TISSUE CULTURE STUDIES IN TAMARIND

(*Tamarindus indica* L.),

A LEGUMINOUS TREE SPECIES

URMIL J. MEHTA

NOVEMBER, 2001

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(*Tamarindus indica* L.),

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A THESIS

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BY

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Dedicated

to my

husband, Dr. J.K. Mehta

and children,

Akshay, Shikha, Shilpa and Shruti

CONTENTS

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(Mrs. Urmil J. Mehta)

Date :
Place: Pune

CERTIFICATE

This is to certify that the work incorporated in the thesis entitle **" Tissue Culture Studies in Tamarind (*Tamarindus indica* L.), a leguminous tree species "**submitted by Mrs. Urmil J. Mehta was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. Mrs. Sulekha Hazra)
Guide

Pune

Date:

Key to abbreviations

°C	Degree Celsius
cv.	Cultivars
v/v	Volume/volume (concentration)
w/v	Weight/ volume (concentration)
ANOVA	Analysis of variance
s.d.	Standard deviation
$HgCl_2$	Mercuric Chloride
MS	Murashige and Skoog medium (1962)
PGR KN	Plant Growth Regulator Kinetin (6-furfuryl amino purine)
BAP	6-Benzyl amino purine
Z	Zeatin
2iP	6-γ,γ-dimethylallylaminopurine
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
NAA	α-Naphthaleneacetic acid
GA_3	Gibberellic acid
PVP	Polyvinyl pyrrolidone
Dicamba	3,6-dichloro-o-anisic acid
2,4-D	2,4-di chlorophenoxy acetic acid
2,4,5-T	2,4,5-trichlorophenoxy acetic acid
Picloram	4-amino-3,5,6-trichloro picolinic acid
IAA	Indole acetic acid
IBA	Indole butyric acid
IPA	Indole propionic acid
ABA	Abscissic acid
TBA	Tertiary butyl alcohol (2-methyl propan-2-ol)
DPX-4 mountant	[189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)]

MOLARITY CONCENTRATIONS OF PLANT GROWTH REGULATORS USED

NAME	Mol. Wt.	Mg.l ^{1}	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	2.0	5.0	10.0	20.0
2-iP	203.25	ìM	0.49	0.98	1.48	1.97	2.46	2.95	3.44	3.94	4.43	4.92	9.84	24.60	49.20	98.40
BAP	225.26	ìM	0.44	0.89	1.33	1.78	2.22	2.66	3.11	3.55	4.00	4.44	8.88	22.20	44.39	88.79
KN	215.22	ìM	0.46	0.93	1.39	1.86	2.32	2.79	3.25	3.72	4.18	4.65	9.29	23.23	46.46	92.93
Z	219.25	ìM	0.46	0.91	1.37	1.82	2.28	2.74	3.19	3.65	4.10	4.56	9.12	22.81	45.61	91.22
TDZ	220.20	ìM	0.45	0.91	1.36	1.82	2.27	2.72	3.18	3.63	4.09	4.54	9.08	22.71	45.41	90.83
NAA	186.21	ìM	0.54	1.07	1.61	2.15	2.69	3.22	3.76	4.30	4.83	5.37	10.74	26.85	53.70	107.41
IAA	175.20	ìM	0.57	1.14	1.71	2.28	2.85	3.42	4.00	4.57	5.14	5.71	11.42	28.54	57.08	114.16
2,4-D	221.04	ìM	0.45	0.90	1.36	1.81	2.26	2.71	3.17	3.62	4.07	4.52	9.05	22.62	45.24	90.48
2,4,5-T	255.49	ìM	0.39	0.78	1.17	1.57	1.96	2.35	2.74	3.13	3.52	3.91	7.83	19.57	39.14	78.28
PIC	241.46	ìM	0.41	0.83	1.24	1.66	2.07	2.48	2.90	3.31	3.73	4.14	8.28	20.71	41.41	82.83
DICAMBA	221.00	ìM	0.45	0.90	1.36	1.81	2.26	2.71	3.17	3.62	4.07	4.52	9.05	22.62	45.25	90.50
IBA	203.24	ìM	0.49	0.98	1.48	1.97	2.46	2.95	3.44	3.94	4.43	4.92	9.84	24.60	49.20	98.41
GA_3	346.40	ìM	0.29	0.58	0.87	1.15	1.44	1.73	2.02	2.31	2.60	2.89	5.77	14.43	28.87	57.74
ABA	264.32	ìM	0.38	0.76	1.13	1.51	1.89	2.27	2.65	3.03	3.40	378	7.57	18.92	37.83	75.67

ABSTRACT

Trees are complex biological systems in themselves and they are part of an even more complex system – the forest ecosystem. As trees grow and reproduce, they interact in many ways with other biological systems and with the environment. The importance of trees in maintaining our ecosystem and their importance in the world economy, need no emphasis. The global demand for wood products and wood as source of energy and raw material for various industries are increasing with the ascent in world population. With industrialization of the developing countries like India, the natural forests are disappearing rapidly. To increase the forest cover major efforts are directed towards afforestation of the denuded and barren lands, commonly known as wastelands. The role of biotechnology and tissue culture lies in enhancing the production of superior planting material. Tree biotechnology has to be an integral part of an overall forest genetics and tree-breeding program. One of the most important things that biotechnology can offer is to cut the long periods required for tree improvement using conventional technology. The trees thus grown through organized program will not only support the ecosystem but also serve as the source of raw material for industries and renewable resource of energy.

Leguminous tree species are well known for their utility to mankind. They are of special interest due to their economic and ecological importance. These trees are particularly considered in afforestation and soil erosion control programs due to their ability to fix atmospheric nitrogen.

Survey of existing literature reveals that although research in tree tissue culture was initiated earlier, research in the area of tree legumes started only two decades ago. Keeping in view the recalcitrant nature of the leguminous tree species, standardization of protocols for *in vitro* morphogenesis remains a challenging proposition. In addition to the advantages shared with conventional vegetative propagation, *in vitro* culture has a number of associated potential and real applications that are unique. These include protoplast culture and fusion for generation of somatic hybrids, *in vitro* screening, generation of useful somaclonal variants, generation of artificial seeds, and long-term storage of germplasm using cryopreservation. Finally, *in vitro* culture currently provides the only route for generation of genetically engineered genotypes of forest trees. Thus much efforts are needed before a model system is realized for the utilization of the various biotechnological approaches to aim for genetic improvement of leguminous tree species. With this background, the present study was conducted in tamarind (*Tamarindus indica* L.) to standardize tissue culture systems for micropropagation and *in vitro* morphogenesis which fall into three broad categories: axillary shoot (or bud) multiplication, organogenesis, and somatic embryogenesis.

Tamarind (*Tamarindus indica* L.), a tree legume, is a multipurpose monotypic genera. It is native to tropical Africa and grows widely in South East Asia and India. Apart from being economically important, due to its hardy nature and wide adaptability to various agroclimatic conditions, it is a suitable candidate for afforestation and soil reclamation/phytoremediation programs.

Very little work has been done on in vitro regeneration and clonal propagation of tamarind. Thus there is a scope to develop reliable protocols for *in vitro* regeneration of tamarind and study the basic processes involved in morphogenesis. The present study on tamarind was initiated with the following objectives:

1. Optimization of conditions for clonal propagation.

2. Optimization of conditions for *de novo* morphogenesis.
3. Optimization of conditions for somatic embryogenesis.

The thesis is divided into six chapters followed by summary and list of references.

CHAPTER 1: GENERAL INTRODUCTION

This chapter covers the current status of research on in vitro morphogenesis in tree legume and tamarind in particular. Influence of plant growth regulators in *in vitro* morphogenesis and methods of micropropagation are also discussed.

CHAPTER 2: MATERIALS AND METHODS

Common materials and methods implemented during the course of this work are described in this chapter. This includes the source of glassware, plasticware, chemicals, etc.; procedure involved in cleaning and preparation of glassware; preparation of media; surface sterilization of explants; histological methods etc. Materials and methods specific to individual experiment have been dealt with in respective chapters.

CHAPTER 3: CLONAL PROPAGATION

This chapter describes the clonal propagation of tamarind plant from:

- Shoot tip and cotyledonary nodes of seedling origin.
- The nodal bud explants obtained from mature trees.

Parameters studied include the effect of various concentrations and combinations of growth regulators and carbohydrates. The shoots obtained were subjected to rooting procedures using different auxins at various concentrations and combinations. The rooted plantlets were transferred to soil. Results of the experiments conducted on micrografting of shoots for improved survival in soil are also included.

CHAPTER 4: *DE NOVO* ORGANOGENESIS

Development of a highly reproducible protocol for regeneration of shoot buds from the mature zygotic embryo axis of tamarind is dealt with in this chapter. Histological studies were conducted to demonstrate the *de novo* origin. These adventitious caulogenic buds differentiated to form shoots. The shoots were rooted and plantlets recovered.

CHAPTER 5: INDUCTION OF SOMATIC EMBRYOGENESIS

This chapter describes the experiments conducted for induction of somatic embryogenesis from immature zygotic embryos. Effect of various growth regulators and carbohydrates at different concentrations were tested. Size dependent response in the explants, influence of culture vessels and different culture conditions were studied. Somatic embryos were induced from the immature zygotic embryo axes. However the development of the somatic embryos were restricted to the bipolar and cotyledonary stage. Repeated attempts to differentiate the embryos remained unsuccessful. The

experiment was extended to the immature embryos collected from five trees to test the possible influence of the genotype on somatic embryo differentiation. Morphology of the somatic embryo and their origin were studied using histological means. Somatic embryos proliferated on subculture. Embryogenic response was very slow and at a very low frequency. Repeated attempts to convert the somatic embryos to plantlets remained futile.

CHAPTER 6: MORPHOGENETIC RESPONSE IN SEEDLINGS GERMINATED IN PRESENCE OF THIDIAZURON

N-Phenyl-N$^{D'}$ 1,2,3-thidiazol-5yl urea (Thidiazuron), a synthetic cytokinin originally described as a defoliant for *Gossypium hirsutum* L. is now identified as a highly potent cytokinin for induction of morphogenesis in woody plant species. Studies included in this chapter show that Thidiazuron was effective in inducing variation during germination of seeds. It triggered morphogenetic activity in the cotyledonary nodal segment. Histological studies, conducted using the nodal segment with the morphogenic buds confirms that the regeneration is via organogenesis.

SUMMARY

This part of the thesis summarizes the main findings of the work conducted on tamarind tissue culture. The information generated from the studies included in this thesis is encouraging and will find application in improvement of tamarind tree via advanced biotechnological approaches.

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RESEARCH WORK PUBLISHED

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PRESENTATIONS AT NATIONAL SEMINARS / SYMPOSIA

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CHAPTER 1: GENERAL INTRODUCTION

1.1 INTRODUCTION

Trees are complex biological systems in themselves and they are part of an even more complex system – the forest ecosystem. As trees grow and reproduce, they interact in many ways with other biological systems and with the environment. The importance of trees in maintaining our ecosystem and their importance in the world economy, need no emphasis. The global demand for wood products and wood as source of energy and raw material for various industries are increasing with the ascent in world population. With industrialization of the developing countries like India, the natural forests are disappearing rapidly. To increase the forest cover major efforts are directed towards afforestation of the denuded and barren lands, commonly known as wastelands. The role of biotechnology and tissue culture lies in enhancing the production of superior planting material. Tree biotechnology has to be an integral part of an overall forest genetics and tree-breeding program. One of the most important things that biotechnology can offer is to cut the long periods required for tree improvement using conventional technology. The trees thus grown through organized program will not only support the ecosystem but also serve as the source of raw material for industries and renewable resource of energy

Leguminous tree species are well known for their utility to mankind. They are of special interest due to their economic and ecological importance. These trees are particularly considered in afforestation and soil erosion control programs due to their ability to fix atmospheric nitrogen.

Survey of existing literature reveals that although research in tree tissue culture was initiated earlier, research in the area of tree legumes started only two decades ago. Keeping in view the recalcitrant nature of the leguminous tree species, standardization of protocols for *in vitro* morphogenesis remains a challenging proposition. In addition to the advantages shared with conventional vegetative propagation, *in vitro* culture has a number of associated potential and real applications that are unique (Dean *et.al.*,1997). These include protoplast culture and fusion for generation of somatic hybrids, *in vitro* screening, generation of useful somaclonal variants, generation of artificial seeds, and long-term storage of germplasm using cryopreservation. Finally, *in vitro* culture currently provides the only route for generation of genetically engineered genotypes of forest trees.

Thus much efforts are needed before a model system is realized for the utilization of the various biotechnological approaches to aim for genetic improvement of leguminous tree species. Accelerated tree programs, combining the most sophisticated traditional and molecular-breeding techniques, along with the efficient and inexpensive large-scale clonal propagation of superior clones, are key elements for the successful restoration and management of future commercial forests.

Currently, most tree-improvement programs are based on the management of genetic resources, including the selection of superior clones from existing forests, conservation of genetic variability, partially controlled propagation and classical breeding for desired traits etc. Although molecular breeding is routine in agriculture, and numerous agri-biotech companies are producing many new genetically engineered field crops, vegetables and ornamental plants, tree species have been left far behind. Plant-transformation techniques and gene isolation and characterization are no longer serious obstacles. Tree species should be a major target for genetic engineering and molecular breeding in the 21st century (Tzfira *et al*.1998).

1.1.1 Limitations of tree breeding

In contrast to other agronomic and horticultural species, the long generation time of tree species has been the main obstacle to traditional breeding. In practice significant progress in the breeding of many tree species is limited because of the long lag time between seed germination and flowering. Most of the relevant tree traits can be assessed only when the tree reaches maturity. Limited knowledge of the genetic maps of most trees is yet another limitation. There is a lack of genes for hybridization and expression of new traits, which are known mainly for novel phenotypes and tolerance for biotic and abiotic stress. Moreover, the identification of suitable parents and the technical difficulties involved in their controlled mating are the limitations of tree breeding. Even though several superior hybrid trees with accelerated growth, altered form and environmental adaptations have been obtained through classical breeding, their maintenance is problematic because of the high heterozygosity of trees (which are mainly propagated by seed). Finally, the size of trees and the area required for field trials create considerable difficulties in assessing their performance.

1.1.2 Prospects for tree breeding

The potential of biotechnology for overcoming many of these limitations for accelerating tree-breeding programs can be realized at several levels:

- clonal propagation of superior genotypes using tissue-culture techniques
- somatic-cell techniques such as somatic hybridization and exploitation of somaclonal variations
- use of induced mutations
- molecular breeding to complement classical breeding (use of marker-assisted selection)
- direct and rapid introduction of specific traits via genetic engineering.

In the recent past technological advances have opened up new vistas for creation of genetic variability and selection of desired traits. The ability to regenerate whole plants from selected or genetically altered cells or tissues is the key to the broad biotechnological potential of plant cell cultures. In the last two decades, significant advances have been made in regenerating plants from different explants of mature tree species.

1.2 MICROPROPAGATION OF TREE SPECIES

Micropropagation is the only aspect of plant tissue culture, which has been most convincingly documented with regard to its feasibility for commercial application, and consequently it has been extensively used for rapid and large-scale propagation of a number of plant species. The economical and ecological importance of leguminous forest trees necessitates the application of this technique for their clonal multiplication (Tomar and Gupta, 1988b). During the past few years, a number of woody legumes have been successfully propagated *in vitro* using juvenile as well as mature plant parts.

Plant tissue culture is an essential component of plant biotechnology. Apart from mass multiplication of elite plants, it also provides the means to multiply and regenerate elite plants from genetically engineered cells. These elite plants may be cloned under aseptic conditions. Micropropagation has been used for clonal multiplication of orchids and ornamentals on a commercial scale for the last 35 years, but very little work has been done in this effect in forest trees. The only practicable method for their propagation has been through seeds, which is not favorable for high productivity due to genetic

heterogeneity. By the time the tree has been evaluated for desirable traits, it passes the juvenile stage and loses the ability to root. In such cases, tissue culture is the most valuable aid for cloning selected trees. So far success in the micropropagation of elite trees has been largely restricted to those species that root readily *in-vitro*. The *in-vitro* propagation of difficult to root species deal mainly with embryonal or young seedling material. Considerable progress has been made over the last two decades on the development of tissue culture methodologies for trees and their possible application to forestry.

1.2.1 Advantages of Micropropagation

Micropropagation of forest trees has several advantages over the conventional propagation methods. These advantages are :-

- Rapid multiplication of any new hybrid/genetically engineered trees/selected variety that arises as a single plant (for field-testing and commercialization).
- Vegetative propagation of difficult-to-propagate species (poor seed-set, less seed viability etc.).
- Propagation of clones all round the year.
- Large number of plants can be produced in a small space.
- Physical and chemical environmental factors can be greatly controlled.
- Production of genetically uniform plants in large number for large-scale planting in seed orchards to facilitate production of superior quality seeds.
- Elimination of viruses from the infected stock using meristem culture.
- Possibilities of rejuvenation of mature tissues.

1.2.2 Various approaches for Micropropagation

The methods that are available for propagation of plants in vitro essentially follow three pathways (George and Sherrington, 1984).

1. Multiplication of shoots from existing meristems - axillary buds/shoot tips
2. Direct morphogenesis from plant organs: (i) organogenesis and (ii) somatic embryogenesis
3. Indirect morphogenesis : (i) organogenesis and (ii) somatic embryogenesis

1. Multiplication of shoots from existing meristems - axillary buds/ shoot tips

In this method, development and multiplication of the growing meristem is achieved. A large number of shoots are produced from these cultures and roots are induced either *in vitro* or *extra vitrum* during horticulture establishment. Clonally propagated plants ensure the true to parent type characteristics and are therefore used for mass propagation of identified elites. This approach is the most exploited one e.g. rosewood (Raghava Swamy *et al*., 1992), *Bauhinia variegata* and *Parkinsonia aculeata* (Mathur and Mukunthakumar, 1992), *Acacia catechu* (Kaur *et al*.,1998), *Acacia mearnsii* (Beck *et al*., 1998a ; b), *Bauhnia vahlii* (Dhar and Upreti, 1999), pistachio (Onay, 2000).

2. Direct morphogenesis from organs

In this method, adventitious budding of shoots or development of embryos are induced on stem, roots, leaves, bulb scales and other organs of plant. Adventitious shoots at high rates can be induced from excised plant parts in many species in cultures. This approach of *de novo* direct organogenesis, has been used in Prosopis cineraria (Kackar *et al*.,1992), *Albizzia. falcataria* (Sinha and Mallick, 1993), *Sesbania grandiflora* (Detrez *et al*., 1994), *Albizzia procera* (Kumar *et al*.,1998), Direct somatic embryogenesis has been reported in *Dalbergia latifolia* (Rao and Lakshmi Sita, 1996), *Hardwickia binata* (Chand and Singh, 2001) etc. This procedure also involves stages, though the mode of regeneration is different. The method reviewed by Thorpe and Patel (1984) involves the induction of localised meristematic activity by treatment with phytohormones. This leads to the induction of a primordium and eventually formation of shoot.

In the adventitious system any part of the plant can be used as an initial explant. The most frequently used explants that lead to successful regeneration for both angiosperm and conifers have been the seed or parts of seedling. These includes the cotyledons, hypocotyl, epicotyl and embryonic axis. The shoot buds initiated on the explants are elongated and excised for rooting as described earlier.

Although this method of propagation has a great potential for multiplication but there are certain limitations too. The main purpose of clonal propagation is to obtain true-to-type genetic copies. However, the adventitious buds differentiate from the non meristematic tissue where the genetic constitution may not be maintained. Therefore the genetic stability of the regenerants is not assured.

The other problem is that in the adventitious system requires a one time induction from the original explant material and may not be capable of producing multiple shoots (Dunston, 1986).

3. Indirect morphogenesis

This method of micropropagation technique is by indirect organogenesis or embryogenesis in callus and suspension cultures. Callus is produced *in vitro* from any plant structure or part such as seeds, stems, roots, leaves, storage organs, or fruits (Street, 1977; Thorpe, 1981) and is maintained for long periods by repeated subcultures. Cell suspension culture is initiated by placing a piece of friable callus or homogenized tissue in a liquid medium and agitate on a rotary shaker at a particular speed so that the cells dissociate from each other.

Organogenetic regeneration from callus involves separate shoot and root induction phase. This process is similar to the initiation of adventitious shoots on explants except that initially callus is obtained from the explant before regeneration via meristemoid formation. Transfer of induced shoots to a root induction medium containing auxin results in a complete plantlet formation. This approach is used in *Bauhinia purpurea* (Kumar, 1992), *Sesbania rostrata* (Pellegrineschi and Tepfer, 1993), East Indian Rosewood (Pradhan *et al*., 1998), loblolly pine (Tang *et al*., 1998), *Acacia sinuata* (Vengadesan *et al*., 2000) etc.

While attempting the callus organogenesis route to plantlet production, several factors should be taken into consideration; (i) whether the shoot has differentiated from the callus or from the differentiated tissue enveloped with a layer of callus or (ii) the regeneration system is reproducible, that is whether the callus can retain the regeneration potential even after several subcultures and finally (iii) whether the callus-regenerated shoots are genotypically identical to the parent tissue or they show somaclonal variation. This is important because in many cases in which shoot formation is induced from callus tissues, aberrant plants are often produced. The frequency of such aberrant types increases with the length of time through which the callus is maintained *in vitro* (Skirvin, 1978). Larkin and Scowcroft (1981) recognized that such a variation could be useful for improvement of plants and might be a very valuable source for introduction of new traits.

The variation which occurs in tissue culture and in the regenerated plants is often heritable. These variations are not desirable for clonal propagation.

The other method of micropropagation is by **asexual embryogenesis** from callus. In this method somatic cells in culture give rise to somatic embryos. **Somatic embryogenesis** offers several distinct advantages over organogenesis (i) somatic embryos are bipolar, bearing both shoot and root meristems, so that in one step both meristems necessary for complete plant development are initiated, (ii) more number of regenerants in embryogenic culture flasks, compared to the multiple shoots originating via organogenesis and (iii) in suspension cultures, embryos usually develop separately from each other and float freely. As a result these need not be manually separated before transfer to the next step for their development into plants. Regeneration of forest trees via somatic embryogenesis is achieved in various species, eg.Douglas fir (El-Nig, 1980), Sandalwood (Lakshmi Sita *et al*., 1982), Norway spruce (Hakman *et al*., 1985), sugarpine (Gupta and Durzan, 1986), loblolly pine (Gupta and Durzan, 1987), *Eucalyptus citriodora* (Muralidharan and Mascarenhas, 1987), *Acacia catechu* (Rout *et al*., 1995), *Acacia nilotica* (Garg *et al*., 1996), *Dalbergia sissoo* (Das *et al*., 1997), *Acacia mangium* (Xie and Hong, 2001), etc. Even though shoot culture is at present the most promising tool for commercial micropropagation of many forest trees, this technique seems to be gradually replaced by somatic embryogenesis in future once this process is fully controllable and automated for various species (Haissig *et. al*., 1987).

1.2.3 Advancement in micropropagation : artificial seed technology

Although the importance of micropropagation is well recognised, and the clonal fidelity has been demonstrated thereafter, in conventional vegetative propagation of different tree species (Haissig *et al*., 1987) unfortunately, *in vitro* propagation method has certain limitations.

- It is labour-intensive involving several *in vitro* steps and gradual acclimatization of the plants from culture tube to the green house and then to the field.
- Costs associated with these steps are very high.
- For seed propagated crops, the cost of tissue culture propagation using the present techniques is expensive.

What is necessary is, a highly mechanised, direct greenhouse or field planting system that would enable production cost to be comparable to that of seeds. This has lead researchers to the concepts of synthetic seeds (Evans and Sharp, 1982; Krikorian, 1982). The idea of somatic encapsulation to produce synthetic seeds is primarily based on the similarity between somatic and zygotic embryos in many respects such as in morphology, physiology and biochemistry. Somatic embryos proceed through similar developmental stages as in zygotic embryogenesis to form both shoot and root apical meristems connected by a common vascular system and attached to cotyledon/s in a normal fashion (Redenbaugh *et al.*, 1986). Novel and basic information has been reported for various plants including sandalwood (Bapat and Rao, 1988), alfalfa (Fujii *et al*., 1989; McKersie and Bowley, 1992), carrot (Molle *et al*., 1993), spruce (Roberts *et al.*, 1993) and celery (Janick *et al*., 1993), groundnut (Padmaja *et al*.,1995), pistachio (Onay *et al*., 1996), mango (Ara *et al*.,1999).

1.3 MORPHOGENESIS

Origin and changes in the specific form (shape, structure, and organization) during the development of an organism and all such changes on and in the organism itself are called **morphogenesis**. It may occur *via* somatic embryogenesis (SE) or organogenesis. The resultant is a structured, physiologically organized and integrated plant. This topic has been reviewed extensively (Thorpe, 1983).

The major preconditions of a tissue for morphogenesis are high potential for cell division and pronounced morphogenetic plasticity. Usually, mature or highly differentiated tissue is morphogenetically determined, which means that the cells are fixed in a particular developmental program. The commitment of competent cells for morphogenesis is affected by many factors i.e. directive induction, including a complex interaction between genotype, the explant (and its stage of development) medium etc. Morphogenesis is triggered usually after competent cells are subcultured into a less complex medium allowing the expression of new developmental potential. This trigger is referred to as permissive induction (Thorpe, 1983).

All morphological processes in which structures with the form of natural non-autonomous organs are formed on the explant (protoplast, cell, callus, and tissue

fragment, plant fragment) are called **organogenesis** Organogenesis can occur directly from the explant without an intervening callus phase or indirectly through callus phase. It involves the formation of primordia followed by development of an organ *viz*. leaf, shoots, root etc.

Early studies, (White, 1939; Nobecourt, 1939; Skoog, 1944; Gautheret 1945; Tsui 1948; Skoog and Tsui, 1951; Jacquiot, 1951; Levine 1950; Wiggans 1954; Nickell, 1955; Miller *et* al., 1956, Skoog and Miller 1957; Murashige 1974, 1977, Thorpe, 1980) using a variety of explants and numerous plant species showed that successful culture and organized development could be achieved by providing the sterilized explant or cultured tissue an appropriate nutrient medium and proper culture conditions. Five classes of compounds generally used in the nutrient media of most of the species are :

i. Inorganic macro and micro nutrients
ii. Carbohydrate as energy source
iii. Vitamins
iv. Reduced nitrogen and
v. Phytohormones

Physiological factors such as the form of medium (liquid or semi solid), pH, light, temperature and humidity are all important in the manipulation of organized development. Thus much of the work done in tissue culture has concerned itself with optimizing these conditions to achieve organ or embryo formation. Over the years, extensive research has been conducted using natural and synthetic plant growth regulators (PGRs) and by varying the culture conditions to induce morphogenetic activity.

Since 1988, TDZ, a potent cytokinin, has been reported to induce adventitious morphogenesis in a number of species, especially woody plants (Lu, 1993; Huetteman and Preece, 1993). A vast amount of knowledge generated on TDZ-induced responses in a wide variety of plant species is compiled by Murthy et al. (1998).

1.3.1 Importance of tissue culture of tropical hardwoods

Some of the important physical characteristics of wood are determined by the thickness of the cell walls and the relative size and proportion of the vessels, tracheids

and fibres. Gymnosperms are known as 'softwoods' because their wood is composed entirely of tracheids. Angiosperms containing both vessel and fibres are 'hardwoods'. This classification however does not mean that all angiosperms have 'harder' wood than gymnosperms. Hardness of wood is a reflection of the sturdiness of the individual cell walls and other factors such as the amount of lignin they contain.

Tree planting in the tropics was initially for industrial purposes. However, its importance for firewood, to reduce soil erosion, to control water run-off, to combat desertification and to provide shelter and shade has also become known. These uses clearly point out the urgent need of either expanding the current area under forestation or to improve the quality of forests by planting uniform, genetically superior varieties with higher yields.

Conventional methods of propagation, for example by cutting, grafts etc. have their limitations especially when there is demand for large number of propagules. Cuttings from various heavy hardwoods have not rooted inspite of repeated experiments under various growth conditions using a wide variety of root induction hormones. The tissue culture method offers an alternate novel possibility and the technique has been successfully applied to many of the tree species.

The possibility of developing genetically improved strains of forest trees, in particular, tree legumes, which are suitable for plantation in currently marginal or sub-marginal sites need exploitation. Tree legumes are greatly under-exploited inspite of the constant demand (Raghava Swamy *et al*.,1992). The potential economic and ecological importance of woody legume species has already been emphasized (Trigiano *et al*., 1992) (Table 1.1).

Table 1.1 Potential economic and ecological importance of woody legume species

(Ref: Trigiano *et al*., 1992)

Soil Improvement	Human consumption or Utilization
• nitrogen fixation	• pods or seeds are high sources of protein
• erosion control, wind break	• gums, oil
• reclamation of mining sites	• secondary products, pesticides
• green manure	• ornamental value
Timber	**Livestock Interactions**
• lumber, carpentry	• fodder or forage
• pulp for paper production	• "living fences" to limit animal movement
• fuel wood or charcoal	• shade to reduce temperatures

Nurse crops

- shade for specialty crops (coffee, tea, cocoa)
- support for specialty vine crops (vanilla, black pepper)

1.3.2 Morphogenesis in tree legumes

In the last two decades significant advances have been made in regenerating plants from callus and explants of legumes. Legumes exhibit diverse responses when cultured *in vitro*. Depending on several factors, regeneration occurs *via* organogenesis and/or embryogenesis either directly or indirectly. Methodologies leading to the diversity of *in vitro* responses are relatively new accomplishment. Till 1992, there were reports of regeneration from at least 75 species from 25 genera in the leguminosae (Parrott *et al*., 1992). With the exceptions of east Indian walnut, *Albizzia lebbeck* (L) Beneth, A. *richardiana* King,, redbud, *Cercis canadensis* L. and sensitive plant, *Mimosa pudica* L., all reports of regeneration were confined to the Papilionoideae, the legume subfamily of greatest economic importance.

The first legumes regenerated were *Crotalaria* (Ramawat *et al.*, 1977), cicer (Mukhopadhyay and Bhojwani, 1978) and indigo (Bharal and Rashid, 1979). In these plants organogenesis could be induced from various vegetative explants. Other early attempts achieved regeneration from legumes that formed somatic embryos after a callus phase. Notable in this group was alfalfa, *Medicago sativa* L. (Saunders and Bingham, 1972, 1975; Bingham *et al.*, 1975) and red clover, *Trifolium pratense* L. (Phillips and Collins, 1979; Beach and Smith, 1979). Several important achievements led to the development of efficient regeneration systems for large seeded legumes that have not regenerated from callus or *de novo* organogenesis, such as soybean, *Glycine max* (L.) Merr (Parrott *et al.*, 1992). These developments centered on the selection of appropriate genotypes and the use of relatively high auxin concentrations for induction of somatic embryogenesis from immature zygotic embryos. Other advances have resulted in simple protocols for embryo maturation and conversion into plants. Application of these principles served as guidelines for experimental approaches leading to regeneration of other legumes.

The reports on the morphogenetic studies in tree legumes are enlisted in Table 1.2.

Table 1.2 Reports on the morphogenetic studies in tree legumes

(1976 to 1989)

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Acacia auriculiformis A. Cunn. ex Benth.	Seedling axillary buds	Indirect organogenesis	Plantlet	Mittal *et al.*, 1989
Acacia koa Gray	Seedling shoot tip	Indirect organogenesis Embryogenesis	Plant in soil –	Skolmen, 1986; Skolmen and Mapes, 1976
A. nilotica (L.) Delile *Albizzia lebbeck* L.	Seedling stem Seedling hypocotyl Hypocotyl, root, leaves, cotyledon	Direct organogenesis Embryoids Caulogenesis	– Plantlet Plantlet	Mathur and Chandra, 1983 Gharyal and Maheshwari, 1981
	Anther	Haploid	Plantlet	Gharyal *et al.*, 1983a
	Seedling root, hypocotyl	Indirect organogenesis	Plantlet	Upadhyaya and Chandra, 1983
	Seedling hypocotyl, leaf, stem	Indirect organogenesis	Plant in soil	Lakshman Rao and De, 1987
	Seedling hypocotyl, cotyledon, root, leaf rachis	Indirect Organogenesis	Plant in soil	Varghese and Kaur, 1988
A. richardiana King.	Seedling hypocotyl	Organogenesis Indirect embryogenesis	– Plantlet	Tomar and Gupta, 1988a
A. amara Boivin. *Albizzia lucida* Benth. *A. richardiana King.*	Seedling hypocotyl	Direct organogenesis	Plant in soil – Plant in soil	Tomar and Gupta, 1988b
Alhagi camelorum Fitch.	Seedling cotyledon, leaf, root, stem	Direct and Indirect organogenesis	–	Bharal and Rashid, 1981
Cassia fistula Linn.	Seedling explants Excised maturing	Callus Seedling formation	– Plant in soil	Bajaj, 1989

Table 1.2 continued

Plant species	Explant	Pathway of morphogenesis	Status	Reference
	Embryos with well-developed cotyledons			
	Anthers	Pollen embryogenesis	–	
Ceratonia siliqua L.	Seedling shoot tip, Mature nodal segment	Shoot proliferation	Plant in soil	Sebastian and McComb, 1986
	Mature shoot tip, nodal stem segment	Shoot proliferation	Plantlet	Thomas and Mehta, 1983
Cercis canadensis L.	Immature embryos	Direct embryogenesis	Germinated, only one embryo formed shoot	Trigiano *et al.*, 1988
Dalbergia sissoo Roxb.	Seedling Excised root	Direct organogenesis	Plantlet	Mukhopadhyay and Mohan Ram, 1981
	Mature Axillary bud	Shoot proliferation	Plantlet	Datta *et al.*, 1983
	Mature nodal explant	Shoot proliferation	Plantlet	Datta and Datta, 1983
	Mature Axillary bud	Shoot proliferation	Plant in soil	Dawra *et al.*,1984
D. latifolia Roxb.	Seedling Hypocotyl	Indirect organogenesis	Plantlet	Sharma and Chandra, 1988
	Mature nodal explants	Shoot proliferation	Plantlet	Mascarenhas *et al.*, 1982
	Somatic callus tissue	Indirect organogenesis	Plantlet	Rao, 1986
	Shoot callus of 5-yr old tree	Indirect organogenesis	Plant in soil	Lakshmi Sita *et al.*, 1986
	Seedling Hypocotyl	Indirect organogenesis	Plantlet	Sudhadevi and Nataraja, 1987a; 1987b
	Mature Shoot callus	Callus regeneration	Plant in soil	Ravishankar and Jagadish Chandra, 1988
	Seedling shoot tip, nodal segment from *in vitro* derived shoots	Shoot proliferation	Plant in soil	Ravishankar and Jagadish Chandra, 1989
	hypocotyl	Direct organogenesis	Plantlet	

Table 1.2 continued

Plant species	Explant	Pathway of morphogenesis	Status	Reference
D. lanceolaria Linn.	Seedling root, hypocotyl, cotyledon, stem, leaf	Indirect caulogenesis, rhizogenesis	Plantlet	Anand and Bir, 1984
Indigofera enneaphylla L.	Cotyledon	Indirect organogenesis	Plantlet	Bharal and Rashid, 1979; 1984
Leucaena leucocephala (Lam.) de Wit	Axillary node 1-2yr. old plant	Shoot proliferation	Plantlet	Kulkarni *et al.*,1984
	Cotyledon and epicotyl	Direct organogenesis	Plantlet	Nataraja and Sudhadevi, 1984
	Seedling nodal segment, Mature nodal segment	Shoot proliferation	Plant in soil	Dhawan and Bhojwani,1985;
	Green house grown 2-3m tall tree axillary bud	Shoot proliferation	Plant in soil	Goyal *et al.*, 1985
	Mature node	Shoot proliferation	Plantlet	Datta and Datta, 1985
	Cotyledon	Indirect organogenesis	Plantlet	Nagamani and Venketeswaran, 1987
L. retusa Benth.	Cotyledon	Indirect organogenesis	--	Nagamani and Venketeswaran, 1987
Mimosa pudica L.	Seedling cotyledon, hypocotyl,leaf, shoot apex	Indirect organogenesis	Plantlet	Gharyal and Maheshwari, 1982
Ougeinia dalbergioides Benth.	Seedling nodal segment	Shoot proliferation	--	Dhawan, 1988
Poinciana regia (Boj.)	Seedling explants	Callus	--	Bajaj, 1989
	Excised maturing embryos with well-developed cotyledons	Seedling formation	Plant in soil	
	hypocotyl segments,	Somatic embryogenesis	--	

Table 1.2 continued

Plant species	Explant	Pathway of morphogenesis	Status	Reference
	excised cotyledons	and shoot buds		
	Anthers	Pollen embryogenesis	–	
Prosopis alba Griseb	Seedling shoot tip, nodal stem segment	Shoot proliferation	Plantlet	Jordan, 1987
P. chilensis (Mol.) Stuntz.	Seedling shoot tip, nodal stem segment	Shoot proliferation	Plantlet	Jordan, 1987
P. cineraria (L.) Druce	Seedling hypocotyl	Indirect regeneration	Plant in soil	Goyal and Arya, 1981
	Shoot tip or nodal stem segment	Shoot proliferation	Plantlet	Goyal and Arya, 1984
P. tumarugo F. Phil.	Seedling shoot tip or nodal stem segment	Shoot proliferation	Plantlet	Jordan, 1987
Pterocarpus santalinus	Seedling explants	Shoot proliferation Indirect Organogenesis		Sarita Patri *et al.*, 1988
Robinia pseudoacacia	Mature nodal stem segment	Shoot proliferation	Plantlet	Chalupa, 1983
	Mature nodal stem segment	Shoot proliferation	Plantlet	Barghchi, 1987
	Zygotic embryo	Embryogenesis	Plantlet	Merkle and Wiecko, 1989
Sesbania bispinosa (Jacq.) W.F. Wight	Seedling hypocotyl, cotyledon	Direct organogenesis	Plant in soil	Kapoor and Gupta, 1986
Sesbania grandiflora	Seedling hypocotyl, cotyledon	Indirect organogenesis	Plantlet	Khattar and Mohan Ram, 1983
Sesbania rostrata	Seedling cotyledon, hypocotyl, zygotic embryo	Organogenesis	Plantlet	Vlachova *et al.*, 1987

Table 1.2 continued

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Sesbania sesban	Seedling hypocotyl cotyledon and hypocotyl	Direct organogenesis Indirect organogenesis	– –	Khattar and Mohan Ram, 1982
Tamarindus indica L.	Seedling explant Mature axillary bud	Shoot proliferation Shoot proliferation	Plantlet Plantlet	Mascarenhas *et al.*, 1981
	Seedling shoot tip axillary bud	Shoot proliferation	Plant in soil	Mascarenhas *et al.*, 1987

Table 1.2 continued

Reports in the 90's and onwards

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Acacia auriculiformis A. Cunn. ex Benth.	Seedling hypocotyl	Indirect organogenesis	Plantlet	Ranga Rao and Prasad, 1991
Acacia catechu Willd.	Immature cotyledon	Indirect embryogenesis	Plant in soil	Rout *et al.*, 1995
	Mature nodal segment	Shoot proliferation	Plant in soil	Kaur *et al.*, 1998
A. mangium Willd.	Seedling axillary buds	Shoot proliferation	Plantlet	Saito *et al.*, 1993
	Mature nodal bud	Shoot proliferation	Plant in soil	Bhaskar and Subhash, 1996
	Juvenile and mature explants	Shoot proliferation	Plant in soil	Monteuuis and Bon, 2000
	Immature zygotic embryos	Indirect embryogenesis	Plant in soil	Xie and Hong, 2001
A. mearnsii de Willd	Seedling shoot tip	Shoot proliferation	Plantlet	Huang *et al.*, 1994
	In vitro seedling explant	Shoot proliferation	Plant in soil	Beck *et al.*, 1998a
	Ex vitro seedling explant			
	Coppice nodal explant	Shoot proliferation	–	Beck *et al.*, 1998b
A. nilotica (Linn.) Willd.	Immature endosperm	Indirect embryogenesis	Plantlet	Garg *et al.*, 1996
A. salicina		Indirect embryogenesis	Plantlet	Zhao *et al.*, 1990
A senegal (L.) Willd.	Seedling Juvenile tree Nodal explant	Shoot proliferation	Plant in soil	Badji *et al.*, 1993
A. sinuata	Seedling hypocotyl	Indirect organogenesis	Plant in soil	Vengadesan *et al.*, 2000
Acacia spp.		Indirect embryogenesis	Plantlet	Jones *et al.*, 1990
A. tortilis subsp *raddiana* (Forsk) Hayne	Seedling cotyledonary node	Shoot buds, shoots	Plant in soil	Nandwani, 1995

Table 1.2 continued

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Albizzia falcataria (L.) Forberg	Cotyledon pieces	Direct organogenesis	Plantlet	Sinha and Mallick, 1993
A. julibrissin Durrazz.	Roots of intact seedlings	Indirect organogenesis	Plant in soil	Sankhla *et al.*, 1994
	Seedling Excised root segments	Direct organogenesis	Plant in soil	Sankhla *et al.*, 1996
A. lebbeck L.	Mature Petiole, stem	Indirect organogenesis	Plant in soil	Gharyal and Maheshwari, 1990
A. procera	Seedling leaflet	Direct organogenesis	Plant in soil	Kumar *et al.*, 1998
Bauhinia purpurea L.	Mature stem segments 15-18 yr. old tree	Callus regeneration	Plant in soil	Kumar, 1992
B. vahlii Wight and Arnott	Seedling cotyledonary nodal segment	Shoot proliferation	Plant in soil	Upreti and Dhar, 1996
	Nodal segment of shoots from mature liana	Shoot proliferation	Plant in soil	Dhar and Upreti, 1999
	Seedling cotyledonary node	Shoot proliferation	Plant in soil	Bhatt and Dhar, 2000
B. variegata L.	Mature nodal explant (6-8 yrs.)	Axillary shoot proliferation	Plant in soil	Mathur and Mukunthakumar, 1992
Caesalpinia pulcherrima Sw.	Mature (20 yrs.) nodal bud	Shoot proliferation	Plant in soil	Rahman *et al.*, 1993
Cassia fistula L. and *Cassia siamea* Lam.	Mature stem	Indirect organogenesis	Plant in soil Plantlet	Gharyal and Maheshwari, 1990
Cercis canadensis L.	Developing zygotic embryos	Direct embryogenesis Rhizogenesis	Plant in soil	Geneve and Kester, 1990
Cladrastris lutea (Michx.) K. Koch.	Immature embryos	Direct embryogenesis	Plant in soil	Weaver and Trigiano, 1991
Dalbergia. Latifolia Roxb.	Mature Nodal explant of 60-80 yr old tree	Shoot proliferation	Plant in soil	Raghava Swamy *et al.*, 1992

Table 1.2 continued

Plant species	Explant	Pathway of morphogenesis	Status	Reference
	Immature zygotic embryos	Direct embryogenesis	Plant in soil	Rao and Lakshmi Sita, 1996
	Seedling hypocotyl segment	Indirect regeneration from cell suspension derived callus	Plant in soil	Pradhan *et al.*, 1998
D. sissoo Roxb.	Cells of cambial origin of mature tree	Cell suspension derived callus regeneration	Plant in soil	Kumar *et al.*, 1991
	Semimature zygotic embryos	Indirect embryogenesis	–	Das *et al.*,1997
	Seedling explant - Cotyledonary node	Shoot proliferation	Plant in soil	Pradhan *et al.*, 1998
Faidherbia= Acacia albida (Del.) A. Chev.	Seedling Roots	Direct organogenesis	Plantlet	Ahee and Duhoux, 1994
Hardwickia binata	Immature cotyledonary explant	Indirect Embryogenesis	–	Das *et al.*, 1995
Mimosa tenuiflora (Willd.) Poiret	Seedling explant nodal axillary bud	Single bud culture	Plant in soil	Villarreal and Rojas, 1996
Parkinsonia aculeata L.	Nodal explants of mature trees (6-8 yrs.)	Axillary shoot proliferation	Plant in soil	Mathur and Mukunthakuma r, 1992
Prosopis cineraria (L.) Druce	Single node segment (4½ yr.old tree	Axillary branching	Plant in soil	Kackar *et al.*, 1991
	Seedling 3-4 month old root segment 4 ½ yr. old tree root segment	Direct organogenesis	Plantlet	Kackar *et al.*, 1992
Pterocarpus santalinus	Seedling explant	Axillary shoot proliferation	Plant in soil	Lakshmi Sita *et al.*, 1992
Robinia pseudoacacia L.	Immature seeds	Indirect embryogenesis	Plant in soil	Arrillaga *et al.*, 1994
Sesbania aculeata (Pers)	Seedling hypocotyl	Adventitious Organogenesis	Plant in soil	Bansal and Pandey, 1993

Table 1.2 continued

Plant species	Explant	Pathway of morphogenesis	Status	Reference
S. bispinosa (Jacq.) W.F. Wight	Cotyledon Mature leaflet	Callus regeneration	Plant in soil	Sinha and Mallick, 1991
S. grandiflora	Seedling hypocotyl, cotyledon	Indirect organogenesis	Plantlet	Shankar and Mohan Ram, 1990
	Cotyledon pieces	Direct organogenesis	Plant in soil	Detrez *et al.*, 1994
S. rostrata	Seedling stem, leaves, roots of micropropaga - ted plant	Callus Caulogenesis	Plantlet	Pellengrineschi and Tepfer, 1993
Swartzia madagascarien sis (Desv.)	Seedling 1st node, 2nd node, shoot apex	Shoots Roots	Plantlet	Berger and Schaffner, 1995
Tamarindus indica L.	Seedling Shoot tip Seedling Cotyledonary node	Shoot proliferation Shoot proliferation	Plant in soil Plant in soil	Kopp and Nataraja,1990 Splittstoesser and Mohamed-Yasseen, 1991
	Nodal tissue of cotyledon	Shoot proliferation	Plant in soil	Jaiwal and Gulati, 1991
	Seedling Shoot tips, nodes of shoot regenerated from shoot tip	Shoot proliferation	Plant in soil	Jaiwal and Gulati, 1992
	Seedling Excised nodal segment	Shoot proliferation	Plant in soil	Kopp and Nataraja,1992
	Seedling hypocotyl segment	*De novo* Organogenesis	Plant in soil	Sonia *et al.*, 1998
	Mature zygotic embryo axis	*De novo* organogenesis	Plant in soil	Mehta *et al.*, 2000

1.4 TAMARIND

The tamarind plant is native to the dry savannas of tropical Africa. In ancient times the tree was introduced to Asia by Arab traders, and with its pleasant acidic tasting fruit, it was so enthusiastically adopted, especially on the subcontinent, that today the plant's botanic and common names both, point to its association with India. The name tamarind derives from the Arabic tamar-ul-hind, which means, "date of India", also known as tamarindo (Spanish and Portuguese) and tamarin, tamarinier, tamarindier (French).

