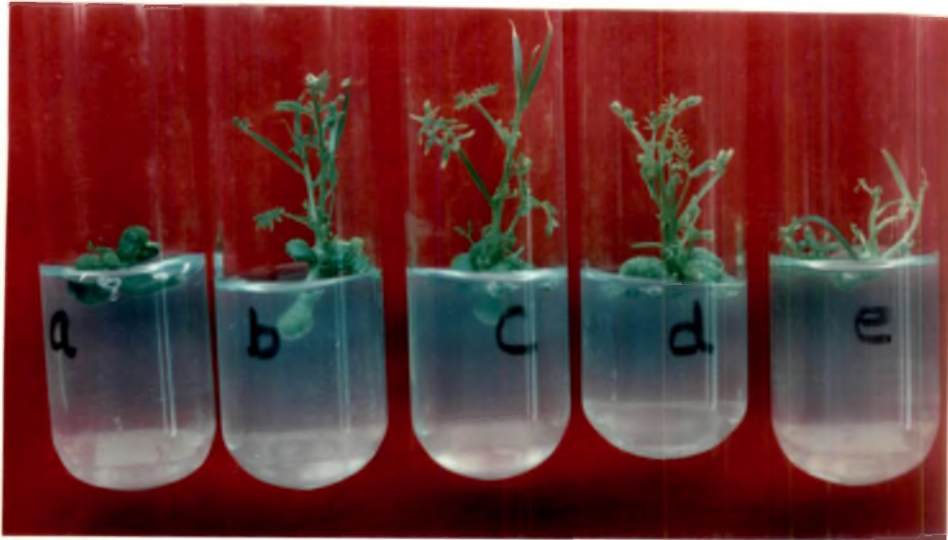
	Treatments mg l ⁻¹ BA + 1 NAA	Explant inoculated	Length of Axillary buds produced	Buds/Explant
1.	MS + 0	5	10	2
2.	MS + 1 BA	5	10	2
3.	MS + 2 BA	5	10	2
4.	MS + 3 BA	5	20	4
5.	MS + 4 BA	5	10	2
6.	MS + 5 BA	5	5	1
7.	MS + 1 + 0.1	5	-	-
8.	MS + 2 + 0.1	5	10	2
9.	MS + 3 + 0.1	5	10	2
10.	MS + 4 + 0.1	5	5	1

CD (5%) : 0.3813

F test : *

Plate 7: Response of mesocotyl explants on MS medium supplemented with different concentrations of auxin and cytokinins.

- a: MS + 0
- b: MS + 1 mg^l⁻¹ BA
- c: MS + 2 mg^l⁻¹ BA
- d: MS + 3 mg^l⁻¹ BA
- e: MS + 4 mg^l⁻¹ BA
- f: MS + 5 mg^l⁻¹ BA
- g: MS + 1 mg^l⁻¹ BA + 0.1 mg^l⁻¹ NAA
- h: MS + 2 mg^l⁻¹ BA + 0.1 mg^l⁻¹ NAA
- i: MS + 3 mg^l⁻¹ BA + 0.1 mg^l⁻¹ NAA
- j: MS + 4 mg^l⁻¹ BA + 0.1 mg^l⁻¹ NAA



supplemented with different sources of cytokinins i.e., BA, Kinetin and Adenine sulphate with different concentrations. Two types of explant sources i.e., axillary bud and internodal segments were selected from the *in vitro* grown multiple shoots and inoculated.

The periodical observations at intervals of 15 days indicated that the explants started showing response and the results were recorded after 60 days culture. Number of multiple shoots produced and number of adventitious shoots produced per explant at different sources of cytokinins were recorded and presented in Table 4.14 and 4.15, respectively (Plate 8a & 8b).

Among the two explant sources, axillary bud showed better response in terms of production of multiple shoots. Among different sources of cytokinins, maximum number of multiple shoots were recorded on MS medium supplemented with BA. Among the different concentrations of BA, 2.0 mg l⁻¹ BA gave maximum number of multiple shoots and this was followed by 3.0, 4.0, 5.0 and 1.0 mg l⁻¹ BAP. The results of the response of Kinetin and Adenine sulphate are not interpreted as these cytokinin sources did not influence the explant towards the production of multiple shoots.