It is a medium sized semi-evergreen tree (Fig.1.1A) with a short strong trunk with grey scaly bark. The leaves are alternate pinnately compound 7-15cm long with pulvinus at the base of the petiole. There are 10-20 pairs of small leaflets arranged opposite, entire, almost sessile, oblong 1-2.5 x 0.5-1.0cm long. Inflorescence is small terminal drooping raceme, 5-10cm long. The flowers are small, scented, and attractive with yellow and red colors (Fig.1.1B). Pods are usually curved, flattened and vary considerably in size and shape (Fig 1.2A). They are constricted, indehiscent, 1-10 seeded. When ripe, the fruits are stiff and brittle. Seeds are obovate, flattened, brown about 1-1.5 cm long (Fig.1.2B) joined to each other with tough fibers running through brown sticky pulp. Tamarind tree is long lived and attains a large size, but the rate of growth after the seedling stage is slow. The tree begins to bear fruit at the age of 13-14 years and continues to yield abundant crops for more than 60 years (The Wealth of India, 1976). The average yield of tamarind fruit from a mature tree is 30-50 kg. annually, although some trees have been identified which yield 180-225 kg. per year. India is perhaps the chief producer of this fruit in the world with an essential production of about 250,00 tonnes per annum (Mascarenhas *et al*., 1987). The entire demand for the pulp in India is met by indigenous production.

Of all the tropical and subtropical fruit trees, none is more widely distributed nor more appreciated as an ornamental than tamarind (Morton, 1989). It is surprising that the tree is poorly exploited commercially, although the fruit is highly priced and common in all local markets. Nothing is known about the genetic variability of the species or about agronomic requirements. Thus extensive germplasm collection and

Fig.1.1

Fig. 1.1 A: Tamarind tree

B: Flowers of tamarind

Fig. 1.2

Fig. 1.2 A : Fruits of tamarind

B : Seeds of tamarind

characterization is needed to find fruits with good flavor and higher percentages of usable pulp (El- Siddig *et al.*, 1999).

Tamarind is particularly adapted to semi-arid tropics, but it can also grow in monsoon regions, provided that the soil is well drained (Purseglove, 1982). It does not do well in ever-wet climate, or under low temperature, and freezing conditions (Maslekar, 1981; Morton, 1989). It is classified as quantitative long day plant, since growth is enhanced by long days, but not prevented by short days (Broschat and Danselmen, 1983). Dry weather is important during the period of fruit development (Chapman, 1984 Ref. El Siddig *et al.*, 1999). It thrives best in deep alluvial soils but it does well even in rock soils with little or no cultural attention (Coronel, 1991 Ref. El Siddig *et al.*, 1999). Tamarind is considered salt-tolerant and can be grown fairly well in soils containing up to 45% exchangeable sodium (Dwivedi *et al.*, 1996).

The main method of propagating tamarind is by seeds. However, it does not breed true-to-type due to heterozygosity since the flowers are cross-pollinated and noticed to be outcrossing. Prolonged juvenile phase has also been observed in plantations due to predominant seed propagation (Karale *et al.*, 1997). Therefore vegetative propagation of superior genotypes is necessary for shortening juvenile phase and for production of uniformly growing elite trees. This can be achieved by conventional horticultural methods of grafting and airlayering or through non-conventional method of propagation by tissue culture (Mascarenhas *et al.*, 1987).

Almost every part of the tamarind tree finds some use. The wood is a valuable timber, but the value of heartwood is small. The sapwood is yellowish often with brownish red streaks. The heartwood is dark brown usually streaked and mottled with black, particularly in old trees. The wood is close-grained, strong, very hard and heavy (weight: 913-1282 kg/m^3). It is moderately refractory, shows cracking and splitting during air seasoning and is responsive to preservation treatment. It is durable under cover and is resistant to insect attack. It bends well and takes on good polish. The wood is used mostly for agricultural implements, tool handles, wheels, mallets, rice pounders, oil mills and turnery. It is suitable for printing blocks and tent plugs. It yields a decorative plank for paneling and furniture. In South India, it is employed for construction work and has been recommended as a substitute for teak and sal (*Shorea robusta*) for beams, rafters,

purlins and trusses. When burned it gives off immense heat and in India it is a choice wood for firing brick kilns. Tamarind wood is also valued as a fuel. Tamarind charcoal is of such high quality that it is preferred for making gunpowder. Tamarind is also useful species for beautifying and shading parks, backyard gardens, city avenues and country roads. In India it is particularly common as an avenue shade tree. Around the tree the ground is usually bare because of shading by the dense canopy, and in India tamarind is planted in strips among forest plantations to act as firebreaks.

The fruit contains a sweetish, acidic pulp, the tamarind of commerce, which is widely used in curries, sauces, chutneys and certain beverages and also a local medicine. It contains both sugars (30-40%) and organic acids such as citric, tartaric, acetic and ascorbic (Vitamin C). It has vitamins, important minerals and calcium. It contains 8% tartaric acid (Kumar *et al*.,1997).

The potential of Tamarind has been recognized for novel food products from agroforestry trees (Leakey, 1999). Studies on the food values of tamarind have also been conducted (Table 1.3).

Table 1.3 Food value per 100 g of edible portion (Ref. Morton, 1989)

	Pulp (ripe)	Leaves (young)	Flowers
Calories	115		
Moisture	28.2-52 g	70.5 g	80 g
Protein	3.10 g	5.8 g	0.45 g
Fat	0.1 g	2.1 g	1.54 g
Fibre	5.6 g	1.9 g	1.5 g
Carbohydrate	67.4 g	18.2 g	
Invert Sugar (70% glucose; 30% fructose)	30-41 g		
Ash	2.9 g	1.5 g	0.72 g
Calcium	35-170 mg	101mg	35.5 g
Magnesium		71mg	
Phosphorus	54-110 mg	140 mg	45.6 mg
Iron	1.3-10.9 mg	52 mg	1.5 mg
Copper		2.09 mg	
Chlorine		94 mg	
Sulphur		63 mg	
Sodium	24 mg		
Potassium	375 mg		
Vitamin A	15 I.U.	250 mcg	0.31mg
Thiamine	0.16 mg	0.24 mg	0.072 mg
Riboflavin	0.07 mg	0.17 mg	0.148 mg
Niacin	0.6-0.7 mg	4.1 mg	1.14 mg
Ascorbic acid	0.7-3.0 mg	3.0 mg	13.8 mg
Oxalic acid		196 mg	
Tartaric acid	8-23.8 mg		
Oxalic acid	trace only		

The hulled seeds contain a large amount of non-starch polysaccharide with ratios of D-galactose: D-xylose: D-glucose of 1:2:3. Non-hulled and crushed seeds have been used since 1943 to supply a crude preparation of polysaccharide, commercially known as tamarind kernel powder (TKP) used extensively as a sizing material in textile industry and weaving industry (Gerard, 1980, Ref. Sano *et al*., 1996). When the powder is boiled, gruel is obtained which on drying in thin layers produces strong, smooth, continuos and elastic films, which is useful for sizing. The fatty oil from the kernel resembles peanut oil and is reported to be useful in the preparation of paints and varnishes and for burning in lamps.

The pulp of the fruit is used in various edible household preparations, while the seeds are thrown away as agricultural waste. Valuable polyose is extracted from these waste seeds to obtain a cheaper indigenous product. Tamarind seed polyose (TSP) is a polysaccharide gum having innumerable qualities and hence is unique with other natural and synthetic gums or products of its kind. The seeds yield 20-25% TSP that is of immense pharmaceutical values (Khanna *et al*., 1999). It can find a number of uses in the field of pharmacy, as it has passed detailed toxicity study in rats and monkeys. Besides binding, suspending, emulsifying and haemostatic property, it can also be used as polymer of choice for sustained delivery of therapeutics. Since 1964, Japan has been using purified refined TSP xyloglucan from hulled seeds in many commercial food products as a gelling, thickening or stabilizing agent (Gidley *et al.*, 1991, Ref. Sano *et al*., 1996) at concentrations of 0.1-2% in foods. Tamarind wastes such as tamarind husk, pulp, seeds, fruit and the effluent generated during tartaric acid extraction are used as supplements to evaluate their effects on alcohol production from cane molasses using yeast cultures. Small amounts of these additives enhanced the rate of ethanol production to 40% improvement in batch fermentation. The use of cheaper tamarind-based waste products, provide a cost-effective means for improving ethanol productivity (Patil *et al*., 1998).

The pulp of the fruits is sweet or sour, cooling, carminative digestive, laxative, antiscorbutic and antibilious. Bark, leaves and seeds are astringent. Tender leaves and flowers are cooling and antibilious. All these parts are used medicinally. It is used in constipation, colic, cough, dyspepsia, fever, flatulence, gastro-intestinal diseases and urinary infection. Pulp of the fruit as well as a poultice of the leaves is recommended for external application to inflammatory swellings, to relieve pain. The ripe pulp of the fruit

is considered as an effective laxative in habitual constipation and enters into many Ayurvedic preparations. An infusion of the leaves is used as a gargle for aphthous sores and sore throat and for washing indolent ulcers. Poultice of flowers is useful in inflammatory affections in conjunctiva.

1.4.1 Diseases of tamarind tree

The tree is affected by a number of rots such as : Saprot (caused by *Xylaria euglona*), brownish Saprot (caused by *Polyporous calcuttensis*) and white rot (caused by *Trametes floccosa*) (Wealth of India, 1976); powdery mildew (caused by *Oidium* sp.) (Siddaramaiah and Kulkarni, 1982), hypocotyl rot (caused by *Sclerotium rofsii*) (Lingaraju *et al*., 1979). Several insects attack the fruits and seeds of tamarind, both on the tree and when stored for marketing. These feed on larvae, which feed on the sap of fruits and the saps of the young twigs and branches. Among the pest, mealy bugs and scales are often causing serious concerns especially in nursery and can be controlled by spraying 0.1% monocrotophos (Kumar *et al*., 1997). *Latoia lepida* [*Parasa lepida*] (slug caterpillar) is reported as a serious pest of *Tamarindus indica* (Babu *et al*., 2000).

1.4.2 Tissue culture studies in tamarind

With the extension of knowledge on biotechnological and tissue culture approaches, micropropagation methods are often considered to supplement conventional propagation methods particularly for breeding. These methods offer the possibility of multiplication and establishment of selected trees on a large scale. Although the potential of these methods are identified and is being extended to various tree species (Merkle and Dean, 2000), very few reports are available on *in vitro* clonal propagation of tamarind. Literature on tissue culture studies in tamarind is summarized below (Table 1.4):-

Table 1.4 Tissue culture studies in tamarind

Explant	Pathway of morphogenesis	Status	Reference
Seedling explant Mature explant	Shoot proliferation Shoot proliferation	Plantlet Plantlet	Mascarenhas *et al.* (1981) Propagation of trees by Tissue Culture. In : AN Rao (ed.) Proc. COSTED Symp. Tiss. Cult. Econ. Imp. Pl., Singapore. pp. 175-179
Seedling explant	Shoot proliferation	Plantlet	Mascarenhas *et al.* (1987) Tamarind. In : JM Bonga and DJ Durzan (eds.) Cell and Tiss. Cult. in Forestry. Vol. 3 pp. 316-326
Seedling explant Shoot tip	Shoot proliferation	Plantlet	Kopp and Nataraja (1990) *In vitro* plantlet regeneration from shoot tip cultures of *Tamarindus indica* L. *Ind. J. For.* **13**: 30-33
Nodal tissue associated with cotyledon	Shoot proliferation	Plantlet	Jaiwal and Gulati (1991) *In vitro* high frequency plant regeneration of a tree legume *Tamarindus indica* L. *Plant Cell Rep.* **10** : 569-573
Seedling explantCotyledonary node	Shoot proliferation	Plantlet	Splittstoesser and Mohamed-Yasseen (1991) *In vitro* shoot regeneration Proc. InterAmer. Soc. Trop. Hort. **35**: 6-8
Seedling explants Shoot tips, nodal explants of shoot regenerated from shoot tip	Shoot proliferation	Plantlet	Jaiwal and Gulati (1992) Micropropagation of *Tamarindus indica* L. from shoot tip and nodal explants. *Nat. Acad. Sci. Lett.* **15**:63-67
Seedling explantExcised nodal segment	Shoot proliferation	Plantlet	Kopp and Nataraja (1992) Regeneration of plantlets from excised nodal segments of *Tamarindus indica* L. *Myforest* **28**: 231-234
Hypocotyl segment	*De novo* organogenesis	Plantlet	Sonia *et al.*, (1998) Direct organogenesis in hypocotyl cultures of *Tamarindus indica*. *Biol.Plant.* **41**: 331-337

Micropropagation of tamarind from seedling derived meristems was reported from this laboratory (Mascarenhas *et al.*, 1981; Mascarenhas *et al*,1987), and also from other laboratories (Kopp and Nataraja, 1990; 1992). Multiple shoot regeneration from cotyledon nodes was observed when thidiazuron was used instead of BAP (Splittstoesser and Mohammed-Yasseen, 1991, ref. rev. Rao *et al*., 1999). However high frequency regeneration was achieved from excised cotyledon of tamarind in medium containing BAP singly (Jaiwal and Gulati, 1991). Rooting in these shoots was induced in presence of IAA. The same authors reported multiple shoot regeneration from shoot tips and nodal explants with BAP (Jaiwal and Gulati, 1992). Other researchers reported (Rao *et. al.*, 1999) micropropagation of tamarind from seedling explants in a medium composition with KN, BAP and IAA. However, rooting of these shoots was obtained in a mixture of three auxins including IAA, IBA and NAA. The only report on *de novo* organogenesis in

tamarind describes production of shoots with small amounts of callus at the proximal end of the hypocotyl explants (Sonia *et al*., 1998). Research on tamarind tree is reviewed (Rao *et. al.*, 1999).

It is apparent from the above literature that very little tissue culture studies have been done in this multipurpose, leguminous tree species. Thus there is a need to develop reliable tissue culture protocols for *in vitro* regeneration of tamarind plants from seedling and mature explants via direct and indirect organogenesis and somatic embryogenesis. The protocols developed for clonal propagation can then be used for rapid propagation of identified elites and genetically modified plants. The protocols developed for de novo organogenesis and embryogenesis will not only be useful for understanding the basic processes of morphogenetic pathways and causes of recalcitrance in legumes but also for genetic modification of this plant for productions of desired biochemicals. Some of the compounds produced by this plant have commercial value. The extended, juvenile phase of tamarind will be reduced by tissue culture (Mascarenhas *et. al*.,1987).

1.4.3 Objectives of the present study

Keeping in view the existing literature, it is apparent that very little work has been done on *in vitro* regeneration and clonal propagation of tamarind. Thus there is a lot of scope to develop reliable protocols for *in vitro* regeneration, and study the basic processes involved in tamarind morphogenesis. The present study on tamarind was initiated with the following objectives:

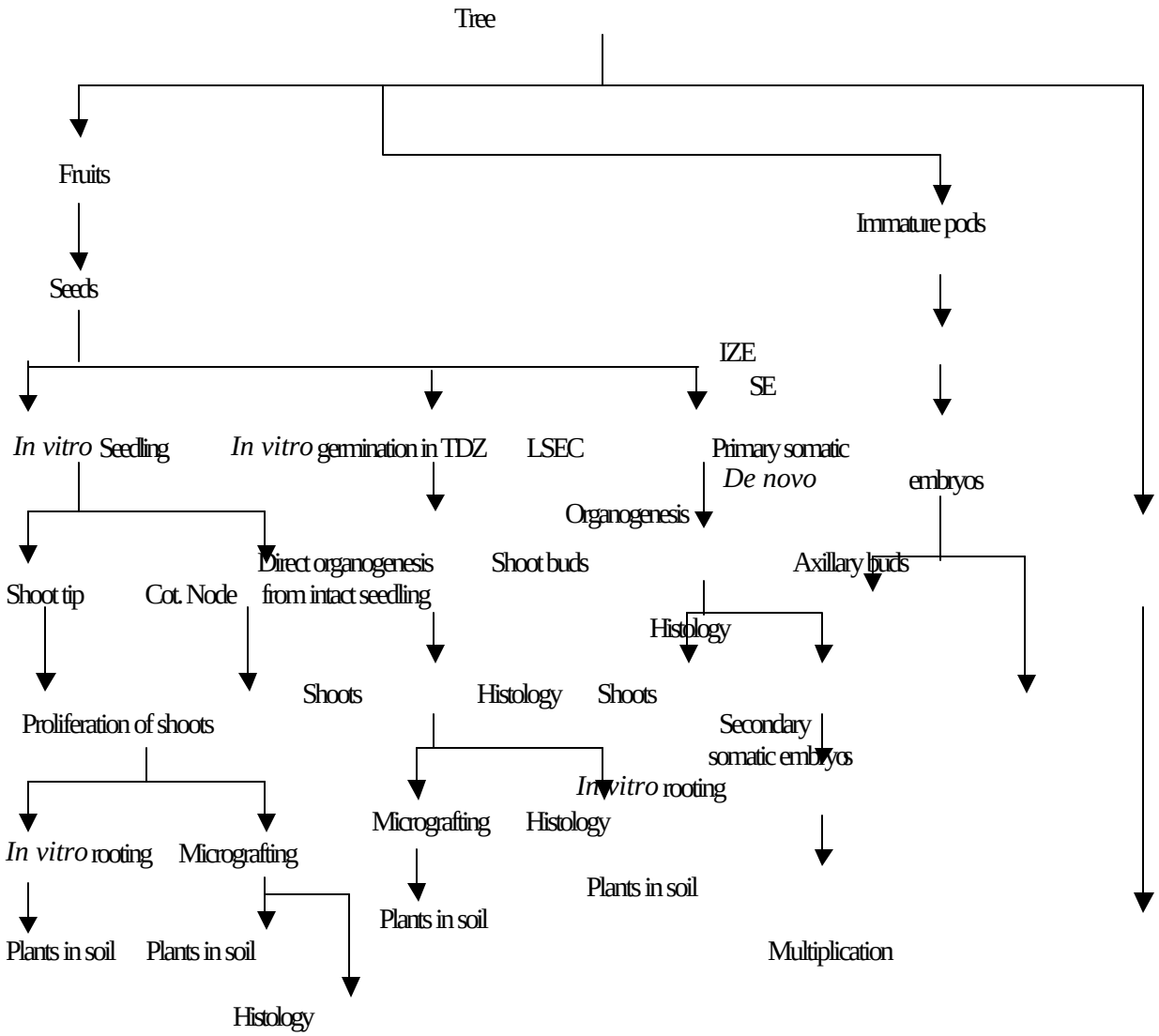
Optimization of conditions for:

(i) Clonal propagation

(ii) *De novo* morphogenesis

(iii) Somatic embryogenesis.

Schematic representation of the research work included in the thesis



Cot. Node - Cotyledonary node
LSEC - Longitudinal section of embryo axis attached to cotyledon
TDZ - Thidiazuron
IZE - Immature zygotic embryo
SE - Somatic embryogenesis

CHAPTER 2: MATERIALS AND METHODS

This chapter describes the materials and the techniques routinely practiced in plant tissue culture. The materials and the methods, which were specific to the particular experiments, have been dealt in detail in the respective chapters.

2.1 CONSUMABLES

Most of the consumables and chemicals were procured from various local suppliers. Few chemicals including the growth regulators and Phytagel were imported.

2.1.1 Glassware

Test tubes (25x150mm), conical flasks (250ml capacity), pipettes (0.1,0.2,1,2,5,10 ml capacity) and measuring cylinders (25ml, 100ml, 1000ml capacity) of Borosil, India were used for culturing the tissues and for preparation of media.

Glassware used for histological studies were coplin jar (60ml capacity), slides (Blue Star, India) and cover slips (Micro-Aid, India).

2.1.2 Plasticwares

Plasticwares including sterile disposable plastic petridishes of 55 and 85 mm diameter were procured from Laxbro Manufacturing Company, Pune. Klin wrap used for sealing the petridishes and microtips were procured from Flexo Wraps (Bombay) and Bio-rad (U.S.A.) respectively.

2.1.3 Filter papers and membranes

Whatman filter paper No.1 was used for making filter paper bridges for liquid media in tubes, while the filter sterilizing membranes were procured from Advanced microdevices (P) Ltd., Ambala, India.

2.1.4 Chemicals

Chemicals used for surface sterilization procedures were Bavistin® (BASF, India), Savlon (Johnson and Johnson Limited, India), mercuric chloride (Qualigens Fine Chemicals, India).

Inorganic salts and vitamins used for preparation of culture media and for other experiments are of Analar grade (BDH, Hi-Media and Qualigens Fine Chemicals, India).

Activated charcoal was procured from Sarabhai M Chemicals, India.

Growth regulators including kinetin (KN), 6-benzylaminopurine (BAP), zeatin (Z), 6-γ,γ-dimethylallylaminopurine (2-iP), N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron, TDZ), α-naphthalene acetic acid (NAA), 3,6-dichloro-o-anisic acid (Dicamba), indole acetic acid (IAA), indole butyric acid (IBA), indole propionic acid (IPA), abscissic acid (ABA) and gibberellic acid (GA_3) were obtained from Sigma or Aldrich (U.S.A.).

Chemicals used for histological studies including formaldehyde solution, glacial acetic acid and xylene were procured from Qualigens Fine Chemicals, India. Ethanol, 2-methyl propan-2-ol (tert butyl alcohol), iron alum was from s.d. fine chemicals, India; paraffin wax (m.p.58-60°C) from (E. Merck, India Ltd.); haematoxylene and eosin stain from (Hi-Media Laboratories Pvt. Ltd., Bombay), and DPX-4 mountant [189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)] was from BDH, India.

2.1.5 Carbohydrates

Sucrose, fructose, glucose, maltose were procured from Hi-Media (India) and Qualigens Fine Chemicals (India).

2.1.6 Gelling agents

Agar agar bacteriological grade), used as gelling agent in the semisolid culture medium was procured from Hi-Media (India) and Qualigens Fine Chemicals (India). Phytagel™ (Gelrite) used in one of the experiment was procured from Sigma Chemicals Co. (U.S.A.).

2.1.7 Source of Explants

Seeds of tamarind were obtained from the mature fruits collected from the trees growing locally. These seeds were either used as explant directly or for developing seedlings as source of explants. Immature zygotic embryos from young pods and actively growing twigs were also collected from the trees growing locally. Immature zygotic embryos were isolated from these young pods and the axillary buds were dissected from the growing twigs.

2.2 EQUIPMENTS

With the exception of pipetman, analytical balance, microtome, microscopes, and camera, all other equipments used in the course of this study are fabricated by different companies in India. The major equipments used include :

Equipment	**Company**
pH meter	Global, India
Magnetic stirrer	Remi, India
Steamer	Ultradent, India
Autoclave	Nat Steel Equipment Pvt. Ltd., Bombay, India
Temperature controlled oven	Pathak Electricals, India
Light microscope	Carl-Zeiss Jena
Microtome	Reichert Jung
Laminar flow	Klenzoids/ Microfilt, India
Camera	Nikon / Zeiss
Analytical balance	Sartorius
Membrane filter sterilizing unit	Laxbro Manufacturing Company, Pune
Pipetman®	Gilson Medical Electronics

2.3 METHODS

Standard plant tissue culture techniques were used for the experiments. Specific methods applied for histological studies and micrografting are described in the relevant chapters.

2.3.1 Preparation of glassware and instruments

Glassware used in our studies were cleaned by boiling in saturated solution of sodium bicarbonate for 1 h followed by washing in tap water. These were then immersed in 30% nitric acid solution for 30 min. These were washed thoroughly with tap water. After rinsing with double distilled water these were allowed to dry on a draining rack.

Tubes and flasks were plugged with absorbent cotton (Seasons healthcare Ltd., Ranga Reddy District, Andhra Pradesh, India). Pipettes were wrapped in brown paper for sterilization by autoclaving. All dissecting instruments were either wrapped singly or were put in closed aluminium cans for sterilization by autoclaving.

Ordinary grade filter paper pieces of approximately 10x20cm were kept in stack alternatively with brown paper pieces of similar size. These were packed in autoclavable plastic bags with 20-25 pieces in each bag and autoclaved. Dissection and transfer of explants were carried out on these papers under aseptic conditions and disposed after use.

Sterilization of the glassware and instruments was carried out by autoclaving at 121°C for 1 h in 15 lbs/(inch)^2.

2.3.2 Preparation of media

Stock solutions of the media ingredients were prepared in glass distilled water. Appropriate aliquotes of these solutions were mixed to prepare the media. Concentrations of the macro and micro element salts and organic constituents of the MS (Murashige and Skoog, 1962) basal medium is listed in Table 2.1. Stock solutions of growth regulators (PGR) were prepared and stored. For media preparation a calculated amount of aliquots were added from these stock solutions. Carbohydrate was weighed and added in required quantity and allowed to dissolve. Unless mentioned, pH of all the media was adjusted to 5.6-5.8 using 1N NaOH or 1N HCl after mixing all the constituents except the gelling agent. The volume was made up with glass distilled water. Gelling agent (agar agar or phytagel) was then added and heated on water bath or steamed for the agar to melt. Molten medium was dispersed into sterile culture tubes, flasks or bottles after thorough mixing. In case of liquid medium no gelling agent was required. For liquid medium in tube a Whatman no.1 filter paper support was inserted

during distribution of medium. Semisolid medium containing agar was used in most of the studies unless otherwise mentioned. All the culture media were autoclaved for 20 min. at 121°C and 15 lbs/(inch)^2. Autoclaved media was poured in sterile petridishes before gelling, as and when required. Auxins used for rooting media and Abscisic acid (ABA) were filter sterilized through millipore membranes (0.22 µM) and added aseptically to the autoclaved liquid medium or in the autoclaved semisolid medium before it gells. In case of charcoal containing medium individual tube was shaken before the setting of medium for uniform distribution of charcoal.

2.3.3 Preparation of explants

Mature seeds, mature buds or young fruits / pods were washed and surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for specified time period. Excess $HgCl_2$ was removed by repeated washings (4-5 times) with sterile distilled water under aseptic conditions. Seeds were soaked in sterile distilled water for 12-16 h prior to culturing them for germination *in vitro*. These seedlings were used as source of explants. For some of the experiments the seeds were used directly after soaking. For isolation of immature zygotic embryos, the young fruits were surface sterilized prior to aseptic dissection.

2.3.4 Initiation of cultures

Cultures were initiated from the explants in laminar air flow cabinets. All the dissections were carried out on sterile filter papers. The instruments used for aseptic dissection or transfer of tissues were pre-autoclaved. During aseptic operations these were flamed intermittently after dipping in rectified spirit. After transferring the tissues, the petridishes were sealed with klin wrap. All cultures were labelled appropriately prior to incubation.

2.3.5 Culture conditions

Most of the cultures were incubated in light in culture room adjusted at 25±2°C with 16h photoperiod at 32 µE $m^{-2}s^{-1}$ light intensity. Some of the cultures were incubated in continuous light (24h), other conditions remaining unchanged. The cultures incubated in dark were also at 25±2°C.

2.4 STATISTICAL PROCEDURES

Statistical methods were used for comparison of treatment means during optimization parameters for micropropagation, organogenetic and embryogenetic response. Completely Randomized Designs were used. The data was subjected to analysis of variance (ANOVA) and treatment means were compared (Panse and Sukhatme, 1967). The data was analyzed using Microsoft Excel package.

2.5 HISTOLOGICAL PROCEDURES

Fixation: The tissues for histological studies were cut into small pieces and fixed in Formaldehyde: acetic acid: ethanol::5:5:90 v/v (FAA) mixture for 48h.

Dehydration and preparation of paraffin block: The FAA fixed tissues were passed through the grades of tertiary butyl alcohol (t-butanol, TBA) dehydration series (Table 2.2). In 100% TBA, samples were kept for 8 hours to overnight. In all other solutions they were kept for 6-8 hours.

Table 2.2 Grades of tertiary butyl alcohol for dehydration

H_2O	Ethanol	t-butanol (TBA)
50	40	10
30	50	20
15	50	35
-	45	55
-	25	75
-	-	100

The tissues were embedded in paraffin wax (m.p.58-60°C) following the method described (Sharma and Sharma 1980) and the paraffin blocks were made using a mould.

Sectioning: Sections of 10 µm thickness were cut on a microprocessor controlled rotatory microtome (Reichert - Jung 2050, Germany) and transfixed on glass slides.

Staining : The sections were dewaxed with xylene for 5-10 min. and were double stained with haematoxylin and eosin by passing the slides through the following series of solvents and solutions in coplin jars.

Xylene
↓2 min

Xylene
↓2 min

Xylene : absolute alcohol (1:1)
↓2 min

absolute ethanol
↓2 min

70% ethanol
↓2 min

40% ethanol
↓2 min

20% ethanol
↓2 min

distilled water
↓2 min

↓

Mordant, 4% iron alum (w/v in distilled water)

↓5 min

distilled water

↓2 min

1% haematoxylin (w/v in distilled water, matured for one month in light)

↓10 min

water

↓2 min

20% ethanol

↓2 min

40% ethanol

↓2 min

70% ethanol

↓2 min

absolute ethanol

↓2 min

absolute ethanol

↓2 min

1% eosin (w/v in absolute ethanol)

↓20 min

absolute ethanol

↓2 min

Xylene : absolute alcohol (1:1)

↓2 min

Xylene

↓2 min

Xylene

↓10-15 min

DPX-4 mountant

The stained sections were covered with cover slips and the permanent slides were examined under the microscope.

2.6 MICROSCOPY AND PHOTOGRAPHY

The morphogenic response in various explants was evaluated under stereo microscope, and recorded in black and white/colour photographs. Histological slides were studied under microscope and photographed. Magnifications of the photomicrographs were noted.

Table 2.1 Composition of macro and micro-element salts and vitamins in Murashige and Skoog basal medium

Ingredients	Amount (mgl^{-1})
Macro-nutrients	
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micro-nutrients	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Vitamins	
Myo-inositol	100
Thiamine-HCl	0.1
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Glycine	2

CHAPTER 3: CLONAL PROPAGATION

Clonal propagation through tissue culture offers an alternative to vegetative practices used in the past and has the potential to provide high multiplication rates of uniform genotypes, resulting in short term gains (Gupta *et al*., 1993). Cloning allows for the immediate and total capture of genetic gain. Cloning of mature trees is generally preferred over juvenile tissues as it is not always possible to determine if the juvenile tissue will have the desired qualities at maturity (Bonga, 1977).

The conventional method of clonal propagation of plants is often difficult, expensive and even unsuccessful. Tissue culture methods offer an alternative means of plant vegetative propagation. Clonal propagation through tissue culture (popularly called **micropropagation**) can be achieved in short time and space.

The advantages of vegetative propagation are: -

(a) faster asexual multiplication in contrast to seed propagation in plants with a long lifecycle.
(b) undesirable juvenile phase associated with the seed raised plants of woody perennials is bypassed by propagating them vegetatively directly from the adult material, and
(c) establishment of gene banks by multiplying variants among clonally propagated plants.

Micropropagation can broadly be described in four stages: -

Stage I - Establishment of culture

In this stage, axenic culture is initiated. For most micropropagation, the explant of choice is apical or axillary bud as the genetic makeup of the plant is preserved in these parts. Other explants used are leaf pieces (*Ficus lyrata, Anthurium spp., Saintpaulia ionantha* etc.), flower heads (*Gerbera jamsonii, Freesia*) etc.

For initiation of culture, **developmental stage of an explant** is an important factor. The age of stock plant, the physiological age of the explant and its developmental stage, as well as its size can determine the success of a procedure. Seedling explants are more responsive compared to the mature plant derived explants. Younger tissues, such as terminal or axillary shoot tips or tips of adventitious shoot regenerate better than older and more mature tissues of the same plant. Seasonal dormancy of the buds must be

considered while initiating the culture. Frequency of sprouting is always higher in the buds taken from plant during its vegetative phase.

Explants of some species e.g. banana, mango, black pepper etc. contain endogenous substances (e.g. **phenolics**) that exudes from the cut surfaces into the medium and inhibit development. These exudates are eliminated by various methods, prior to or during initiation of cultures. The explants are washed repeatedly after surface sterilization to eliminate both, adhering surface sterilant and the phenolics released from the cut surface. Frequent changes of medium can also overcome this inhibition. Antioxidants such as ascorbic acid or citric acid, are used either in a preliminary washing solution or in the medium itself. Absorbent material such as activated charcoal or polyvinyl pyrollidone (PVP) incorporated in the medium supports sprouting of buds by eliminating the inhibitory substances.

Stage II - Proliferation of shoots in culture

Proliferating cultures provide shoots for subsequent propagation as well as material that is required to maintain the stock. Multiplication of shoots is achieved by subculturing the shoot cultures at regular intervals.

Variant plants may arise from the cultures maintained *in vitro* for a long period. For the purpose of micropropagation, using the shoots from the cultures maintained for several passages and having higher **subculture numbers** is avoided to ensure genetic fidelity.

One of the physiological aberrations that may cause serious problem in stage II is **vitrification** (glassiness). Leaves become irreversibly translucent with varying degrees of distortion and swelling. At times, this is followed by necrosis and death of shoots. Sutter and Langhams (1979) attributed this to the absence of epicuticular wax.

This phenomenon has been associated with high cytokinin levels in the medium or in liquid cultures (Yeoman, 1986). Plants showing glassiness do not survive well.

Stage III – Rooting

Rooting in shoots can be induced either *in vitro* or *extra vitrum*. The major cost in plant propagation by tissue culture is manual labor. This is especially pronounced in the rooting stage when individual shoots are manipulated. It is preferable that all manipulations of separated shoots, such as in the rooting stage, be performed under non-aseptic conditions.

In majority of the culture systems, shoots are obtained by transfer of cultures from the propagation medium, to an appropriate elongation medium. This can be achieved by transplanting isolated shoots to a medium devoid of or containing low cytokinins. However, instead of handling isolated shoot, it is economical to transfer clusters of shoots to a medium that allows good elongation of all of the shoots.

For rooting in vitro, shoots are cultured in auxin containing medium or subjected to pulse treatment of auxin prior to transferring to an auxin free medium to induce root formation or growing shoots on low auxin concentration.

For rooting *in vivo / ex vitro,* the shoots are excised as small cuttings (microcuttings), treated with auxin and planted in soil. These are then placed in a high humidity chamber for rooting.

Rooting *ex vitro* has many advantages: -

- It is easier to stick a cutting than to plant a rooted plantlet.
- Labor-intensive *in vitro* operations of single shoots are avoided.
- The root system produced *ex vitro* simultaneously establishes in soil.
- Possibility of damaging the roots while transferring the plant to soil is avoided. These damages cause root or stem diseases.
- For difficult-to-root plants it is easier and cheaper to create good conditions *ex vitro* than *in vitro*.

Stage IV – Hardening of tissue culture raised plants

This stage involves transfer of plantlets from aseptic condition to the environments of the greenhouse and ultimately to the final location. At this stage the shoots may be rooted or unrooted (microcuttings) but in either case the plantlet or

propagule must undergo a period of **acclimatization** to enable it to survive. Plants are transferred to soil in polybags, pots or protrays. Use of plug systems with natural or artificial substrates offers possibilities to robotize the most labor requiring stage of micropropagation.

Important factors after transplanting :-

- Maintenance of **high relative humidity** to protect the plants from desiccation and enable them to initiate new roots and shoots.
- A loose, aerated, well-drained **suitable rooting medium**, which allows new roots to develop quickly.
- **Protection from various pathogens** until some resistance has developed.

Often problems are encountered while using adult plant material for regenerative propagation *in vitro*. In such cases a model study is set by using seedling (juvenile) material. Surface sterilized seeds are sown *in vitro*; and the seedlings obtained are used for *in vitro* propagation via proliferation of shoots by axillary or adventitious shoot formation. The information generated is then extended to selected adult plants (Pierik, 1976).

One of the most striking differences between herbaceous and woody species, is that the latter are far more difficult to clone *in vitro*. The reasons for this are (Pierik, 1975; Bonga and Durzan, 1982; Kunneman-Kooij, 1984) : -

- Woody species have a relatively weak regenerative capacity when compared to herbaceous species.
- Research with trees and shrubs was initiated later.
- The induction of rejuvenation is generally difficult in woody species.
- The multiplication rate is much lower with woody species than herbaceous species.
- Dormancy plays a role in the case of trees and shrubs; buds do not open and stem elongation fails to take place.
- Woody species are more liable to be effected by excretion of toxic substances into the nutrient media.
- Trees and shrubs are selected for cloning when they are adult. Adult material is often very difficult to propagate *in vitro*.
- Genetic variation in trees is generally greater than in agricultural and horticultural crops, giving rise to variable results.

- Green house material is not available for adult plants of woody species and explants are taken from field-grown trees. Variation in the explants due to the different growth conditions and annual fluctuations in the climate demands continuous modification of protocol.

The work carried out on clonal propagation on tamarind is included in this Chapter. The experiments carried out and the results obtained are divided into four parts. Part 'A' includes the work on seedling explants. Part 'B' describes the work on mature-tree-derived axillary buds, part 'C' describes the experiments on micrografting of *in vitro* raised shoots and part 'D' describes the studies carried out to demonstrate the differences in variation exhibited at the early stage of plant development from seed.

A : Clonal propagation from seedling explants

3.1A INTRODUCTION

Seedling explants are, in general, more responsive than explants derived from mature trees (Pradhan *et al*., 1998). As most of the woody species segregate for phenotypic traits when propagated by seeds, the plants regenerated *in vitro* from seedling explants would display inherent genetic variation. This could, however, be controlled using an elite seed population as was selected by Pradhan *et al.*(1998) for their study on propagation of *Dalbergia sissoo*.

Reports of successful micropropagation of various leguminous species is reviewed extensively (Parrott *et al*., 1992). In forest tree species, the success has been largely restricted to seedling materials (Summer and Wetzstein,1984). *In vitro* protocols, using seedling explants, have been employed for rapid propagation and manipulation of woody legumes, such as *Albizzia* (Gharyal and Maheshwari, 1981), *Acacia nilotica* (Dewan *et al*., 1982), *Sesbania sesban* (Khattar and Mohanram, 1982), *Sesbania grandifolia* (Khattar and Mohanram, 1983), *Leucaena leucocephala* (Nataraja and Sudhadevi, 1984; Dhawan and Bhojwani, 1985), *Dalbergia latifolia* (Sudhadevi and Nataraja, 1987a,b), *Acacia tortilis* subsp. *raddiana* (Nandwani, 1995), *Swartzia madagascariensis* (Berger and Schaffner, 1995), *Mimosa tenuiflora* (Villarreal and Rojas, 1996), *Bauhinia vahlii*

(Upreti and Dhar, 1996), *Acacia mearnsii* (Beck *et al*., 1998a) and *Dalbergia sissoo* (Pradhan *et al*., 1998).

Repeated attempts have been made to standardize protocol for clonal propagation of tamarind seedling explants (Table 1.4). With an ultimate objective to establish a method for clonal propagation of mature tamarind tree, in the present investigation initial studies were conducted with the seedling explants. This is primarily to generate information on the nature of responses, exhibited *in vitro* by the tissues of this plant, which can be extended for studies with mature explants. The plants raised from tissue culture of seedling derived explants will also be useful to confirm the previous observations (Mascarenhas *et.al*., 1987) regarding reduction of the juvenile phase of seedling raised tree.

3.2A EXPERIMENTAL PROTOCOL

3.2A. 1 Preparation of explant

Tamarind seeds were collected from the mature pods of locally grown trees or from Agriculture College, Pune. The seeds were washed with liquid detergent and surface sterilized using 0.1% w/v $HgCl_2$ for 15 min. Excess $HgCl_2$ was removed by repeated washings with sterile distilled water under aseptic conditions. Seeds were soaked overnight in sterile distilled water at room temperature and washed thoroughly before culturing them in half strength Murashige and Skoog (MS) basal salts and vitamin with 2% sucrose and 7g.l^{-1} agar. Cultured seeds were incubated in dark for 4-6 weeks.

Germination and growth of seedlings are asynchronous (Fig. 3A.1). From 20 d onwards some of the seedlings show presence of first pair of leaves. Due to the asynchronous germination and growth seedlings reached this size in 20-60 days. Seedlings of 30-42 days were used as source of explant. Shoot tip after excising away

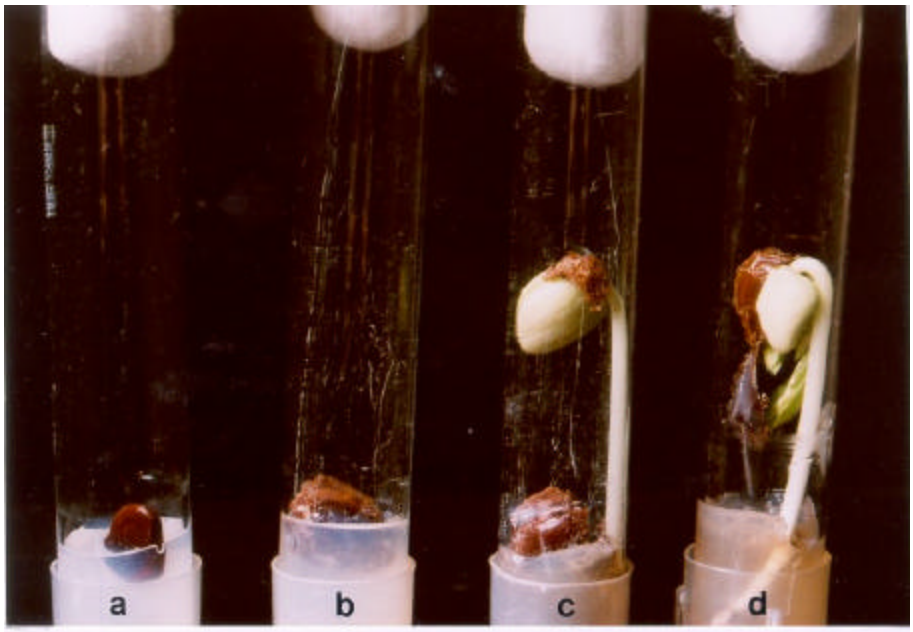


Fig. 3A.1

Fig. 3A.1 : Asynchronous germination of tamarind seeds after 20 days in culture

(a) Unswollen seed
(b) Swollen, cracked seed
(c) Seedling with differentiated hypocotyl
(d) Seedling with differentiated plumule and hypocotyl – source of explants.

the first pair of leaves and the cotyledonary node after excising away the cotyledons was isolated from the hypocotyl 2-2.5cm below the node. These explants were used for the experiments (Fig. 3A.2).

3.2A. 2 Multiplication and elongation of shoots

3.2A.2.1 Growth regulators

MS basal medium supplemented with growth regulators including KN, BAP, Z and 2iP singly at concentrations 0.02, 0.1, 0.5, 2 and 10 $mg.l^{-1}$ were tested. TDZ at concentration 0.02, 0.1, 0.5 and 2 $mg.l^{-1}$ was tested. Kinetin and BAP were tested in combinations. The concentrations were 0.02, 0.1, 0.2, 0.5, 1 and 2 $mg.l^{-1}$.

After the incubation of 4 weeks, number of explants responded in culture and total number of shoot primordia formed in each explant were scored. The average height of the elongated shoots was determined and the cultures showing opening of leaves in the shoots were scored. The data was processed for statistical analysis.

3.2A.2.2 Carbohydrates

MS basal media with BAP (0.02, 0.1, 0.2, 0.5, 1 and 2 $mg.l^{-1}$) were supplemented with 2% Glucose, Fructose or Sucrose to study the effect of various carbohydrates.

At the end of the incubation period of 4 weeks, number of explants responded in culture and the number of shoots more than 1cm in each explant were scored. Average height of the elongated shoots was determined. Data was processed for statistical analysis.

The observations were noted by nondestructive method, and the incubation period of these cultures was extended for two more weeks to obtain elongated shoots (3-6 cm). The elongated shoots were isolated from the explant and used for rooting experiments. The original explant devoid of the elongated shoots was recultured on fresh media.

3.2A.2.3 Subculture

For multiplication of shoots induced from the existing meristems of tamarind, two methods were applied. In the **first method** the elongated shoot was excised leaving one

node with the original explant. The original explant with this node was recultured for further sprouting and elongation. The elongated shoot was used either for rooting or for subculture. In the **second method** the elongated shoot was cut into 2-3 pieces (each measuring ~2cm). One had the tip and the others were nodal segments. These explants were cultured vertically in agar gelled medium containing 0.1 or 0.2 $mg.l^{-1}$ BAP with 2% sucrose in tubes. The elongated shoot tip and the shoots developed from the nodal meristems were used for rooting or further multiplication.

Cultures were incubated in light for 6 - 8 weeks to obtain elongated shoots.

3.2A. 3 Rooting of elongated shoots

The elongated shoots (3-4 cm) were cultured in liquid half strength MS medium supplemented with auxin mixture (IAA, IBA, IPA and NAA at 1 $mg.l^{-1}$ each). Following 72 h incubation in dark, these were transferred to medium devoid of PGRs. In another experiment, auxins, IAA or IBA at 1 and 2 $mg.l^{-1}$ with 0.7% agar were used singly for 45 days. Number of shoots rooted along with the number of roots per shoot and its length was noted. In the third experiment the shoots were shifted to half strength MS medium supplemented with 0.25% activated charcoal following 45 days exposure in IAA or IBA at 1 and 2 $mg.l^{-1}$ with 0.7% agar. Cultures were incubated for 4-6 weeks in light.

3.2A. 4 Transfer of plant to soil

Rooted plantlets were washed thoroughly under running tap water to remove the medium adhering to the root system prior to transfer of these plantlets to 10×5cm polybags / pots containing soil:sand (1:1). The plantlets were maintained in light at 25±2°C in the trays. Trays were covered with glass sheets and pots were covered with polythene bags to minimize the loss of moisture. After 20 days the glass sheet covers were removed partially or polythene cover was cut from one corner and plants were maintained for 30 days prior to transfer to pots in greenhouse.

3.3A RESULTS AND DISCUSSION

3.3A.1 Preparation of explant

The seedlings showing the presence of first pair of leaves were the source of explants (Fig. 3A.2 A, B). The shoot tip explant containing the apical meristem and first nodal segment without the pair of leaves and the decotyledonated node (having the pair of

dormant axillary buds) with a portion of elongated hypocotyl (Fig. 3A.2 C, D) were used as explants.

Shoot tips, isolated from less differentiated seedlings did not respond in culture. Therefore the seedlings showing first pair of leaves was chosen as the explant donor. Seedlings reached that required stage of growth at different days intervals.

3.3A.2 Multiplication and elongation of shoots

MS medium has frequently been used successfully for shoot induction in tree tissue culture studies (Dunston and Thorpe, 1986). The superiority of MS over other salt formulations has been demonstrated in other leguminous trees such as *Prosopis cineraria* (Shekawat *et al*., 1993), *Swartzia madagascarensis* (Berger and Schaffner, 1995), *Bauhinia vahlii* (Upreti and Dhar, 1996). MS basal medium was used in our studies in Tamarind.

The shoot tip as well as the cotyledonary nodal explant, both sprouted within 8-10 days of culture in MS medium with 2% sucrose and gave rise to shoots in 4 weeks (Fig. 3A.3A) when cultured in light.

3.3A.2.1 Effect of Growth Regulators

Initiation and multiplication of shoots require appropriate medium containing either a cytokinin or a combination of cytokinin and auxin. In tree species, Kinetin or BAP or their combination has been used to achieve multiplication of the shoot buds. Nodal explants of *in vitro* grown seedlings and fungicide pretreated 3 and 9-month-old greenhouse grown *Acacia mearnsii* plants were cultured on MS medium supplemented with 2 $mg.l^{-1}$ BAP. *In vitro* grown seedling resulted in optimal shoot production with 85% of the explants producing shoots with an average of 2 shoots per node. The 3 month old and 9 month old greenhouse grown plants showed response

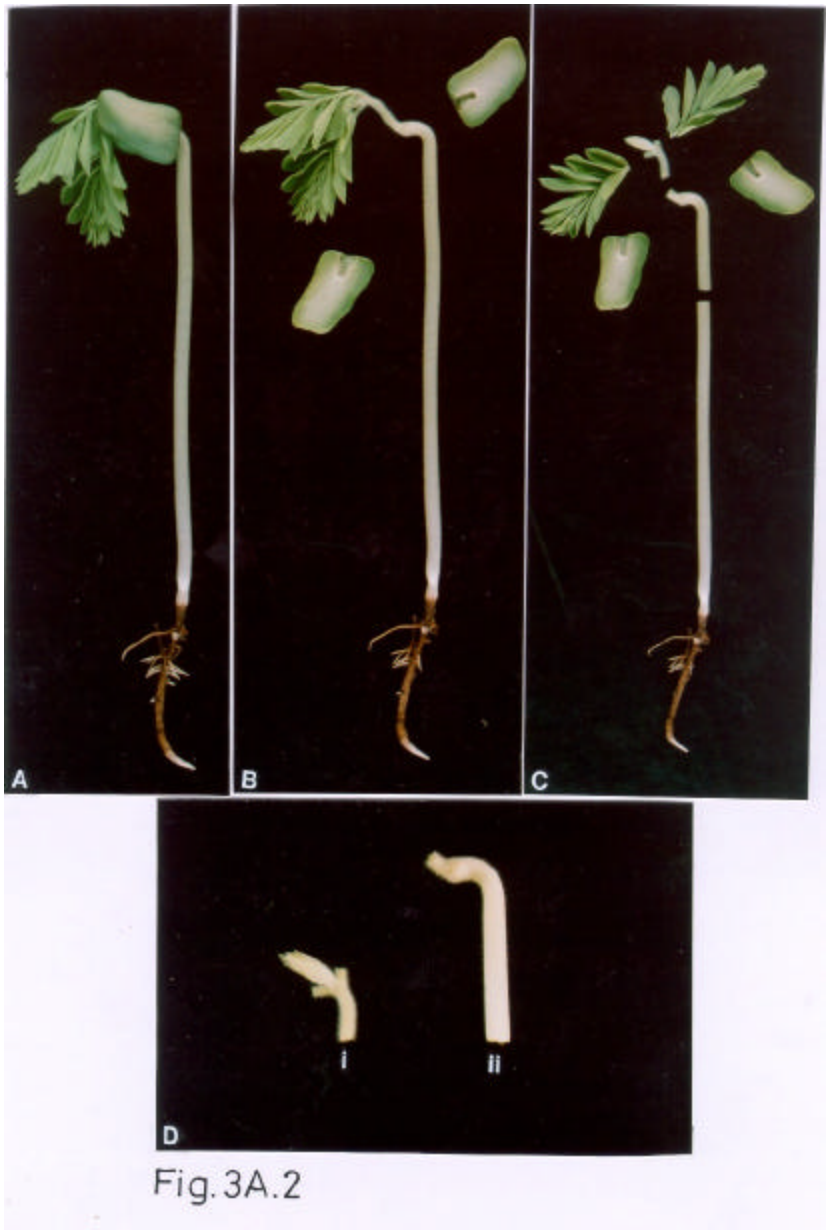


Fig. 3A.2

Fig. 3A.2 : Preparation of seedling explant for culture

A. Tamarind seedling at the stage of development used for isolation of explant

B. Decotyledonated seedling of tamarind

C. Dissected seedling with various parts.

D. Isolated explants used for initiation of culture.

(i) Shoot tip with apical meristem and first pair of axillary nodes.

(ii) Cotyledonary node explant with portion of hypocotyl.

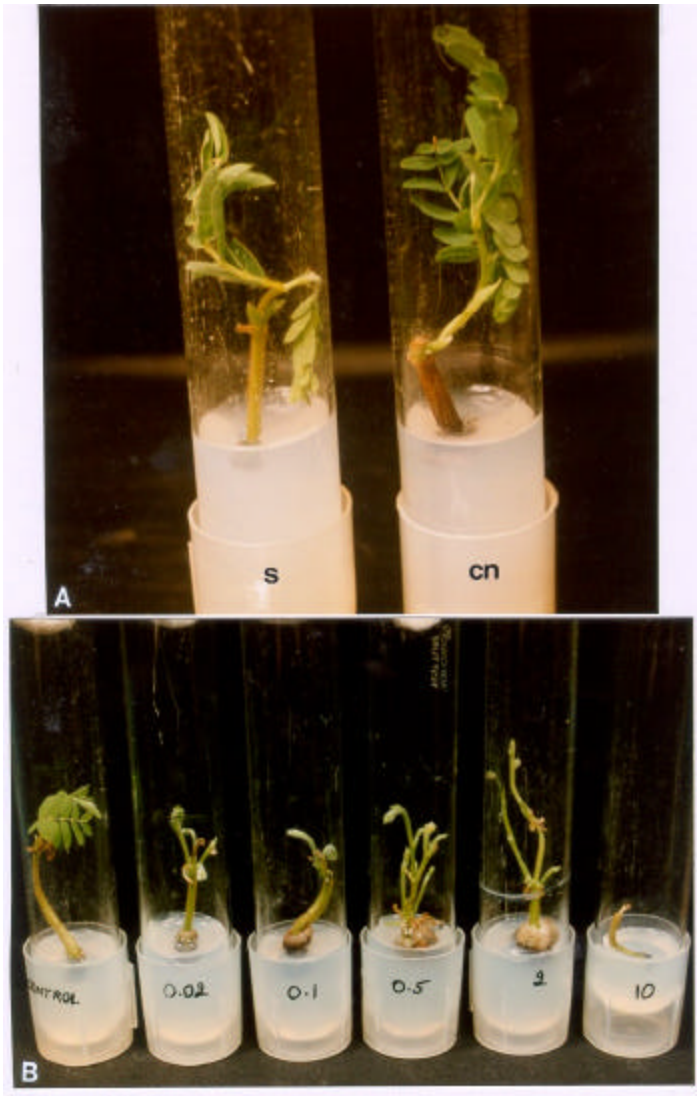


Fig. 3A.3

Fig. 3A.3A: Sprouting and differentiation of shoot tip (s) and cotyledonary node (cn) explant giving rise to shoots after four weeks in culture.

Fig. 3A.3B: Effect of varying concentration of BAP on shoot tip cultures. Figures on tube holders indicate concentration of BAP in $mg.l^{-1}$

with 15% and 45% cultures at the same concentration of BAP (Beck *et al*., 1998a). In *Dalbergia sissoo* Roxb, 99% shoot proliferation and maximum number of shoots per cotyledonary node explant (7-9 shoots) was achieved using 8.9µM BAP (Pradhan *et al*., 1998).

In an attempt to micropropagate *Acacia tortilis* subsp. *raddiana* shoot multiplication was achieved from cotyledonary nodal explants in MS medium containing 1 $mg.l^{-1}$ NAA and BAP 5 $mg.l^{-1}$ (Nandwani, 1995). In an attempt to obtain shoot multiplication for *in vitro* propagation of *Swartzia madagascarensis* 37 shoots/explants could be induced in MS medium containing 2.2 µM BAP (Berger and Schaffner, 1995). Micropropagation of *Bauhinia vahlii* Wight and Arnott- a leguminous liana could be achieved by Upreti and Dhar (1996) when the cotyledonary nodes from *in vitro* germinated seedlings were cultured on MS medium containing 1µM thidiazaron. Jaiwal and Gulati (1992) used BAP $5x10^{-6}$M to culture shoot tip and nodal explants from elongated shoot of tamarind and obtained 3 shoots/explant.

In the present study, shoot tips and cotyledonary nodes of tamarind seedlings cultured in MS medium with various concentrations of cytokinins responded in 8-10 days by sprouting and elongating respectively. At the end of the incubation period of 30 days it was observed that the explants responded in all the cytokinins tested. The frequency of response ranged between 40-100% (Table 3A.1). The average number of shoots/explant for shoot tip varied significantly. Highest response of 2.6±0.3 was obtained in media containing BAP 0.5 $mg.l^{-1}$ whereas BAP 10 $mg.l^{-1}$ was least effective (Fig. 3A.3B). Elongated shoots with opened leaves are appropriate for rooting and survival in soil. In BAP 0.02 $mg.l^{-1}$ elongation of shoot was maximum giving an average height of shoot 2.0±1.2cm, but the number of shoots per explant was only 1.9±1.3. In presence of KN, the shoots did not multiply but in all the concentrations except in 10 $mg.l^{-1}$, leaf opening was noted. However, the number of cultures showing opened leaves varied significantly with varying concentrations of KN. In KN 0.5 $mg.l^{-1}$ all the shoots had opened leaves. BAP inhibited the process of leaf opening and it was restricted in media with 0.5 $mg.l^{-1}$ and above. For clonal propagation, increased number of shoots is desirable. Therefore, media containing 0.02-2 $mg.l^{-1}$ BAP were viewed as the potential combinations. In the media with TDZ 0.02 $mg.l^{-1}$ the frequency of response was 100% and it decreased with

increasing concentration of this growth regulator. There was no significant change in shoot multiplication and the elongation of shoot. Leaf opening was reduced. In 2iP and lower concentrations of Z the frequency of sprouting response was 100% but there was no shoot multiplication. In Z 2 $mg.l^{-1}$ the frequency of response was similar to BAP 0.5 $mg.l^{-1}$ but the number and average height of shoots per explant was less than BAP 0.5 $mg.l^{-1}$. In contrast to 100% leaf opening in 0.5 $mg.l^{-1}$ KN, in BAP at the same concentration, none of the shoots had opened leaves (Fig. 3A.3B) although the number of multiple shoots was highest.

Table 3A.1 : Effect of different cytokinins on shoot tips of tamarind seedling

Medium (PGR conc. mg. l^{-1})	Frequency of response (%) Mean ±s.d	Av. no. of shoots/ Explants Mean ±s.d	Av. Ht. of shoot (cm) Mean ±s.d	Cultures showing leaf opening (%) Mean ±s.d
KN 0.02	**100.0± 0.0**	0.9±0.1	1.1±0.2	87.5±17.7
KN 0.1	90.0±14.1	1.0±0.0	1.3±0.4	45.0± 7.1
KN 0.5	80.0±28.3	1.0±0.0	1.7±1.0	**100.0± 0.0**
KN 2	**100.0± 0.0**	0.9±0.1	1.2±0.2	45.0± 7.1
KN 10	80.0±28.3	0.8±0.3	1.5±0.7	0.0± 0.0
BAP 0.02	**100.0± 0.0**	1.9±1.3	**2.0±1.2**	43.6±51.5
BAP 0.1	**100.0± 0.0**	1.2±0.9	1.4±0.4	22.2±15.7
BAP 0.5	90.0±14.1	**2.6±0.3**	1.6±0.3	0.0± 0.0
BAP 2	70.0±14.1	1.7±0.5	1.5±0.2	0.0± 0.0
BAP 10	50.0±70.7	0.3±0.4	0.6±0.9	0.0± 0.0
TDZ 0.02	**100.0± 0.0**	1.0±0.0	1.0±0.0	90.0±14.1
TDZ 0.1	50.0±70.7	0.5±0.7	0.6±0.9	50.0±70.7
TDZ 0.5	40.0±28.3	1.5±0.7	1.1±0.4	16.7±23.6
TDZ 2	40.0± 0.0	0.5±0.7	0.6±0.8	0.0± 0.0
2iP 0.02	90.0±14.1	1.0±0.0	1.5±0.7	80.0±28.3
2iP 0.1	**100.0± 0.0**	1.0±0.0	1.0±0.1	40.0±56.6
2iP 0.5	**100.0± 0.0**	1.0±0.0	1.0±0.0	70.0±42.4
2iP 2	**100.0± 0.0**	1.0±0.0	1.4±0.5	80.0±28.3
2iP 10	**100.0± 0.0**	1.0±0.0	0.9±0.2	0.0± 0.0
Z 0.02	**100.0± 0.0**	1.0±0.0	1.1±0.2	90.0±14.1
Z 0.1	**100.0± 0.0**	1.1±0.1	1.1±0.3	58.3±58.9
Z 0.5	**100.0± 0.0**	1.7±0.4	1.1±0.0	36.4± 9.1
Z 2	90.0±14.1	1.9±0.2	1.1±0.1	25.0±35.4
Z10	60.0±56.6	1.0±0.0	1.5±0.8	0.0± 0.0
MS	70.0±14.1	1.0±0.0	1.2±0.2	87.5±17.7
ANOVA	NS	S 1%	NS	S 1%

Data from 10 replicates

Table 3A.2: Effect of different cytokinins on cotyledonary nodes of tamarind seedling

Medium (PGR conc. mg. l^{-1})	Frequency of response (%) Mean ±s.d	Av. no. of shoots / Explants Mean ±s.d	Av. ht. of shoot (cm) Mean ±s.d	Cultures showing leaf opening (%) Mean ±s.d
KN 0.02	90.0±14.1	1.1±0.5	1.5±0.6	76.2±13.5
KN 0.1	**100.0± 0.0**	0.9±0.4	1.9±0.7	83.3±23.6
KN 0.5	90.0±14.1	1.0±0.0	2.3±0.7	90.0±14.1
KN 2	90.0±14.1	1.1±0.2	3.4±2.8	70.0±42.4
KN 10	90.0±14.1	1.5±0.1	2.5±1.6	29.8±18.5
BAP 0.02	**100.0± 0.0**	1.6±0.3	**3.9±2.6**	88.9±15.7
BAP 0.1	**100.0± 0.0**	1.5±0.7	3.1±1.9	65.0±49.5
BAP 0.5	90.0±14.1	**2.4±1.7**	2.4±0.7	8.3±11.8
BAP 2	90.0±14.1	2.2±1.4	2.1±0.4	0.0± 0.0
BAP 10	70.0±14.1	1.9±0.2	1.1±0.1	0.0± 0.0
TDZ 0.02	70.0±42.4	1.4±0.2	1.7±1.0	66.7± 0.0
TDZ 0.1	87.5±17.7	0.9±1.0	3.1±1.2	30.0±42.4
TDZ 0.5	65.0±21.2	0.5±0.0	0.8±0.0	0.0± 0.0
TDZ 2	50.0±14.1	0.3±0.4	0.4±0.6	0.0± 0.0
2iP 0.02	**100.0± 0.0**	1.3±0.1	2.2±1.7	77.4± 8.4
2iP 0.1	**100.0± 0.0**	1.2±0.0	1.7±0.6	91.7±11.8
2iP 0.5	90.0±14.1	1.3±0.1	1.5±0.8	80.0±28.3
2iP 2	**100.0± 0.0**	1.1±0.1	2.8±2.0	66.7±47.1
2iP 10	90.0±14.1	2.1±1.9	1.3±0.2	2.9± 4.2
Z 0.02	80.0±28.3	1.4±0.0	2.1±0.1	85.7±20.2
Z 0.1	90.0±14.1	1.3±0.1	2.5±0.5	90.0±14.1
Z 0.5	**100.0± 0.0**	2.0±1.1	2.7±1.5	64.3±50.5
Z 2	87.5±17.7	2.1±0.1	1.8±0.7	9.1±12.9
Z10	**100.0± 0.0**	1.1±0.6	1.0±0.1	0.0± 0.0
MS	70.0±42.4	1.1±0.1	3.1±2.1	**100.0± 0.0**
ANOVA	NS	NS	NS	S 1%

Data from 10 replicates

Similar to the shoot tip explant, in all the cytokinins the cotyledonary nodes gave rise to shoots at varying frequencies (Table 3A.2). However although there were a pair of meristems in each explants but often only one meristem sprouted and elongated. In medium without PGR all the shoots had opened leaves (Fig.3A.4A). Parallel with the observation noted in the shoot tip, in cotyledonary node maximum number of shoots/explant (2.4±1.7) was obtained in BAP 0.5 $mg.l^{-1}$ and maximum elongation of shoot (3.9±2.6cm) was achieved in BAP 0.02 $mg.l^{-1}$. In BAP 2.0 $mg.l^{-1}$ although the number of multiples were close to BAP 0.5 $mg.l^{-1}$ but none of the shoots had opened leaves (Fig, 3A.4B). More number of cultures with opened leaves was noted in KN (Fig.3A.4C) and 2iP (Fig.3A.5A). In medium with TDZ, not only the multiplication, elongation and leaf opening was poor but there was dedifferentiation and profuse callusing in the part of the explant in contact of medium (Fig. 3A.5B). In Z 0.5 $mg.l^{-1}$ and 10 $mg.l^{-1}$ the frequency of morphogenetic response was 100%. This was similar with the effect in BAP 0.02 and 0.1 $mg.l^{-1}$. However the number of shoots/explant was higher in BAP 0.5 $mg.l^{-1}$. Similar to BAP in higher concentrations leaf opening was poor in Z (Fig. 3A.5C) at higher concentration.

The opening of leaves in cultures growing in KN containing media were found to be lesser than MS, but the difference was not significant. All the rest of the PGRs (BAP, TDZ, 2iP and Z) showed significantly lower percentage in the opening of leaves, which were completely inhibited at higher concentrations.

In the previous experiment with various PGRs it was observed that KN and BAP are comparatively more favorable for growth, multiplication, elongation and leaf opening in tamarind shoot cultures. To determine the optimum concentration of KN and BAP required to obtain maximum response, in the following experiments influences of these two growth regulators were studied in ranges between 0.02-2 $mg.l^{-1}$ in both shoot tip and cotyledonary nodes.

It was observed that KN at 1 $mg.l^{-1}$ induced morphogenic response in all the explants as against 46% and 71% in shoot tip and cotyledonary node respectively in medium devoid of growth regulator. In other concentrations of KN none of the data was conclusive. Statistical analysis shows very high deviations in all the values. In 0.5

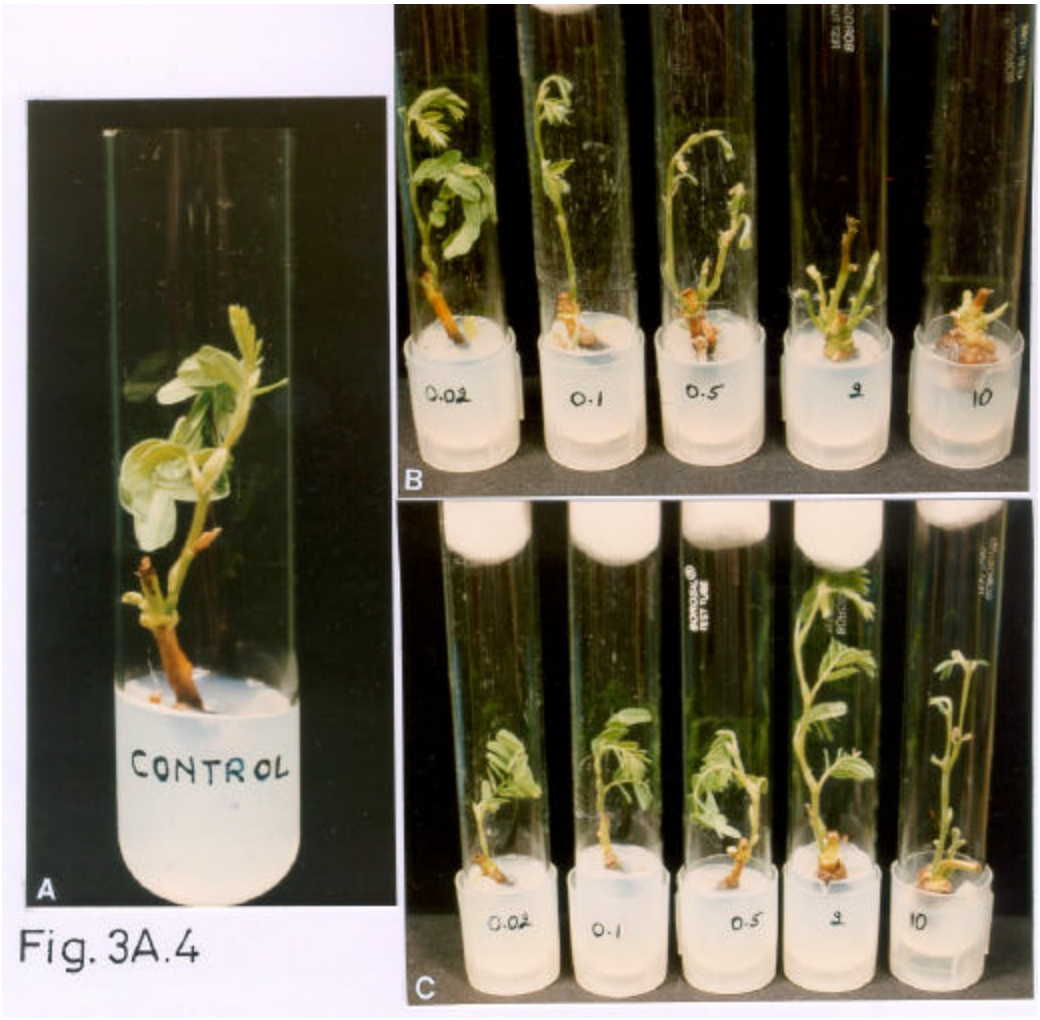


Fig. 3A.4

Fig. 3A.4 : Sprouting, elongation and leaf opening in cotyledonary nodal explants :

(A) Shoot with opened leaves in medium devoid of growth regulator,

(B) Multiplication of shoots, reduction in shoot height with increase in concentration of BAP. Leaves did not open in higher concentrations.

(C) Shoot cultures with opened leaves in various concentrations of KN

Concentration of the growth regulator in $mg.l^{-1}$ indicated on the tube holder.

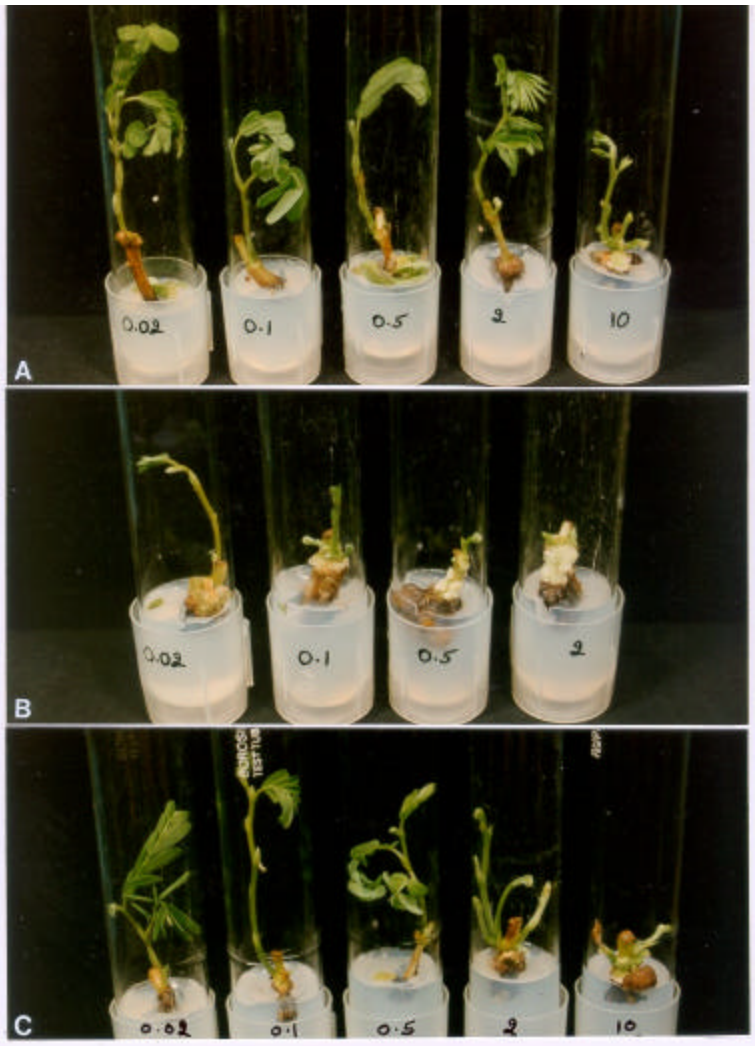


Fig.3A.5

Fig. 3A.5 Sprouting, elongation and leaf opening in cotyledonary nodal explants in presence of :

(A) Cultures with opened leaves in 2iP

(B) Poor multiplication, elongation and leaf opening in TDZ. Cultures growing in TDZ showed dedifferentiation and profuse callusing in the part of explant in contact of medium.

(C) Reduction in shoot height and leaf opening in the shoots with increasing concentration of Z

Concentration of the growth regulator in $mg.l^{-1}$ indicated on the tube holder.

$mg.l^{-1}$ all the shoots had opened leaves (Fig.3A.6A). No significant changes were noted in the other responses (Table3A.3).

Table 3A.3 : Effect of Kinetin concentration on tamarind seedling explants

Medium (PGR conc. in $mg.l^{-1}$)	Shoot tip				Cotyledonary node			
	Frequency of response (%) Mean±s.d	Avg. no. of sh. / expl. Mean±s.d.	Avg. ht. of shoot (cm) Mean±s.d.	Cultures showing leaf opening (%) Mean±s.d	Frequency of response (%) Mean± s.d	Avg. no. of sh. / expl. Mean±s.d.	Avg. ht. of shoot (cm) Mean±s.d.	Cultures showing leaf opening (%) Mean ±s.d
K0.02(18)	66.7±57.7	0.6±0.5	0.8±0.7	58.3±52.0	73.0±31.9	1.1±0.3	1.4±0.5	84.1±16.7
K0.1 (18)	64.2±45.9	**1.0±0.0**	1.7±0.8	63.3±32.2	70.8±50.5	0.9±0.3	2.0±0.6	88.9±19.3
K0.2 (20)	80.0± 0.0	**1.0±0.0**	1.7±0.4	81.3±8.2	95.0± 7.1	**1.4±0.1**	**2.4±0.1**	53.6±5.1
K0.5 (18)	61.7±37.5	0.9±0.1	1.5±0.7	**100.0±0.0**	76.7±25.2	1.0±0.0	2.3±0.5	76.7±25.2
K1 (19)	**100.0± 0.0**	**1.0±0.0**	**2.2±0.7**	68.3±2.4	**100.0± 0.0**	**1.4±0.1**	2.2±0.2	73.2±2.5
K2 (18)	75.0±43.3	0.9±0.1	1.4±0.4	63.3±32.2	60.0±52.9	0.8±0.7	2.2±2.8	46.7±50.3
MS (28)	45.6±30.2	0.8±0.4	1.1±0.2	85.4±17.2	71.0±42.0	1.1±0.1	2.2±1.3	**93.2±13.6**
ANOVA	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**

Figures in parenthesis indicates number of replicates

The effect of BAP on various responses in the seedling explants is included in Table 3A.4. In medium with BAP 0.2 $mg.l^{-1}$ maximum number of shoot tip and cotyledonary node explants responded. However, the difference was not significant when compared with the response obtained in medium devoid of growth regulators. In medium containing BAP 1 $mg.l^{-1}$ the apical meristem was stimulated to produce (2.2±0.7) shoot/explant. This was significantly higher than in medium devoid of growth regulators. This indicates that BAP 1 $mg.l^{-1}$ induces proliferation of apical meristem in tamarind. In other concentrations of BAP the number of shoots/explant was higher than in MS but this increase was not significant. In medium with BAP 0.1 $mg.l^{-1}$, the average height of shoot tip derived shoot was 2.9±1.5cm. However the number of shoots/explant was 1.1±0.4 which was lower than in BAP1 $mg.l^{-1}$. Effect of BAP on leaf opening is inhibitory in higher concentrations.

Cotyledonary node explant cultured in medium with BAP 1 $mg.l^{-1}$ also showed the maximum number of shoots/explant (2.6±1.0), like in shoot tip explant. These shoots were more elongated in lower concentrations of BAP (Table 3A.4). The

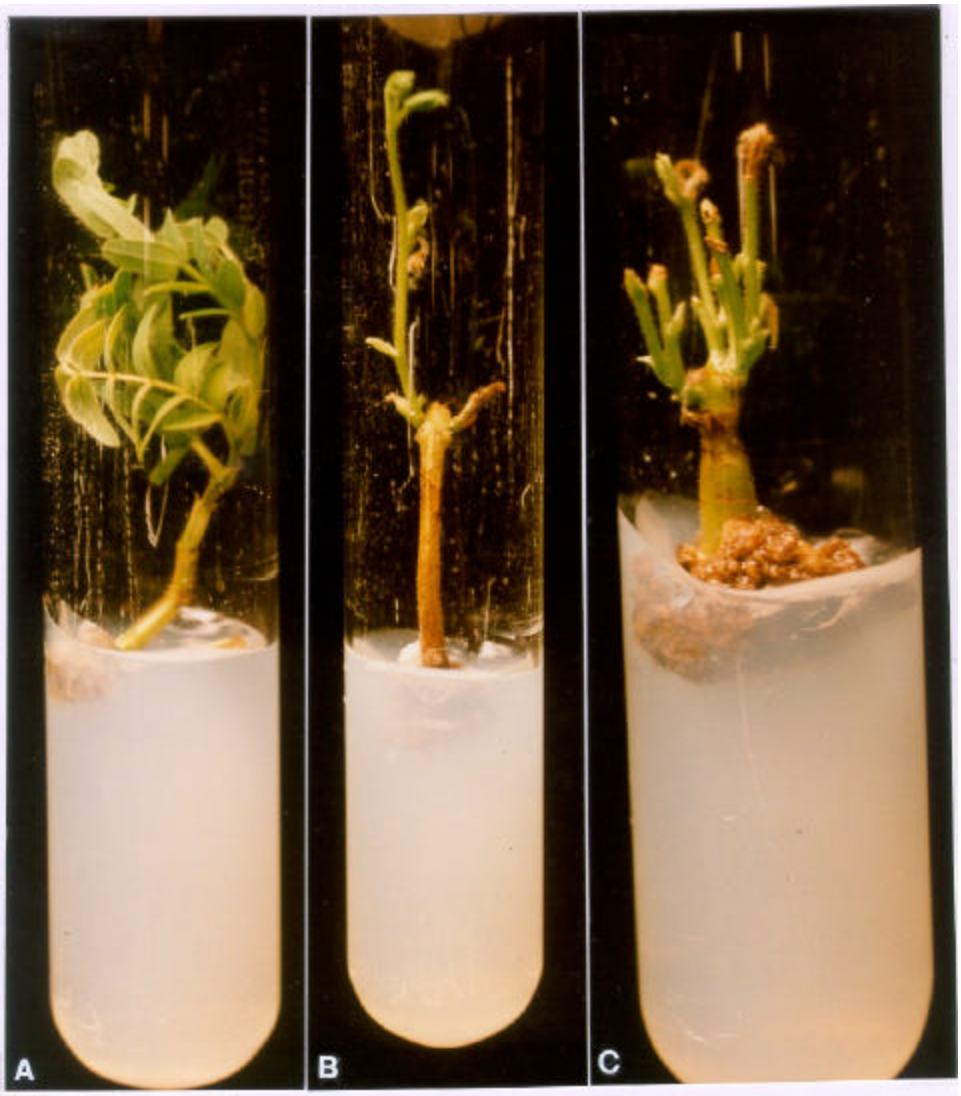


Fig. 3A.6

Fig. 3A.6 : Elongated shoots of tamarind from :

(A) **Shoot tip** in KN 0.5 $mg.l^{-1}$ showing opening of leaves.

(B) **Cotyledonary nodal** explant showing elongation of shoot in MS medium supplemented with BAP 0.2 $mg.l^{-1}$.

(C) **Cotyledonary nodal** explants showing multiplication of shoots in MS medium supplemented with BAP 1 $mg.l^{-1}$. Leaves did not open.

leaf opening considerably decreased as compared to MS in lower concentration of BAP and in higher concentrations (1, 2 $mg.l^{-1}$) it was completely inhibited. Thus it was noted that shoot multiplication and elongation did not occur together in a single medium (Fig.3A.6B, C).

Table 3A.4 : Effect of BAP concentrations on tamarind seedling explants

Medium (PGR conc. in $mg.l^{-1}$)	Shoot tip				Cotyledonary node			
	Frequency of response (%) Mean±s.d.	Avg. no.of sh./expl. Mean± s.d.	Avg. ht. of shoot (cm) Mean±s.d.	Cultures showing leaf opening (%) Mean ±s.d	Frequency of response (%) Mean±s.d.	Avg. no. of sh./expl. Mean± s.d.	Avg. ht. of shoot (cm) Mean±s.d.	Cultures showing leaf opening (%) Mean ±s.d
B0.02(32)	75.7±28.9	1.3±0.7	2.3±1.0	54.8±41.2	73.8±25.2	1.5±0.4	**2.8±1.4**	60.2± 24.2
B0.1 (32)	73.9±27.5	1.1±0.4	**2.9±1.5**	56.0±38.7	81.7±28.2	1.8±0.5	2.7±1.1	44.9± 32.3
B0.2 (14)	**85.4± 17.2**	1.2±0.5	2.7±0.6	0.0±0.0	**91.5±11.8**	1.8±0.3	2.7±1.0	16.7± 83.6
B0.5 (32)	65.6±27.8	1.6±0.7	1.8±0.8	0.0±0.0	80.4±22.4	2.1±1.0	2.0±0.7	13.3± 21.7
B1 (14)	58.8±31.2	**2.2±0.7**	2.3±1.3	0.0±0.0	65.8±29.9	**2.6±1.0**	1.8±0.5	0.0± 0.0
B2 (32)	48.0±35.7	1.1±0.6	1.7±1.2	0.0±0.0	67.4±28.0	2.6±0.8	1.7±0.4	0.0± 0.0
MS (24)	50.6±28.3	0.8±0.4	1.1±0.1	**91.7±14.4**	50.6±36.5	1.1±0.1	2.6±1.4	**100.0± 0.0**
ANOVA	NS	S 5%	NS	S 1%	NS	S 5%	NS	S 1%

Figures in parenthesis indicate number of replicates

An interactive study on the combination of KN and BAP was conducted on shoot tip and cotyledonary node explants. In all the combinations of KN and BAP the shoot tip responded to produce shoots or meristematic buds. However the frequency of response varied. These variations were not significant statistically. In shoot tip explants 2.8±2.0 caulogenic buds/explant were induced in KN 1 $mg.l^{-1}$+BAP 2 $mg.l^{-1}$. In KN 1 $mg.l^{-1}$ + BAP 0.02 $mg.l^{-1}$ average height of shoot was 3.6 ±1.3 cm. However in this combination, number of buds induced/explant was only 1.0. Statistical analysis of the data shows that BAP plays a significant role in shoot tip morphogenesis in tamarind. It accentuates proliferation of meristem in shoot tip and inhibits their differentiation significantly by reducing the elongation of shoots and opening of leaves (Table 3A.5). Supplementation of KN in BAP containing media remained ineffective in overcoming these inhibitory influences eventhough KN singly at 0.5 $mg.l^{-1}$ supported leaf opening as shown in the previous experiment (Table3A.3). These effects of shoot multiplication and elongation in media supplemented with KN can be seen in Fig. 3A.7A, B.

Fig. 3A. 7

Fig. 3A.7 Effect on KN and BAP combination on multiplication and elongation of shoots derived from shoot tip explants.

(A) Multiple buds formed in KN 0.5 $mg.l^{-1}$ + BAP 0.5 $mg.l^{-1}$. Presence of KN at this concentration was ineffective in inducing either elongation or leaf opening.

(B) Elongated shoots formed in KN 1 $mg.l^{-1}$ + BAP 0.02 $mg.l^{-1}$. BAP at lower concentration did not inhibit elongation of shoot and leaf opening.

Table 3A.5 : Effect of Kinetin and BAP combinations on multiplication and elongation of shoot tip explants

Medium (PGR conc. in mg.l^{-1})	Frequency of response (%) Mean±s.d.	Av. no. of sh. meristem / expl. Mean±s.d.	Av. no. of sh./expl. (>1cm) Mean±s.d.	Av. ht of sh. (cm) Mean±s.d.	Cultures showing leaf opening (%) Mean±s.d.
KN 0.02+BAP 0.02	60.0±28.3	1.3±0.4	1.2±0.4	2.5±0.2	58.3±11.8
KN 0.1+ BAP 0.02	**90.0±14.1**	1.3±0.1	1.3±0.1	1.9±1.1	**75.7± 6.1**
KN 0.2+ BAP 0.02	80.0± 0.0	1.3±0.4	1.2±0.4	2.5±0.4	45.8±29.5
KN 0.5+ BAP 0.02	70.0±42.4	1.0±0.0	1.0±0.0	2.4±0.1	25.0±35.4
KN 1+ BAP 0.02	50.0±42.4	1.0±0.0	1.0±0.0	**3.6±1.3**	62.5±53.0
KN 2+ BAP 0.02	**90.0±14.1**	1.0±0.0	1.0±0.0	3.5±1.9	47.5±38.9
KN 0.02+ BAP 0.1	70.0±14.1	1.4±0.1	1.2±0.2	2.1±0.2	25.0±35.4
KN 0.1+ BAP 0.1	70.0±14.1	1.3±0.1	1.3±0.1	2.5±0.1	20.0±28.3
KN 0.2+ BAP 0.1	80.0±28.3	1.7±0.4	1.6±0.6	2.0±0.4	25.0±35.4
KN 0.5+ BAP 0.1	70.0±42.4	1.0±0.0	1.0±0.0	2.7±0.0	0.0± 0.0
KN 1+ BAP 0.1	80.0±28.3	1.3±0.5	1.3±0.5	2.4±0.3	10.0±14.1
KN 2+ BAP 0.1	80.0±28.3	1.4±0.3	1.4±0.3	2.8±0.5	30.0±42.4
KN 0.02+ BAP 0.2	87.5±17.7	1.4±0.3	1.2±0.6	2.5±0.4	12.5±17.7
KN 0.1+ BAP 0.2	70.0±14.1	1.1±0.2	1.1±0.2	2.8±1.1	20.0±28.3
KN 0.2+ BAP 0.2	70.0±42.4	1.5±0.7	1.5±0.7	2.7±0.2	12.5±17.7
KN 0.5+ BAP 0.2	60.0± 0.0	1.7±0.9	1.7±0.9	3.0±0.9	7.1±10.1
KN 1+ BAP 0.2	80.0±28.3	1.4±0.3	1.4±0.3	2.7±0.1	16.7±23.6
KN 2+ BAP 0.2	80.0±28.3	1.5±0.2	1.5±0.2	2.4±1.0	14.3±20.2
KN 0.02+ BAP 0.5	**90.0±14.1**	1.8±0.0	1.7±0.1	2.4±0.1	6.3± 8.8
KN 0.1+ BAP 0.5	**90.0±14.1**	**2.5±0.4**	1.7±0.7	1.9±0.2	0.0± 0.0
KN 0.2+ BAP 0.5	80.0±28.3	1.0±0.0	1.0±0.0	2.2±0.3	43.3±33.3
KN 0.5+ BAP 0.5	**90.0±14.1**	2.2±0.8	**2.2±0.8**	1.8±0.2	4.6± 6.4
KN 1+ BAP 0.5	80.0±28.3	1.9±0.1	1.9±0.1	1.8±0.2	0.0± 0.0
KN 2+ BAP 0.5	67.5±10.6	1.2±0.2	1.2±0.2	2.3±0.5	0.0± 0.0
KN 0.02+ BAP 1	70.0±14.1	1.2±0.2	1.2±0.2	2.9±2.2	12.5±17.6
KN 0.1+ BAP 1	87.5±17.7	1.7±0.1	1.4±0.6	1.5±0.7	0.0± 0.0
KN 0.2+ BAP 1	80.0±28.3	1.9±1.0	1.4±0.3	1.5±0.6	0.0± 0.0
KN 0.5+ BAP 1	**90.0±14.1**	2.2±0.5	1.6±0.6	1.7±0.6	0.0± 0.0
KN 1+ BAP 1	70.0±14.1	1.7±0.1	1.7±0.1	1.5±0.4	0.0± 0.0
KN 2+ BAP 1	70.0±14.1	1.7±0.1	1.5±0.3	1.4±0.1	0.0± 0.0
KN 0.02+ BAP 2	50.0±14.1	2.6±1.3	1.6±1.3	1.4±0.4	0.0± 0.0
K 0.1+ BAP 2	70.0±14.1	2.1±0.6	1.7±0.1	1.1±0.1	0.0± 0.0
KN 0.2+ BAP 2	60.0±28.3	2.8±0.4	1.5±1.4	1.8±0.4	0.0± 0.0
KN 0.5+ BAP 2	63.3± 4.7	1.4±0.1	1.7±0.2	2.4±0.2	0.0± 0.0
KN 1+ BAP 2	80.0±28.3	2.8±2.0	1.4±0.1	1.4±0.1	0.0± 0.0
KN 2+ BAP 2	50.0±42.4	1.5±0.7	1.5±0.7	1.5±0.7	0.0± 0.0

ANOVA	NS	Tr. NS BAP S 1% KN NS BAP x KN NS	NS	Tr. NS BAP S 1% KN NS BAP x KN NS	Tr. S 5% BAP S 1% KN NS BAP x KN NS

Data from 10 replicates

The cotyledonary nodes responded in all the media combinations containing KN and BAP (Table 3A.6), and either one or both the buds sprouted to form shoots. In the combinations containing KN 2mg.l^{-1} and BAP 2mg.l^{-1}, on an average 3.2±0.3 buds/explants were noted. In concurrence with our earlier observation in shoot tip BAP reduced the leaf opening significantly. In medium containing KN 2 mg.l^{-1} + BAP 0.2 mg.l^{-1} height of shoot was 3.4±1.0cm, followed by 3.1±0.4cm in medium containing KN 0.2 mg.l^{-1} + BAP 0.5 mg.l^{-1}. The data on height of shoot is not significant statistically.