The internoded explant behaved differently. In the earlier stages of observation it was noticed that there was slight callusing at the cut ends of the internode and later adventitious shoots were produced from the callused cells. Further, it was noticed that BA alone had given better result than other two sources of cytokinin, Kinetin and Adenine Sulphate, clearly

Table 4.14. Effect of different sources of cytokinin on MS media on multiple shoot production from *in Vitro* grown axillary bud

(Observation after 60 days)

Sl No.	Treatments (BA/kin/Ad SO ₃) (mg l ⁻¹)	No. of explants	No. of multiple shoot produced	Number of Multiple shoots per explant
1.	MS	5	1	1
2.	MS + 1	5	2	-
3.	MS + 2	5	20	4
4.	MS + 3	5	15	3
5.	MS + 4	5	10	2
6.	MS + 5	5	10	2
7.	MS + 8	5	5	1
8.	MS + 10	5	1	-

CD (5%) : 0.8154

F test : *

* The data interpreted represents the response of BA only.

Plate 8a: Effect of BA on MS media on multiple shoot production from *in vitro* grown axillary buds.

- a: MS + 0
- b: MS + 2 mg^l⁻¹ BA
- c: MS + 3 mg^l⁻¹ BA
- d: MS + 4 mg^l⁻¹ BA
- e: MS + 5 mg^l⁻¹ BA
- f: MS + 8 mg^l⁻¹ BA



Table 4.15. Effect of different sources of Cytokinin in MS media on multiple shoot production from *in vitro* grown internodes

(Observations after 60 days)

Treatments (BA/kin/AdSO ₄) (mg l ⁻¹)		No. of explants	No. of adventitious shoots produced	Number of Ad. Sh/explant
1.	MS	5	5	1
2.	MS + 1	5	2	0.4
3.	MS + 2	5	10	2
4.	MS + 3	5	10	2
5.	MS + 4	5	5	1
6.	MS + 5	5	15	3
7.	MS + 8	5	5	1
8.	MS + 10	5	2	0.4

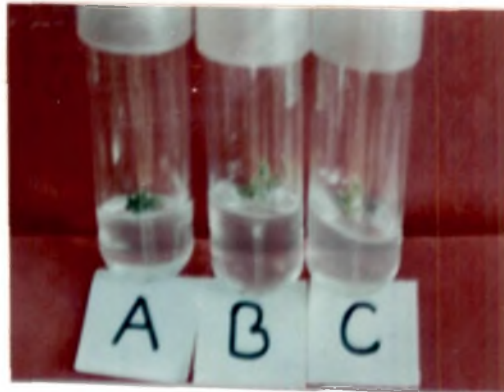
CD (5%) : 1.123

F test : *

* The data interpreted represents the response of BA only.

Plate 8b: Response of BA on MS media on the adventitious shoot production from *in vitro* grown internodes.

- a: MS + 0
- b: MS + 2 mg l⁻¹ BA
- c: MS + 3 mg l⁻¹ BA
- d: MS + 4 mg l⁻¹ BA
- e: MS + 5 mg l⁻¹ BA
- f: MS + 8 mg l⁻¹ BA



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suggesting that the explants of *Acacia auriculiformis* responded well in presence of BA as Cytokinin source for adventitious shoot production.

4.12. *IN VITRO* MAINTENANCE OF EXPLANT SOURCE FOR FUTURE GENERATION ON STANDARDISED MEDIUM

A comparison was made to see the influence of B₅ medium supplemented with 3.0 mg l⁻¹ BAP with 0.1 mg l⁻¹ NAA combination on the development of multiple shoots from explants of different generations. Explants taken from *in vitro* developed seedling during the I generation produced on an average of 5 multiple shoots per explant and the average length of the multiple shoot at the end of 30 days was approximately 3.2 cms. However, during the II generation (multiple shoots from the I generation were transferred to the same medium), the number of multiple shoots produced per explant was 10 and also the average length of the shoot was 5.5 cms. The values indicated that the production of multiple shoots were maximum when explants were transferred from *in vitro* grown multiple shoots to a fresh medium supplemented with same concentration of growth regulator (Table 4.16; Plate 9).

4.13. INDUCTION OF ROOTING IN *IN VITRO* GROWN SHOOTLETS

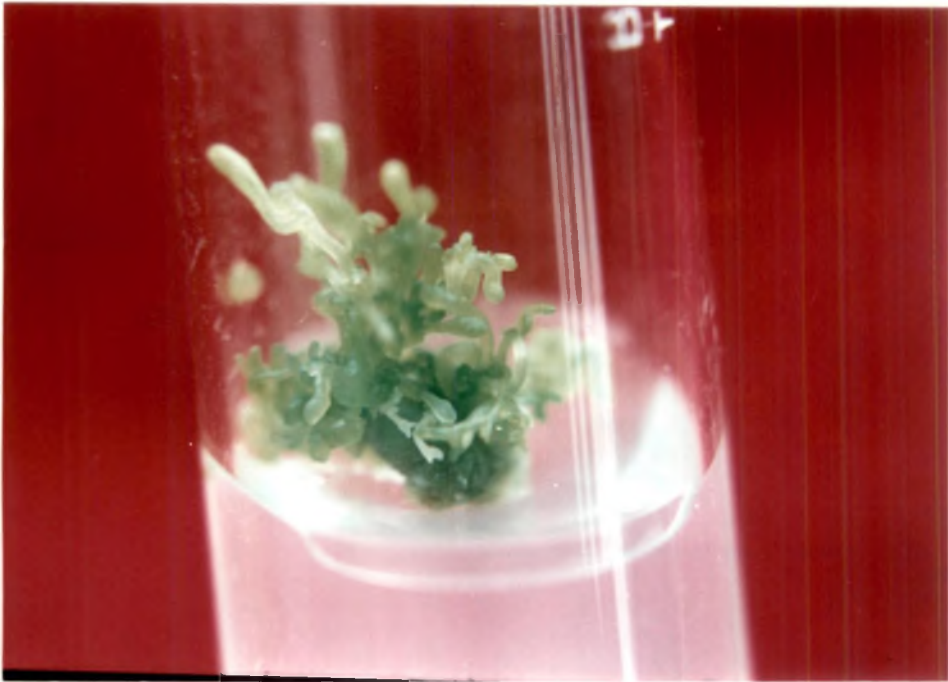
Half strength MS basal media has been found to be good for the induction of rooting in majority of the *in vitro* grown shootlets of tree species. Keeping this in background, the experment is designed to induce rooting on half strength basal medium supplemented with two sources of root inducing hormones that is IAA and NAA (0.1 and 0.25 mg l⁻¹ respectively). Uniform

Table 4.16. Response of explants from I and II generation on multiple shoot production

Treatments mg l ⁻¹	Explant inoculated	No. of explants inoculated	Multiple shoots produced	Shoots/ explant	Average shoot length
B ₅ + 3 BA + 0.1 NAA	MC+SB	12	62	5	3.083
B ₅ + 3 BA + 0.1 NAA	Shootlet	12	120	10	5.0
No. of shoots		Shoot length			
CD (5%) : 0.85		CD (5%) : 0.87			
F test : **		F test : *			

Plate 9: Response of explant from 1 generation on further multiple shoot production.

$B_5 + 3 \text{ mg l}^{-1} \text{ BA } 0.1 \text{ mg l}^{-1} \text{ NAA}$



length of shootlets measuring around 1.5 - 2 cm was selected and inoculated on the root inducing medium. The cultures were maintained at constant temperature and light.

The periodical observations indicated that there was initiation of root proliferation after a period of 60 days. Among the root inducing hormones tested, IAA at 0.25 mg l⁻¹ gave good response and this was followed by NAA at 0.1 mg l⁻¹. The rooted seedlings were maintained for further growth and acclimatization to *in vivo* conditions (Table 4.17; Plate 10).

Table 4.17. Influence of different sources of auxins on induction of root from developed shoots

Treatments mg l ⁻¹	Explants inoculated	No. of roots produced	Roots/explant
1) CONTROL	5	5	1
2) 1/2 MS + 0.1 IAA	5	10	2
3) 1/2 MS + 0.25 IAA	5	15	3
4) 1/2 MS + 0.1 NAA	5	10	2
5) 1/2 MS + 0.25 NAA	5	5	1

CD (5%) : 4.83
F test : *



Plate 10: Response of two auxins on root induction.

- A: $1/2$ MS + 0.1 mg l^{-1} IAA
- B: $1/2$ MS + 0.25 mg l^{-1} IAA
- C: $1/2$ MS + 0.1 mg l^{-1} IAA
- D: $1/2$ MS + 0.25 mg l^{-1} IAA
- E: Control.