Table 3A.6 : Effect of Kinetin and BAP combinations on sprouting, multiplication and elongation of the meristems in cotyledonary node explants

Medium (PGR conc. in mg.l^{-1})	Frequency of response (%) Mean±s.d.	Av. no. of sh. meristem / expl. Mean±s.d.	Av. no. of sh./explants (>1cm) Mean±s.d.	Av. Ht. of shoot (cm) Mean±s.d.	Cultures showing leaf opening (%) Mean±s.d.
KN 0.02+BAP 0.02	90.0±14.1	2.0±0.3	1.5±0.1	2.4±1.3	29.8±18.5
KN 0.1+ BAP 0.02	**100.0± 0.0**	1.9±0.1	1.7±0.4	1.7±0.3	15.0±21.2
KN 0.2+ BAP 0.02	90.0±14.1	1.8±0.3	1.6±0.1	2.1±0.1	58.3±11.8
KN 0.5+ BAP 0.02	90.0±14.1	1.6±0.5	1.3±0.4	1.9±1.2	68.8± 8.8
KN 1+ BAP 0.02	90.0±14.1	2.1±0.2	1.5±0.7	2.0±0.4	20.0±28.3
KN 2+ BAP 0.02	70.0±42.4	2.3±1.0	1.6±0.6	2.7±0.3	**75.0±35.4**
KN 0.02+ BAP 0.1	90.0±14.1	1.6±0.3	1.6±0.3	2.5±0.1	21.4±10.1
KN 0.1+ BAP 0.1	90.0±14.1	1.3±0.0	1.2±0.0	2.4±0.4	45.0± 7.1
KN 0.2+ BAP 0.1	**100.0± 0.0**	2.2±0.6	1.7±0.4	1.5±0.0	20.0±28.3
KN 0.5+ BAP 0.1	90.0±14.1	1.8±0.6	1.6±0.3	1.8±0.4	28.6±20.2
KN 1+ BAP 0.1	90.0±14.1	1.9±0.2	1.6±0.1	2.3±1.0	14.6± 3.0
KN 2+ BAP 0.1	80.0±28.3	2.5±0.2	1.3±0.1	2.4±0.2	33.3±47.1
KN 0.02+ BAP 0.2	**100.0± 0.0**	2.4±0.9	**2.4±0.9**	2.3±0.0	27.0±22.5
KN 0.1+ BAP 0.2	90.0±14.1	1.9±0.5	1.7±0.7	2.5±0.0	22.2±15.7
KN 0.2+ BAP 0.2	90.0±14.1	2.0±0.3	1.9±0.5	2.2±1.0	18.8±26.5
KN 0.5+ BAP 0.2	90.0±14.1	2.6±0.3	1.8±0.3	2.5±1.2	12.5±17.7
KN 1+ BAP 0.2	80.0±28.3	1.9±0.4	1.9±0.4	2.0±1.0	20.0±28.3
KN 2+ BAP 0.2	90.0±14.1	2.0±0.8	1.3±0.1	**3.4±1.0**	7.1±10.1
KN 0.02+ BAP 0.5	90.0±14.1	2.0±0.6	1.6±0.5	2.5±0.5	0.0± 0.0
KN 0.1+ BAP 0.5	70.0±14.1	2.1±0.2	1.8±0.4	2.2±0.0	8.3±11.8
KN 0.2+ BAP 0.5	90.0±14.1	1.4±0.3	1.3±0.1	3.1±0.4	24.3± 6.1
KN 0.5+ BAP 0.5	90.0±14.1	2.2±0.0	2.1±0.2	1.6±0.4	11.1±15.7
KN 1+ BAP 0.5	90.0±14.1	2.3±0.1	1.6±0.5	2.3±1.3	0.0± 0.0
KN 2+ BAP 0.5	**100.0± 0.0**	1.8±0.0	1.8±0.0	2.5±0.4	0.0± 0.0
KN 0.02+ BAP 1	**100.0± 0.0**	2.4±0.0	1.5±0.1	2.2±0.4	7.1±10.1
KN 0.1+ BAP 1	83.3±23.6	2.4±1.2	1.0±0.0	2.5±0.4	0.0± 0.0
KN 0.2+ BAP 1	90.0±14.1	2.5±1.0	1.4±0.6	1.9±0.7	0.0± 0.0
KN 0.5+ BAP 1	90.0±14.1	2.8±0.7	1.6±0.2	1.6±0.5	0.0± 0.0
KN 1+ BAP 1	90.0±14.1	2.0±0.6	1.6±0.1	2.6±0.9	8.3±11.8
KN 2+ BAP 1	**100.0± 0.0**	2.2±0.3	1.8±0.4	1.9±0.1	0.0± 0.0
KN 0.02+ BAP 2	**100.0± 0.0**	3.0±1.7	1.7±0.7	1.7±0.9	0.0± 0.0
KN 0.1+ BAP 2	**100.0± 0.0**	2.3±0.4	1.1±1.0	2.5±1.4	0.0± 0.0
KN 0.2+ BAP 2	77.5± 3.5	2.5±0.3	2.0±0.4	1.4±0.4	0.0± 0.0
KN 0.5+ BAP 2	90.0±14.1	2.5±0.4	1.8±0.7	1.9±0.0	0.0± 0.0
KN 1+ BAP 2	77.5± 3.5	2.8±0.4	2.0±0.0	1.4±0.2	0.0± 0.0
KN 2+ BAP 2	90.0±14.1	**3.2±0.3**	1.6±0.3	1.1±0.1	0.0± 0.0
ANOVA	NS	Tr. NS BAP S 1% KN NS BAP x KN NS	NS	NS	Tr. S 5% BAP S 1% KN NS BAP x KN NS

Data from 10 replicates

From the experiments with KN and BAP singly and also in combinations it is apparent that BAP plays a significant role in shoot morphogenesis from the existing

meristems of tamarind. At higher concentration it induces bud multiplication but inhibits shoot differentiation and leaf opening. Effective concentrations of BAP vary between 0.2-2 mgl^{-1}. Keeping this in view, BAP in varying concentrations was used for testing various carbohydrates in tamarind morphogenesis.

3.3A.2.2 Effect of Carbohydrates

Carbohydrate as carbon source is one of the important component of the plant tissue culture medium. Sucrose is the most commonly used carbon source in the media.

In cow pea (*Vigna ungiculata* L. Walp) experiment was conducted with various carbohydrates, including sucrose, fructose and glucose (Pellegrineschi, 1997) to compare their influences and identify the one which is most favorable for *in vitro* plant regeneration. In presence of fructose, tissue growth was lower than in sucrose and no shoot regeneration occurred. Glucose induced a similar response as fructose, producing a small amount of callus without any shoot differentiation. These responses could be explained by the different roles played by these sugars in plant metabolism, sucrose, for example, being the major form in which carbon is translocated in the plant.

The effect of carbohydrate particularly the chemical composition of the carbon source has been studied very little because most of the earlier studies strongly suggested the use of sucrose as carbon source. Regulation of *in vitro* organogenesis from Albizzia root explants by carbohydrate source modifications was reported (Maataqui *et al*., 1998). They observed that sucrose was not suitable in the Albizzia system whereas maltose, lactose and xylose were more potent. Proliferation and growth of cultures was strongly influenced by sucrose concentrations in the studies on micropropagation of adult *Swainsona formosa* (Jusaitis, 1997). Significant interactions of sucrose concentrations with cytokinin were observed for shoot-height, bud-number and vigor. Shoot-number increased linearly with increasing sucrose concentration both in presence and in absence of BAP. However, incorporation of BAP reduced elongation, particularly at sucrose concentrations between 20-40 $g.l^{1}$. Substitution of sucrose with fructose and glucose improved the growth of soybean (Saka *et al*., 1980) and *Swainsona galegifolia* (Emmayanti *et al*., 1994) cultures respectively.

Studies on seedling explants of tamarind with different carbohydrates showed that average number of shoots developed in each shoot-tip explant was 2.6±1.2 in medium supplemented with Glucose 2%, followed by sucrose 2% (2.2±0.7). In both cases concentration of BAP was 1 $mg.l^{-1}$. The difference in response between sucrose 2% and glucose 2% was not significant statistically (Table 3A.7).

Table 3A.7 : Effect of carbohydrate source on seedling explant cultures

Medium (PGR conc. in $mg.l^{-1}$)	No. of explants tested	Shoot tip			No. of explants tested	Cotyledonary node		
		Frequency of response (%) Mean±s.d.	Av. no. of shoots/ Explant Mean±s.d.	Av. Ht. of shoot (cm) Mean± s.d.		Frequency of response (%) Mean±s.d.	Av. no. of shoots / Explant Mean±s.d.	Av. Ht. of shoot (cm) Mean±s.d.
BAP 0.02+S2%	14	63.8±33.0	1.0±0.0	2.7±1.0	14	66.7±23.6	1.6±0.4	2.4±0.4
BAP 0.1+S2%	14	70.0±24.5	1.0±0.0	3.6±1.4	14	86.7±16.3	1.9±0.4	2.6±1.1
BAP 0.2+S2%	11	**85.4±17.2**	1.3±0.5	2.7±0.6	9	68.8±47.3	1.3±0.9	2.1±1.7
BAP 0.5+S2%	13	66.7±11.8	1.3±0.5	2.1±1.0	9	62.5±47.9	1.6±1.5	1.3±1.1
BAP 1+S2%	14	58.8±31.2	2.2±0.7	2.3±1.3	14	65.8±29.9	2.6±1.0	1.8±0.5
BAP 2+S2%	14	45.8±41.7	0.8±0.6	1.7±1.7	14	66.7±23.6	2.5±0.4	1.6±0.3
BAP 0.02+Gl2%	14	68.8±37.5	1.0±0.0	3.2±1.1	13	75.0±31.9	1.4±0.4	3.3±1.8
BAP 0.1+Gl2%	14	66.7±23.6	1.1±0.1	2.3±0.6	14	87.5±25.0	1.4±0.3	4.1±0.7
BAP 0.2+Gl2%	14	60.4±31.5	1.2±0.2	3.4±1.2	14	91.7±16.7	1.6±0.5	3.7±3.0
BAP 0.5+Gl2%	14	81.3±37.5	1.9±0.2	1.9±0.8	14	93.8±12.5	1.8±0.6	2.5±0.7
BAP 1+Gl2%	14	77.1±31.5	**2.6±1.2**	1.6±0.3	13	83.3±33.3	2.7±0.9	2.2±1.1
BAP 2+Gl2%	14	59.6±36.2	1.5±0.6	1.2±0.3	14	85.4±17.2	1.8±0.9	1.9±0.8
BAP 0.02+Fr2%	14	54.6±17.5	1.0±0.0	**4.0±1.7**	14	80.4±14.2	1.2±0.3	4.5±1.4
BAP 0.1+Fr2%	14	80.4±14.2	1.3±0.7	3.3±2.3	14	95.0±10.0	1.5±0.4	**4.6±1.3**
BAP 0.2+Fr2%	14	66.7±23.6	1.9±0.8	2.5±1.1	14	87.5±25.0	1.8±0.3	3.3±1.2
BAP 0.5+Fr2%	13	50.0±43.0	1.2±0.9	1.6±1.1	13	**100.0± 0.0**	2.1±0.9	2.4±0.2
BAP 1+Fr2%	14	40.8±33.4	1.5±1.7	1.5±1.2	14	83.3±33.3	**2.8±0.4**	2.1±0.4
BAP 2+Fr2%	14	65.0±23.8	1.5±0.7	1.6±0.4	14	86.7±16.3	2.4±1.3	1.6±0.5

ANOVA	--	**NS**	**Tr. S 5% BAP S 1% CHO NS CHOxBAP NS**	**Tr. S 5% BAPS 1% CHO NS CHOxBAP NS**	--	**Tr. NS BAP NS CHO S 5% CHOxBAP NS**	**Tr. S 5% BAP S1% CHO NS CHOxBAP NS**	**Tr. S 1% BAP S 1% CHO S 1% CHOxBAP NS**

However the analysis highlighted that the effect of BAP was beneficial for the induction of more number of shoots per explant irrespective of the type of carbohydrate used. Fructose showed still less number of shoots per explant. In all the carbohydrates tested, BAP at lower concentrations (0.02 - 0.2 $mg.l^{-1}$) significantly increased the height of

shoots, whereas at higher concentrations (0.5 - 2 $mg.l^{-1}$) limited the shoot elongation (Fig. 3A.8A,B,C). Fructose, alongwith BAP (0.02 $mg.l^{-1}$) concentration showed the maximum height of shoot (4.0±1.7 cm) followed by 3.6±1.4 cm in BAP 0.1 $mg.l^{-1}$ with sucrose. It is apparent from the data that in shoot tip explant neither glucose 2% nor fructose 2% improves morphogenetic response when compared to sucrose 2%. Therefore, sucrose was kept as the carbohydrate source for all the remaining studies.

Influence of sucrose, fructose and glucose in sprouting, multiplication and elongation of cotyledonary node meristems was not similar to the shoot tip (Table 3A.7, Fig. 3A.9A,B,C). Data on number of shoots/explant and shoot length were statistically significant. Analysis of the data on cotyledonary nodal explants show that carbohydrates play a significant role in sprouting and elongation of the meristematic buds. Multiplication of shoots is influenced by BAP. Glucose appears to be better than sucrose for sprouting and elongation whereas fructose is still better than glucose.

The basal cut end of the explant (shoot tip or cotyledonary node) showed some amount of callus at the media surface. The amount of callusing increased linearly with the increasing concentration of BAP irrespective of the carbohydrate used. The amount of callus produced in the media containing glucose was more while those with fructose was least (Fig. 3A.9B).

3.3A.2.3 Subculture

For multiplication of shoots induced from the existing meristems of tamarind, two methods were applied. The original explant (**first method**) one with node on reculturing sprouted and elongated (Fig. 3A.10). The elongated shoot was used either for rooting or for subculture. In the **second method** the elongated shoot was cut into 2-3 pieces (each measuring ~2cm). The piece with shoot tip elongated and was used

Fig.3A.8

Fig. 3A.8 Effect of different carbohydrates on **shoot tip** explants growing in MS basal medium with varying concentrations of BAP:
Alphabets A-F on the tube holder indicate 0.02, 0.1, 0.2, 0.5, 1 and 2 mg.l^{-1} concentration of the growth regulator.
(A) 2% sucrose (B) 2% glucose (C) 2% fructose
More callusing in glucose followed by sucrose and fructose on the explants at the region in contact of the medium

Fig. 3A.9 Effect of different carbohydrates on **cotyledonary node** explants growing in MS basal medium. with varying concentrations of BAP

Alphabets A-F on the tube holder indicate 0.02, 0.1, 0.2, 0.5, 1 and 2 mg.l^{-1} concentration of the growth regulator.

(A) 2% sucrose (B) 2% glucose (C) 2% fructose

More callusing in glucose followed by sucrose and fructose on the explant at the region in contact of the medium.

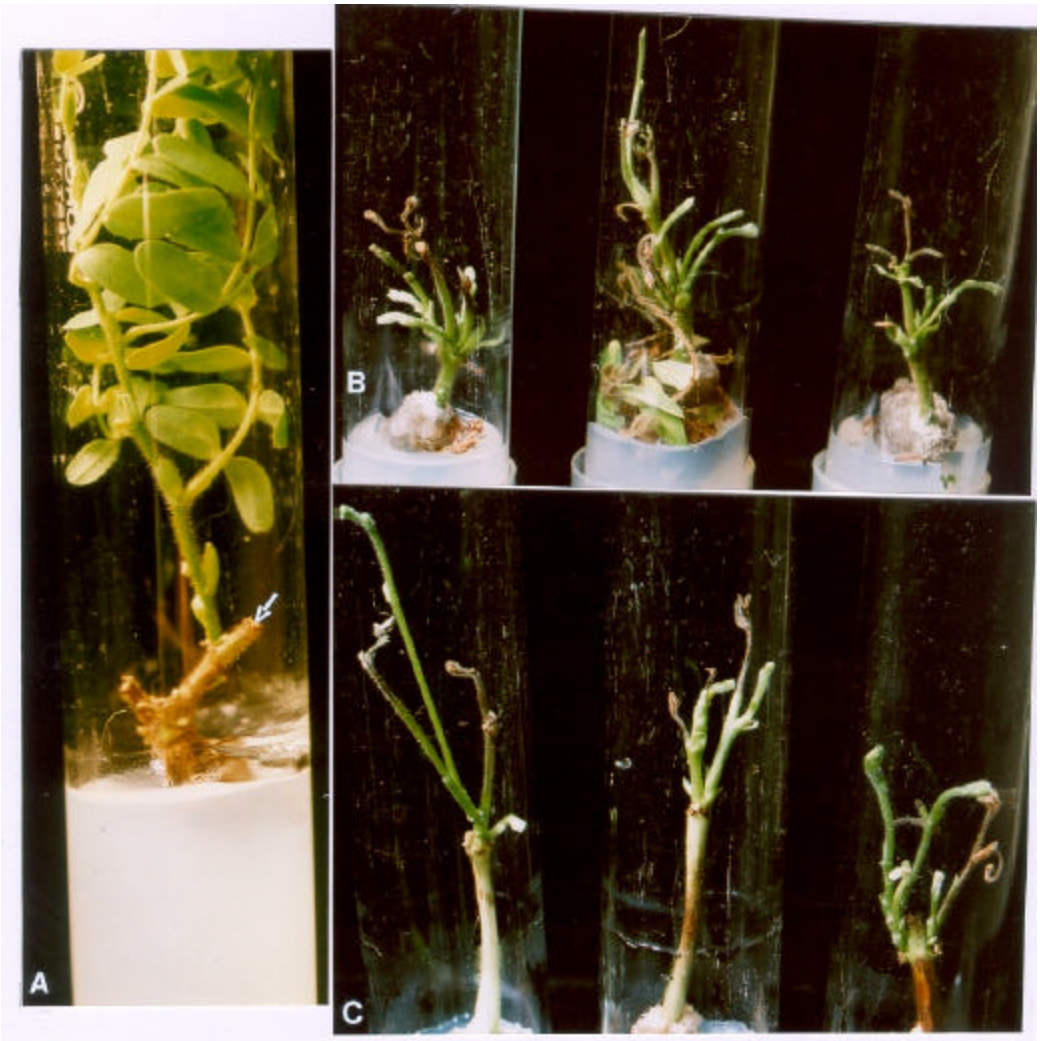


Fig. 3A.10

Fig.3A.10 A. Second crop of shoot raised from the original cotyledonary node explant in MS basal medium supplemented with 0.2 mg.l^{-1} BAP and 2% sucrose.
Arrow shows the remaining basal portion of the first shoot

B. Induction of multiple shoots in BAP 1 mg.l^{-1} on elimination of apical dominance due to the burning of the shoot tip.

C. Cotyledonary node explant showing burning of tip and induction of multiples in BAP 1 mg.l^{-1}

for rooting or further multiplication. The axillary buds in this shoot tip explant did not sprout due to apical dominance exerted by the existing tip. The pieces of axillary nodes sprouted and elongated to produce shoots. Growth and differentiation of shoots were always associated with profuse callusing and dedifferentiation at the cut end in contact of medium. Thus explants with less than two nodes could not be used. Due to the inherent callusing nature of the species proliferation of shoot and production of multiples was hampered. Thus the shoot cultures could not be maintained for more than four passages. Various efforts to control this dedifferentation remained futile. Callusing at the basal end of the *Leucaena leucocephala* explants in BAP-containing medium (Dhawan and Bhojwani, 1985) was noted earlier. The degree of callusing was directly related to shoot growth. In tamarind such correlation could not be established since callusing was not measured. In this tissue, a lot of callusing was observed in the basal portion of the explants. To avoid the engulfing of the explant by growth of callus, care was taken to keep the explants to a minimum size of at least 2-3 cm.

Shoots initiated in 0.02-0.5 $mg.l^{-1}$ BAP either in shoot tip explant or in cotyledonary node elongated in the same medium on extended incubation. However, elongation was faster in medium with lower BAP.

In some cultures the tip of the shoot turned brown, and necrotic. In these cultures multiple shoots initiated from the lower nodes, which elongated into shoots. This phenomenon was noted in both shoot tip and cotyledonary node cultures (Fig. 3A.10 B, C). Proliferation of shoots from the lower nodes was due to elimination of apical dominance by tip necrosis.

3.3A.3 Rooting of shoots

Mascarenhas *et al*(1981;1987) have demonstrated that a mixture of auxins (IAA, IBA, IPA & NAA) at 1 $mg.l^{-1}$ each was required for the root initiation in the tamarind shoots. This method was used in our initial experiments with shoots originated from the shoot tip and the cotyledonary node explants. Rooting was preceded by callusing at the cut end of the shoots prior to root initiation in liquid MS medium with filter paper bridge and the root elongation was poor. Transfer of these shoots with root initials in MS half

strength medium with 0.25% activated charcoal did help in elongation of roots (Fig. 3A.11A). With this method 60% of the shoot tip and cotyledonary node derived shoots rooted in 20 days which were transferred on half strength MS basal medium supplemented with 2% sucrose and 0.25% activated charcoal for further development of the roots.

Single auxins (IAA, IBA, NAA) have been used in semisolid medium for rooting of in vitro raised shoots in other tree species (Dhawan and Bhojwani, 1985; Raghava Swamy *et al*., 1992; Nandwani, 1995; Upreti and Dhar, 1996, Beck *et al*., 1998a). Jaiwal and Gulati (1991, 1992) achieved rooting in all the tamarind shoots in agar gelled MS medium with IAA singly. Sonia *et al*.(1998) used IBA at $5x10^{-6}$ M concentration for rooting the shoots obtained via direct organogenesis in hypocotyl cultures of *Tamarindus indica*. The frequency of rooting was 87%.

Our results (Table 3A.8) were in concurrence with Jaiwal and Gulati (1991, 1992). All the shoots rooted in IAA 1 $mg.l^{-1}$ in 45 days with 2 - 4 roots/plant having average root length of 4.1cm and showing laterals. An increase in the IAA concentration to 2 $mg.l^{-1}$ led to a decrease in rooting percentage (40%), average number of roots/plant and the root length (Fig. 3A.11B). IBA at 1 $mg.l^{-1}$ induced rooting in 80% of the shoots showing 2 - 4 roots/explant having 4.6cm length on an average with laterals emerging out. Raised level of IBA 2 $mg.l^{-1}$ exhibited 60% rooting efficiency. Though 2-7 roots/plant were observed but they were short and stunted (Fig. 3A.11C). The callusing at the cut end was more in the medium with higher concentrations of auxin.

Table 3A.8 : Effect of auxins on rooting of shoots

Auxins conc. in $mg.l^{-1}$	% rooting			Av. no. of roots/plant (45 days)	Av. length of roots/plant (45 days) (cm)
	23d	30d	45d		
IAA 1	40	80	100	2.4	4.1
IAA 2	0	0	40	2	1.2
IBA 1	20	60	80	2.5	4.6
IBA 2	40	40	60	2.7	1

Data from 10 replicates

Fig. 3A.11

Fig 3A.11 Rooting of *in vitro* raised shoot.

A. Rooted shoot in MS medium supplemented with 0.25% charcoal. The shoots were exposed in auxin mixture for 72h prior to transfer in this medium.

Callusing noted in the basal region of shoot near the media surface.

B. Rooting of shoots in IAA 1 and 2 $mg.l^{-1}$. Limited root elongation in higher concentration

C. Rooting of shoots in IBA 1 and 2 $mg.l^{-1}$. Root elongation restricted in higher concentration

After induction of rooting either in IAA or in IBA, transfer of shoots into half strength MS basal medium with 2% sucrose and 0.25% activated charcoal, restricted callusing at the root-shoot junction (Fig. 3A.12 A, B, C) and supported growth of shoot and root.

3.3A.4 Transfer of plants to soil

On transplantation of rooted shoots, initially for 6-7 days all plants survived. Gradually the shoots dried up and subsequently got infected with fungus. The fungal infection always started from the apex.

Plants raised by rooting the shoot in auxin mixture (Fig. 3A.11A) did not survive on transfer to soil. In this plants due to bigger callus mass at the base, it was not clear whether the root developed from the callus or there was vascular connection between shoot and root. This was also observed by Sonia *et al.*(1998) in tamarind and Datta and Datta (1983) and Datta *et al.* (1983) in *Dalbergia sissoo*. The intervening callus at the basal end of the shoot seen prior to the root initiation may be the cause of low survival of these plantlets.

The poor survival of the rooted plants in soil had prompted us to test IAA and IBA in agar medium for improved root system, which in turn will give increased survival rate. Jaiwal and Gulati (1991) achieved survival of 70% plants rooted in IAA. Sonia et al. (1998) reported 75% survival of plants rooted in IBA. In contrast to these observation, we achieved only 21% survival of the plants (Fig. 3A.13). These plants were raised by induction of roots in agar medium containing 1 or 2 $mg.l^{1}$ IBA followed by transfer to MS medium with 0.25% charcoal. Mortality of these plants was primarily due to browning followed by fungal infection.

On examining the root system of infected and dying plants it was noted that there was very poor or no root growth after transferring plant to soil. It is presumed that tamarind being a slow growing plant, the root takes a long time to grow and establish in soil. Therefore due to absence of nutrition and moisture from soil, the plant cease to grow and the leaves get infected.

Fig. 3A.12

Fig 3A.12 Rooting of shoot in half strength MS basal medium supplemented with 0.25% charcoal after 45 days preexposure in auxin :
(A) IAA 1 mg.l^{-1} (B); IBA 1 mg.l^{-1} and (C) IBA 2 mg.l^{-1}
Restriction of calluing and healthy root formation on transferring auxin pretreated shoots in charcoal.

Fig. 3A.13

Fig 3A.13 : Seedling derived *in vitro* raised plants growing in soil.

3.4A CONCLUSIONS

Seedling explants, shoot tip and cotyledonary node, responded in culture to give rise to multiple shoots in BAP. In lower concentrations shoots elongated and had opened leaves. The shoot tip explant gave rise to 2.2±0.7 shoots per explant while cotyledonary nodal explant gave rise to 2.6±1.0 shoots per explant in BAP 1mg.l^{-1} supplemented with 2% sucrose. Multiple shoots that appeared in shoot tip explants were not due to proliferation of apical meristem. In both, shoot tip and cotyledonary node explants the shoots originated from the nodal meristems. Elongation of shoot was observed at 0.02-0.1 mg.l^{-1} BAP concentrations. Further supplementation with various concentrations of KN remained ineffective in inducing multiplication and elongation in the same medium.

Rooting of the excised shoots was achieved in 60% of the cultures when a pulse treatment of auxin mixture was used. In presence of IAA or IBA, 80- 100% shoots rooted. But a survival rate of only 21.4% was achieved, when these plants were transferred to soil. Poor survival in soil could either be due to lack of vascular connection between the shoot and root, or due to transient cessation of root and shoot growth and very slow root growth on transfer to soil.

B : Clonal propagation from mature explants

3.1B INTRODUCTION

Cloning of mature trees is preferred over cloning of embryos or seedlings because often it is not possible to determine if these explant sources have the genetic potential to develop the desired qualities later in their lifecycle (Bonga, 1987).

Direct regeneration or clonal propagation is generally via bud culture or apical meristem culture. The latter generally involves only the actual apical dome of the shoot, preferably without a few leaf primordia. These are used for obtaining disease free clones. Clonal propagation via bud culture involves the entire rudimentary vegetative shoot. Explant for propagation is the shoot node with the axillary bud. This ensures the genetic uniformity of a clone as regeneration is from existing meristem.

Rapid clonal multiplication / propagation from mature explants of tree legumes could be achieved in *Dalbergia latifolia* (Mascarenhas *et al*., 1982, Datta and Datta, 1983,

Raghava Swamy *et al*., 1992;), *Leucaena leucocephala* (Kulkarni *et al*., 1984, Dhawan and Bhojwani, 1985), *Prosopis cineraria* (Kackar *et al*., 1991), *Dalbergia sissoo* (Dawra *et al*., 1984), *Bauhinia variegata* and *Parkinsonia aculeata* (Mathur and Mukunthakumar, 1992), *Caesalpinia pulcherrimi a* (Rahman *et al*., 1993), *Acacia catechu* (Kaur *et al*., 1998), and *Bauhinia vahlii*, a leguminous liana (Dhar and Upreti, 1999). Ravishankar Rai and Jagdish Chandra (1988) showed regeneration of plantlets from shoot callus of mature trees of *Dalbergia latifolia*.

This section describes the experiments conducted to standardize a protocol for clonal propagation of tamarind using explants from mature trees. The explants for this study were collected from the locally grown trees of tamarind.

3.2B EXPERIMENTAL PROTOCOL

3.2B. 1 Preparation of explant

Vegetative growth in the locally grown tamarind trees starts in March / April. The flowering starts about 3-4 weeks later. In the same branch, flower buds appear from some of the axils and vegetative growth is noted in others (Fig. 3B.1A). The twigs, which were healthy sturdy and 3-4 mm thick were excised from the mature tree and were used as source of explant (Fig. 3B.1B). These twigs measured about 15-30 cm. The leaves of these twigs were cut off with a sharp blade or scissors leaving a small portion (about 5-6 mm) of petiole attached to the twig so that the axillary bud is not damaged. Very young buds at the tip of the twig were discarded and the buds at the middle and lower portions of the twigs were used. The twig was cut into 2-2.5 cm pieces (depending on the internodal distance). Each piece had atleast one axillary node and a portion of internodal segment. Direct contact of the medium with the axillary bud is avoided by leaving long (1.5 – 2cm) internode below the bud.

The nodal bud explants were washed thoroughly with liquid detergent and surface sterilized using 0.1% w/v $HgCl_2$ for 10 min. Excess $HgCl_2$ was removed by repeated washings with sterile distilled water under aseptic conditions.

Fig. 3B.1

Fig. 3B.1 A : A branch of tamarind tree showing both flower buds and new vegetative growth in the month of April / May

B: Excised twig 3-4 mm thick – source of explant

3.2B. 2 Culturing of buds for sprouting and elongation of shoots

3.2B. 2.1 Plant Growth Regulators

Explants were cultured in tubes containing MS basal medium supplemented with 2% sucrose and cytokinins including KN, BAP, Z, 2iP, TDZ either singly (0.2-2 $mg.l^{-1}$) or in combination to induce bud break in them. The concentrations and combinations of PGRs are included in Table 3B.1. The cultures were incubated in light for 4 weeks. Number of buds showing bud break, were scored and the frequency of response was determined in percentage.

Following the initial culture of 4 weeks the sprouted buds were shifted to corresponding fresh medium of same composition and concentration (Table 3B.2) for further growth and differentiation, in light. Number of buds showing differentiation into shoots with 2-3 nodes (~1-2cm in length) were scored and frequency of response were determined.

In the second experiment, to induce bud break and shoot development MS medium with 2% sucrose supplemented with KN, BAP and Z at various concentrations (0.1, 0.2, 0.5 $mg.l^{-1}$) were used for culturing the initial buds. Cultures were incubated in light. The number of buds showing sprouting / bud break, elongation into shoots and opening of leaves was scored and frequency of response was calculated. Height of the elongated shoots was also noted.

For further refinement of the process, MS medium with 2% sucrose supplemented with BAP at 0.1, 0.2, 0.3, 0.4 and 0.5 $mg.l^{-1}$ was used for culturing the buds. Cultures were incubated in light. The same parameters recorded in the earlier experiment were noted in this experiment too.

Charcoal is known to support morphogenesis by absorbing inhibitory substances leached from the tissues in culture. So influence of charcoal on sprouting and elongation of buds was tested by incorporating 0.25% charcoal in MS medium supplemented with BAP (0.2 and 2 $mg.l^{-1}$) and 2% sucrose. Cultures were incubated in light.

3.2B. 2.2 Carbohydrates

In the above experiments with mature plant-derived explants, 2% sucrose was used. To identify the appropriate carbon source in this experiment sucrose was substituted by fructose, glucose or maltose at the same concentration (2%). All media contained BAP 0.2 $mg.l^{-1}$. Cultures were incubated in light. The experiment was repeated thrice. Number of buds showing bud break / sprouting, number of buds elongated into shoots of height more than 1cm and number of shoots with opened leaves were recorded. Height of the elongated shoots was also noted.

3.2B. 2.3 Influence of pH

The nodal explants were cultured in MS basal medium supplemented with BAP 0.2 $mg.l^{-1}$ at different pH 5.8, 6.0, 6.2, 7.0. The pH was adjusted prior to autoclaving of the media. Cultures were incubated in light. The experiment was repeated twice using 20 replicates per treatment. All the parameters were recorded as in earlier experiments.

3.2B. 2.4 Influence of photoperiod during incubation

The buds from mature trees were cultured in MS basal medium supplemented with BAP 0.2 $mg.l^{-1}$ and 2% sucrose. The pH of the medium was adjusted at 5.8 prior to autoclaving. Cultured buds were incubated in two different light regimes - 16h and 24h photoperiod for 4 weeks. The experiment was conducted twice.

After the incubation of cultures for 4 weeks, the number of buds sprouted in culture was scored. The frequency of response was determined.

3.2B. 3 Elongation of sprouted buds

The buds sprouted in 0.2 $mg.l^{-1}$BAP were transferred to various combinations of KN (0.2 and 0.5 $mg.l^{-1}$) and BAP (0.2 and 0.5 $mg.l^{-1}$) for further elongation of shoots. Cultures were incubated in 24h light. Number of explants responded in culture were scored and expressed in percentage. The shoot height was measured. The response was compared with the response obtained in BAP 0.2 $mg.l^{-1}$ singly.

The elongated shoots were excised and used for further subculturing and rooting experiment following the method used for seedling (Section 3.2A.2.3). However the

original nodal explant after excising the shoot was recultured on fresh medium with BAP 0.2 or 0.5 $mg.l^{-1}$ for a further crop of elongated shoot.

3.2B. 4 Rooting of elongated shoots

The elongated shoots were cultured on MS medium supplemented with different auxins, IAA and IBA at 0.2, 0.5, 1 and 2 $mg.l^{-1}$ either singly or in combination at similar concentrations. The media were solidified using 0.7% agar. Cultures were incubated in light for 4-6 weeks. Ten replicates per treatment were used.

3.3B RESULTS AND DISCUSSION

3.3B.1 Preparation of explant

Sprouting of the tamarind buds was initiated in the old brown stem / twig (Fig. 3B.2A in the month of March / April). Due to the rough surface of the woody twig of previous year, surface sterilization methods were not effective. Subsequently buds taken in the month of April / May from the 3-4 mm thick, actively growing green twigs (Fig. 3B.2B), were found to be appropriate for effective surface sterilization for establishment of culture. Thinner green twigs did not survive the sterilization treatments and turned brown. The excised nodal segments of 2-2.5 cm were used as explant. Explants with lesser length tend to callus in culture.

3.3B.2 Culturing of buds for sprouting and elongation of shoots

3.3B.2.1 Effect of Plant growth regulators

Similar to seedling explants, the mature explants were also subjected to different PGRs for bud sprouting (Fig. 3B.3A) and elongation (Fig. 3B.3B). Beck *et al.* (1998b) used MS medium supplemented with BAP at 2 $mg.l^{-1}$ for rejuvenation and micropropagattion of adult *Acacia mearnsii*, using coppice material. In order to achieve *in vitro* vegetative propagation from mature tree derived buds of *Leucaena leucocephala* (Lam.) de Wit, Dhawan and Bhojwani, (1985) used BAP $3x10^{-6}$M.

Fig. 3B.2

Fig. 3B. 2 A: Mature and brown stem / twig in the month of March / April showing sprouting of buds.
B: Actively growing green twigs collected in the month of April / May

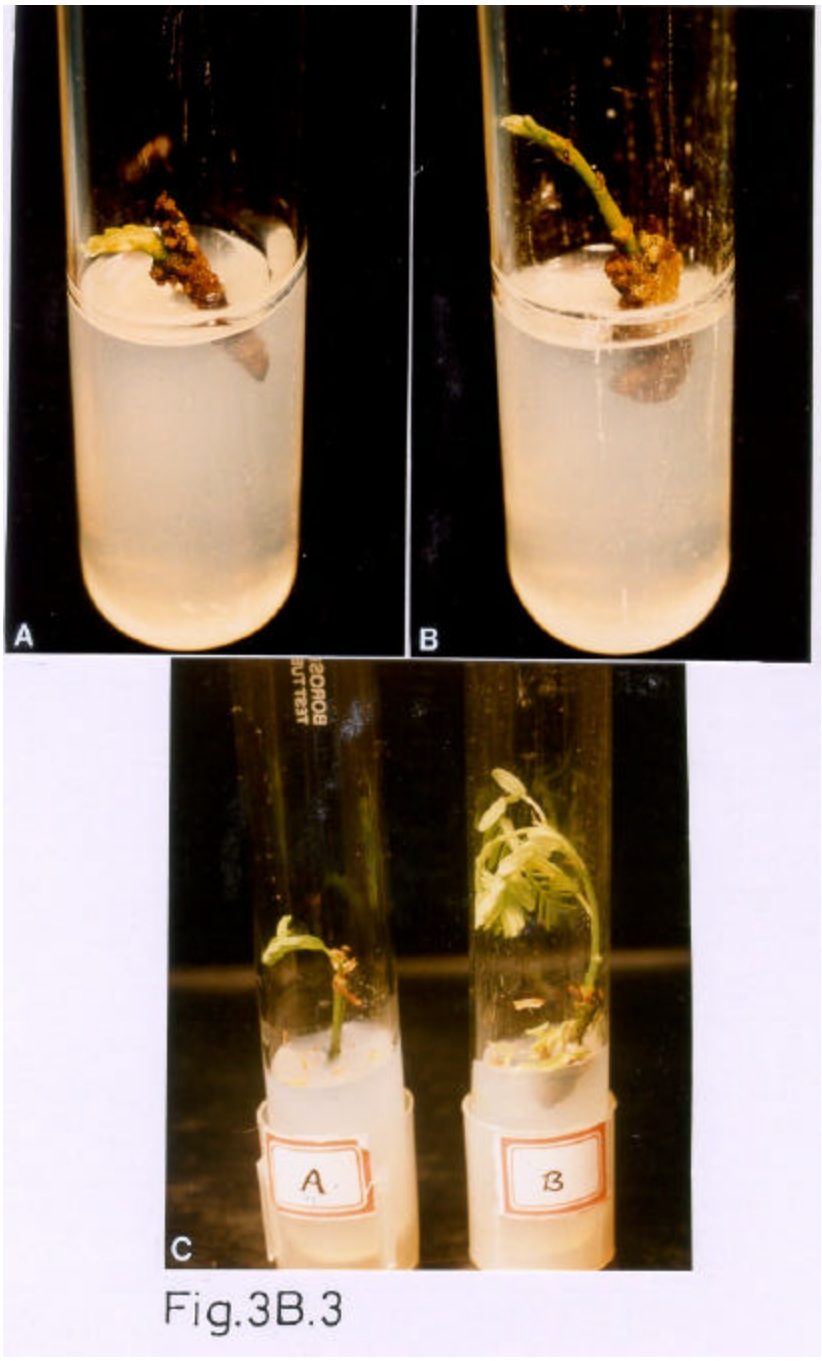


Fig. 3B.3 Sprouting (A) and elongation (B) of bud excised from mature nodal bud explant after 4 weeks in culture
(C) Bud cultured in medium without PGR (Alphabet A on tube holder) and bud cultured in presence of BAP showing shoot elongation (Alphabet B on tube holder)

Optimum bud break (100%) and shoot multiplication of 5.6 fold was attained. Datta and Datta, (1985) observed that in *Leucaena leucocephala* BAP 2 $mg.l^{1}$ induced multiple (3-6) shoots from axillary bud primordia of each explant after 30 days of culture. Micropropagation of *Bauhinia variegata* and *Parkinsonia aculeata* from nodal explants of mature tree could be achieved in MS medium supplemented with 13.3µM and 8.9µM of BAP respectively.

Morphogenic differentiation of the explants was markedly influenced by the nature and combination of growth regulators (Rahman *et al*., 1993) in *Caesalpinia pulcherrima*. These researchers showed that BAP 4.5µM + NAA 5.5µM produced maximum number of shoots, (5.8/explant). Kaur *et al*. (1998) also showed that maximum shoot multiplication (8.66 ±1.71) was achieved on MS medium fortified with BAP 4 $mg.l^{1}$ and NAA 0.5 $mg.l^{1}$ along with other additives including adenine sulphate, ascorbic acid and glutamine in *Acacia catechu*. A combination of KN 0.5 $mg.l^{-1}$, BAP 1 $mg.l^{1}$, CH 500 $mg.l^{1}$, calcium pantothenate 0.1 $mg.l^{1}$ and biotin 0.1 $mg.l^{-1}$ was used by Mascarenhas *et al*. (1982) for shoot bud initiation in *Dalbergia sissoo*. Shifting the primary explants on KN 0.1 $mg.l^{1}$, BAP 0.2 $mg.l^{1}$ with calcium and biotin 0.1 $mg.l^{1}$ each, induced multiple shoot formation (3 shoots/explants). Kulkarni *et al*. (1984) used KN 0.2, BAP 0.3, calcium pantothenate 0.1 and biotin 0.1 $mg.l^{1}$ to obtain multiple shoot induction from mature tree derived buds of *Leucaena leucocephala*. In an attempt to achieve *in vitro* micropropagation of elite rosewood (*Dalbergia latifolia* Roxb.), (Raghava Swamy *et al*., 1992) used two media. Shoot cultures were initiated from axillary shoot buds in MS basal medium supplemented with BAP 1 $mg.l^{1}$ and NAA 0.05 $mg.l^{1}$ while multiplication of the shoots was obtained on MS (reduced major elements) or Woody Plant Medium supplemented with BAP 1 $mg.l^{-1}$ and KN 0.5-1 $mg.l^{1}$. Kacker *et al*. (1991) could achieve rapid in vitro propagation through axillary branching of the shoots initiated from single node segments from actively growing branches of elite tree of *Prosopis cineraria* cultured on MS medium containing 3 $mg.l^{-1}$ of β Napthoxy acetic acid and NAA. *In vitro* regeneration of *Bauhinia vahlii* Wight and Arnott was achieved in MS medium supplemented with 2.5 µM KN + 100 $mg.l^{1}$ adenine sulphate (Dhar and Upreti 1999). Proliferation, shoot number and shoot length were 58%, 4.5, and 3.5 mm respectively.

Preliminary experiments in tamarind, were conducted using different cytokinins either singly or in combination for sprouting of mature tree derived axillary buds (Table 3B.1). Higher percentage of sprouting was observed in medium with BAP singly or in combination with kinetin, zeatin or 2iP. On transferring the buds in fresh medium of same composition, it was apparent that, the highest percentage of buds elongating into shoots were obtained in BAP 0.2 $mg.l^{-1}$ which reduced with increasing concentrations and was even restricted at BAP 2 $mg.l^{-1}$ (Table 3B.2). The sprouted buds did not grow further in absence of BAP (Fig. 3B.3C). Buds cultured on MS medium devoid of any growth regulators, showed 71.4% sprouting (Table 3B.1) but they did not elongate at all in this medium even after culturing in fresh medium (Table 3B.2). There was no elongation of sprouted buds with KN, 2iP and TDZ singly. KN in combination with Z (at lower concentration), 2iP and TDZ; Z and 2iP in combination with TDZ (above 0.5 $mg.l^{-1}$); combination of higher concentrations of BAP with Z, 2iP and TDZ; combination of higher concentrations of Z and 2iP-were either ineffective or marginally effective in supporting elongation of the sprouted buds.

Table 3B.1 : Effect of different cytokinins singly or in combination on Tamarind mature bud sprouting

Medium (PGR conc. in mg.l^{-1})	No. of buds Cultured	No. of buds sprouted	Sprouting frequency (%)
KN 0.2	11	10	90.9
KN 0.5	10	9	90.0
KN 1	11	10	90.9
KN 2	11	9	81.8
KN 0.2+BAP 0.2	11	11	**100.0**
KN 0.5+BAP 0.5	11	10	90.9
KN 1+BAP 1	11	9	81.8
KN 2+BAP 2	11	8	72.7
KN 0.2+Z 0.2	10	7	70.0
KN 0.5+Z 0.5	10	10	**100.0**
KN 1+Z 1	10	8	80.0
KN 2+Z 2	10	10	**100.0**
KN 0.2+2ip 0.2	11	8	72.7
KN 0.5+2iP 0.5	11	9	81.8
KN 1+2iP 1	11	10	90.9
KN 2+2iP 2	11	9	81.8
KN 0.2+TDZ 0.2	11	5	45.5
KN 0.5+TDZ 0.5	11	4	36.4
KN 1+TDZ 1	10	2	20.0
KN 2+TDZ 2	11	3	27.3
BAP 0.2	11	11	**100.0**
BAP 0.5	10	10	**100.0**
BAP 1	11	10	90.9
BAP 2	11	9	81.8
BAP 0.2+Z 0.2	10	10	**100.0**
BAP 0.5+Z 0.5	10	10	**100.0**
BAP 1+Z 1	10	10	**100.0**
BAP 2+Z 2	10	9	90.0
BAP 0.2+2iP 0.2	11	9	81.8
BAP 0.5+2iP 0.5	11	11	**100.0**
BAP 1+2iP 1	11	11	**100.0**
BAP 2+2iP 2	10	10	**100.0**
BAP 0.2+TDZ 0.2	11	9	81.8
BAP 0.5+TDZ 0.5	10	7	70.0
BAP 1+TDZ 1	10	9	90.0
BAP 2+TDZ 2	10	8	80.0
Z 0.2	10	8	80.0
Z 0.5	10	8	80.0
Z 1	10	7	70.0
Z 2	10	7	70.0
Z 0.2+2iP0.2	10	8	80.0
Z 0.5+2iP0.5	10	9	90.0
Z 1+2iP 1	10	7	70.0
Z 2+2iP 2	10	7	70.0
Z 0.2+TDZ 0.2	10	7	70.0
Z 0.5+TDZ 0.5	10	5	50.0
Z 1+TDZ 1	10	2	20.0
Z 2+TDZ 2	10	4	40.0
2iP 0.2	10	5	50.0
2iP 0.5	10	6	60.0
2iP 1	10	7	70.0
2iP 2	10	8	80.0
2iP 0.2+TDZ 0.2	10	6	60.0
2iP 0.5+TDZ 0.5	10	0	0.0
2iP 1+TDZ 1	10	0	0.0
2iP 2+TDZ 2	10	0	0.0
TDZ 0.2	10	7	70.0
TDZ 0.5	10	2	20.0
TDZ 1	10	8	80.0
TDZ 2	10	4	40.0
MS	63	50	71.4

In some of the combinations, all axillary buds responded by sprouting whereas in some the response was poor. At this stage it was not clear whether the sprouted bud will differentiate into shoot or only to leaf. Although the data was not conclusive to identify any particular cytokinin singly or in combination, but some of the general observation were:

(i) The bud break in MS medium devoid of PGRs was 71%.

(ii) BAP at lower concentration either singly or in combination had positive influence on bud break and sprouting.

(iii) KN and ZN in some combinations favored bud break.

(iv) Sprouting in presence of KN is in high frequency but the shoot growth was not vigorous.

(v) 2iP singly did not accentuate bud break.

(vi) TDZ neither supported sprouting nor differentiation.

(vii) Wound associated callusing and callusing on the surface of the explants in contact of medium was frequent in tamarind tissues. This effect is minimal in cultures grown in presence of KN and maximum in presence of TDZ singly or in combination.

These observations can also be seen in Fig. 3B.4, 5, 6, 7.

Similar to other woody legumes, growth of tamarind tissue in culture is slow. Therefore after incubation for 4 weeks only bud break could be achieved but these did not differentiate into shoot although some cultures show leaf differentiation. On culturing of sprouted buds in fresh medium all buds did not differentiate into shoots (Table 3B.2). Some buds gave rise to a leaf and a few dried and degenerated. The number of buds showing, shoot formation with two to three nodes were scored.

Fig. 3B.4

Fig. 3B.4 Axillary buds cultured in KN 0.2-2mgl^{-1} singly or in combination with BAP, Z, 2iP and TDZ at similar concentrations

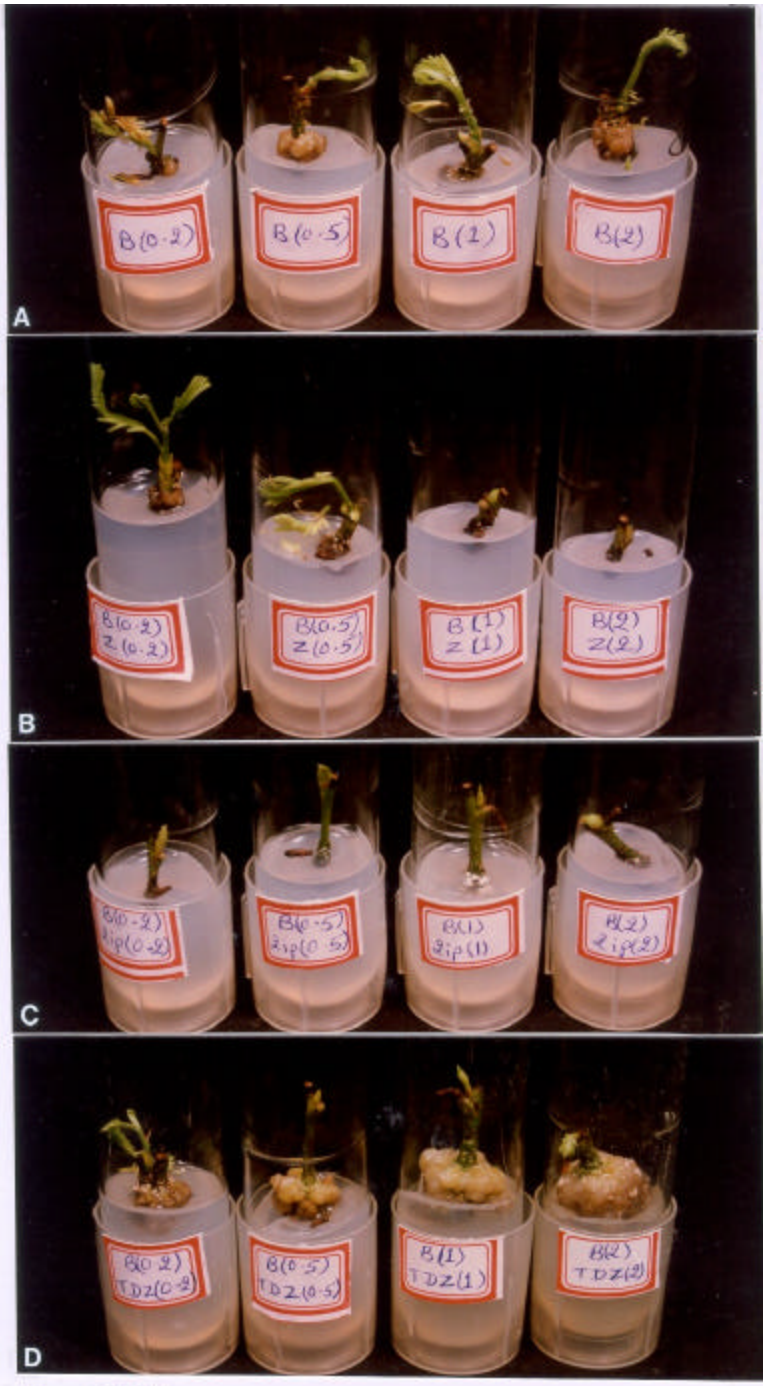


Fig. 3B.5

Fig. 3B.5 Axillary buds cultured in BAP 0.2-2mg.l^{-1} singly or in combination with Z, 2iP and TDZ at similar concentrations

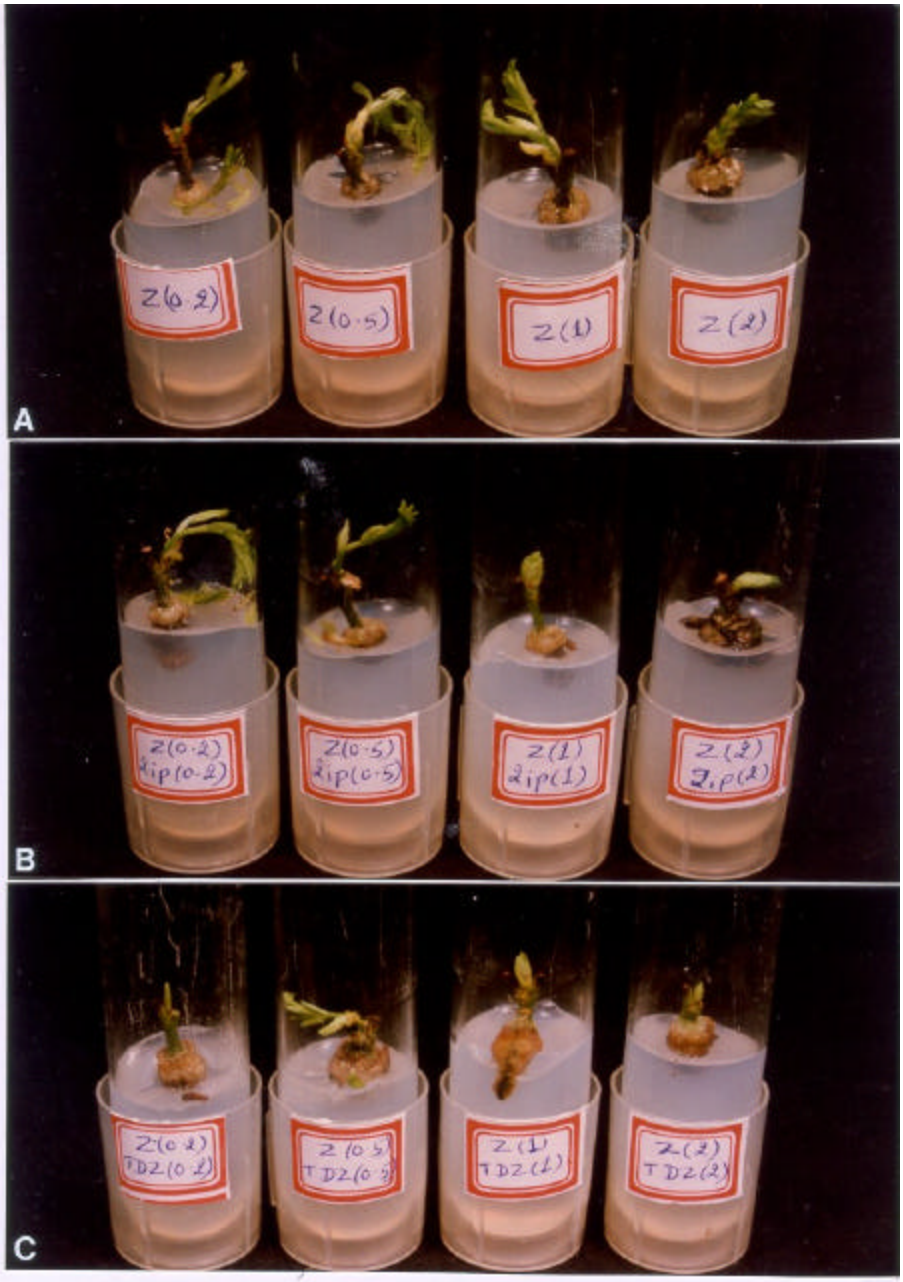


Fig.3B.6

Fig. 3B.6 Axillary buds cultured in Z 0.2-2mg.l^{-1} singly or in combination with 2iP and TDZ at similar concentrations

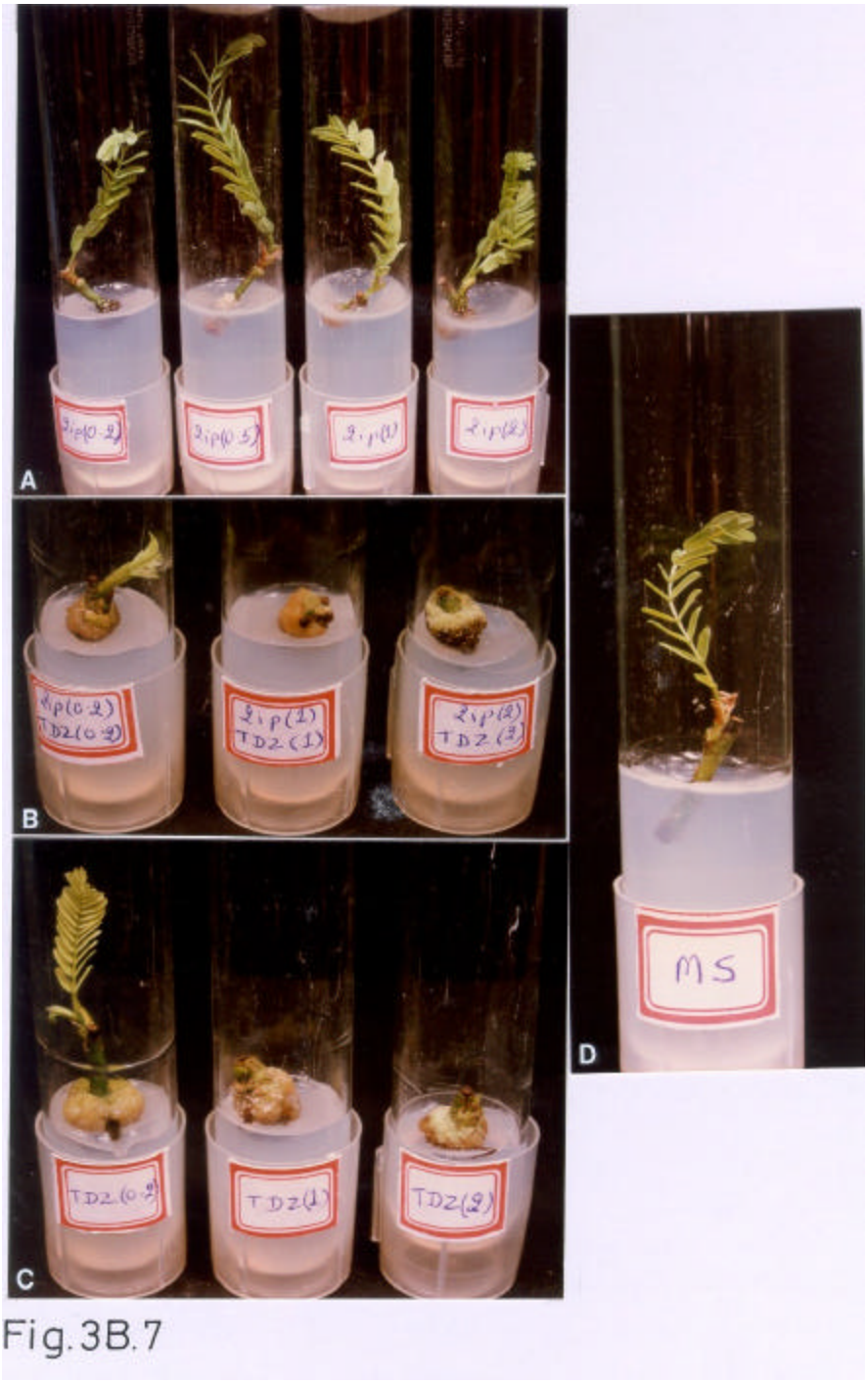


Fig. 3B.7

Fig. 3B.7 Axillary buds cultured in 2ip 0.2-2mg.l^{-1} singly or in combination with TDZ at similar concentrations
Axillary buds cultured in TDZ 0.2-2 mg.l^{-1}.
Axillary buds cultured in medium without PGR

Table 3B.2 : Response of sprouted buds on culturing in fresh medium of same composition

Medium (PGR conc. in $mg.l^{-1}$)	No. of sprouted buds cultured	No. of buds elongated to shoots	Frequency of response (%)
KN 0.2	11	0	0.0
KN 0.5	9	0	0.0
KN 1	10	0	0.0
KN 2	10	0	0.0
KN 0.2+BAP 0.2	17	3	17.6
KN 0.5+BAP 0.5	16	1	6.3
KN 1+BAP 1	19	0	0.0
KN 2+BAP 2	16	2	12.5
KN 0.2+Z 0.2	5	0	0.0
KN 0.5+Z 0.5	7	0	0.0
KN 1+Z 1	8	0	0.0
KN 2+Z 2	7	2	28.6
KN 0.2+2iP 0.2	10	0	0.0
KN 0.5+2iP 0.5	10	0	0.0
KN 1+2iP 1	6	0	0.0
KN 2+2iP 2	9	0	0.0
KN 0.2+TDZ 0.2	6	0	0.0
KN 0.5+TDZ 0.5	5	0	0.0
KN 1+TDZ 1	7	0	0.0
KN 2+TDZ 2	6	0	0.0
BAP 0.2	11	4	**36.4**
BAP 0.5	10	2	20.0
BAP 1	11	2	18.2
BAP 2	8	1	12.5
BAP 0.2+Z 0.2	10	2	20.0
BAP 0.5+Z 0.5	9	1	11.1
BAP 1+Z 1	9	0	0.0
BAP 2+Z 2	10	0	0.0
BAP 0.2+2iP 0.2	9	0	0.0
BAP 0.5+2iP 0.5	10	2	20.0
BAP 1+2iP 1	10	1	10.0
BAP 2+2iP 2	10	0	0.0
BAP 0.2+TDZ 0.2	10	1	10.0
BAP 0.5+TDZ 0.5	8	0	0.0
BAP 1+TDZ 1	10	1	10.0
BAP 2+TDZ 2	10	0	0.0
Z 0.2	14	5	35.7
Z 0.5	10	2	20.0
Z 1	11	2	18.2
Z 2	15	4	26.7
Z 0.2+2iP 0.2	2	0	0.0
Z 0.5+2iP 0.5	2	0	0.0
Z 1+2iP 1	1	0	0.0
Z 2+2iP 2	2	0	0.0
Z 0.2+TDZ 0.2	2	0	0.0
Z 0.5+TDZ 0.5	2	0	0.0
Z 1+TDZ 1	1	0	0.0
Z 2+TDZ 2	4	0	0.0
2iP 0.2	3	0	0.0
2iP 0.5	2	0	0.0
2iP 1	6	0	0.0
2iP 2	7	1	14.3
2iP 0.2+TDZ 0.2	6	1	16.7
2iP 0.5+TDZ 0.5	1	0	0.0
2iP 1+TDZ 1	4	0	0.0
2iP 2+TDZ 2	3	0	0.0
TDZ 0.2	5	0	0.0
TDZ 0.5	0	0	0.0
TDZ 1	4	0	0.0
TDZ 2	6	0	0.0
MS	49	0	0.0

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From the response of the sprouted buds cultured in various cytokinins, it appeared that BAP, KN and Z, singly or in combination, are favorable for sprouting and differentiation of mature buds. Therefore, the following experiment was carried out to study the effect of these three cytokinins on frequency of sprouting and elongation, average shoot height and frequency of cultures showing leaf differentiation.

The media containing BAP showed highest sprouting of buds as compared to KN and Z. The frequency of cultures showing elongation was higher in BAP 0.1-0.5 $mg.l^{-1}$ as compared to KN and Z at similar concentrations, and also compared to MS basal medium (Table 3B.3). In BAP 0.5 $mg.l^{-1}$, the frequency of response was 28.7±17.4 %. However the difference in response was not significant among the BAP concentrations. The shoot height is also similar in all these concentrations (Fig.3B.8A). The shoots in BAP 0.1 $mg.l^{-1}$ showed maximum height of 1.4±0.2 cm.

Table 3B.3 : Effect of Kinetin, Benzylaminopurine and Zeatin on sprouting, elongation, and leaf opening of mature buds

Medium (PGR conc. in $mg.l^{-1}$)	Sprouting frequency (%) Mean ± s.d.	Cultures elongated* (%) Mean ± s.d.	Average shoot height* (cm) Mean ± s.d.	Cultures showing leaf opening (%) Mean ± s.d.
KN 0.1	86.7±15.3	10.1± 4.7 ^{bc}	1.1±0.1 ^{ab}	34.4±28.2
KN 0.2	85.0± 8.7	3.8± 3.4 ^{c}	0.7±0.6 ^{abc}	41.8±29.5
KN 0.5	90.0±10.0	5.2± 5.0 ^{c}	0.7±0.6 ^{abc}	45.0±38.8
BAP 0.1	95.0± 8.7	27.1±12.2 ^{a}	**1.4±0.2** ^{a}	23.7±15.1
BAP 0.2	**96.7± 2.9**	22.5±12.4 ^{ab}	1.1±0.1 ^{ab}	19.2±12.3
BAP 0.5	95.0± 5.0	**28.7±17.4** ^{a}	1.1±0.1 ^{ab}	10.9± 9.9
Z 0.1	83.3±10.4	2.1± 3.6 ^{c}	0.7±1.2 ^{abc}	26.5±15.1
Z 0.2	81.7± 2.9	0.0± 0.0 ^{c}	0.0±0.0 ^{c}	36.4±17.1
Z 0.5	90.0± 8.7	1.8± 3.0 ^{c}	0.3±0.6 ^{bc}	**49.7±37.2**
MS	90.0± 5.0	0.0± 0.0 ^{c}	0.0±0.0 ^{c}	43.5±37.2
ANOVA	NS	S 1%	S 5%	NS

Data from 60 replicates

* Similar letters indicate the data is not significant

The increase was not significantly different from those obtained in KN (0.1-0.5 $mg.l^{-1}$), BAP (0.2 and 0.5 $mg.l^{-1}$) and Z 0.1 $mg.l^{-1}$ but it was significantly different

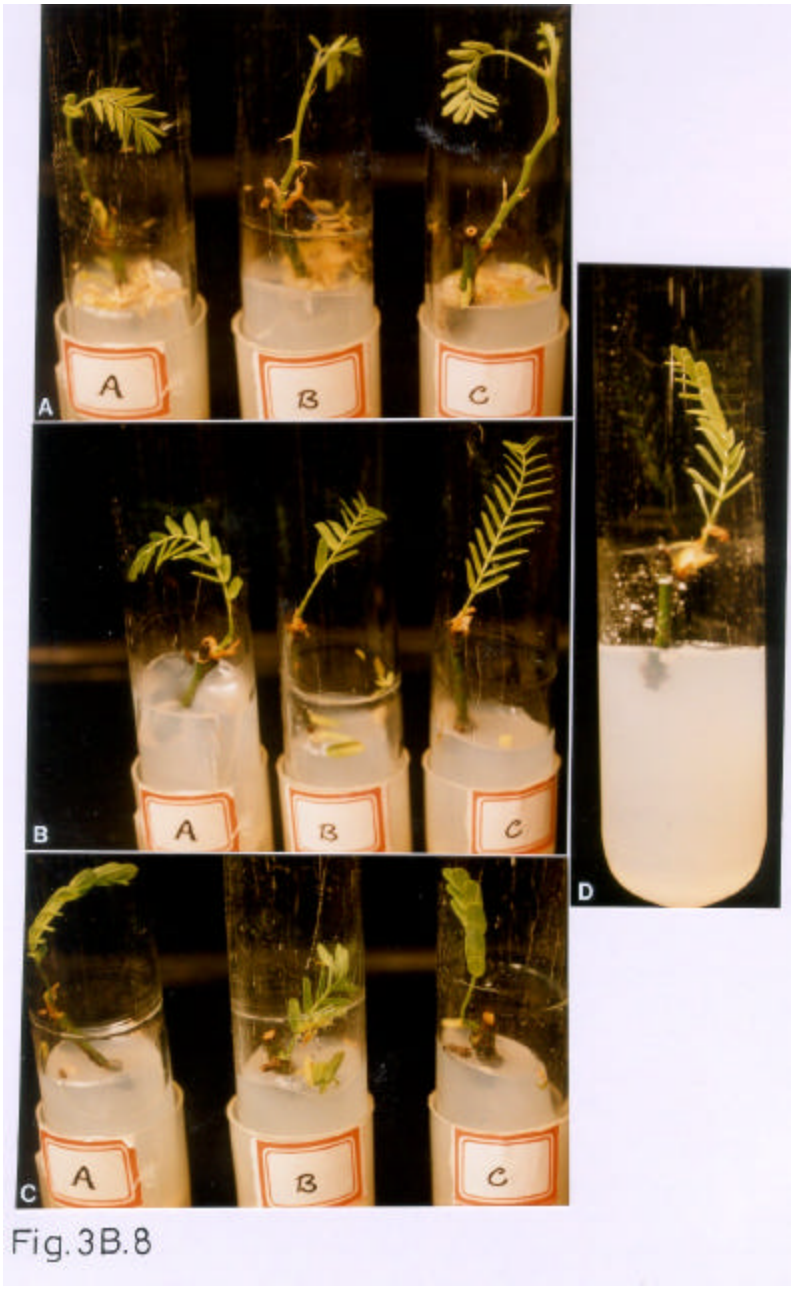


Fig. 3B.8

Fig.3B.8.: (A) Sprouting of buds and shoot differentiation in medium containing BAP.
Alphabet A-C on tube holders indicate 0.1,0.2 and 0.5 mg.l^{-1} of BAP
(B) Sprouting of bud showing leaf opening in medium containing KN.
Alphabet A-C on tube holders indicate 0.1,0.2 and 0.5 mg.l^{-1} of KN.
(C) Sprouting of bud showing leaf opening in medium containing Z.
Alphabet A-C on tube holders indicate 0.1,0.2 and 0.5 mg.l^{-1} of Z.
(D) Sprouting of bud showing leaf opening in medium devoid of PGR.

Only in medium with BAP shoot elongation was noted whereas in other cytokinins and in medium devoid of PGR leaves opened directly without shoot differentiation.

from Z (0.2 and 0.5 $mg.l^{-1}$) and MS basal medium. In case of percentage of cultures showing leaf opening, BAP showed a decrease with increasing concentration. More number of shoots showed opening of leaves directly in cultures grown in KN, Z or even MS basal medium (Fig.3B.8B,C,D). The degree of opening of leaves varied (being a qualitative trait). The nature of leaf opening was of two types. In one the shoots elongated and its leaves opened. In other, the shoot did not elongate but the leaf of the bud opened directly, as in the case of MS (Fig. 3B.8D).

The data generated from the experiment conducted to refine BAP concentration (Table 3B.4) shows that frequency of buds sprouting in medium devoid of growth regulator is 83%. It increased to 93-95% in media with BAP 0.1-0.5 $mg.l^{-1}$. Presence of BAP was essential for differentiation of bud to form shoot. More number of buds (37.8±22.0 %) elongated into shoots (Fig. 3B.9A) at BAP 0.5 $mg.l^{-1}$. Optimum shoot height of 1.4±0.3 cm was also achieved in the same medium. Although the bud in the MS medium did not form shoot but differentiated into leaf directly. These leaves were fully opened in 43% of the culture. The leaves of shoots differentiated in media containing BAP were not open in all the cultures. Among the BAP containing media optimum leaf opening was seen in cultures grown in BAP 0.2 $mg.l^{-1}$.

Table 3B.4 : Effect of different BAP concentrations on percent response of sprouting, elongation, shoot height and leaf opening of mature buds

Medium (PGR conc. in $mg.l^{-1}$)	**No. of buds inoculated**	**Sprouting frequency (%) Mean ±s.d.**	**Cultures elongated (%) Mean ± s.d.**	**Average shoot height (cm.) Mean ± s.d.**	**Cultures showing leaf opening (%) Mean ± s.d.**
BAP 0.1	62	93.6± 5.5	10.1± 4.9	1.1±0.2	26.1± 6.7
BAP 0.2	61	93.4± 3.0	26.0±12.5	1.2±0.4	39.6±26.5
BAP 0.3	62	**95.3±4.6**	37.2±20.6	**1.4±0.3**	30.4±17.0
BAP 0.4	61	95.0± 5.0	25.8±19.0	1.2±0.2	17.0±18.6
BAP 0.5	64	92.5± 4.3	**37.8±22.0**	**1.4±0.3**	12.3±16.9
MS	63	82.8± 3.9	0.0± 0.0	0.0±0.0	**43.0±35.6**
ANOVA		S 5%	NS	S 1%	NS

3.3B.2.2 Effect of carbohydrates

Data generated from the experiment on influence of various carbohydrates on mature bud culture demonstrate that frequency of mature buds sprouting in culture is

Fig.3B.9

Fig.3B.9 : (A) Differentiation of axillary buds to form shoots in presence of BAP.
Alphabets on tube holders indicate serial concentrations of BAP (0.1,0.2,0.3,0.4,0.5 $mg.l^{-1}$). The last tube 'F' is shoot in medium devoid of PGR in which leaf opened directly.

(B) Effect of carbohydrate supplement on sprouting elongation and leaf opening of mature buds cultured in MS basal medium with 0.2 $mg.l^{-1}$ BAP. Alphabets on tube holders indicate different carbohydrates at 2%
'A' -fructose, 'B' -glucose, 'C' -maltose, 'D' -sucrose.

(C) Effect of media pH on sprouting elongation and leaf opening of mature buds cultured in MS basal medium with 0.2 BAP and sucrose 2%. Alphabets on tube holders indicate media pH serially (5.6, 6.0, 6.2, 7.0).

optimal in medium with sucrose (Table 3B.5). Substitution of sucrose with fructose, glucose or maltose of similar concentration does not alter the frequency significantly. However the frequency of shoot elongation was 72.4±15.7% in presence of glucose

Table 3B.5 : Effect of, Fructose, Glucose, Maltose and Sucrose on sprouting, elongation, shoot height and leaf opening of mature buds

Medium (PGR conc. in $mg.l^{-1}$)	Sprouting frequency (%) Mean ± s.d.	Cultures elongated (>1cm) (%) Mean ± s.d.	Average shoot height (cm) Mean ± s.d.	Cultures showing leaf opening (%) Mean ± s.d.
MS+BAP 0.2 +Fruc. 2%	53.3±28.9	38.1±39.3	0.8±0.8	28.6±28.6
MS+BAP 0.2 + Gluc. 2%	60.0±15.0	**72.4±15.7**	**1.6±0.4**	**71.7± 4.4**
MS+BAP 0.2 + Malt. 2%	53.3±30.6	0.0± 0.0	0.0±0.0	2.8± 4.8
MS+BAP 0.2 + Suc. 2%	**68.3± 2.9**	36.8±8.8	1.1±0.1	29.3± 1.3
ANOVA	NS	S 5%	S 5%	S 1%

Data from 60 replicates

followed by 38.1±39.3% in fructose and 36.8±8.8% in sucrose. The increase in frequency of response by substituting sucrose with glucose was significant. Maltose was ineffective for elongation of shoot and formed leaves directly (Fig.3B.9B). Maltose inhibited the elongation and opening of leaves. Even for leaf opening, glucose was most effective. In 71.7±4.4% cultures with glucose, the leaves opened. The frequency of leaf opening in glucose was significantly higher than sucrose. This indicates that variation of carbohydrate did not influence the frequency of sprouting of buds but substitution of sucrose with glucose did support frequency of shoot elongation and leaf opening.

3.3B.2.3 Effect of pH

Tamarind tissues and leaves are sour to taste thereby indicating that the tissue is acidic in nature. So it was presumed that higher pH of the medium in which the buds are cultured may have some effect on their sprouting and shoot elongation. However the data

(Table 3B.6), and figure (Fig.3B.9C) does not show any significant differences with variation in pH of the medium.

Table 3B.6 : Effect of pH on sprouting and elongation of mature buds

pH	Sprouting frequency (%) Mean ± s.d.	Cultures elongated (>1cm) (%) Mean ± s.d.	Average shoot height (cm) Mean ± s.d.	Cultures showing leaf opening (%) Mean ± s.d.
5.8	77.5±10.6	**30.9±27.0**	1.1±0.0	92.9±10.1
6.0	**82.5± 3.5**	15.1± 3.6	**1.3±0.3**	**100.0± 0.0**
6.2	75.0± 0.0	10.0± 4.7	1.1±0.2	**100.0± 0.0**
7.0	72.5±10.6	14.7±11.9	1.0±0.0	66.7±47.1
ANOVA	**NS**	**NS**	**NS**	**NS**

Data from 40 replicates

3.3B.2.4 Effect of photoperiod

The cultures incubated in 24h photoperiod showed higher frequency of sprouting (89.4±0.6 %) as compared to 16h photoperiod, thus indicating a possible need for higher light requirement (Table 3B.7). However the difference was not significant statistically.

Table 3B.7: Effect of photoperiod on sprouting of mature buds

Photoperiod	No. of buds inoculated	No. of buds sprouted	Sprouting frequency (%) Mean ± s.d.
16 h	230	155	67.6±13.0
24 h	198	177	**89.4± 0.6**

3.3B.3 Elongation of sprouted buds

Earlier studies (Mascarenhas *et.al*., 1981) demonstrated that combination of KN and BAP was beneficial for sprouting and elongation of tamarind axillary buds.To test the role of KN and BAP in combination on elongation of shoots, the buds sprouted in 0.2 $mg.l^{-1}$ BAP were transferred to medium combinations containing different concentrations of KN and BAP. Data (Table 3B.8) shows that most of the sprouted buds elongated in

BAP 0.2 $mg.l^{-1}$ singly followed by the combination of KN 0.2 $mg.l^{-1}$ and BAP 0.5 $mg.l^{-1}$. In the medium with BAP 0.2 $mg.l^{-1}$ the average height of the shoots was 2.5±1.6 cm. Whereas in medium containing KN 0.2 $mg.l^{-1}$ + BAP 0.2 $mg.l^{-1}$ the shoots attained the optimum height 2.6±1.4 cm. Supplementing the initial medium with KN 0.2 $mg.l^{-1}$ did not increase the height significantly. Further enrichment of the medium either with KN 0.5 $mg.l^{-1}$ or with BAP 0.5 $mg.l^{-1}$ was inhibitory and the heights of the shoots were significantly lower in these two media. However, in the medium with KN and BAP both at 0.5 $mg.l^{-1}$, elongation of shoots was similar to the medium with 0.2 $mg.l^{-1}$ BAP singly or in combination with KN 0.2 $mg.l^{-1}$.This suggests that BAP 0.2 $mg.l^{-1}$ is optimum for shoot elongation.

Table 3B.8 : Effect of Kinetin and Benzylaminopurine in combination on differentation and elongation of buds sprouted in BAP 0.2 $mg.l^{-1}$

Medium (PGR conc. in $mg.l^{-1}$)	**No. of explants inoculated**	**No. of explants elongated**	**Cultures elongated (%) Mean ± s.d.**	**Height of shoots (cm) Mean ± s.d**
BAP 0.2	46	35	**72.9±14.8**	2.5±1.6
KN 0.2 + BAP 0.2	47	30	63.7±37.4	**2.6±1.4**
KN 0.5 + BAP 0.2	49	33	67.4±26.6	1.8±0.8
KN 0.2 + BAP 0.5	48	33	67.6±23.1	1.7±0.8
KN 0.5 + BAP 0.5	47	31	65.5±28.9	2.3±1.1

ANOVA	NS	S 1%

The elongated shoots obtained from different experimental batches were excised and used for further subculturing or rooting experiment while the original basal explant when cultured on fresh medium, showed sprouting and elongation of a second crop of shoot (Fig. 3B.10). This response was not very consistent.

Fig.3B.10

Fig. 3B.10. Elongation of a second crop of shoot developed on reculturing of the original explant after excision of the elongated shoot. Arrow indicates the point of excision of elongated shoot.

3.3B.4 Rooting of elongated shoots

IAA and IBA either singly or in combination were ineffective for induction of rooting, in the shoots at the end of 4 weeks of incubation. The shoots turned brown and dried off. Few shoots growing in IBA 1mg.l^{-1} singly and in IAA 0.5 mg.l^{-1} + IBA 0.5 mg.l^{-1} were green, and had little whitish callus at the basal cut end. Transferring these shoots to half strength MS basal medium supplemented with 0.25% activated charcoal did not support root induction. At the end of 15 days, no rooting was observed and the shoots dried.

3.4B CONCLUSIONS

Tamarind axillary buds collected at particular time of the year from the specific region of twigs sprouted when cultured in MS basal medium devoid of PGR and containing 2% sucrose. The response was obtained when the cultures were incubated in light. This response could be enhanced by, supplementing the medium with BAP 0.1-0.5 mg.l^{-1}. Longer photoperiod and glucose in place of sucrose were favorable for sprouting and differentiation of the buds. Callusing was noted at the cut end of the explants in all the cultures with or without PGR indicating callogenic nature of the plant. Efforts to induce healthy roots in these shoots remained unsuccessful.

From the results of the experiments conducted to standardize a protocol for clonal propagation from mature tree-derived explants, it is apparent that BAP plays an important role in induction and differentiation of the shoots from the existing meristems. Glucose is beneficial for elongation of shoot and leaf opening although callusing is increased in these cultures. However this method of micropropagation will need further refinement for rapid propagation of elites.

C: Micrografting

3.1C INTRODUCTION

Micrografting consists of grafting of a shoot apex from a donor plant or a microshoot onto a young plant grown in the greenhouse/nursery or onto a decapitated seedling under aseptic growth conditions (Jonard, 1986). Micrografting has been successfully applied in the production of virus-free clones of citrus (Murashige *et al*., 1972), pistachio (Abousalim and Mantell, 1992) tissue culture regenerants in citrus (Pasguale *et al*., 1999), tea (Prakash *et al*., 1999), and cotton (Luo and Gould 1999, Banerjee *et al*., 2000). Micrografting techniques have also been used in the recovery of putative transformants in grain legumes like *Vicia narbonensis* (Pickardt *et al*., 1995) and chickpea (Krishnamurthy *et al*., 2000). Root formation could not be induced in the somatic-embryo-derived multiple shoot clones of *Vicia narbonensis* (Pickardt *et al*., 1995). Similar observations were also made in the multiple shoot from mature seed-derived embryos of *Cicer arietinum* (Krishnamurthy *et al*., 2000). A major problem associated with somatic embryogenesis is poor conversion to plants. The embryo differentiated from endosperm callus of *Citrus grandis* did not develop to the dicotyledonous stage. The endosperm callus of *Citrus sinensis* produced dicotyledonous embryo, which developed shoot but not roots. Complete plants were established by micrografting of the shoots onto the diploid rootstock seedling (Gmitter *et al.,* 1990). In case of the kiwi fruit, micrografting shortened the time required for transfer of *in vitro*-derived kiwi fruit plants to the field (Ke *et al*., 1993). During research on rejuvenaton and cloning, Pullman and Timmis (1992) achieved establishment of juvenile-like shoot cultures and plantlets from 4-16 year old Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) trees using *in vitro* grafting techniques. Micrografting has been used to rejuvenate plants by grafting of shoot tips from mature plants onto juvenile rootstocks as in Sequoia (Huang *et al*., 1992), Hevea (Perrin *et al*., 1994), Avocado (Pliego-Alfaro and Murashige, 1987) Pistachio (Barghchi and Alderson, 1989) and *Acacia tortilis* (Forsk.) Hayne Subsp. *raddiana* (Savi) Brenan (Detrez., 1994). Ollitrault (1992) grafted somatic embryos of citrus on 1-year-old *Citrus volkameriana* Ten. and Pasq. seedlings. Micrografting has also facilitated the study of the histological nature of graft unions (Abousalim and Mantell, 1992; Gebhardt and Goldbach, 1988).

3.2C EXPERIMENTAL PROTOCOL

3.2C.1 *In vitro* grafting

3.2C.1.1 Preparation of scion

The seedling explant derived shoots used for this experiment were pooled from different experiments of KN and BAP medium and BAP 0.2 $mg.l^{-1}$ supplemented with different carbohydrates, after prolonged incubation. The elongated shoot above 3 cm obtained either from shoot tip or from the cotyledonary node explants of the seedlings were excised and were identified as shoot tip explant. Elongated shoots of more than 5-6 cm was cut into 2-3 pieces, to obtain one shoot tip of 3 cm and one or 2 pieces (nodal segment) of about 3 cm. Pieces of shoots, without shoot tip had 2-3 nodes and was identified as nodal cuttings. Near the base of the shoot approximately 0.5 cm length was cut in tangent to have a tapering end to ensure good contact with rootstock and callusing from the wound. Thus the scion was ready.

3.2C.1.2 Preparation of rootstock

In vitro dark grown 10-15 days old seedlings were decapitated about 3 cm above the root-shoot joint served as rootstocks. It was ensured that there was no shoot meristem in the rootstock.

3.2C.1.3 Procedure for *in vitro* grafting

A longitudinal slit about 1 cm. deep was made in the center of the cut end of each rootstock with a sharp blade dipped in IBA 20 mg / 100 ml solution. The basal end of the scion with wounded sides, was also dipped in IBA solution and was inserted into slits of the rootstocks in a manner, so that the inner cut surface of the rootstock and wounded outer surface of the scion were in direct contact. The graft was transferred vertically in culture bottle containing MS basal medium with 2% sucrose and 0.75% agar. Before closing the bottle, the scion was adjusted carefully once again to ensure that the wounded surfaces are well in contact. The culture bottles were handled carefully thereafter to avoid disturbing the graft.

3.2C.1.4 Culture conditions

All the grafts were incubated at 25±2°C under 16h photoperiod of light intensity 16µE m^{-2} s^{-1}. Observations were noted after 45 days of incubation.

3.2C.1.5 Histology of the graft union

Histological studies were carried out to confirm the graft union. About 1 cm long segment of the graft-union of a 35-day old graft was excised and fixed in FAA (formalin : glacial acetic acid : alcohol : : 5:5:90 by volume) for 48h at room temperature. Tissues were dehydrated and embedded in paraffin according to Sharma and Sharma (1980). Serial sections 8-10 µm were cut using a Reichert-Jung 2050 rotary microtome. Sections were double-stained with haematoxylin-eosin and mounted with DPX (Loba Chemie).

3.2C.1.6 Hardening of the grafted plants

Grafted plants were carefully removed after 60 days from the bottles. Roots were washed thoroughly to remove the adhering medium. Plants were treated with 1% fungicide solution (Bavistin) for 5 min. and transferred to disposable plastic pots containing a mixture of autoclaved soil:sand (1:1). The pots were kept in a glass chamber and covered with a glass sheet to minimize loss of moisture. The chamber was kept at 25°C in light intensity of 32µE $m^{-2}s^{-1}$ and 16h photoperiod. These grafted plants were intermittently sprayed with 1% fungicide solution (Bavistin) and irrigated with sterile water twice every week and were supplied with the nutrient solution (1/10 concentration of MS major salts + full minor salts) once a week for 30 days. These plants were acclimatized by removing the glass sheet cover for increasingly extended period of time each day for another 30 days. The established and growing plants were transferred to green house.

3.2C.2 *Ex vitro* grafting

3.2C.2.1 Procedure for *ex vitro* grafting

Procedure for *ex vitro* grafting was similar to *in vitro* grafting except in this the seedlings used for rootstock were germinated in the pot directly. Scions were prepared according to section 3.2C.1.1 and rootstock according to section 3.2C.1.2. Procedure was

according to 3.2C.1.3. The grafts were incubated in light for 6 weeks. Following which, the plants were transferred to the greenhouse.

3.3C RESULTS AND DISCUSSION

Studies on micropropagation of *Prosopis cineraria* showed that though 80% of the shoots rooted, only 30% could be survived on transplantation (Kacker *et al*., 1991). The shoots of *Albizzia spp*. obtained via *de novo* organogenesis from hypocotyl explants (Tomar and Gupta, 1988b) did not readily form adventitious roots. After subculturing on IAA 1M 30-40% rooting could be achieved. Lack of efficient rooting methods (40% rooting with IBA 500ppm) for the micropropagation of cashewnut, prompted Das *et al*. (1996) to use *Agrobacterium rhizogenes* treatment to achieve 80-90% rooting efficiency.

To supplement these techniques, *in vitro* and / or *ex vitro* grafting methods can be used for recovery of plants from in vitro raised shoots. The method may find its application in recovery of putative transformed regenerants.