DISCUSSION

V. DISCUSSION

Since prehistoric times, man depends on forests for timber as well as for fuel wood. In addition to these two major uses, a number of forest products also are used to satisfy the ever increasing demands of man for the raw materials for his other need based modern industries. However, the forest area in India is declining at an alarming rate and now it constitutes only about 14-19% of geographical area. To reduce the pressure on absolute forest in India, number of forestry programmes like social forestry, agroforestry etc. are being popularised. The selection of forest species for these programmes is aimed at proper selection of that the tree species which will satisfy the demands like timber, fuel, fodder and also other forest products. For successful afforestation programme, a continuous supply of healthy sapplings is a must under normal conditions to obtain uniform healthy transplantable sapplings at a large number is difficult. In addition to this, planting is normally done immediately after a good rainfall in rainy season and the sapplings are allowed to establish during rainy season for better survival. This indicates the need for a large number of well developed seedlings at a particular season for mass planting programme.

Tissue culture techniques are used for mass multiplication of crop and tree species. Vegetative propagation by micropropagation is useful because it helps in the maintenance of genetic characters. Successful multiplication using in vitro micropropagation technique through tissue culture is already standardised for many important crop and forest species (Bonga and Durzan, 1982; Ahuja and Muhs, 1985).

Acacia auriculiformis A. Cunn. ex Benth is a leguminous, nitrogen fixing tree. It belongs to the genera, *Racosperma* (Pedley, 1986) and subfamily *Mimosoideae*. This is most adoptable species for all kinds of tree planting programmes in tropical, humid and sub humid low land regions and *Acacia auriculiformis* is used as fuel wood, wood, green manure. Pulp of this, is used for the production of unbleached kraft pulp for bags, wrapping paper, liner board and high quality neutral sulphate semichemical pulp for corrugating medium and higher grade products. *Acacia auriculiformis* is also identified in agroforestry and in erosion control.

A natural hybrid of *Acacia auriculiformis* has been recently identified. This hybrid is found superior in respect of higher growth rate and good quality timber compared to *Acacia auriculiformis*. The maintenance of the superiority is possible only by adopting alternate propagation techniques. With this background, in this study, experiments were designed with the following objectives.

1. To study the physiological and biochemical characters of *Acacia auriculiformis*, *Acacia mangium* and their hybrid.
2. To standardise the explant source and culture media and culture conditions for the production of large number of multiple shoots of *Acacia auriculiformis*.
3. To standardise the rooting media for the production of good root system for trans planting and further hardening.

4. To develop of a protocol for mass multiplication by tissue culture of *Acacia auriculiformis* and extending this with minor modification for the mass multiplication of *Acacia auriculiformis* hybrid will help in developing a protocol for the micropropagation of natural hybrid.

5.1. PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERS OF THE NATURAL HYBRID AND ITS PARENTS

The parents of natural hybrid is believed to be *Acacia auriculiformis* and *Acacia mangium*. In the present study, a few physiological and biochemical parameters were studied in these three species. The hybrid is taller as compared to its parents. Similarly the volume of the wood of one and a half year old trees were 0.064 cft, 0.207 cft and 0.035 cft for the species *Acacia auriculiformis*, hybrid and *Acacia mangium*, respectively. This clearly indicates that hybrid is superior over its parents in the production of wood. The leaf size and leaf area of the individual leaf of hybrid was in intermediate between *Acacia auriculiformis* and *Acacia mangium*. The soluble protein content and chlorophyll content of leaf ranges from 1.349 mg/gm. fresh weight to 3.167 mg/gm fresh weight in this species and the maximum value was obtained in *Acacia auriculiformis* followed by the hybrid and the lowest value was in *Acacia mangium*. These set of data clearly indicate the superiority of the growth and the volume of the wood produced. However, in other physiological characters the hybrid was intermediate between the two parents.

5.2. STANDARDISATION OF EXPLANT SOURCE AND CULTURE MEDIA

With an objective to obtain large number of plants from the established tree species, growth regulators were applied to the detopped branches to induce bud growth so that such buds can be used as explant sources. Different concentrations of auxins, cytokinins and gibberellins were used for swabbing the detopped branch to cover about eight buds from the detopped end. None of these growth regulator combinations was effective in induction of buds and their growth more compared to the untreated branch. However, branches treated with a combination of 10 ppm NAA, 10 ppm BA and 0.2 ppm GA was effective in inducing bud elongation to a larger extent compared to other treatments. However, a few of the elongated buds died of in this treatment. This suggests that the above said treatment is effective in inducing the growth of the bud only. However, it seems such buds must be used immediately after higher elongation rate to obtain the maximum response.

With an objective to use the axillary buds directly from the matured trees as an explant source, buds measuring from 0.5-1.5 cms. were excised and cultured over B₅, MS and WPM medium. Buds did not show any response either in terms of growth and multiple shoot production even 30 days after inoculation indicating that axillary buds from matured trees is not a good source for multiplication. While attempting mass multiplication by micropropagation, a number of earlier workers suggested that the response of the tissue for multiple shoot production as well as growth in the culture medium reduces with the age of the plant. Rao et al., (1977) were successful

in getting shoot buds from the hypocotyl segments from seedlings of *Santalum album* grown *in vitro*. But stem segments from adult trees did not respond. Multiple shoots were induced from both explants from field grown and *in vitro* grown Guava and it was observed that number of shoots produce was more in *in vitro* grown plants (Mohammad *et.al.*, 1987). Shoot apices of Jack from two month old seedling or six month old grafted plants and from fresh stem shoots of 5, 10 and 30 years old trees when cultured, maximum multiplication rate was observed in shoot apices from two to six month old seedlings (Rajmohan *et.al.*, 1988). Similar type of result was obtained by Rodriguez *et.al.*, (1988) when nodal segments from embryogenic material of 45 days old seedlings and two year old adult tree were cultured. Nodal segments from 45 days old seedlings responded better than that from the tree. Timirbaran *et al.*, (1992) were successful in getting highest number of micro-shoots per explant (35.12) from cotyledonary nodes and shoots tips of germinated seed lings rather than nodal explants of field grown Tea plants. However, a number of workers were successful in obtaining response even when explants from matured trees were used. Nadgir *et.al.* (1984) reported the production of multiple shoots from nodal segments of mature trees of *Dendrocalamus strictus*, *Bambusa arundinacea* and *B.vulgaris*. Better response was obtained with nodes from root suckers of mature *Acacia albida* trees when Gassama *et.al.* (1987) tried with nodes from both matured and juvenile plants. Yadav *et.al.* (1991) were successful in getting rooted plants of *Morus nigra* from nodal explants of 12 year old mulberry tree. Similar type of result was obtained by Singh *et.al.* (1993) in case of mature trees of *Acacia mlotica*. Rao K.S. (1988), achieved, rapid multiplication of axillary

meristems and direct shoot development from nodal explants of matured *Eucalyptus tereticornis* Sm. Micropropagation of *Frazinus angustifolia* was successfully achieved both from mature and juvenile plant material using shoot tip and nodal explants by Perez *et.al.* (1994). Buds from 15 year old trees of *Tilia cordata* resulted in best shoot development (Young *et.al.*, 1994).

Seeds of *Acacia auriculiformis* germinated on half strength MS medium and from 14 days old seedling, mesocotyl, mesocotyl with shoot bud, internode and shoot bud alone were used as explant source and inoculated on B₅, MS and WPM. Amongst the explant sources, mesocotyl with a shoot bud was highly responsive in growth and production of axillary buds compared to other explant sources. The next best explant was shoot bud. All other explant sources showed poor response. Amongst the three culture media used in the experiment, the growth of the explant and the production of axillary buds was more in B₅ medium closely followed by MS. The response of these explants to WPM was very poor even 30 days after inoculation.

These results, indicated that the best explant sources is mesocotyl with a shoot bud and in all other explants, mesocotyl with a shoot bud and axillary buds were used as the explant source. Among the various explants used hypocotyls from young seedlings resulted in best response interms of multiple shoot production. Vegetative propagation of sandal wood was carried out using hypocotyl segments as the explants by Rao *et.al.* (1977). Tomar *et.al.* (1988) achieved in obtaining maximum shoots per explant from the hypocotyl segments of 10-12 day old seedlings of *Albizia amara*, *A.lucida* and

and *A.richardiana*, number of earlier reports indicated that apical bud followed by axillary bud can be used as the best explant source for mass multiplication of the tree species. Bruneau (1987) reported that axillary buds taken from clones of *Paulownia tomentosa*, *P.elongata*, *P.tortumii* and *P.coreana* resulted in the production of multiple shoots when cultured. Best results were obtained when Gassama *et.al.* (1987) tried with the axillary buds from stool shoots and root suckers of mature *Acacia albida* trees. *In vitro* mass propagation of *Lagerstroemia indica* f.alba Pender was successfully carried out by Lee *et.al.* (1987) through the culturing of axillary buds. Best shoot development was observed by Youn *et.al.* (1988) when the axillary buds of matured *Lilia cordata* were cultured. Kim *et.al.* (1988) achieved micropropagation of Jujuba cultivars through axillary bud culture. Multiple shoot production from axillary buds of *Acacia auriculiformis* was achieved by Aradhana *et.al.* (1989).