3.3C.1 *In vitro* grafting

As described in the previous sections of this chapter, rooting could be induced in the tamarind shoots multiplied *in vitro*, but survival of these rooted shoots in soil was poor. To overcome this limitation, micrografting of shoots was carried out successfully. The shoots used in this experiment were taken from the cultures grown in MS basal medium, with varying concentrations of cytokinins and / or different carbohydrates. After 1 week some shoots turned brown and wilted. This indicated that the graft was rejected. This could primarily be due to lack of contact between the cambial tissue of the scion and the rootstock.

Scions which did not wilt or turn brown, and showed elongation of the apical part within a few days of culture in media, were considered to have established graft unions. Callus formation in the cut ends of the slit of the rootstock started in about 10 days and the slit started opening outwardly. In about 15 days, there were signs of new growth. In 20-30 days callus formation took place in the area of union and filled the gap, thus holding the scion firmly in the slit of the rootstock (Fig.3C.1A,B). When shoot tips were used as scions, 70.0 ± 13.3 % survival of micrograft was noted while 43.1 ± 6.8 %

survival was noted when nodal cuttings were used (Table 3C.1). The decrease in the survival percentage of nodal cuttings as scion in the graft may be due to the lack of sprouted bud/opened leaves. It may also be due to the exposed wound at the upper-end of the segment, thus effecting the loss of moisture from the explant.

Moore (1984a) outlined three phases for the development of a compatible graft : (1) cohesion of stock and scion, (2) proliferation of callus at the graft interface and (3) re-differentiation of vascular tissue across the graft interface. However, the latter phase is not absolutely essential for a successful graft as indicated in a number of reports (Herrero 1951; Muzik 1958; Moore 1984a, 1991). Moore and Walker (1983) observed that adjacent callus masses, graft successfully in the absence of

Table 3C.1 : Influence of the type of scion in *in vitro* micrografting

Type of scion used	No. of micrografts	No. of successful micrografts	Frequency of response (%) Mean±s.d.
Shoot tip	40	27	**70.0 ± 13.3**
Nodal cutting	26	11	43.1 ± 6.8

vascular differentiation. Incompatibility in grafts may not be synonymous with an unsuccessful graft as the reciprocal grafts have proved to be successful. The subject was reviewed quite exhaustively (Moore, 1991).

On transfer of twenty-five micrografted tamarind plants to soil, sign of new growth was observed within 20 days in them. A survival of about 52 % was achieved in 45 days (Fig. 3C.2). Thirteen micrografted plants are growing in the greenhouse. Field trials will be conducted with these plants to determine the period of juvenility.

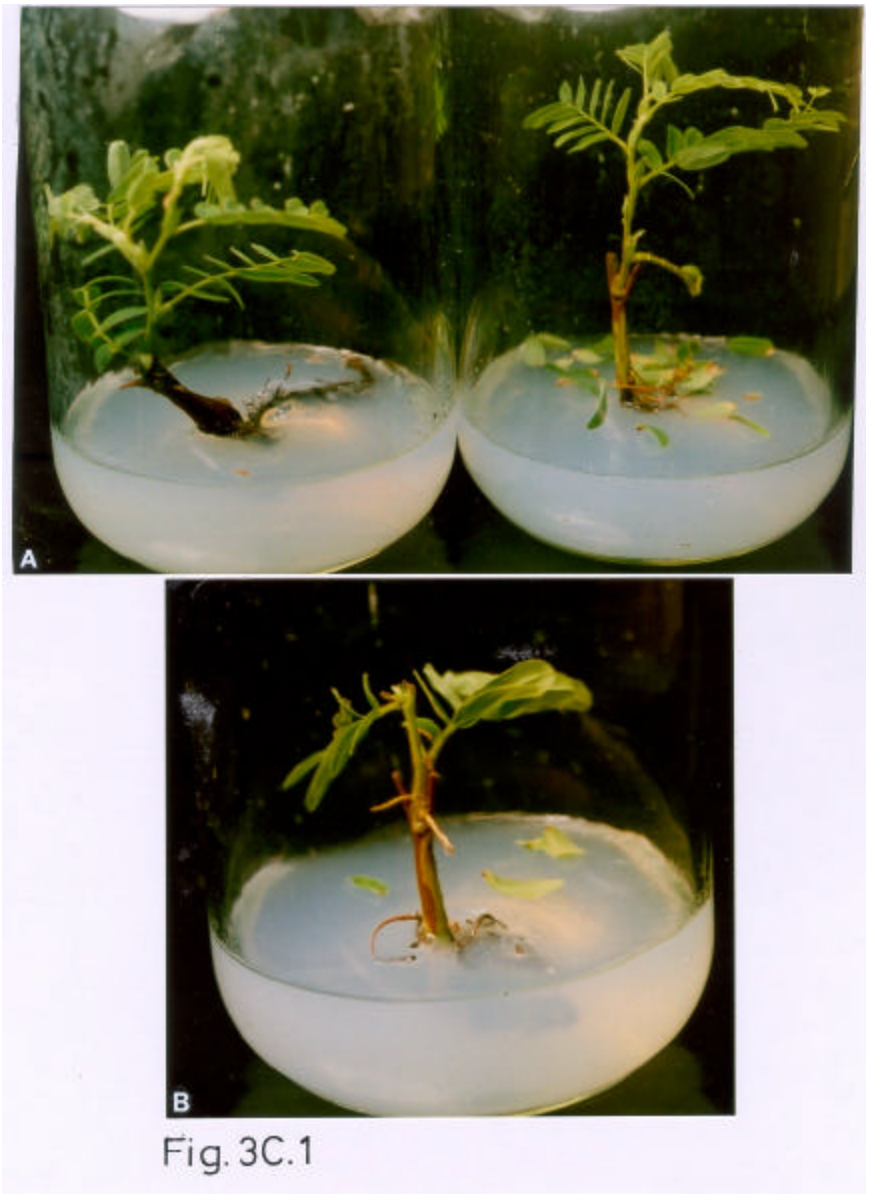


Fig.3C.1: *In vitro* grafted plants.

(A) **Shoot tip** from shoot cultures used as scion and grafted on the rootstock of *in vitro* raised seedling.

(B) **Nodal segments** from axenic shoot used as scion and grafted on the rootstock of *in vitro* raised seedling.

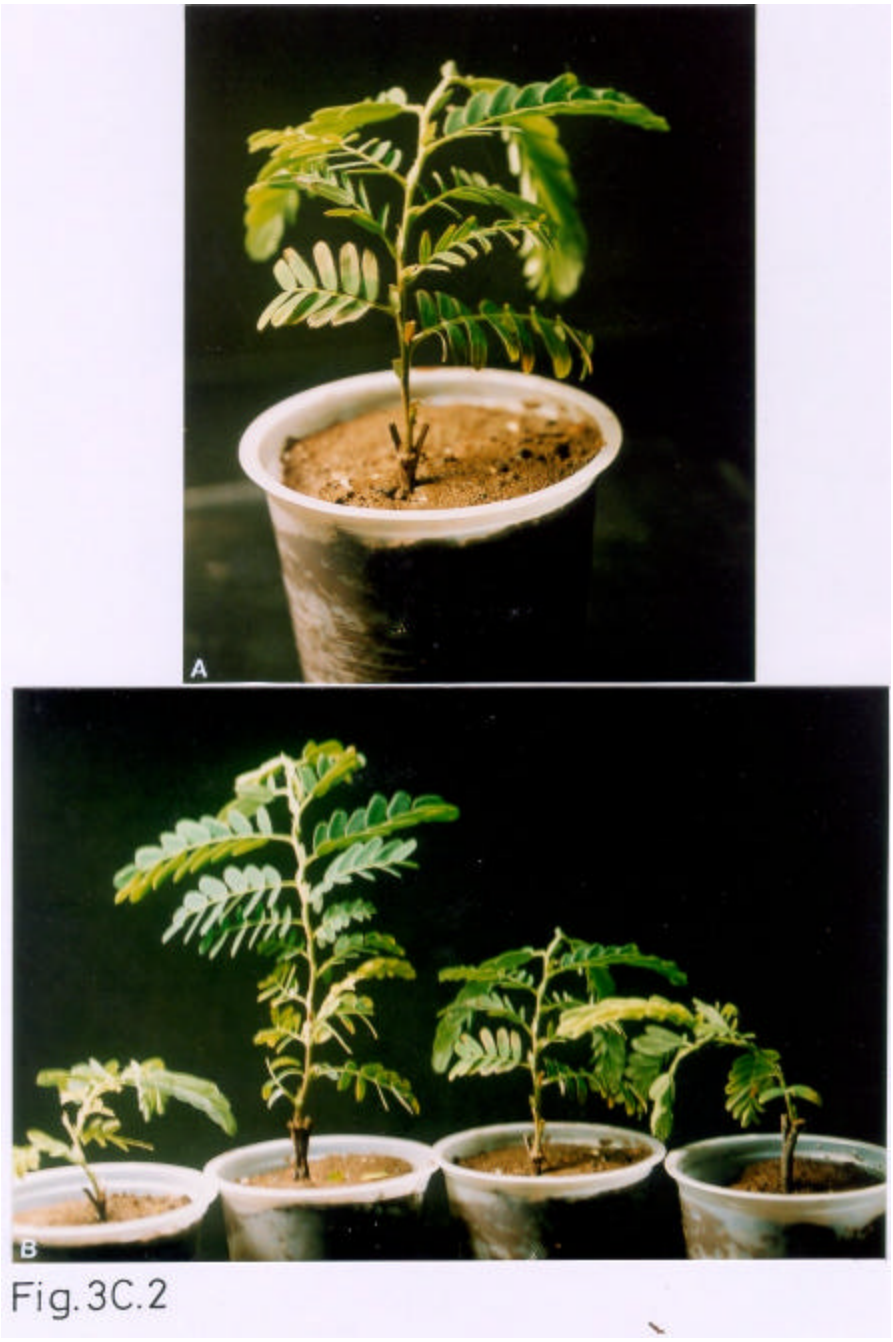


Fig. 3C.2

Fig.3C.2 : (A) The graft union can be seen in the plant raised *in vitro* by micrografting of axenic shoot.
(B) Plants micrografted *in vitro* survived in soil and showed healthy growth.

3.3C.2 Histology of the graft-union

From the histological examination it was found that 4-6weeks were required for the graft union to take place. Light microscopy (Fig.3C.3A,B) showed callus proliferation three weeks after grafting. In tea, callus proliferation was noted two weeks after grafting (Prakash *et al*., 1999). In pistachio, cell division occurred as early as 3 days after micrografting and vascular continuity was established across micrograft 3 weeks after grafting (Abousalim and Mantell, 1992). Section taken from the tissue of tamarind grafts after 35 days (Fig.3C.3C) confirms multiplication of parenchymatous cells by scion and rootstocks and their integration to form a union. Redifferentiation of vascular tissue across the graft interface was not visible in this. Therefore, the successful graft in the absence of vascular differentiation in tamarind is possibly due to the growth of adjacent callus masses. It is known (Moore and Walker, 1983) that vascular redifferentiation is not essential for the establishment of a graft.

3.3C.3 *Ex vitro* grafting

In the present study, only 10% of the *ex vitro* grafts survived (Fig.3C.4A, B). It has been reported (Hartmann and Kester, 1976) that during scion-rootstock union, the new callus tissue that arises from the cambial region is composed of thin walled, turgid cells, which easily desiccate and die. Therefore it is important that air, moisture around the graft union is kept at high to promote proliferation of cells in the graft region. In case of *ex vitro* micrografting of tamarind, the low frequency of successful grafting could be due to loss of moisture in the scion. Till the union of the graft is established, the scion needs moisture for survival.

Ex vitro grafting has some advantages. It is less expensive and less time consuming as transplantation from *in vitro* to greenhouse is omitted. It took 45 days from the *ex vitro* grafting to shifting the survived graft to greenhouse while in the case of *in vitro* grafting, they took nearly 4 months.

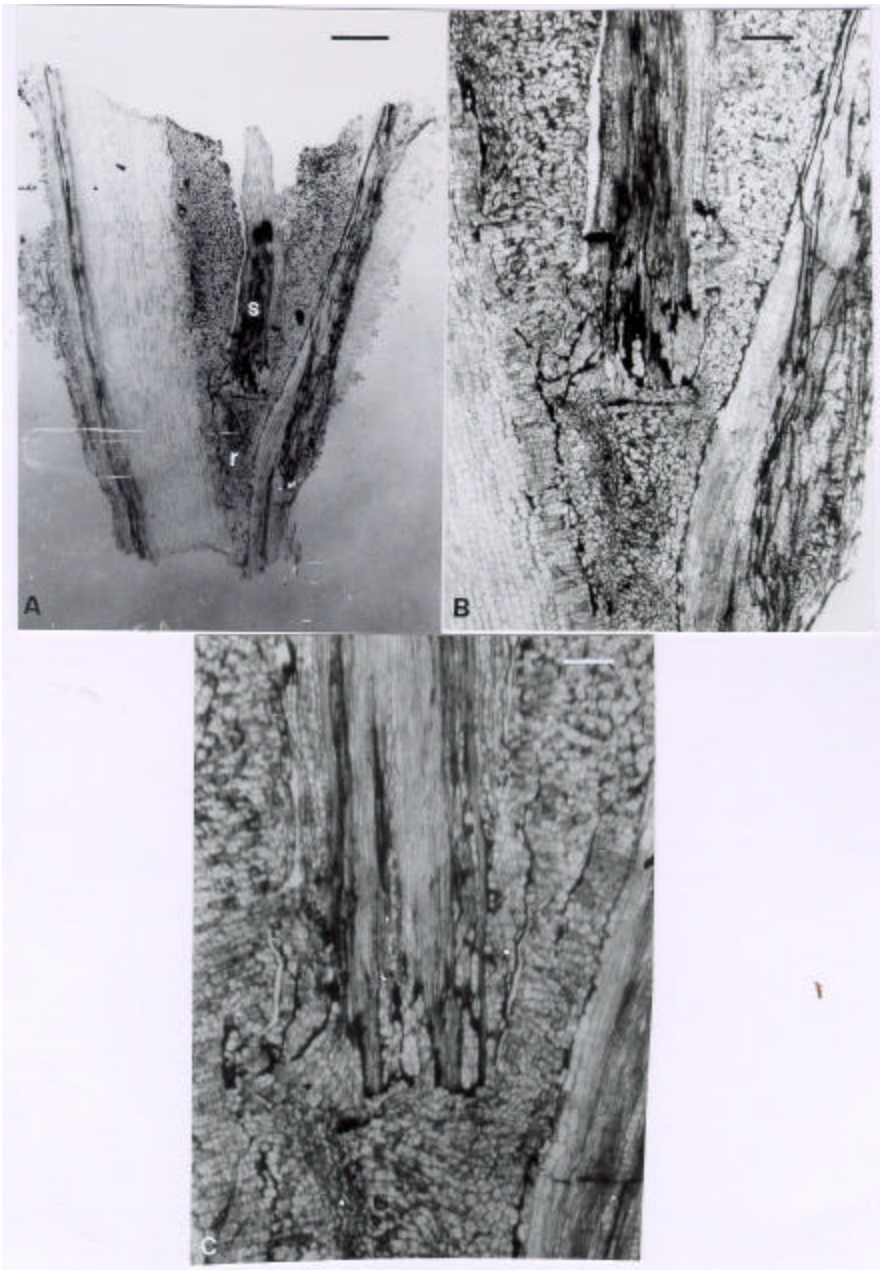


Fig. 3C.3

Fig. 3C.3 (A) : Longitudinal section of the segment showing the insertion of scion (s) in the wedge of the rootstock (r) after 35 days of the union (bar = 1000µm)

(B) : Magnified view of the graft union (bar = 250µm)

(C) : Light micrograph of the graft union showing complete filling of the gap by proliferation of parenchymatous cells at the site of union, between the scion and rootstock (bar = 166µm)

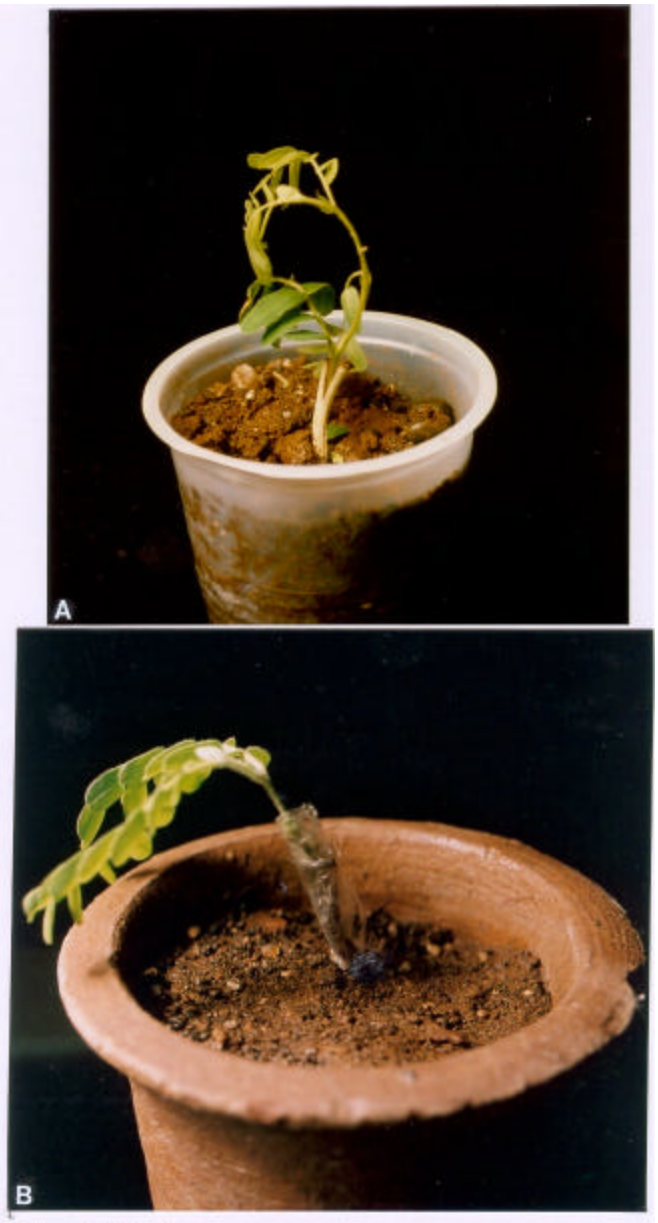


Fig. 3C.4

Fig.3C.4 : (A) The graft union in the *ex vitro* micrografted plant can be seen. The shoots of the plants grafted *extra vitrum* were less vigorous compared to the ones grafted *in vitro*.

(B) Micrograft with the site of graft covered with polyfilm. In case of *ex vitro* grafting, to hold the scion in place and to prevent moisture loss the site of the graft was kept covered for one month.

It should be possible to further enhance the survival percentage of *ex vitro* grafts by manipulating various factors such as source, size of scion and rootstock and growth conditions.

3.4C CONCLUSIONS

In an attempt to increase the survival of *in vitro* raised plants in soil, the tissue culture raised shoots were used as scions for *in vitro* and *ex vitro* micrografting. The rate of successful *ex vitro* grafting was 10 %. *In vitro* grafting frequency was 70 % on using shoot tips as scion whereas it was 43% when nodal cuttings of elongated shoots were used. Fifty two percent of these *in vitro* micrografted plants survived, on transfer to soil.

D : Variation in germination and seedling growth among tamarind seeds

3.1D INTRODUCTION

Tamarind is a highly outbreeding species, in which cross-pollination takes place primarily with the help of insects and air. Due to this reason it shows variability in the tree characters like height, spread, volume and physico-chemical characters of fruits such as fruit length, girth, average weight, average volume, weight : volume ratio, pulp content, number of seed/fruit, rag and shell content, fruit shape, color of pulp, variability in bearing habit and yield etc. (Chaoji, 1995; Karale *et al*., 1999a); floral abnormalities (Karale *et al*., 1999b).

Seedling derived explants are often used for standardization of protocols for clonal propagation, *de novo* organogenesis, embryogenesis etc. The information generated from seedling culture become useful for studies on mature plant derived explants. For using seedling explants in clonal propagation the seeds are germinated *in vitro* and the age of the seedling is counted from the day of culture. The age of the donor seedling is kept constant to maintain the consistency in the experiment.

This is possible only when the germination is synchronous. However, in the highly out-breeding species due to wide variation among the seeds the germination may not be synchronous. Secondly, the responses noted in the explants in culture may also vary. Similarly, explants collected from the seed raised trees may also show variation in response among the explants collected from different trees.

On observing high variation from batch to batch in our experiments conducted to standardize protocols for clonal propagation of tamarind, with seedling (Section 3A) and mature explants (Section 3B), we resorted to a simple experiment to adjudge the variation among seeds on the basis of germination behavior.

3.2D EXPERIMENTAL PROTOCOL

Sterilized seeds were cultured on half strength MS basal medium supplemented with 2% sucrose and incubated in dark. The experiment was repeated six times. Number of seeds germinated into seedlings with elongated hypocotyl and first pair of leaves was scored at 10 days interval starting from 20^{th} day. Observations were taken till 60 days. The data was expressed in the graph to compare the response from batch to batch. The experiment was conducted with seeds collected from the same tree.

3.3D RESULTS AND DISCUSSION

Seed raised plants exhibiting variation in morphological character, fruit quality and quantity etc. is common, as the seeds are the resultants of sexual reproduction involving two plants.

The present investigation demonstrates the differences exhibited at the early stage of plant development from seed. In our studies on clonal propagation using seedling (Section 3A.) it was necessary to obtain similar explants for replication. Due to asynchronous germination and seedling growth similar explants in sufficient number could not be obtained on a particular day after germination. Therefore in our studies the explants were taken from the seedlings of similar morphology. The seedlings with elongated hypocotyl and emerged first pair of leaves (Fig.3A.2A) were used for our experiments on clonal propagation. Tamarind seedlings with this morphological stage could be obtained from 20 days onwards. Thus, culturing of the explants was carried out

as and when the seedling reached the specified morphological stage. Thus, the usual practice of using seedlings of similar age was not applied in the experiments on clonal propagation from seedling explants.

The frequency, at which the seedlings reached the stage on twentieth day and every tenth day thereafter, was shown in the graph (Fig. 3D.1A) and bar diagram (Fig. 3D.1B). From the graph it is apparent that the number of seedlings which reached the appropriate stage on 20^{th}, 30^{th}, 40^{th}, 50^{th} and 60^{th} day varied among the batches. Comparing the treatment mean (Fig. 3D.1B), it was observed that the viable seeds germinated till 60^{th} day and attained the specified morphology between 20^{th} and 60^{th} day. The frequency was low on the 60^{th} day. As shown in the bar diagram, seeds of this tree produced optimum number of seedlings of this stage on 40^{th} day. Our experiments were carried out using the explants from 30 – 40 day seedlings from the day of seed culture. The wide variation in response noted in the results (Section 3A.3) on repeating the experiment using seedling explants may be attributed to the variation within the seeds.

Chapter 5 of this thesis on Somatic Embryogenesis demonstrates the variation in embryogenic response in immature zygotic embryos collected from various trees. These data exhibit the influence of genotype on tamarind morphogenesis.

3.4D CONCLUSION

The staggered pattern of germination of tamarind seeds and their variation in attaining the required stage may be the reason for the variation in response demonstrated by the seed derived explants.

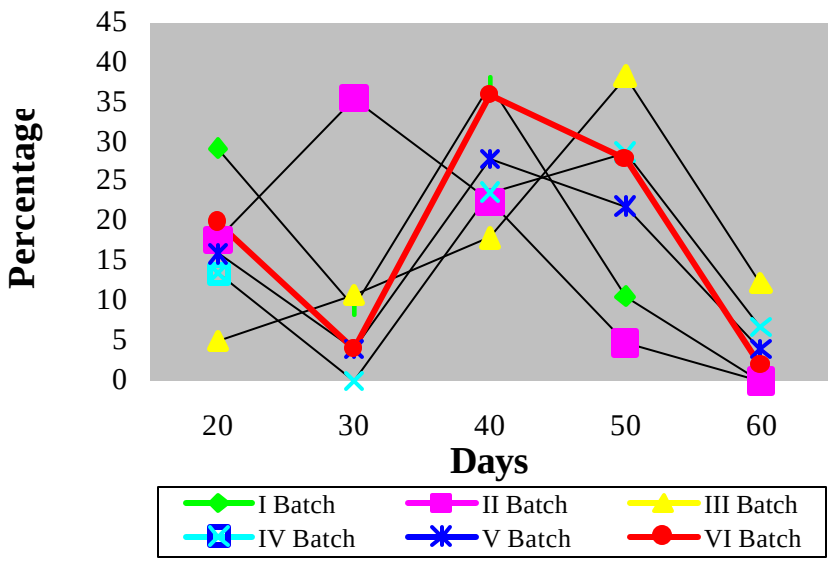


Fig. 3D.1A Growth of seedlings *in vitro*

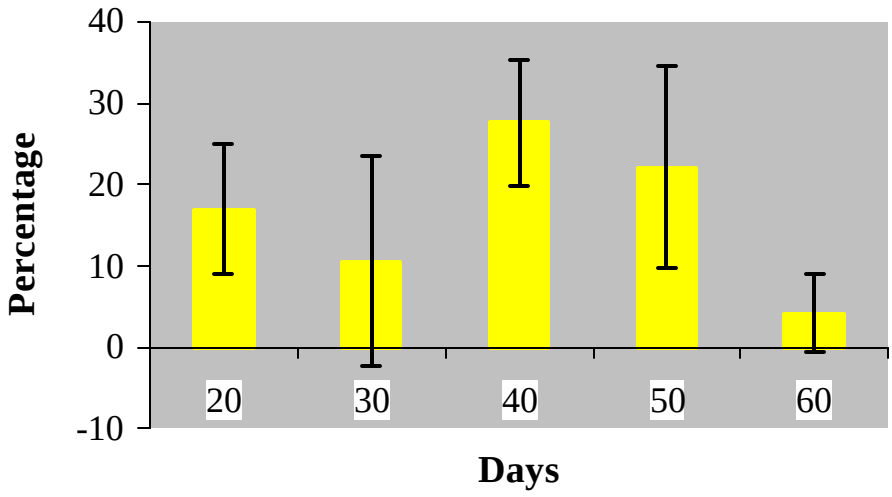


Fig 3D.1B Growth of seedlings *in vitro*

CHAPTER 4: *DE NOVO* ORGANOGENESIS

4.1 INTRODUCTION

Origin and changes in the specific form (shape, structure, organization) during the development of an organism and all such changes on and in the organism are called **morphogenesis**. The recent adaptation of descriptive terms from animal development has stimulated a better understanding of morphogenesis from cells and tissue cultures. Cells and tissue cultures are viewed as first acquiring competence, which is associated with, altered differential gene regulation and expression. The commitment of competent cells for morphogenesis is affected by many factors including complex interaction between genotypes, the explant (and its stages of development), medium etc. Morphogenesis is triggered usually after competent cells are subcultured into a less complex medium allowing the expression of new developmental potential. This trigger is referred to as permissive induction (Thorpe, 1983).

Morphogenesis *in vitro* occurs in two different patterns i.e. Organogenesis and embryogenesis. **Organogenesis** is the process by which cells and tissues are manipulated to undergo changes, which lead to the production of unipolar structure namely a shoot or root primordium, whose vascular system is often connected to the parent tissues. In contrast, **somatic embryogenesis** leads to the production of bipolar structure containing a root/shoot axis with a closed independent vascular system. Both of these can occur directly on explant or indirectly via callus (Thorpe, 1994).

Research on morphogenesis *in vitro* began in the early 20^{th} century when Haberlandt theorized that the entire plant could be produced from single living cell (Haberlandt, 1902). Early studies on tobacco callus culture by Miller and Skoog (1953) indicated that morphogenesis *in vitro* can be manipulated using auxins and cytokinins. In subsequent studies, critical factors determined were the types, concentrations, and ratios of various plant growth regulators (Sutter, 1988). Pioneering work by Steward (1958) and Reinert (1958) on induction of embryogenesis in carrot suspension cultures introduced more questions than answers concerning the role of plant growth regulators and other controlling factors in morphogenesis.

With the advent of micropropagation and genetic engineering, plants from hundreds of species have been grown *in vitro* (Bajaj, 1986). Still many species remain recalcitrant in culture conditions. It is important that regeneration of whole plants from

single cells or simple tissues of recalcitrant species be accomplished if the possibilities of genetic engineering are to be fully implemented. Until recently, standardization of successful methods for regeneration has proceeded empirically. The literature abound with methods have taken years to develop because of the lack of the basic understanding of the regulatory processes of morphogenesis. Furthermore, knowledge gained from successful manipulation of one species or cultivar is often not applicable to other species or cultivars even if the plants are closely related genetically. Development of successful schemes for regeneration *in vitro* of recalcitrant plant species would benefit from improved understanding of fundamental regulatory mechanisms of morphogenesis.

Since the discovery of phytohormones and the hypothesis of regulation of morphogenesis by auxin/cytokinin ratio (Skoog and Miller, 1957), significant progress has been made leading to important applications, in agronomy and industry and to the promising technology of haploidization and of protoplast fusion leading to genetic engineering.

Changes in the macro-micro nutrients and incorporation of various substances such as charcoal vitamins, amino acids, polyamines, polypeptides, steroids, carbohydrates etc. or diverse plant extracts can affect morphogenesis. Variation in physical conditions such as light (quality and quantity), pH, water potential, temperature, gaseous atmosphere, container shape etc. also influences plant growth and differentiation (Tran Thanh Van, 1981). It is believed that neither the out burst of miscellaneous factors nor the unique hypothesis of auxin-cytokinin ratio can bring one closer to a basic understanding of the whole process of morphogenetic differentiation as long as the target cells are scattered among a heterogeneous mass of cells. From these considerations, emerges the need for having a common cell origin for all morphogenetic patterns in order to localize more closely the target cells and the role of morphogenetic signals (Tranh Thanh Van, 1981). This has resulted in voluminous literature describing various factors that influence morphogenetic response in plant tissues but the regulatory processes of morphogenesis still remains unknown.

Organogenesis *in vitro* consists of many aspects such as phytohormone perception, dedifferentiation of differentiated cells to acquire organogenic competence, re-entry of quiescent cells into cell-cycle and organization of cell division to form specific

organ primordia and meristems (Sugiyama, 1999). Organogenesis *in vitro* depends on the application of exogenous phytohormones, in particular auxin and cytokinin, and also on the ability of the tissue to respond to these phytohormone changes during culture. Organogenesis *in vitro* is generally composed of three distinct phases of different dependency on exogenous phytohormones: the first phase in which cells are dedifferentiated to acquire organogenic competence; the second phase in which dedifferentiated cells canalized and determined for specific organ formation in response to exogenous phytohormones; and the third phase in which organ morphogenesis proceeds independently of exogenous phytohormones. Genes that are presumed to play critical roles in each phase of organogenesis *in vitro* are being identified largely through genetic analysis and some of them have been already isolated.

Plant tissues cultured *in vitro* are able to differentiate and form organ *de novo*. Such organs include roots, shoots and flowers (Thorpe, 1980). The earliest report on controlled shoot formation *in vitro* was by White (1939). He observed that shoots were formed on callus of *Nicotiana glauca* X *Nicotiana longsdorfii* hybrid when it was submerged in a liquid medium, but not when cultured on the surface of nutrient agar. In the same year, the first observation on root formation from callus was noted by Nobecourt (1939) using carrot callus. White's observation was confirmed and extended by Skoog (1944) who showed that auxins could stimulate root formation and inhibit shoot formation. Gautheret (1945) made a similar conclusion on the role of auxin in rooting. In addition, Skoog (1944) found that the inhibitory effect of auxin on shoot formation could be partially overcome by increasing concentration of sucrose and organic phosphate in the medium.

In manipulating organogenesis *in vitro*, many growth-active substances, phytohormones as well as other compounds have been included in the medium. Although large number of plant species respond to suitable auxin/cytokinin by forming shoots and roots, in a number of cases the permissive phytohormones balance leads to the induction of organogenetic tissue only (Hicks, 1994). This will then develop into organs in a medium with an altered phytohormonal balance. Stewards *et al.* (1964) pointed out that various growth regulating stimuli might need to be applied to cells, not only in right amounts, but also in the right sequence and under the right culture conditions. Despite a vast lore of information on hormonal control, largely through trial and error, knowledge

of the fundamental biology underlying *in vitro* induction of organogenesis remains scanty (Hicks, 1994). For example the identity of specific root or shoot forming gene remains unknown. Gene expression associated with organ specific inductive events is poorly characterized. The mechanism of action of auxins and cytokinins in organogenesis is still a mystery.

While genetic engineering of tree legumes is the long-term goal, development of suitable protocols for regeneration of plantlets via organogenesis and somatic embryogenesis is the short-term goal. Although the majority of tree species have consistently proved recalcitrant or difficult to regenerate *in vitro*, success has been achieved in some of the tropical legumes (Lakshmi Sita, 1999). These include micropropagation by direct and indirect organogenesis. In certain species adventitious shoots which arise directly from the tissues of the explant can provide a reliable method of propagation. However, direct regeneration depends on the nature of the explant as well as the genotype (George, 1993). Propagation by all methods of indirect organogenesis carries a risk of somaclonal variation. Regenerated plants may differ genetically from each other and from the stock plants. The chances of this variability occurring are greatest in plants produced by indirect shoot formation and appear to be less in those resulting from indirect embryogenesis (George, 1993). Direct as well as indirect organogenesis however has a potential as a method of regeneration and propagation for genetically transformed plants.

In many tree legumes like *Albizzia*, *Acacia*, *Prosopis*, and *Sesbania*, direct *de novo* organogenesis is reported. Gharyal and Maheshwari (1981) achieved caulogenesis from various explants namely hypocotyl, root, cotyledon and leaflets from 10-15 days old *in vitro*-raised seedlings of *Albizzia lebbeck*. Tomar and Gupta (1988b) obtained shoot regeneration from 1cm long hypocotyl segments (without axillary buds) excised from 10-12-days-old seedlings of *A. amara*, *A. lucida* and *A. richardiana*. The shoot buds were induced directly without callusing within 10-15 days of culturing. Sinha and Mallick (1993) reported successful *in vitro* regeneration and multiplication of *A. falcataria* through organogenesis. They used segments of juvenile cotyledons (approx. 8 mm) obtained from 15-day old *in vitro* seedlings, as explants. Sankhla *et al.* (1996) developed a high frequency *in vitro* regeneration protocol for silktree (*A. julibrissin* Durazz.) using excised root explants from 15-20 days old seedlings. Kumar *et al.* (1998) described an

efficient procedure for plant regeneration by shoot organogenesis from leaf explants of *in vitro* raised shoot cultures. Ahee and Duhoux (1994) reported development of a technique of *in vitro* clone production based on adventitious shoot formation from excised root explants of *Faidherbia* =*Acacia albida*. Kackar *et al.* (1992) achieved direct regeneration and multiplication of *Prosopis cineraria* using root segments as explants obtained from 3-4 month old seedling and four-and-a-half year old tree. Kapoor and Gupta (1986) obtained differentiation of *Sesbania bispinosa* plantlets from 1cm long hypocotyl segments and cotyledon explants from 12 days old seedlings. Detrez *et al.* (1994) reported direct organogenesis from cut surface of cotyledon explants obtained from 24/36 h-old post-imbibition dark-grown seedlings of *S. grandiflora*.

Among the tree legumes, indirect *de novo* organogenesis via callus was achieved in very few plants. Some of them are *Albizzia*, *Bauhinia*, *Dalbergia*, and *Sesbania*. Upadhyaya and Chandra (1983) described shoot and plantlet formation from cotyledon derived callus of Albizzia lebbeck. While Varghese and Kaur (1988) showed shoot regeneration from calli obtained from hypocotyl, cotyledon, root, leaf and rachis (0.5 – 0.6cm. long) excised from 7-10-day-old sterile seedlings of the same species. Sankhla *et al.* (1994) obtained adventitious shoots from roots of intact seedlings of *Albizzia julibrissin* after the formation of large masses of callus. Kumar (1992) demonstrated micropropagation through indirect organogenesis achieved from callus induced in the stem cuttings (ca. 1cm) collected from young branches of 15-18 year old sexually mature 'elite' *Bauhinia purpurea* trees. Anand and Bir (1984) obtained caulogenic differentiation in 40% of cotyledon and leaf callus, while hypocotyl and stem calli exhibited 55% shoot differentiation in *Dalbergia lanceolaria*. Ravishankar and Jagadish Chandra (1988) achieved organogenesis in callus derived from shoot segments. Pradhan *et al.* (1998) obtained effective plant regeneration from cell suspension-derived callus of *Dalbergia latifolia*. Kumar *et al.* (1991) demonstrated regeneration of plantlets achieved from cell suspension-derived calli of cambial origin from mature elite trees of *D. sissoo*. Sinha and Mallick (1991) described plant regeneration from cotyledon and leaf-derived calli of *Sesbania bispinosa*. While Khattar and Mohan Ram (1983), in a preliminary report, published shoot bud differentiation from callus derived from hypocotyl and cotyledon explants of *S. grandiflora*. Shanker and Mohan Ram (1990) published the procedure for whole plantlet regeneration via callus regeneration and maintenance of cultures through serial transfers of *S. grandiflora* 4 different media. Vlachova *et al.* (1987), achieved shoot

formation in calli of *Sesbania rostrata* at low frequency but Pellegrineschi and Tepfer (1993) regenerated plantlets from the calli in the same species at a higher frequency.

It is often observed that it is easier to induce regeneration from seedling explants. However, it is desirable to get regeneration from explants derived from mature trees whose desirability is well-established (Winton, 1978; Bonga, 1977; Lakshmi Sita, 1986). The potential of micropropagation of tamarind has been emphasized by several researchers (Mascarenhas *et al*.,1981; Mascarenhas *et al*., 1987; Jaiwal *et al*.,1991; Gupta *et al*.,1991). Methods for manipulation of tamarind tissue *in vitro* have been restricted to shoot multiplication from meristematic buds and nodal explants of seedlings (Mascarenhas *et al*., 1987), or from cotyledonary nodal tissue of seedlings (Jaiwal *et al*.,1991) and to a limited extent, shoot tips and nodal buds from mature trees (Mascarenhas *et al*.,1981). At the time of initiation of this work, there was no report on *de novo* organogenesis in tamarind and there were very few reports in other leguminous tree species. In the course of our work, the first report on tamarind *de novo* organogenesis appeared from a parallel group (Sonia *et al*., 1998). However, they reported direct regeneration of multiple shoot buds from the proximal end of the hypocotyl explants obtained from 12-day-old seedling of *Tamarindus indica*, while in the present investigation, we achieved direct adventitious regeneration from the non-meristematic, cut surface of mature embryo axis explant of tamarind.

4.2 EXPERIMENTAL PROTOCOL

4.2.1 Initiation of culture

4.2.1.1 Preparation of explant

Tamarind seeds were collected from the mature pods of five trees growing locally. The seeds were washed with liquid detergent and surface sterilized using 0.1% (w/v) $HgCl_2$ for 15 min. Excess $HgCl_2$ was removed by repeated washings with sterile distilled water under aseptic conditions. Seeds were soaked in sterile distilled water for 4-5 h at room temperature and washed thoroughly before culturing them in half strength Murashige and Skoog (MS) basal salts and vitamin with 2% sucrose and 5 $g.l^{-1}$ agar. The pH of the medium was adjusted to 5.6-5.8 prior to addition of agar. Cultures were incubated in dark.

From 5th day onwards, the seed with cracked seed coat were used as the source of explants. In the preliminary experiments various explants were tested for morphogenic response. These include intact embryo axis, longitudinal section of embryo axis, and longitudinal section of embryo axis with an attached cotyledon (LSEC).

In the subsequent experiment only LSEC was used. To obtain this explant, the seed coat was removed, and cotyledons were separated carefully to obtain two explants, each consisting of a longitudinal section of the embryo axis with an attached cotyledon (LSEC).

4.2.1.2 Identification of the potential explant.

In preliminary (probing) experiments all the three explants were cultured in various media formulations containing auxins and cytokinins singly and in combinations. Auxins tested were NAA and 2,4-D and cytokinins tested were KN,BAP and 2ip. The concentration of cytokinins and NAA ranged from 0.1 – 15mg/l whereas 2,4-D was from 0.1 – 10mg/l. The objective was to identify the potential explant in order to initiate the studies on morphogenesis. Therefore to some extent the experiment was random in nature. Only few explants were t ested in each of these combinations.

Except the intact embryo axis other explants were cultured in two orientations. Explants were either cultured with adaxial or abaxial side in direct contact of medium.

4.2.1.3 Optimization of culture media for caulogenesis

In the subsequent experiment, various media combinations of cytokinins including kinetin, BAP and 2iP and/or auxins NAA and 2,4-D were tested for induction of morphogenetic response in the LSEC explants of tamarind. The plant growth regulators were incorporated in MS basal medium supplemented with sucrose (2%-6%). The media were solidified with 0.5% agar. Number of explants in each treatment in this experiment varied. The mature embryo axes derived explants were cultured in petridishes (85×15mm) with 20ml media per dish. The cultures were incubated for 4 weeks in light. At the end of this period, the explants showing caulogenic response was noted. The various media

combinations tested and the number of explants used in each treatment are incorporated in Table 4.1.

On the basis of the results noted in these preliminary experiments, 10 media combinations composed of MS basal medium with BAP 2-15 $mg.l^{-1}$, NAA 0.1 to 1 $mg.l^{1}$ and sucrose 2% and 4% were identified. These media were tested repeatedly to select the combination suitable for optimum response. The frequency of response in different media was determined in percentage and the data was subjected to statistical analysis.

4.2.2 Histological studies

The explant, LSEC with the caulogenic buds, was subjected to histological studies. Pieces of explants ranging from 5-7mm in length were fixed in FAA (formalin: glacial acetic acid: alcohol::5:5:90 by volume) for 48 h at room temperature. Tissues were dehydrated in graded tertiary butyl alcohol and embedded in paraffin (m.p 58-60°) following the method described (Sharma and Sharma 1980). Serial sections, 10 µm, were cut using a Reichert - Jung 2050 rotary microtome. Sections were double stained with haematoxylin - eosin and mounted with DPX (Loba Chemie). These were studied under microscope to identify the origin of the shoot buds.

4.2.3 Elongation of shoots

The explant, LSEC with clusters of shoot buds were transferred to 250 ml Erlenmeyer flasks containing 50ml MS basal medium with or without agar supplemented with either kinetin or zeatin 0.2 $mg.l^{-1}$and with BAP 0.5 $mg.l^{1}$. All media were supplemented with calcium pantothenate 0.1$mg.l^{1}$, biotin 0.1$mg.l^{1}$ and sucrose 2%. Calcium pantothenate and biotin were used earlier in the medium for proliferation and elongation of axillary meristem derived shoos of tamarind (Mascarenhas *et al*., 1987). The cultures were incubated for 6 weeks in light. Cultures in liquid medium were incubated on a gyratory shaker (80 rpm).

After 4 weeks the elongated shoots (25mm and above) were isolated from the cluster and tested for root induction. The main explant bearing the remaining small shoots

and buds were transferred to fresh liquid MS medium of similar composition for elongation of a second batch of shoots.

4.2.4 Rooting of shoots

Rooting was induced in the elongated shoots using the method described in earlier reports from this laboratory (Mascarenhas *et al*., 1981; 1987) with minor modifications. In brief, the method involves incubation of shoots for 72 h in half strength MS liquid medium containing 1 $mg.l^{-1}$ each of IAA, IBA, IPA and NAA, prior to culturing them for 4 weeks in MS half strength medium devoid of growth regulators and containing 2% sucrose. These shoots were shifted to half strength MS basal medium supplemented with 2% sucrose, 0.25% charcoal and 0.5% agar. The cultures were incubated in light for 4 weeks. Macarenhas *et al*. (1987) used White's basal medium (Whites, 1962) for induction and elongation of the roots.

4.2.5 Transfer of rooted plant to soil

Rooted plantlets were washed thoroughly under running tap water to remove the medium adhering to the root system prior to transfer to 10×5cm. polybags or pots containing soil:sand (3:1). The plantlets were maintained in light at 25±2°C in the trays covered with glass sheets to minimize the loss of moisture. After 20 days the glass sheet covers were removed and plants were maintained for 10 days prior to transfer to pots in greenhouse.

4.3 RESULTS AND DISCUSSION

4.3.1 Initiation of culture

4.3.1.1 Preparation of explant

Pre-soaking of surface sterilized seeds in sterile distilled water for few hours facilitates imbibition and swelling of the seeds. Similar approach was used by Detrez *et al*., (1994) in *Sesbania grandiflora* seeds for easy isolation of explants from post-imbibed seeds. Tamarind seeds cultured on half strength MS basal medium and incubated in dark, started swelling in 4 days. The multiple cracks appear on the seed coat from 5^{th} day onwards (Fig. 4.1A). Complete removal of seed coat under aseptic conditions became

simpler due to the cracks developed in the seed coat. Presence of the seed coat partially or fully, resulted in browning of the medium and inhibited growth in culture.