5.3. STANDARDISATION OF GROWTH REGULATOR COMBINATION FOR MULTIPLE SHOOT PRODUCTION

Two sets of experiments were conducted to standardise the ratio of auxin and cytokinin requirements to induce maximum multiple shoot production from the mesocotyl explants. In the first experiment, at a fixed concentration of 0.1 ppm NAA, seven concentrations of BA were tested to see the response.

Observations recorded at both 10th and 30th day after inoculation indicated that cytokinin was effective in promoting the elongation of the bud as well as induction of multiple shoots. In general, with increasing

concentrations of BA, the growth of the bud as well as the thickness of the main axis increased, upto 2-3 ppm of BA. Although the elongation of the apical bud was more upto 5.0 ppm of BA, the stem thickness reduced with increase in BA concentrations after 3.0 ppm. The maximum number of axillary buds per explant was 3 on 10th day after inoculation in the treatment with 3.0 ppm BA with 0.1 ppm NAA which is most effective in inducing 5 multiple shoots per explant. Observations recorded on the morphology of the leaf also indicated that in the presence of BA at 1,2 and 3 ppm with 0.1 ppm NAA, the leaf showed full opening compared to other treatments.

In another set of experiments, the influence of various concentrations of BA with a fixed concentration of NAA of 0.25 ppm on bud growth and multiple shoots production was studied. Compared to the earlier experiments in which 0.1 ppm NAA was used, higher concentration of NAA of 0.25 ppm induced poor response in terms of the growth of the bud as well as multiple shoots production. In the presence of 0.25 ppm NAA in B_5 media with varying concentrations of BA, the growth of the main axis was less compared to the earlier experiments. Interestingly, the length of the branches from the main axis increased. However, there was no production of multiple shoots. These set of experiments clearly indicated that presence of 3 ppm BA with 0.1 ppm NAA was the best treatment to induce maximum growth and maximum number of multiple shoot production from *Acacia auriculiformis*.

In general, a higher ratio of cytokinin to auxin was effective in the induction of multiple shoots in many tree species. Even in other forestry species, a higher ratio of cytokinin to auxin resulted in the production of

large number of multiple shoots. For instance, in *Lagarstroemia indica* f. *alba*, BA and NAA concentration 0.05 ppm and 0.01 ppm respectively resulted in the production of multiple shoots from axillary bud as an explant source. Similar observations were made by the author Bruneau (1986) in *Paulownia*. Rajmohan *et al.* (1988) reported that the explants from in vitro grown seedlings which were cultured on establishment medium, were cultured on media containing 0.2 ppm NAA and BA at 5.0, 7.5, 10.0, 12.5, or 40.0 ppm. These treatments resulted in multiple shoot production.

Reports from Mathew *et al.* (1990) revealed that there was best multiple shoot induction from explants of *Syzygium aromaticum* seedlings in two media supplemented with 3.0 ppm BAP and 0.5 ppm NAA among all other combinations, in both the media.

5.4. STANDARDISATION OF GROWTH REGULATOR CONCENTRATION FOR MASS MULTIPLICATION FROM AXILLARY BUD EXPERIMENTS OF MATURED TREES OF *ACACIA AURICULIFORMIS*

Earlier experiments conducted in this study clearly indicated that mesocotyl with a shoot bud was more effective in responding in terms of multiple shoot production when inoculated over B₅ medium supplemented with 3.0 ppm BAP with 0.1 ppm NAA. With an objective to get multiple shoot production from explant obtained from matured trees, axillary buds were selected and inoculated to B₅ medium supplemented with 0.1 ppm of NAA and varying concentrations of BA from 1 to 10 ppm was not effective in inducing multiple shoots from the axillary buds of matured trees.

In another experiment the concentration of NAA in the media was increased to 0.25 ppm and the influence of varying levels of BA on the multiple shoot production from the excised axillary buds were studied. Even increase in the NAA concentration by 2.5 times compared to earlier experiment was not effective in the induction of multiple shoot production from the axillary buds. This set of experiments indicates that axillary buds from mature trees do not respond to various concentration of auxins and cytokinins supplemented in B₅ media.

Bruneau (1987) reported that axillary buds from clones of *Paulownia tomentosa*, *P. elongata*, *P. fortunei* and *P. coreana* resulted in multiple shoot production. When Gassama *et.al.* (1987) tried with the axillary buds from stool shoots and root suckers of mature trees, axillary buds from root suckers of mature trees had best response. It as reported by Youn *et.al.* (1988) that buds from matured trees of *Tilia cordata* when cultured on three different media with different concentrations of BA resulted in the best shoot development.