4.3.1.2 Identification of potential explant

Of the three explants tested only LSEC (Fig. 4.1B) responded when cultured with the adaxial side of the cotyledon in contact of the media in combinations containing BAP and NAA. Inspite of splitting the cotyledons carefully with controlled pressure, the embryo axis divided into two uneven longituclinal sections, with the split being just below the plumule. As the split was oblique, often one explant had the plumule whereas the other was devoid of it.

The cotyledon of the LSEC explants turned green within 4 days of culture. The split embryo axis showed elongation and broadening within 6 days. After 3 weeks of incubation in light buds in form of small protuberances appeared on the expanded cut surface of the axis (Fig. 4.2A) in contact with the medium. These were distributed throughout the cut surface including epicotyl and hypocotyl. The caulogenic buds with meristematic domes and green leaf primordia were seen when

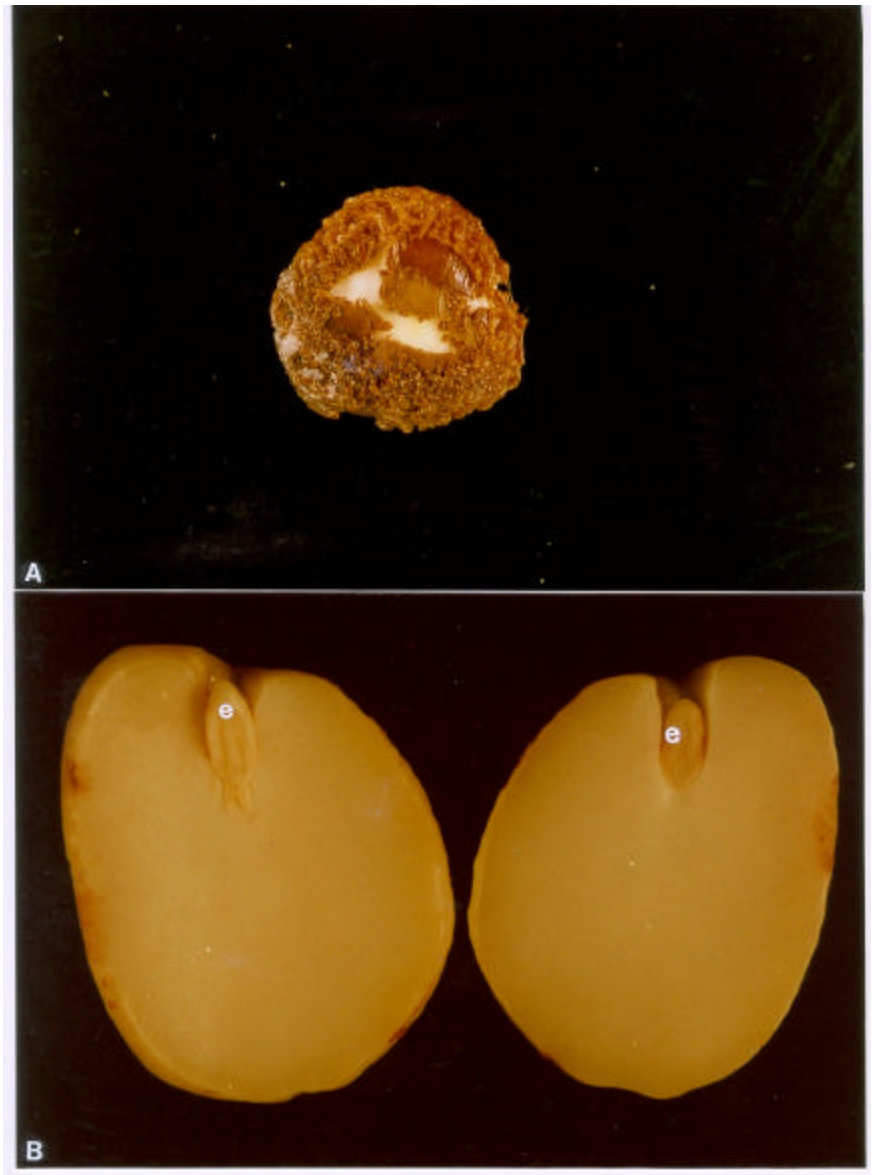


Fig. 4.1

Fig. 4.1 A : Swollen seed with cracks developed on seed coat

B : Longitudinally splitted embryo axis (e) with attached cotyledon (LSEC). The explant used for *de novo* organogenesis.

the explants were examined under the microscope (Fig.4.2B). Both the LSEC explants with or without plumule, responded in culture.

Among the other two explants the intact embryo axis expanded a little but callused. The sections of embryo axes which were without cotyledons expanded, but morphogenic response was not observed on the cut surface even in the cultures in which the cut surface was in contact of the medium. This indicates the importance of attachment of the cotyledon with the embryo axis to obtain a caulogenic response.

In the present study, shoot buds appeared on the expanded cut surface of the embryo axis in 3 weeks while it was in contact with the medium. In contrast, the LSEC explants, which lost contact with the medium, failed to respond. In cultures even where abaxial side of the cotyledon was in contact of the medium, and the cut surface of the embryo axis was in the upper side, caulogenic response was not seen. No response was noted in the other side of the responding explants and in explants in any other orientation. This shows that the contact of the cut surface of the explant with the medium was essential for the caulogenic response from the wounded surface only.

Orientation of the explants plays important role in regeneration. Gulati and Jaiwal (1990) reported that regeneration from cotyledons of *Vigna radiata* (L) Wilczek occurred only on those explants which possessed the petiole tissue embedded in medium. In *Tamarindus indica*, Jaiwal and Gulati (1991) observed that the shoot forming response decreased drastically when (i) the distal end of the cotyledon was embedded in the medium; (ii) the abaxial surface of the cotyledon was in contact with medium and (iii) the cotyledon was embedded longitudinally at a right angle to the surface of the medium. However, when the adaxial surface of the explant was in contact of the medium, the regeneration frequency and the number of shoots per explant increased greatly than when the proximal end of the cotyledon was embedded in the medium. Shri and Davis (1992) observed the response of cotyledon-like structure (CLS) formation in chickpea (*Cicer arietinum*) only in immature cotyledon explants placed with the adaxial surface down, while no response was found in explants placed with the abaxial surface down. In peanut, adaxially oriented

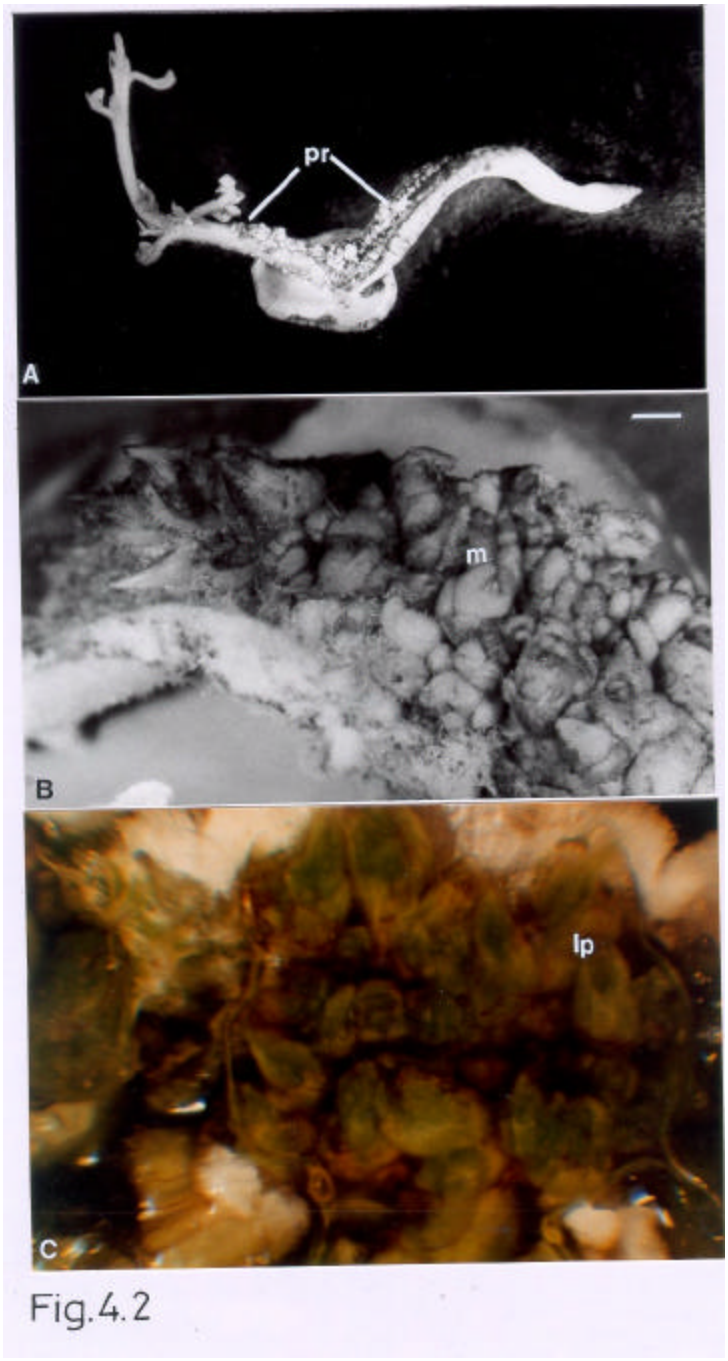


Fig. 4.2

Fig 4.2 A : Protuberances (pr) on the elongated cut surface of LSEC explant

B : Magnified view of the meristematic buds (m) developed on the cut surface of the elongated, splitted embryo axis (bar = 500μm)

C : Magnified view of the shoot buds showing green leaf primodia (lp).

cotyledons gave a better embryogenic response than explants with abaxial surface facing the medium (Ozias- Akins, 1989).

Goh *et al.* (1994) found that wounding response was necessary to trigger shoot-bud differentiation from excised leaves of mangosteen (*Garcinia mangostana* L.) in the presence of BAP. The role of wounding appeared to establish regeneration sites in explants, enabling BAP to induce shoot bud formation. Detrez *et al.* (1994) reported that the regeneration ability of the cotyledon tissues could be increased in response to additional wounding performed with the needle on the epidermal surface of the cotyledon explants (sampled from dark-grown 36-h-old imbibed seedlings) of *Sesbania grandiflora*.

4.3.1.3 Growth Regulators

The most frequently used cytokinin for shoot induction in angiosperm trees is BAP, whereas NAA has been preferred for its synergistic effect on shoot induction. It is apparent that explants obtained from mature, field-grown trees are more likely to require an auxin for shoot induction than explants derived from juvenile or pre-conditioned status (Litz and Gray, 1992). It is suggested by these authors that competent cells are already present in pre-conditioned or juvenile tissue that have been used for explants. These cells are triggered to differentiate when exposed to appropriate PGR.

Keeping in view the influence of BAP in combination with auxins (more often NAA or IAA), BAP and NAA were tested singly or in combinations for morphogenetic response in LSEC. All the media formulations used in the experiments and the medium finally refined and optimized after several replications, are included in the Table 4.1. The responses noted in the LSEC explants are also included. Benzyl amino purine at 0.1 – 15 $mg.l^{-1}$ singly (M2 – M7) did not restrict germination completely, but failed to induce adventitious buds at the cut surface of the embryo axes. Differentiation of root meristem was totally restricted in presence of higher concentrations (5-15 $mg.l^{-1}$) of BAP. Naphthalene acetic acid singly, neither induced morphogenic response nor did it suppress differentiation of radicle in any of the concentrations tested (0.1-15 $mg.l^{-1}$) (M8 – M13). Therefore rooting was noted in all the concentrations. Plumule differentiation was

checked in presence of this GR. In the higher concentrations (5-15 $mg.l^{-1}$), radicle differentiation was associated with profuse callusing.

Combination of BAP and NAA was effective in inducing *de novo* caulogenesis in *Albizzia procera* (Kumar *et al*., 1998). In 60% of the leaf explants, de novo shoot regeneration was noted when cultured in MS medium, supplemented with 10µM BAP and 1µM NAA. Jaiwal and Gulati (1991) demonstrated synergistic effect of BAP and NAA in proliferation of meristematic buds from cotyledonary node meristems of tamarind. Although BAP alone could induce the meristematic activity, but the number of shoots formed on each explant increased three-fold on further supplementing the medium with IAA. This indicates the synergistic effect of BAP with auxin. Similar synergistic effect of BAP and NAA was observed in *Dalbergia latifolia* Roxb. (Lakshmi Sita *et al*.1986; Rao, 1986). Synergism of KN and IAA was observed in induction of *de novo* organogenesis in *Albizzia lebbeck* callus (Upadhyaya and Chandra, 1983). Keeping these evidences in view, the influence of cytokinin in combination with auxins in induction of morphogenetic response, BAP and NAA were tested in combination.

Culturing of LSEC explants in media combinations (M14 – M23) containing both BAP and NAA was found effective in inducing morphogenetic response along the cut surface of the embryo axes. Microscopic examination after 20 days of culture revealed presence of small green protuberances along the cut surface of the explant (Fig 4.2B,C). These structures were distributed all along the cut surface from plumular to radicle end till the vasculature was exposed. As mentioned earlier, the split along the axis is uneven. Thereby one section has both plumule and radicle whereas the other section had only radicle. The organogenic response was observed in both the explants (Fig.4.3A, B).

Fig. 4.3

Fig.4.3 : Shoot buds (s) differentiated from the protruberances appearing on,
(A) LSEC explant which had only the radicle end
(B) LSEC explant which had both plumule and radicle

Table 4.1 : Various media combinations tested for induction of organogenic response

Medium No.	NAA $mg.l^{-1}$	2,4-D $mg.l^{-1}$	BAP $mg.l^{-1}$	KN $mg.l^{-1}$	2iP $mg.l^{-1}$	Sucrose % $gm.l^{-1}$	No. of explants cultured	Response
1	-	-	-	-	-	4	8	Germination
2	-	-	0.1	-	-	4	10	Germination
3	-	-	0.5	-	-	4	10	Limited Germination
4	-	-	1	-	-	4	10	Limited Germination
5	-	-	5	-	-	4	10	No Germination
6	-	-	10	-	-	4	10	No Germination
7	-	-	15	-	-	4	10	No Germination
8	0.1	-	-	-	-	4	10	Root proliferation
9	0.5	-	-	-	-	4	10	Root proliferation
10	1	-	-	-	-	4	10	Root proliferation
11	5	-	-	-	-	4	6	Root with profuse callusing
12	10	-	-	-	-	4	8	Root with profuse callusing
13	15	-	-	-	-	4	10	Root with profuse callusing
14	0.05	-	2	-	-	2	30	Organogenic response
15	0.05	-	5	-	-	2	52	Organogenic response
16	0.05	-	10	-	-	2	30	Organogenic response
17	0.1	-	2	-	-	2	30	Organogenic response
18	0.1	-	5	-	-	2	82	Organogenic response
19	0.1	-	10	-	-	2	52	Organogenic response
20	0.5	-	2	-	-	2	116	Organogenic response
21	0.5	-	5	-	-	2	30	Organogenic response
22	0.5	-	10	-	-	2	30	Organogenic response
23	0.5	-	10	-	-	4	257	Organogenic response
24	0.1	-	0.1	-	-	4	10	No organogenic response Only germination
25	0.1	-	0.5	-	-	4	10	No organogenic response Only germination
26	0.1	-	1	-	-	4	10	No organogenic response Only germination
27	0.1	-	5	-	-	4	8	Organogenic response
28	0.1	-	10	-	-	4	10	Organogenic response
29	0.1	-	15	-	-	4	10	Organanogenic response
30	0.5	-	0.1	-	-	4	10	No organogenic response Only germination
31	0.5	-	0.5	-	-	4	10	No organogenic response Only germination
32	0.5	-	1	-	-	4	10	No organogenic response
33	0.5	-	5	-	-	4	10	Organogenic response
34	0.5	-	10	-	-	4	10	Organogenic response, (same as in M 23)
35	0.5	-	15	-	-	4	10	Organogenic response with callusing
36	1	-	0.1	-	-	4	10	No organogenic response

Table 4.1 continued

Medium No.	NAA mg.l^{-1}	2,4-D mg.l^{-1}	BAP mg.l^{-1}	KN Mg.l^{-1}	2iP mg.l^{-1}	Sucrose % gm.l^{-1}	No. of explants cultured	Response
37	1	-	0.5	-	-	4	10	No organogenic response
38	1	-	1	-	-	4	10	No organogenic response
39	1	-	5	-	-	4	10	Organogenic response with profuse callusing
40	1	-	10	-	-	4	10	Organogenic response with profuse callusing
41	1	-	15	-	-	4	10	Organogenic response with profuse callusing
42	-	0.5	10	-	-	2	10	No germination. No callus
43	-	3	10	-	-	2	10	No germination. No callus
44	-	3	-	-	-	2	10	No germination. No callus
45	-	6	-	-	-	2	10	No germination. No callus
46	-	1	1	-	-	6	2	Callus at radicle end
47	-	1	2	-	-	6	2	Callus at radicle end
48	-	1	3	-	-	6	2	Callus at radicle end
49	-	1	5	-	-	6	2	Callus at radicle end
50	-	-	10	5	-	2	2	Callus at radicle end
51	-	-	-	5	-	2	2	No response
52	-	-	-	-	5	2	2	Germination. No callus
53	-	-	-	5	5	2	2	Callus at radicle end
54	-	-	10	-	5	2	75	Sparse organogenesis
55	-	-	10	5	5	2	10	Germination. Brown callus on cut surface

In media combinations M14 – M19 with lower concentrations of NAA (0.05 and 0.1 mg.l^{-1}) with BAP 2,5,10mg/l, the morphogenic response was sporadic and few buds could be noted. With increase in NAA in M20 – M22 appearance of caulogenic protuberances was dense as more number of buds were formed on each explant. Due to the dense growth of buds the number of buds formed on each explant could not be determined. The frequency of response observed in these media were determined in percentage. It was apparent that the magnitude of response varied among these combinations and did not follow any pattern although the PGRs were increased gradually. These variations in response could be attributed to the inherent differences noted in the seeds during germination (Chapter 3 D).

The medium containing 0.5 mg.l^{-1}NAA, 10 mg.l^{-1} BAP and 2% sucrose (medium number 22) induced numerous caulogenic buds in 34% of the cultures. Increase of sucrose concentration from 2 to 4% (medium number 23) accentuated the frequency of meristematic response dramatically to 48% in the LSEC explants. Further increase in the concentration of sucrose to 6% induced browning of the media, which was detrimental for the growth of the shoots. Tomar and Gupta (1988a) made similar observations in *Albizia*

richardiana where they noted 4% sucrose as optimal for shoot bud differentiation and higher levels of sucrose inhibited differentiation.

After observing the positive influence of 4% sucrose in place of 2%, more combinations of BAP and NAA (M24 – M41) with 4% sucrose were tested with LSEC explants using 10 explants for each combination. Lower concentrations of BAP (0.1-1 $mg.l^{-1}$), was ineffective in inducing morphogenic response, irrespective of the NAA concentration which ranged from 0.1 – 1 $mg.l^{-1}$ (M24 – 26, M30 - 32, M36 – 38). Presence of 4% sucrose was ineffective in lower concentrations of growth regulators. Organogenic response was observed in media with BAP 5–15 $mg.l^{-1}$ irrespective of the NAA or sucrose concentration (M27-29, M33 – 35, M39 – 41). In the six media (M27-29, M33 – 35) with NAA 0.1 and 0.5 $mg.l^{-1}$, BAP 5,10 or 15 $mg.l^{-1}$ and sucrose 4%, organogenic response was observed in 2,3,3, and 3 explants out of 8,10,10 and 10 explants in M27, 28,29 and 33 respectively. On extrapolation of these data to percentages, these values were 25%, 30%, 30% and 30%. These values are much less than the 50% response obtained in the medium M-34 with NAA 0.5 $mg.l^{-1}$, BAP 10 $mg.l^{-1}$ and sucrose 4%. This combination is similar to M-23 in which 48% response was obtained earlier. Increasing BAP concentration to 15mg/l in M-35 the frequency of morphogenetic response did not accentuate. On the contrary caulogenesis was associated with callusing. Similarly in media (M39-41) with higher concentration of both BAP (5-15 $mg.l^{-1}$) and NAA (1 $mg.l^{-1}$), induction of caulogenic buds was associated with profuse callusing. The buds induced in presence of higher concentrations of growth regulators dedifferentiated rapidly on transfer to the following medium used for differentiation and elongation of shoots.

Media with 2,4-D singly or in combination with BAP were also tested for morphogenic (organogenesis or embryogenesis) response (M42-49). Although 2,4-D is more often used for induction of somatic embryogenesis, but induction of caulogenesis in presence of 2,4-D is reported. Shoot formation in *Albizzia amara* (Roxb.) hypocotyl was achieved in presence of 2,4-D (Tomar and Gupta 1988b). In *Leucaena leucocephala*, shoot formation was obtained from the cotyledon and epicotyl explants when cultured in medium containing 2,4-D and kinetin along with 10% coconut water (Nataraja and Sudhadevi, 1984). Kumar *et al.* (1991) demonstrated differentiation of shoot buds in *Dalbergia sissoo* from callus in medium containing 2,4-D and BAP. In *Bauhinia*

purpurea, Kumar (1992) induced organogenic callus in medium with 2,4-D. Shoots were regenerated from this callus on culturing in medium with KN. However, in the present investigation 2,4-D singly or in combination with BAP failed to induce morphogenesis in the LSEC explants

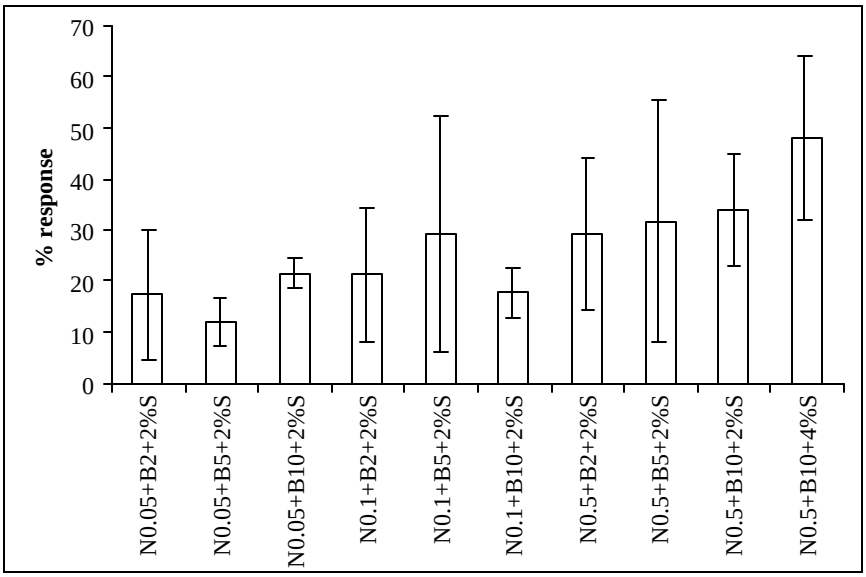
Media with cytokinins like KN and 2iP singly or in combinations with BAP were also tested. (M50 – M55) with LSEC. Medium combination of 0.2 $mg.l^{-1}$ KN with 0.5 $mg.l^{-1}$ BAP was effective in inducing proliferation of shoot buds in the seed ling or mature plant derived axillary meristems of tamarind (Mascarenhas *et al.*1981,1987).

It was observed that among all these combinations only 10 media (M14 – M23) consistently induced morphogenic response in LSEC, although the frequency of response varied greatly. The experiment was repeated using large number of replicates using these combinations. The data generated from these 10 media combinations was subjected to statistical analysis using ANOVA (Table 4.2). The data was expressed in the bar diagram (Fig. 4.4). It shows that the caulogenic response in medium number 23 with 4% sucrose is optimum when compared to the other 9 combinations (medium number 14-22), that had 2% sucrose.

Table 4.2 - ANOVA

Source of Variation	degrees of freedom	sum of squares	mean sum of squares	**F**	F-table
Treatments	9	3079.650	342.1834	3.232	~2.1
Error	45	4764.179	105.8706		
Total	54	7843.829			

Significant at 5%



N – NAA, B – BAP, S – Sucrose

Concentration of plant growth regulators are in $mg.l^{-1}$; error bar indicates standard deviation

Fig. 4.4

4.3.2 Histological studies

Histological studies were conducted after four weeks of culture, primarily to identify the morphology of the small green protuberances that appeared on the cut surface of the explants. Secondly to confirm the origin of the buds on the cut surface of the elongated axis. It is apparent from the histological studies (Fig.4.5A) that the protuberances were morphogenic buds and not somatic embryos. It also confirmed that these were adventitious buds, which originated directly from the subepidermal region that was exposed due to the split in the embryo axis (Fig.4.5B). Mitotic activity was localized on the cut surface of the explants from which the meristematic buds appeared directly without forming callus. Appearance of the buds was asynchronous. Therefore buds at various stages of development could be seen in the same section (Fig. 4.5C).

Direct *de novo* organogenesis in hypocotyl cultures of tamarind is reported by Sonia et al, (1998). Histological studies revealed that mitotic activity was localized in the epidermal and subepidermal cell layers of the explants. In our study the cut surface of the explant was devoid of the epidermal layer and the mitotic activity was noted in the cells directly exposed to the medium. This resulted in formation of a meristematic zone. The shoot buds developed directly from this meristematic zone in 3 weeks.

4.3.3 Shoot bud elongation

Exposure of LSEC explants in the initiation medium (M-23) for more than 4 weeks resulted in dedifferentiation of shoot buds. Transfering explants with shoot buds to the elongation medium containing 0.5 $mg.l^{-1}$ BAP, 0.2 $mg.l^{-1}$ KN, 0.1 $mg.l^{-1}$ calcium pantothenate, 0.1 $mg.l^{-1}$ biotin, 2% sucrose and 0.8% agar could partially circumvent this. This elongation medium was used in our earlier work, for differentiation and elongation of shoots from existing meristems, used for clonal propagation of tamarind (Mascarenhas *et al*., 1981; 1987). Murashige and Skoog's basal medium supplemented with GA_3 5×10^{-6} M was used by Jaiwal and Gulati (1991) to elongate the tamarind shoots induced in the cotyledonary nodal tissue of tamarind. The shoots were induced in presence of BAP 5×10^{-6} M. However shoot

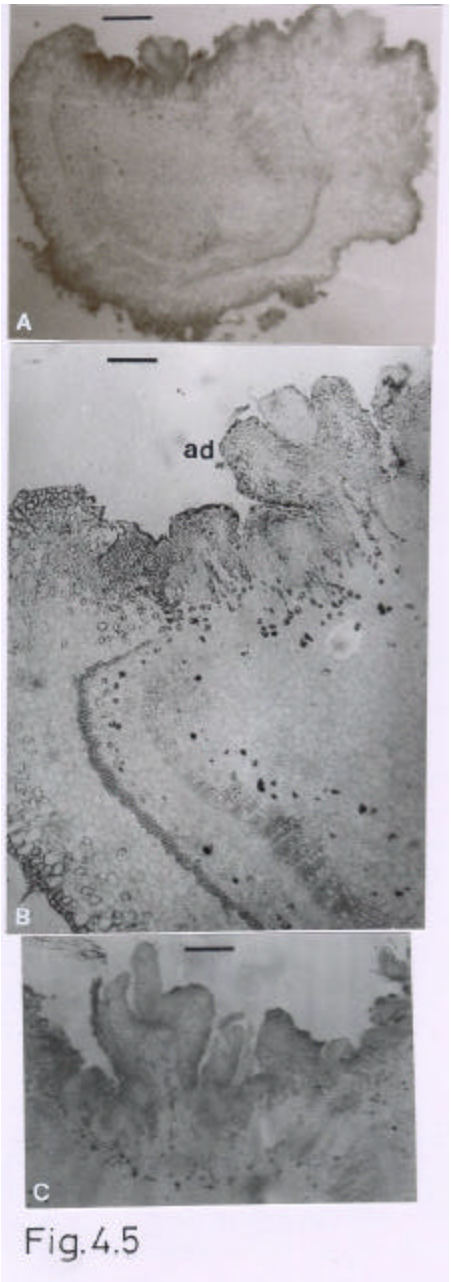


Fig.4.5

Fig. 4.5 A: Light micrograph showing transverse section of the elongated axis with caulogenic buds originating from cut surface of the explant (bar = 450µm)

B : Magnified light micrograph showing the formation of adventitious (ad) buds from subepidermal region of the explant (bar = 80µm)

C : Caulogenic buds at various developmental stages appearing in the same section confirm the asynchronous initiation (bar = 310µm)

buds induced in the hypocotyl explants in the similar medium, did not require a separate medium and elongated on extended incubation (Sonia *et al*, 1998). In the present investigation, it was observed that in 7.9 ± 8.4% cultures few shoot buds did elongate in the elongation medium containing KN, but the rate of shoot differentiation was slow and dedifferentiation was fast, thereby engulfing the smaller shoot buds into callusing. Substitution of KN with zeatin (Z) and elimination of agar in the elongation medium was partially effective in achieving faster elongation at a higher frequency of 15.8 ± 10.8 (Table 4.3). Due to the fast differentiation of the buds to shoots in this medium, dedifferentiation of the buds was reduced but not restricted. Therefore out of the numerous caulogenic buds that developed in the explant, only a few elongated. Shoot differentiation and elongation was asynchronous (Fig 4.6A) and 1-12 shoot elongated from each explant within 6 weeks (Fig.4.6B). The length of the shoots ranged from 1 to 5cm. The elongated shoots exerted domination and restricted differentiation of the other buds. Removal of the elongated shoots from the cluster and reculturing of the explants gave rise to more shoots. Shoot formation reduced to 3-4 per culture in the second passage of 4 weeks. The remaining shoot buds dedifferentiated into callus.

Table 4.3 : Comparative effect of kinetin and zeatin on elongation of shoots developed on longitudinal section of embryo axes with cotyledon

Medium (PGR conc. in $mg.l^{-1}$)	No. of explants cultured	No. of explants responded	Frequency of response (Mean± s.d.)
KN 0.2+BAP 0.5	31	2	7.9±8.4
Z 0.2+BAP 0.5	35	5	15.8±10.8

4.3.4 Rooting of elongated shoots

Clonal propagation of tamarind from the existing meristems of seedlings was reported from this laboratory (Mascarenhas *et al*.,1981, Mascarenhas *et al*.,1987). Use of auxin mixture containing IAA, IBA, IPA and NAA, each at 1 $mg.l^{1,}$ for a pulse treatment of 72 h in White's basal medium (Whites, 1962) for root induction in *in vitro* grown tamarind shoots has already been demonstrated. With minor

Fig. 4.6

Fig. 4.6 : (A) Asynchronous differentiation of the meristematic buds. Shoots at various stages of development seen in the same culture.

(B) Shoots elongating from the LSEC explants on transferring to liquid MS medium containing Z 0.2 $mg.l^{-1}$ and BAP 0.5 $mg.l^{-1}$.

modification same method was followed for rooting of the shoots developed by *de novo* organogenesis. Elongated shoots were isolated from the clusters and used for rooting. In all, 170 shoots were kept for rooting. Several of these shoots were lanky and the leaves were unopened. Root initiation was observed in 108 shoots (63.5%) in 10-15 days. The roots of these plants became healthier on extended incubation for 15-20 days in light (Fig.4.7A) or by culturing the shoots with root initials in medium containing charcoal 0.25%.

From the existing literatures on tissue culture of woody legumes it is observed that use of BAP singly or in combination with auxins or other cytokinins is most prevalent in the media formulations used for induction of shoots either from existing meristem or from other organs via *de novo* organogenesis. On the contrary auxins used in the rooting media for tamarind by other researchers or for other leguminous plants vary widely. The various auxins used for rooting in tissue culture raised shoots of tamarind and some of the other leguminous tree species is reviewed in **Section 3.3A.3.**

4.3.5 Transfer of the rooted plantlets to soil

The rooted plants were transferred to potting mixture in earthen pot. Although the plantlets had very healthy root system, but majority of the plantlets had unopened leaves. On transfer to pots the shoots of the plants with unopened leaves deteriorated rapidly and dried from the tips. On the contrary, plants with fully or partially opened leaves stabilized in soil within 30 days (Fig.4.7B). Twenty-four plants survived on transfer to pots and stabilized in soil in the green house.

4.4 CONCLUSIONS

At the time of initiation of this work, there were no reports on *de novo* organogenesis in tamarind and very few reports in other leguminous tree species. In the course of our work, the first report on tamarind *de novo* organogenesis appeared (Sonia *et al*., 1998) showing organogenesis from the proximal end of the hypocotyl explants. In contrast to that, in the present investigation, we achieved *de novo* organogenesis via direct adventitious regeneration from the non-meristematic, cut

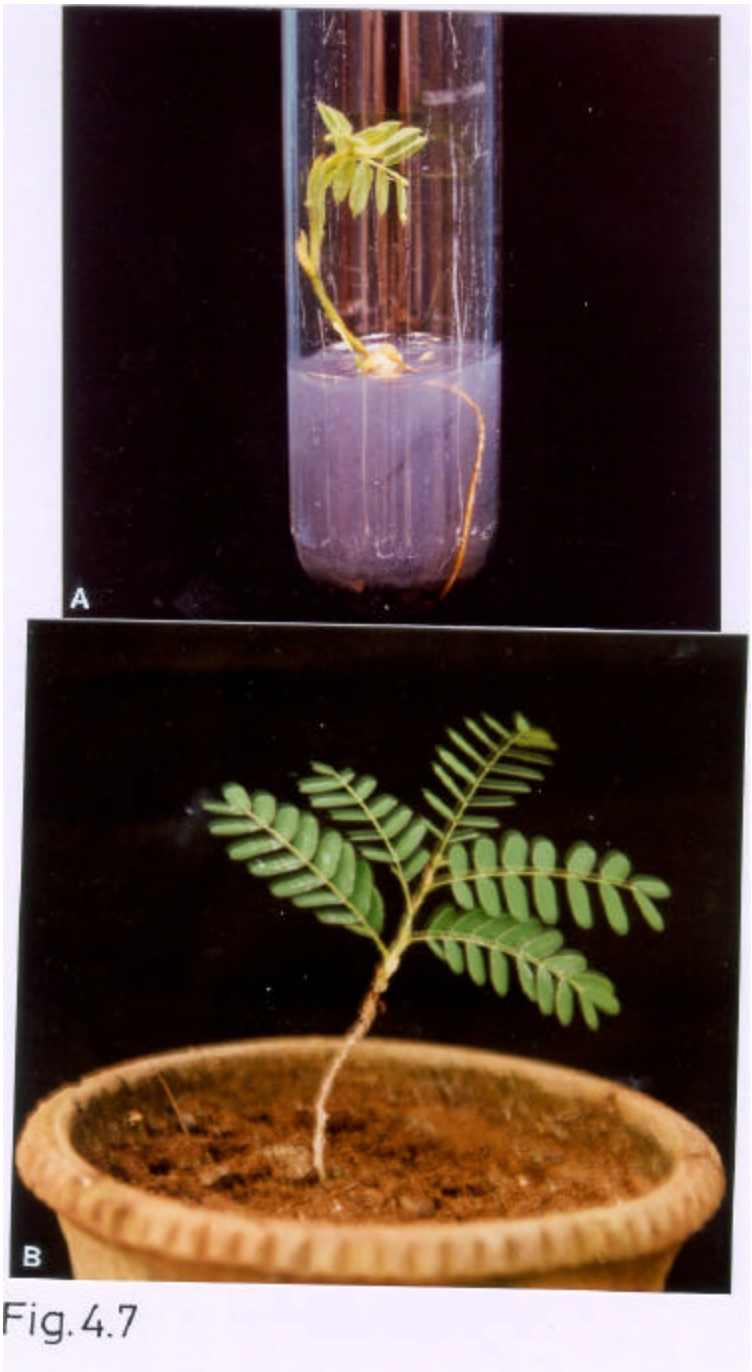


Fig. 4.7

Fig. 4.7 A : Elongation of root in half strength MS medium supplemented with 0.25% charcoal after pulse treatment of auxin mixture for 72 h.

B : Plant in soil

surface of mature embryo axis explant of tamarind. The attachment of the cotyledon and the orientation of the explant were important for morphogenesis

The study included in this piece of work demonstrated induction of de novo caulogenesis from the cut surface of of 48% - 50% LSEC explants on MS medium supplemented with NAA 0.5 $mg.l^{-1}$ and BAP 10 $mg.l^{-1}$ and 4% sucrose. The shoots could be elongated and rooted to form plantlets. Histological studies confirmed the sub-epidermal origin of the caulogenic buds from the explants, which later on turned into shoot buds. The rooted shoots survived in soil.

This type of a regeneration protocol for bud formation associated with the wounding site is often used for Agrobacterium-mediated transformation to generate genetically modified plants with desired characteristics.

Part of this work has been reported in the following publication :

Regeneration of plants via adventitious bud formation from mature zygotic embryo axis of tamarind (*Tamarindus indica* L.)

- Urmil J. Mehta, K.V. Krishnamurthy and Sulekha Hazra

Curr. Sci., **78** : 1231-1234, 2000

CHAPTER 5: INDUCTION OF SOMATIC EMBRYOGENESIS

5.1 INTRODUCTION

The ability to induce somatic embryogenesis (SE) in plants appears to be a universal trait, the occurrence of which depends on the interaction of an appropriate tissue with an appropriate induction stimulus. Induction of embryogenesis in plants, one of the significant achievements of experimental embryology, has become an experimental tool of biotechnology. This phenomenon was first described by Steward *et al.* (1958) in carrot four decades ago. Thereafter, it always remained a topic of interest for the researchers. Thus remarkable progress has been made in the area of research and application of embryogenesis.

Somatic embryogenesis has been defined by Haccius (1978) as a non-sexual developmental process, which produces a bipolar embryo from somatic tissues. They are believed to originate from single cells, whereas organs regenerate through collective organization of many cells. Therefore plants derived from somatic embryos tend to be genetically alike, while those regenerated through organogenesis may result in a genetic mosaic. Somatic embryos have always been assumed to have a single cell origin, although embryogenesis has never been demonstrated conclusively from a single isolated cell. Williams and Maheswaran (1986) suggested that somatic embryos could arise either from single cells or from groups of cells. Proof of a single cell origin would be the presence of a suspensor with the somatic embryo; whereas a multicellular origin could be indicated by the fusion of the somatic embryo base with a broad area of parental tissue. Several aspects of SE starting from historical to molecular aspects have been discussed in recent times (Thorpe, 1995; Jain *et al.*, 1995; Bajaj, 1995). The successful induction and subsequent recovery of viable plants although not a routine, has been possible in many plants and has been reported in several herbaceous dicots, monocots and woody trees including gymnosperms. Sufficient information has accumulated during the last three decades, on the induction of an embryogenic state followed by histodifferentiation, maturation and germination of somatic embryos (Merkle *et al.*, 1995). The potential of embryogenesis derive from number of factors that involve high regenerative capacity, the ability to regenerate from single cells in both gametophyte and sporophyte tissues, the bipolarity of embryos and compactness and broad metabolic potential of embryogenic tissue (Janick, 1993).

Applications of SE may be summarized as follows: -

1. **Clonal propagation** : Somatic embryos offer potential advantages over conventional micropropagation system including
   - high proliferation rate: As many as 1.35 million embryos per litre suspension culture (Janick, 1993)
   - singulation: Each embryo being separate package can be handled without the physical separation required from organogenesis or axillary branching systems
   - bipolarity: Well-developed embryo contains root and shoot meristems. Thus conversion to seedling can be obtained in single step
   - promise of clonal propagation : via synthetic seed technology.

2. Crop improvement:

Somatic embryogenesis offers a number of options for crop improvement.

- Somatic embryogenesis can be utilized as a regeneration technique for cell **selection of spontaneous or induced mutations**. Selection for salt tolerance and disease resistance has proven efficient in embryogenically competent callus tissues of citrus (Litz *et al.*,1985). Hammerschlag (1990) and Hammerschlag and Ognjanov (1990) reported *in vitro* selection of embryogenic cultures of peach (*Prunus persica*) for resistance to the toxins produced by *Xanthomonas campestris* pv *pruni*, the causative agent of bacterial leaf spot, and *Pseudomonas syringae* pv *syringae*, the cause of bacterial canker.

- Chimerism is one of the major hurdles to be overcome in the course of the development of a particular woody plant mutant. Somatic embryos obtained from single irradiated cells help **avoiding chimeras** during selection stages. After the mutagenic treatment, well developed somatic embryos can be germinated into whole plants. It ensures that shoot as well as root system is of the mutated type. Once mutant embryogenic cell lines are obtained, mass production of plants can be envisaged all year round. This steady supply can be of economical importance for newly released cultivars.

- Plants regenerated from somatic embryos display a high genetic uniformity. Thus large populations can be obtained from highly heterozygous and outbreeding

species. This uniform material is an ideal population for mutagenesis treatment. Especially, if certain experiments have to be repeated, the constant supply of **even-aged embryos** or plants is advantageous (Heinze and Schmidt, 1995).

- Immature embryos of interspecific plants from incompatible crosses (Merkle *et al*., 1990) may be rescued by culturing them for secondary somatic embryogenesis and simultaneously the plant is multiplied. This has been exploited as means of obtaining plants from intergeneric hybrid embryos (McGrahanhan *et al.*, 1986; Ozias-Akins *et al*., 1992). It is a widely used technique to **rescue embryos** that normally abort in wide crosses and is an established technique to obtain viable plants from **incompatible interspecific crosses**.

- Breeding at the **haploid** ploidy level offers advantages, as a drastic reduction of time needed to produce **homozygous plants and expression of recessive genes**. Haploid plants can be obtained *in vivo* through gynogenesis, androgenesis, genome elimination, semigamie and by chemical and physical treatments such as heat shock, X-rays and UV light (Pierik, 1987). The frequency of these events is however, low.

  Theoretically, all steps necessary in a haploid breeding scheme can be conducted by using somatic embryogenesis (Raemakers *et al*., 1995). In numerous species large number of haploid embryos can be obtained by culture of microspores or anthers (von Aderkas and Dawkins, 1993; Aslam et al., 1990; Loh and Ingram, 1983). The haploid embryogenic lines can be multiplied and maintained by secondary somatic embryogenesis. Embryos of the selected lines will be used to double the ploidy level. In *Brassica napus* (Loh and Ingram, 1983), *B. campestris* (Aslam *et al*, 1990) and citrus (Hikado *et al*., 1982), this was accomplished by culturing somatic embryos for secondary embryogenesis in the presence of colchicine. In *Asparagus officinalis* (Feng and Wolyn, 1991) and *Camellia japonica* (Kato, 1989) the ploidy level of embryos doubled spontaneously in a low frequency. Selection of doubled somatic embryos in a state that they are still embryogenic, allows the rapid multiplication by secondary somatic embryos.

- Because of the absence of vascular connections between the nucellus and other maternal tissues, polyembryogenic species are generally free of infections that might have affected the parent plant. Similarly, plants derived via embryogenesis from nucellus or nucellus callus would also be **free of pathogens** including viruses (Janick, 1993).

3. **Production of metabolites** : Seeds are storehouses of many important products such as starch, fats, oils, proteins. Therefore cell/organ cultures have been suggested as a means to synthesize desirable metabolites (Al-Abta *et al*., 1979). Lipid synthesis has been followed in embryo cultures in number of species including cocoa, jojoba, borage, rape seed, *Brassica napus* (Nehlin *et al*., 1996), carrot (Janick, 1991; Weber *et al.*, 1992) and in peanut (Mhaske and Hazra, 1994).

4. **Germplasm preservation** : In some species like cocoa, coconut, mango and rubber seeds, the traditional organ used for preservation of germplasm are desiccation sensitive and thus cannot be stored by traditional techniques. This can be overcome by cryopreservation of mature or immature zygotic or somatic embryos (Janick, 1993). Such cyrogenic storage systems are under development in several forest species and most studies show this to be a feasible method of storing embryogenic tissues (Kartha *et al*., 1988; Dumet *et al*., 1993; Laine *et al*., 1992; Klimaszewska *et al*., 1992).

5. **Transformation:** Somatic embryogenesis is the most often used tissue culture system in woody plants for genetic transformation (Handley *et al*., 1995). In woody plants Agrobacterium mediated transformation has been used to regenerate transformants of mango (Mathews *et al*., 1992), *Carica papaya* (Fitch *et al*., 1993), Juglans (McGranahan *et al*., 1990), *Picea mariana* (Mill.) BSP, *Pinus strobus* L and *Populus spp*. (Tian *et al*., 1999). Direct DNA-mediated transfer methods such as biolistics have been used on embryogenic cultures of *Picea glauca* (Ellis *et al*., 1993), *Liriodendron tulipifera* (Wilde *et al*., 1992) and *Carica papaya* (Fitch *et al*., 1990), *Picea mariana* (Mill.) BSP, *Pinus strobus* L and *Populus spp*. (Tian *et al*., 1999). Each of these transformation methods has been used in SE tissue culture system, a

recent report being in the walnut somatic embryogenesis-based transformation system (Escobar *et al*., 2000). The advantages in using such system are, more number of regenerants can be obtained originating from few or single cells which increases the likelihood of achieving transformed cells. Secondly chimeric transformants cycled in repetitive systems can lead to obtaining wholly transformed individuals (Baker and Wetzstein, 1992).