The average length of shootlets produced 30 DAI was relatively more when MS medium was supplemented with 1 ppm BA and 0.1 ppm NAA closely followed by treatment of 2 ppm BA and 0.1 ppm NAA. These results suggest that young developing *in vitro* grown axillary buds are highly responsive to multiple shoot production.

When axillary buds from *in vitro* developed main axis was used as the explant sources, a large number of multiple shoots were developed. Enthused

by these results, the response of internode segments as well as axillary buds on the main axis were used as explant sources. With an objective to increase the rate of multiple shoot production, the axillary buds and internode segments produced *in vitro* from mesocotyl explants were further inoculated on MS medium with different concentrations of cytokinin. Kim *et.al.* (1988), reported that axillary buds from *in vitro* grown *Zyzyphus* cv. showed best shoot and root growth. Aradhana *et.al.* (1988) also reported the development of plantlets from axillary buds of *in vitro* grown *Acacia auriculiformis* seedlings.

In this study, the different levels of cytokinins used were BA, Kinetin and Adenine sulphate with concentrations 0, 1, 2, 3, 4, 5, 8 and 10 ppm without auxin. Both the explant sources were not effective in the production of multiple shoots even 30 DAI. However, axillary bud explants showed a marked elongation indicating that it is a better explant compared to internode. This data also indicated that the medium supplemented only with cytokinin is not effective in the induction of multiple shoots. It also indicates that BA is the potent cytokinin. It was reported by Umer *et.al.*(1993), that successful multiple shoot production was induced from explants of *Chloroilon switenia* (BC) in a medium supplemented with BA among the three cytokinins used (BA, Kinetin and Adenine sulphate).

Data from all the experiments tend to conclude that,

1. Mesocotyl with a shoot bud from *in vitro* grown seedlings respond best in B₅ medium when supplemented with 3.0 mg l⁻¹ and 0.1 mg l⁻¹ NAA.

2. Axillary buds from *in vitro* grown shootlets respond better to MS medium when supplemented with 1.0 mg l⁻¹ BA and 0.1 mg l⁻¹ NAA. [S

Multiple shoots with a length of more than 5 mm or 1 cm were transferred to rooting for the induction of roots. The other shootlets which were less than 1 cm height were again transferred to B₅ medium supplemented with 3.0 mg l⁻¹ BA and 0.1 mg l⁻¹ NAA. The objective of the experiment is to study the multiplication ratio of mesocotyls segments obtained from *in vitro* grown seedlings and multiple shoots obtained from these mesocotyl segments. In other words, this experiment gives the relative response of two generation of *in vitro* grown explants over one generation grown explants. The result indicated that multiple shoots from *in vitro* grown mesocotyls respond better. The conclusion from this experiment is that the response of second generation explants is more than compared to the first generation explant in multiple shoot production.

5.5. STANDARDISATION OF CULTURE MEDIUM FOR THE INDUCTION OF ROOTING

Shootlets or multiple shoots of length more than 1 cm produced *in vitro* were transferred to half strength MS medium incorporated with two auxin sources of two concentrations each (0.1 mg l⁻¹ and 0.25 mg l⁻¹). Among the two auxins tested, IAA and NAA, IAA was found effective at concentration of 0.25 mg l⁻¹ in the production of more number of roots. However, 60 days were needed to produce good root system so that the plants can bear the shock during hardening. It was reported by Paramjit *et.al.* (1982), that successful rooting of the shoots of *Albizia lebback* L. were achieved with

2 mg l⁻¹ of IAA. Hisajima *et.al.* (1987) achieved rooting of *Leucana leucocephala* at 5 µM IAA. Ramesh *et.al.* (1990) transferred shoots grown in the medium containing IAA at 6 µM. for two weeks and successfully achieved tap root production.

Using these protocols, axillary buds from one year old sapplings of hybrid were selected and inoculated over B₅ medium supplemented with 3 ppm BA and 0.1 NAA. The result indicated that the apical meristem respond to a medium in the production of 5-6 multiple shoots at the end of 30 days after inoculation.

SUMMARY

VI. SUMMARY

Experiments were conducted to standardise the protocols for *in vitro* micropropagation of *Acacia auriculiformis* and to extend this for mass multiplication of natural hybrid of *Acacia auriculiformis*. Preliminary studies conducted for understanding the differences of a few physiological and biochemical characters between *Acacia auriculiformis* and *Acacia mangium* and their putative hybrid indicated that the hybrid shows very high growth rate compared to their parents. Chlorophyll content and soluble protein content of hybrid was in between the parents, *Acacia auriculiformis* and *Acacia mangium*. Leaf chlorophyll content and protein content per unit weight of the leaf was maximum in *Acacia auriculiformis*.

Application of mixtures of growth regulator to the branches of established trees to induce bud growth to use it as an explant in micropropagation were not effective.

Standardisation of the best explant source for micropropagation indicated that amongst the explant sources used like cotyledon, shoot bud, mesocotyl with shoot bud, mesocotyl, mesocotyl with shoot bud obtained from *in vitro* grown seedlings was found to be highly responsive and more fruitful for continuous maintenance of growth.

Studies to standardise culture media for growth and multiple shoot production indicated that among B₅, MS and WPM, B₅ used was superior when mesocotyl with shoot bud was used as an explant source. MS medium

was also found to be better for increasing growth and its maintenance. A series of experiments were conducted to standardise the best combination of auxins and cytokinins for the induction of multiple shoot production from mesocotyl with shootbud in B_5 medium, indicated that BA at 3 mg l^{-1} with 0.1 mg l^{-1} NAA was effective in inducing maximum number of multiple shoots per explant. However, multiple shoot production was observed even at higher concentration of BA upto 6 mg l^{-1} (3, 4, 5 and 6).

Experiments conducted with an objective to increase the number of multiple shoots by using 0.25 mg l^{-1} NAA instead of 0.1 mg l^{-1} NAA with different levels of cytokinin were not effective in inducing multiple shoot production. The results of these experiments clearly suggested that mesocotyl with shoot bud on B_5 medium supplemented with 3 mg l^{-1} BA and 0.1 mg l^{-1} NAA induces on an average of 5 shoots per explant. Using this growth regulator concentration, matured axillary buds from established or adult trees were inoculated with an objective to induce multiple shoots. However, buds from matured trees did not respond to these treatments. Even varying cytokinin sources were not effective in inducing multiple shoot production.

Efforts to increase the multiplication ratio of axillary buds developed *in vitro* from the mesocotyl with a shoot bud were excised and inoculated to MS medium supplemented with two sources of auxin, NAA and 2,4-D with 1 and 2 mg l^{-1} of BA. Maximum number of multiple shoots from the explant were observed when MS medium was supplemented with 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. It was also interesting that the average number of shoots produced were 10 per explant.

Axillary buds and internodes from *in vitro* grown shootlets were used as explants and their response on MS medium supplemented with three sources of cytokinin namely BA, Kinetin and Adenine Sulphate were tested. Amongst these three sources, multiple shoot production was maximum from axillary buds on medium supplemented with BA at concentrations 0, 2, 3, 4, 5 and 8 mg l⁻¹. These results indicated that Cytokinin supplied in the form of BA is the potent source of Cytokinin for multiple shoot production.

Subculturing of multiple shoots less than 10 mm in a medium incorporated with 3 mg l⁻¹ BAP & 0.1 mg l⁻¹ NAA were effective in the production of multiple shoots on B₅ medium. This again confirms that the above medium can be used for subculturing the explants for multiple shoot production.

Shootlets more than 10 mm were transferred to rooting media i.e. half strength MS medium with two sources of auxins at two concentrations. Among the two auxins tested, IAA was found to be optimum at a concentration of 0.25 mg l⁻¹ for the induction of rooting.

Salient findings from this study are:

1. The best explant source is mesocotyl with shoot bud and axillary bud.
2. The best media for mesocotyl with shoot bud is B₅ and MS is the best media for axillary bud.
3. The optimum growth regulator for best multiple shoot production was 3 mg l⁻¹ of BAP with 0.1 mg l⁻¹ of NAA for mesocotyl with shoot bud explant source over B₅ medium.

4. Axillary buds from matured trees were found to be irresponsive
5. The proliferation media is nothing but MS media supplemented with 1 & 2 mg l⁻¹ of BAP with 0.1 mg l⁻¹ of NAA was responsible in multiple shoot production from axillary buds collected *in vitro* grown seedlings.
6. Subculturing in B₅ media supplemented with 3 mg l⁻¹ BAP & 0.1 mg l⁻¹ NAA was effective. So this treatment has been confirmed for multiple shoot production from mesocotyl with shoot bud as explant source.
7. Prominent roots were observed in media supplemented with 0.25 mg l⁻¹ IAA followed by 0.1 mg l⁻¹ of NAA & IAA.

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