6. **Basic studies** : Somatic embryogenesis also provides an important tool for the analysis of morphological, molecular and biochemical events that occur during induction and maturation.

The process of embryo initiation and development is called **embryogenesis**. It is distinct from organogenesis in that the product formed is autonomous and is not connected with any other structure *via* vessels. In practice all the processes in which shoot and root poles develop more or less synchronously are included in this term (Thorpe, 1983). Several types may further be distinguished based on the origin of the material (**table 5.1**)

Table 5.1 : Classification of embryos (Bhojwani and Razdan, 1983).

Type	Origin
Zygotic embryos	Formed by fertilized egg or the zygote.
Somatic embryos	Formed by the sporophytic cells, except the zygote, either *in vitro* or *in vivo*
Adventitious embryos	Somatic embryos arising directly from other embryos or organs.
Parthenogenetic embryos	Formed by the unfertilized egg.
Androgenetic embryos	Formed by the male gametophyte (microspores, pollen grains).

The first report of SE appeared when Reinert (1958) demonstrated the phenomenon using carrot callus. Embryogenesis that occurs directly from cells of the explant without a callus phase is identified as **direct embryogenesis**. **Indirect embryogenesis** involves callus proliferation from explants prior to differentiation of embryos. It would be convenient to distinguish a third type viz. **repetitive embryogenesis**, where embryos arise directly from primary somatic embryos (Thorpe, 1983). Somatic embryos formed either directly on explants or indirectly through a callus phase often show a tendency to undergo secondary

embryogenesis to give rise to new embryos. This phenomenon has been referred in literature as **repetitive** or **accessory embryogenesis** (Ammirato, 1987), **recurrent embryogenesis** (Lupotto, 1983) and **proliferative embryogenesis** (Stamp and Meridith, 1988).

Once embryogenic determination has occurred the commitment of a group of cells to integrated development as an embryo depends on the maintenance of coordinated embryogenic behavior and the suppression of any tendency of individual cells to act independently (Williams and Maheswaran, 1986). Secondary embryogenesis then can be explained as an expression of cells, which have broken away from the integrated control. Secondary embryogenesis is considered as one of the major causes for absence of synchrony in populations of somatic embryos (Ammirato, 1987). In manipulating embryogenesis, two media components in particular play crucial roles. These are auxin and nitrogen (Kohlenbach, 1978). Halperin and Witherell (1964) first recognized the importance of auxin. Further studies showed that the process of SE normally takes place in two stages. First the induction of cells with **embryogenic competence** (referred to as embryogenic masses or clumps, proembryos, proembryogenic tissue) in the presence of high concentrations of auxin. Second, development of the embryogenic masses into embryos in absence or in presence of a lowered concentration of auxin. 2,4-Dichlorophenoxyacetic acid is the most commonly used auxin. Reduced nitrogen in the form of NH_4 is also required (Kohlenbach, 1978; Wetherell, 1979). There are a few recent reports on induction of somatic embryogenesis in presence of cytokinins like Thidiazuron (Qureshi and Saxena 1992, Saxena *et.al*.,1992) and BAP (Malik and Saxena 1992).

A substantial amount of information has become available in the last four decades since SE was first recognized. Nevertheless, the successful induction of somatic embryos and subsequent recovery of viable plants is not routine or efficient for the majority of species. Currently the trend is towards the recognition that embryo development is comprised of variety of different stages involving specific patterns of gene expression (Goldberg *et al.*, 1989; Hughes and Galau, 1989; Kermode, 1990). The standard use of simple two step media sequences to promote the induction and development stages of embryogenesis originally identified by Kohlenbach (1978) is proving inadequate to accommodate the multiple and distinct phases that undergo in the course of their ontogeny and subsequent development. Therefore, the more closely the pattern of somatic embryo gene expression matches that of zygotic embryos, the greater chance of obtaining highly efficient regeneration systems. Such

normalization of gene expression patterns will be achieved through the optimization of media and culture protocols for each individual stage of embryo development.

All somatic cells within a plant contain the entire set of genetic information necessary to create a complete and functional plant. Temporal and spatial expression of genes is tightly regulated to permit the differentiation of various organ systems as the plant develops (Goldberg, 1986). The induction of SE must then consist of the termination of the existing gene expression pattern in the explant tissue, and its replacement with an embryonic gene expression program in those cells of the explant tissue which will give rise to somatic embryos. This concept was first embodied by Sharp *et al.* (1982) and Evans *et al.* (1981), who used the term "IEDC", for "induced embryogenic determined cell" to describe an embryonic cell that had originated from a nonembryonic cell. Cells from plant embryos, which already express an embryonic gene expression program, were termed "PEDC", for "preembryogenic determined cells" (Sharp *et al.*, 1982). For the purpose of regeneration, once obtained, both IEDCs and PEDCs are functionally equivalent, and both may be referred as "EDCs" for "embryogenic determined cells" (William and Maheswaran, 1986) or simply, "ECs" for "embryogenic cells" (Carman, 1990; Merkle *et al*., 1990). The later term is preferred, because the formation of SE is not inevitable fate for an EC, indicating an existing plasticity in the cells not adequately conveyed by the term "determined" (Carman, 1990).

Treatments to obtain SE thus depend on whether the explant tissue consists of PEDCs or non ECs. In the first case, a stimulus for cell division may be sufficient for the formation of a somatic embryo on a tissue explant, in a process referred to as direct embryogenesis, as the somatic embryos appear to rise directly from the explant tissue. In contrast, non EC tissue must undergo several mitotic divisions in the presence of an auxin during the induction of the EC state. Cells resulting from these mitotic divisions are manifested as a callus, and the term indirect regeneration is used to indicate that a callus phase intervenes between the original explant and the appearance of somatic embryos.

Any cells, which can develop into somatic embryos, are said to possess embryogenic competence. Whether these cells are target cells which respond to special signals or whether most cells have this capability is not yet known. The selection of specific developmental stages of explant material, conditioning media, sequential transfers and appropriate environmental conditions are generally necessary for successful embryogenesis (Table 5.2). The primary events required for cells to enter the developmental program of embryogenesis

are unknown, but the techniques are available to begin to address this question (Tulecke, 1987).

Table 5.2 : General protocol for SE and plant propagation (Tulecke, 1987)

Stages of the process	Factors involved
1. Selection of material	Genetic base, tissues used, stages of development
2. Conditions	Light, dark, temperature
3. Media	Conditioning, inductive, sequence, frequency of transfer
4. Somatic embryogenesis	Direct, adventive, repetitive Indirect, suspension of cells, protoplasts, screening
5. Somatic embryos	Maturation, selection, dormancy, cold treatment, abnormalities, growth requirements
6. Germination	Primary root, cotyledon expansion and greening, epicotyl growth, leaves
7. Propagation in soil	Transfer to non-sterile environment, dilute nutrient solution, day length, ambient humidity, fungicides
8. Acclimatization	Gradual leaf development in ambient temperature and humidity, hardiness to atmospheric fluctuations
9. Evaluation	Test for clonal or variant characters, selection and screening of plants

The general concept about the key molecular events during induction of embryo development from somatic plant cells is described in a recent review (Dudits *et al.*, 1995). Unlike other organisms, in plants, the cellular totipotency extends not only to the cells in the zygotic embryos but also to the somatic cells. Flexibility of the differentiation program makes it possible to generate the embryogenic cell stage in fully differentiated cell under defined conditions. The reset of the whole ontogenic program by initiation of SE requires an essential reprogramming of the gene expression pattern. After abolishment of the previous differentiated cell functions, totipotent somatic cells with metabolic states similar to that of the fertilized egg cells are formed during the

hormone induced cell divisions. A large number of empirical observations on various embryogenic tissue culture systems and the results of biochemical, physical and structural studies support a general concept, which emphasizes the central role of hormone or stress-induced activation of signal transduction systems. Consequently the internally transmitted signal trigger a series of cell divisions that leads to the formation of either dedifferentiated callus tissues or somatic embryos.

The induction of SE in woody perennials is restricted due to the characters that make them more intractable for studies. These are :
(1) **Short seasonal period** for the availability of any particular tissue or a developmental stage for culture, (2) **long period required for regeneration**, (3) frequent production of **phenolic compounds**, etc. (Tulecke, 1987). Inspite of these limitations SE has been achieved in a number of angiosperms, both dicotyledons and monocotyledons, including legumes (Parrott *et al.*, 1995). Still most woody plants, appear to be recalcitrant and routine regeneration of plants be it either organogenesis or SE is rare. Problems and potentials of tissue culture in forest tree improvement have been discussed extensively (Haissig *et al*., 1987). Although axillary shoot culture is the most promising short-term (2-5 years) possibility for commercial propagation, it is presumed that in the long-term (10-25 years), SE is likely to replace shoot culture.

Tree legumes have been considered highly recalcitrant for tissue culture. While reports on organogenesis have been more frequent, reports on SE have been less (Lakshmi Sita, 1999). Gharyal and Maheshwari (1981) reported SE in *Albizzia lebbeck*. They could recover plantlets from hypocotyl explants via direct embryo formation. Tomar and Gupta (1988a) obtained somatic embryos via callus from hypocotyl of *Albizzia richardiana*, which could be later converted to plantlets. Trigiano *et al.* (1988) could achieve only germination but no conversion of the somatic embryos in *Cercis canadensis* originated directly from the immature zygotic embryos. Later, Geneve and Kester (1990) obtained whole plantlet from the embryos of similar origin. In *Cladrastis lutea* (Weaver and Trigiano, 1991) somatic embryo originated directly from the immature cotyledons. But their conversion to plantlet was very low. In *Acacia koa* (Skolmen, 1986) somatic embryoids formed from seedling hypocotyl and seedling tips-derived cell suspension cultures. Conversion of these somatic embryos could not be obtained. Plantlets could be regenerated via indirect embryogenesis from immature cotyledon-derived callus of

Acacia catechu (Rout *et al*., 1995), immature embryo-derived callus of *Acacia mangium* (Xie and Hong, 2001), immature endosperm-derived nodular callus of *Acacia nilotica* (Garg *et al.*, 1996). Conversion of somatic embryo could not be achieved in *Hardwickia binata* (Das *et al*., 1995) and *Dalbergia sissoo* (Das *et al.*, 1997), respectively. However in *Dalbergia latifolia*, Rao and Lakshmi Sita (1996) could induce direct somatic embryos in the immature zygotic embryos and converted these embryos to plantlets. Arrillaga *et al.* (1994) showed that immature seeds of *Robinia pseudoacacia*, on callus regeneration produced somatic embryos, which converted to plantlets; while Merkle and Wiecko (1989) demonstrated plantlet conversion of somatic embryo obtained at a low frequency from immature seeds in the same species.

Although SE in woody species is not limited to zygotic embryo explants, the developing zygote generally shows a high potential for SE (Williams and Maheswaran, 1986). From the above literature in tree legumes, it appears that immature zygotic embryo, has the optimum potential for induction of SE.

Keeping this in view, attempts were made to standardize a protocol for somatic embryogenesis in tamarind using immature zygotic embryos (IZE). This explant has a major disadvantage, as the IZEs are available only for a limited period in the year. In this part of India, tamarind trees start flowering from the month of May and the period lasts till July. Though the flowering is profuse in tamarind, the fruit set under natural condition is very low ranging from three to five percent (Karale *et al*., 1999). The fruits become visible from the month of July. A very high degree of variations were noted in the fruit length, breadth and number of seeds. Pods ranging from 1-12 cm in length and collected during August till December were used in the experiments conducted in the years 1997 and 1998. These experiments were preliminary in nature to identify the right season for collection of IZE, appropriate size of the IZE, effective growth regulators, approximate concentration of sucrose, method of dissection, method of surface sterilization of hairy pods, incubation period etc. The data incorporated in this chapter were raised from the experiments conducted in the seasons of 1999 and 2000 with the IZE isolated from the pods of particular length, collected during the identified period of the season.

There are a few reports on *in vitro* regeneration of plants via organogenesis using meristematic tissues of tamarind. However, there is no report on SE.

5.2 EXPERIMENTAL PROTOCOL

5.2.1 Preparation of explant

Young pods of Tamarind, ranging from 2-12 cm in length, 1-1.5cm breadth and 0.8-1.5cm thickness having 1-8 compartmentalized ovules were harvested from the trees growing locally (Fig. 5.1A). The young pods (Fig.5.1B) of tamarind are hairy. The pods were scrubbed under running tap water to eliminate the dust particles trapped in the hairs. The pods were washed with detergent for 10 min. After washing thoroughly with distilled water, these were treated for half an hour with 0.1% solution of a fungicide, Bavistin®. This was followed by treatment with 4% (v/v) Savlon for 10 min. The pods were washed with distilled water and transferred to sterile bottles. From this step onwards the process was carried out under aseptic conditions. The pods were treated with 0.1% $HgCl_2$ for 15 min. and washed 4-5 times with sterile distilled water, to remove adhering $HgCl_2$. These young fruits were dissected longitudinally (Fig. 5.2A,B) and immature seeds were isolated. The white and tender seed coat was removed from the seeds to obtain the immature zygotic embryo (Fig.5.3) explant (IZE) from the liquid/gelatinous endosperm.

5.2.2 Initiation of culture

Murashige and Skoog's (1962) basal medium supplemented with 6% sucrose was used and different factors were tested. The media were supplemented with 6% sucrose and gelled using 0.22% Phytagel™ unless otherwise mentioned. Cultures were incubated for 60 days.

5.2.2.1 Growth regulators

Varying concentrations (0.5, 1, 3, 5 and 10 $mg.l^{-1}$) of different growth regulators including 2,4-dichlorophenoxy acetic acid (2,4-D), α-naphthalene acetic acid (NAA), 2,4,5-trichlorophenoxy acetic acid (2,4,5-T), di-chloro-o-anisic acid (dicamba) and 4-amino-3,5,6-trichloro picolinic acid (Picloram) were supplemented in MS basal medium.

Fig. 5.1

Fig. 5.1 (A) Tamarind tree laden with fruits / pods
(B) Immature pods of tamarind

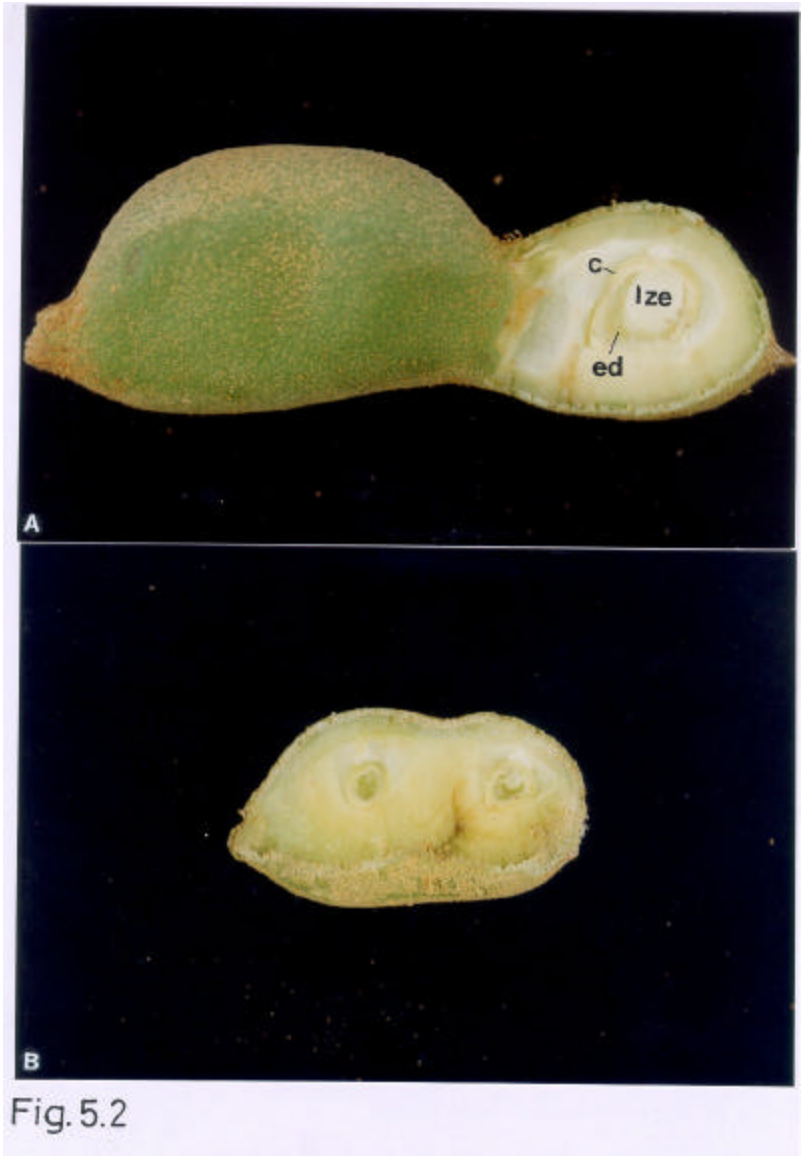


Fig. 5.2

Fig. 5.2 : (A) Tamarind pod partially dissected to expose ovule. Immature zygotic embryo (IZE) surrounded by gelatinous endosperm (ed) and seed coat (c).
(B) Dissected tamarind pod showing two different sizes of immature zygotic embryos (IZE) in the same pod.

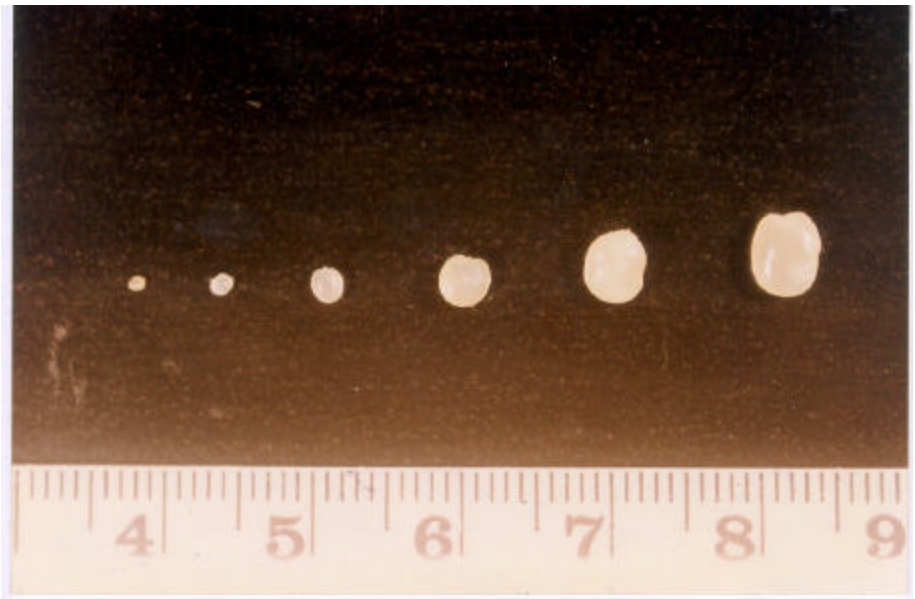


Fig. 5.3

Fig. 5.3 Various sizes (1-6mm) of immature zygotic embryos (IZE) used as explant for induction of somatic embryogenesis

All the growth regulators were procured from Sigma Chemicals, U.S.A. The medium was distributed in tubes (25×150mm). Each tube contained 20ml medium. Four IZE were cultured per tube in these media. Experiment was repeated 4 times using 20 explants in each. Experiments with varying concentrations of dicamba were repeated further.

Immature Zygotic embryos were cultured in MS basal medium supplemented with varying concentrations (0.5, 1, 3, 5 and 10 mg.l^{-1}) of Thidiazuron (TDZ). The medium was distributed in tubes. For IZEs were cultured in each tube.

5.2.2.2 Identification of explant of optimum size

Immature zygotic embryos were dissected from the pods. The sizes of the embryos were determined with reference to a scale placed close to the site of dissection in the laminar airflow. Immature zygotic embryos ranging from 1-10mm (Fig. 5.3) were isolated from the sterilized pods and cultured on MS basal medium containing 5 mg.l^{-1} dicamba. Embryos smaller than 1mm or larger than 10mm were discarded.

5.2.2.3 Carbohydrates

Sucrose at concentrations ranging from 2-8% were tested to determine the optimum concentration for embryogenic response. Two carbohydrates including glucose and fructose at different concentrations were substituted for sucrose and tested for induction of optimum embryogenic response in the IZEs. Explants were cultured in MS basal medium supplemented with dicamba 5mg/l and 2%, 4%, 6%, 8% of each of these carbohydrates. Cultures were divided into two groups. One group was incubated in dark whereas the other group was incubated in light. Sucrose at 6% was used as control.

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5.2.2.4. Influence of culture conditions (gelling agents, light, dark, culture vessel)

In the first experiment addressed to optimize the culture conditions, the influence of light regime was tested. Immature zygotic embryos were cultured in tubes containing 5 mg.l^{-1} dicamba. These were incubated in two light regimes, 16 h and 24 h light conditions

Second experiment was conducted to study and compare the effects of phytagel and agar, light and dark and test tubes and petridishes. Immature embryos ranging in size from 1-6mm were cultured in MS medium supplemented with dicamba 5mg.l^{-1} and sucrose 6%. Media were gelled using either agar (Qualigens Fine Chemicals, India) or Phytagel™. Media with agar or phytagel were distributed in Borosil make glass tubes (25×150mm) or in disposable plastic petridishes (55mm×15mm). In tubes, 20ml media were distributed whereas in 55mm petridishes approximately 10ml medium was poured. Four immature zygotic embryos were cultured in each tube and six IZEs were cultured in each petridish. The cultures were divided into two groups. One group was incubated in dark whereas the other group was incubated in 16h light at 25±2°C for 60 days.

5.2.3 Explants from different trees

To determine the reproducibility of the protocol, immature pods were collected from five different trees and IZEs isolated from these, were cultured in MS basal medium supplemented with dicamba 5 mg.l^{-1} and 6%sucrose. Phytagel 0.22 % was used for gelling. Twenty millilitre medium was distributed in tubes. Embryos ranging in size 1-6mm in size were cultured. Four IZEs were cultured per tube. Cultures were incubated in 16h light at 25±2°C. The trees were identified as I, II, III, IV, V.

5.2.4 Secondary/Repetitive SE

Culture showing somatic embryos, were shifted to fresh medium along with the original explant for further proliferation in MS basal medium supplemented with dicamba 5 mg.l^{-1}, 6%sucrose and 0.22 % phytagel. Subsequently these were subcultured in fresh medium of same combination at an interval of 60 days.

5.2.5 Histological studies

The IZE of size 3 and IZE explant showing embryogenic response were subjected to histological studies. Tissues were fixed in FAA for 48 h at room temperature. These were dehydrated (Section 2.2.5) and embedded in paraffin. Serial sections, were cut and were double stained with haematoxylin – eosin. The stained sections, mounted in DPX, were studied under microscope.

5.2.6 Maturation / Germination / Conversion of somatic embryos

Somatic embryos were subjected to various culture conditions to achieve maturation, germination and conversion. These were transferred on MS basal medium, with or without charcoal, with 10 % sucrose, and with lower concentration of dicamba. Media supplemented with different cytokinins including KN, BAP, zeatin, TDZ alone or in combinations were also tested. Supplementation of ABA was also tried.

The recalcitrance of tree legumes is often mentioned in the reviews and reports. Although a large number of trees have been regenerated via somatic embryogenesis, reports on somatic embryogenesis in woody legumes is rather limited (Lakshmi Sita, 1999). The limited success in regeneration of leguminous tree species via somatic embryogenesis have been attributed to various factors which limit the studies on this direction. While working on somatic embryogenesis in tamarind we encountered several of these factors. After some futile efforts for a brief period to induce SE in seedling explants like mature embryo axis, LSEC, cotyledons etc. attention was diverted towards the use of immature zygotic embryos for induction of embryogenesis. In the local agroclimatic conditions green, tender fruits of tamarind are visible from July. The pods remain green till December although the cover of the pod becomes hard. Extensive preliminary studies were conducted in the fruiting seasons of 1997 and 1998. Intact IZEs with and without seed coat were isolated from the pods of all sizes during the months from July to December. Auxins and sucrose at various concentrations were tested. It was noted that the embryos of 1-6mm sizes respond in more than one auxin. Embryogenic response appears in 3 to 8 weeks. The data of these experiments are not included in this thesis. The information generated from these experiments were helpful in planning and designing the experiments carried out in the two subsequent fruiting seasons of 1999 and 2000.

5.3 RESULTS AND DISCUSSION

5.3.1 Explant source

Initiating the developmental program for SE from a cell or a group of cells frequently depends on the nature of the explant source. Thus refers to the conditions under which the source plant was grown and the stage of development of the plant part

from which the explant was taken. Juvenile tissues of certain types appear to be more suitable for induction of SE (Tulecke, 1987). The developing zygotic embryo explant generally shows a high potential for SE (Williams and Maheswaran, 1986). In tree legumes, baring a few reports (Gharyal and Maheshwari, 1981; Tomar and Gupta, 1988a; Garg *et al.*, 1996), most reports of SE are from zygotic embryo.

The immature zygotic embryos even when dissected from a single pod were not always of the same size and ranged between four to five sizes. So the macroscopically visible size of ~1mm was the least size selected while the maximum size of ~10mm was taken for the preliminary experiments only. IZEs ranging from 1-6mm responded in cultures. Therefore embryos of these sizes were used as initial explant in the present studies. Explants of the required size were obtained from the pods collected in the first week of September to the first week of October. The IZE of 1-6mm were usually found in the ovules of 8-10mm length.

The IZE consisting of embryo axis and tender cotyledons were isolated after removal of the white and tender seed coat and liquid gelatinous endosperm. These explants responded in culture. The IZEs with the seed coat did not respond in culture and turned brown and necrotic. The embryo axis and cotyledons when isolated and cultured separately were not responsive.

5.3.2 Initiation of culture

5.3.2.1 Effects of growth regulators

Inspite of the vast literature on somatic embryogenesis, the nutritional requirements for SE are not well understood. They are neither specific nor exclusive, since various recipes, produce results.

Induction of SE with many woody species has resulted predominantly from the use of 2,4-D. Trigiano *et al.*(1988) used 2 or 3 $mg.l^{-1}$ 2,4-D with varying ammonium ion concentrations in Schenk and Hildebrandt basal medium, while Geneve and Kester (1990) used 5µM 2,4-D in modified WPM medium to achieve SE in *Cercis canadensis*. Weaver and Trigiano (1991) used 9µM 2,4-D for 8 weeks followed by 25µM of NAA for 3 weeks to obtain SE induction effect in *Cladrastis lutea*. During the

SE induction from immature zygotic embryos of other legumes like peanut, 3 $mg.l^{-1}$ of 2,4-D was employed (Hazra *et al*., 1989), whereas Sagare *et al*. (1993) used 3 $mg.l^{-1}$ of 2,4,5-T in chickpea. In non-leguminous tree species, *Eucalyptus citriodora*, 3 $mg.l^{-1}$ NAA has been used for induction of direct SE from cotyledons while 5 $mg.l^{-1}$ NAA supplemented with 500 $mg.l^{-1}$ casein hydrolyzate and 500 $mg.l^{-1}$ glutamine was used for the maintenance of embryogenic cultures (Muralidharan *et al*., 1989). Another herbicide with auxin-like properties, picloram, has proven to be very effective for maintenance of embryogenic calli and plant regeneration for long periods (upto 4 years) in cultures of diploperennial Teosinte (*Zea diploperinnis* Iltis, Doebley and Guzman) (Pedrosa and Vasil, 1996).

Though embryogenesis was mostly induced by the use of auxins, cytokinins have also been employed to obtain the required effect. The use of cytokinin alone to obtain somatic embryo formation from IZEs is well established in a wide range of species including both gymnosperms (Norgaard and Krogstrup, 1991) and angiosperms (Maheswaran and Williams, 1984). BAP alone, was used by Tomar and Gupta (1988a) to achieve SE in callus cultures of *Albizzia richardiana*. Somatic embryogenesis occurred on medium containing TDZ singly, in geranium (Qureshi and saxena, 1992) and in peanut (Saxena *et.al*., 1992). A recent report describes use of TDZ along with IAA, for SE in *Acacia mangium*, a tree legume (Xie and Hong, 2001).

A medium with combination of KN and NAA was found to be beneficial in inducing indirect SE in *Acacia catechu* and *Hardwickia binata* (Raut *et al.*, 1995; Das *et al.*, 1995). KN and 2,4-D supplemented medium supported SE in callus-derived cultures of *Dalbergia sissoo* (Das *et al.*, 1997). Merkle and Weiko (1989) and Arrillaga *et al*. (1994) used 2,4-D and BAP in combination to induce indirect SE in *Robinia pseudoacacia* L. while Garg *et al*. (1996) achieved indirect SE in *Acacia nilotica* with the same growth regulators, further supplemented with casein hydrolysate (CH).

The results of the experiments conducted to identify the suitable auxin for SE in tamarind is included in Table 5.3. Varied responses were noted in the explants. In MS medium the cotyledons and radicle turned brown and necrotic. In some explants plumule was elongated, whereas in some, the hypocotyl region elongated. However, these axes neither differentiated nor produced somatic embryos (Fig. 5.6A).

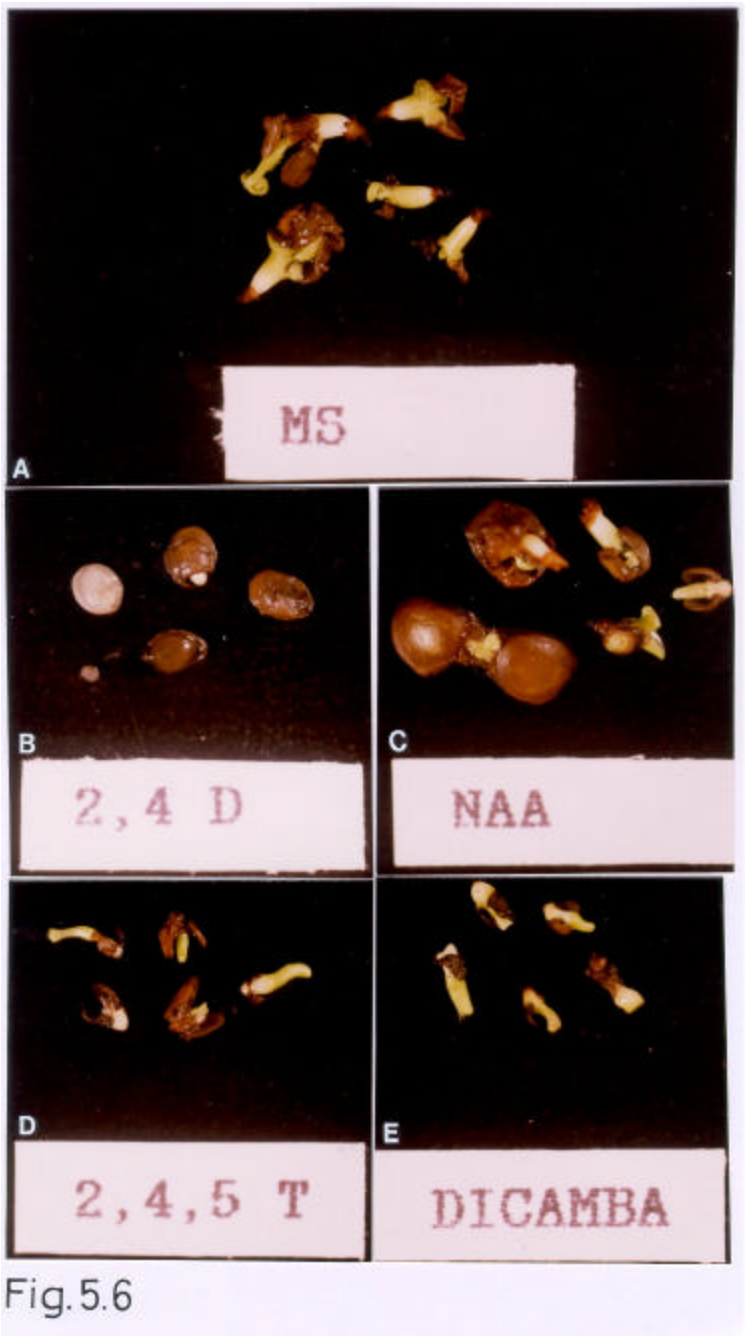

Fig. 5.6

Fig. 5.6 : (A-E) Response in the IZE cultured on MS basal medium, and or various auxins for 60 days. Concentration of auxin used in each medium was 1mg.l^{-1}.

Table 5.3 : Effect of different plant growth regulators on induction of somatic embryogenesis in immature zygotic embryos of Tamarind

Medium (PGR conc. in $mg.l^{-1}$)	Total number of explants cultured	Total number of explants responded	Frequency of response (%) Mean ± s.d.	Frequency of response in form of multiple embryos (%) Mean ±s.d.
2,4-D 0.5	80	1	1.3 ± 1.8	1.3 ± 1.8
1	80	2	2.5 ± 3.5	1.3 ± 1.8
3	80	1	1.3 ± 1.8	0.0 ± 0.0
5	80	1	1.3 ± 1.8	0.0 ± 0.0
10	80	0	0.0 ± 0.0	0.0 ± 0.0
NAA 0.5	80	6	7.5 ± 10.6	1.3 ± 1.8
1	80	7	8.8 ± 12.4	1.3 ± 1.8
3	80	5	6.3 ± 8.8	1.3 ± 1.8
5	80	1	1.3 ± 1.8	0.0 ± 0.0
10	80	0	1.3 ± 1.8	0.0 ± 0.0
2,4,5-T 0.5	80	5	11.3 ± 1.8	1.3 ± 1.8
1	80	10	12.5 ± 3.5	1.3 ± 1.8
3	80	3	3.8 ± 1.8	0.0 ± 0.0
5	80	0	1.3 ± 1.8	0.0 ± 0.0
10	80	0	0.0 ± 0.0	0.0 ± 0.0
Dicamba 0.5	99	7	6.6 ± 3.0	0.0 ± 0.0
1	95	11	11.1 ± 6.0	0.0 ± 0.0
3	151	12	7.4 ± 3.0	1.2 ± 1.3
5	234	50	17.5 ± 10.4	3.1 ± 2.8
10	95	7	6.8 ± 5.1	0.8 ± 1.4
Picloram 0.5	80	10	12.5 ± 0.0	0.0 ± 0.0
1	80	10	14.1 ± 11.1	0.0 ± 0.0
3	80	4	4.7 ± 2.2	0.0 ± 0.0
5	80	0	0.0 ± 0.0	0.0 ± 0.0
10	80	0	0.0 ± 0.0	0.0 ± 0.0
MS	80	0	0.0 ± 0.0	0.0 ± 0.0

ANOVA	S 5%	NS

Some common responses were noted in IZEs. In all the auxins, at all the concentrations, radicle differentiation was restricted and the cotyledons turned brown. Only the embryo axis responded differently in various media. Initially in the responding IZEs, a white, glistening mass was formed at the plumule end. The texture of this mass was similar to the embryogenic mass described in peanut (Chengalrayan *et.al.*, 1994) but these masses were of various shapes and sizes. Often these were tubular in shape with swelling around the middle region. Subsequently embryos developed from these masses. Therefore any growth or swelling in the IZEs was scored as response (Table 5.3). In 2,4-D the response in axis was very poor (1.3-2.5%) and the cotyledons did not open (Fig. 5.6B). However in 1.3% of the responding embryos multiple embryos were also seen. Compared to 2,4-D, in NAA more number of embryo axes (1.3-8.8%) responded to elongate partially and in some of the cultures a pair of fleshy leaves differentiated from the plumule of the embryo axis (Fig. 5.6C). Similar to 2,4-D, multiple embryos were seen in some of the responding embryos. In 2,4,5-T and Dicamba apparently the response in the embryo axis was similar (Fig. 5.6D,E) but the frequency of response varied greatly. In these two auxins the plumule of IZE swelled in 30-40 days to form structures similar to torpedo/ cotyledonary stage somatic embryos (Fig. 5.7A,B,C,D). The base of these structures were constricted near the cotyledonary node and swollen around the middle region. Tips of these structures were either tapering or funnel shaped. Initially it gave the impression that the plumule of the zygotic embryo axis is converted into single somatic embryo. However, this possibility was later ruled out after histological studies. On extended incubation of these structures in the same media for 30-40 days somatic embryos developed from the cotyledonary junctions and the swollen plumule end. The swollen IZE together with these newly developed embryos are identified as multiple embryos (Fig.5.8A,B,C,D). In 2,4,5-T 0.5 and 1 $mg.l^{-1}$, 11.3 to 12.5% embryos responded. Frequency of response decreased with increase in 2,4,5-T concentration. No response could be noted in 10 $mg.l^{-1}$ of this auxin. In 0.5 and 1 $mg.l^{-1}$, multiple embryos were also noted. In dicamba, the frequency of response varied from 6.6 to 17.5%. Dicamba at 5 $mg.l^{-1}$ was optimal. At 10 $mg.l^{-1}$ although 6.8% embryos demonstrated the sign of growth but rest of the embryos turned brown and necrotic indicating the toxicity of higher concentrations. In contrast to 2,4-D, NAA and 2,45-T, dicamba at concentrations 3, 5 and 10 $mg.l^{-1}$, induced multiple embryo formation in 0.8 – 3.1% cultures. The frequency of response and frequency of multiple embryo formation both

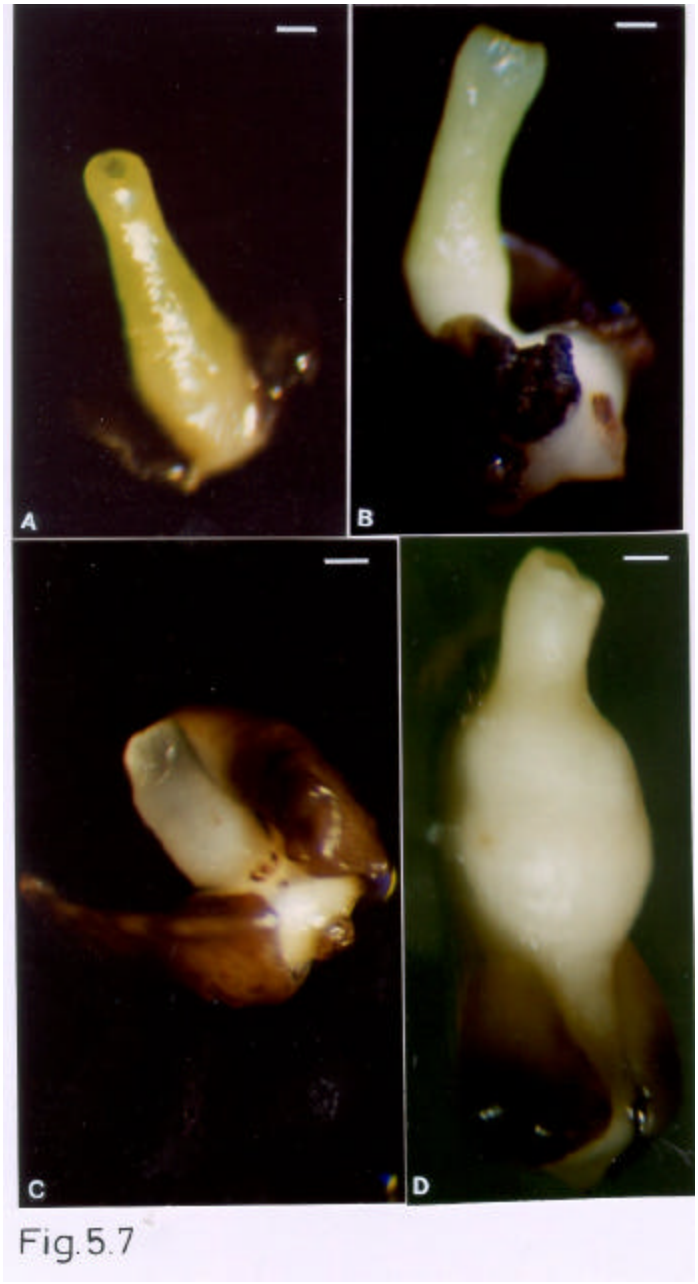

Fig. 5.7

Fig. 5.7 : On culturing in 5 $mg.l^{-1}$ dicamba cotyledons of the IZEs turned brown and necrotic and the plumule developed to form structures similar to,

(A) torpedo stage somatic embryo (bar = 500µm)

(B) and (C) bipolar somatic embryo with fused cotyledons. The base of the structures were constricted. Hypocotyl of IZE was swollen and radicle did not differentiate (bar = 500µm)

(D) horn shaped somatic embryos with broad hypocotyl (bar = 500µm)

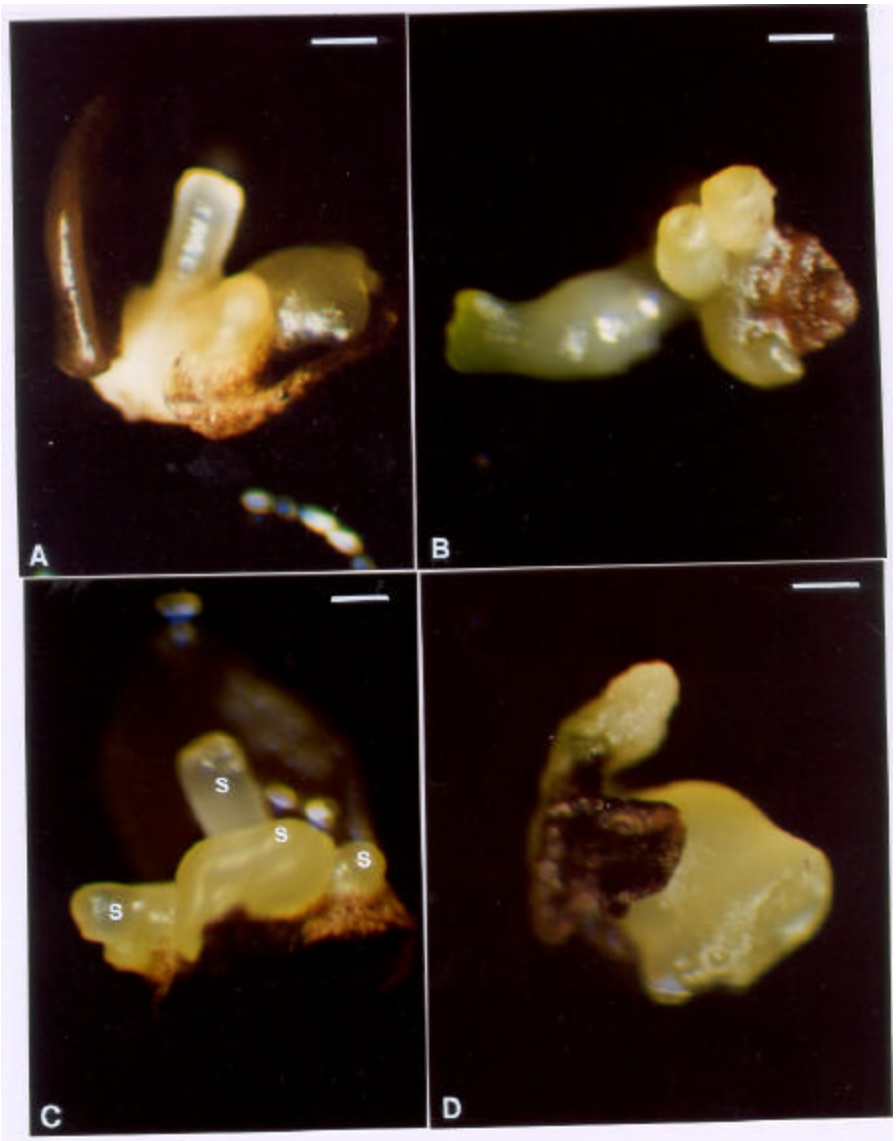

Fig. 5.8

Fig. 5.8 : Somatic embryo like multiple structures (A - D) developed from the IZE in 5 $mg.l^{-1}$ dicamba (bar = 660 µm).
The structures (s) initiated from the plumular region of the IZE and from the cotyledonary nodal region.

were highest at dicamba 5 $mg.l^{-1}$. Similar to the first three auxins, in picloram, only lower concentrations were more effective in inducing response (Fig.5.9A) in the IZE, and were totally ineffective at higher concentrations. Thus it was observed that dicamba at 5 $mg.l^{-1}$, was optimum for induction of embryogenic response in 17.5% of the cultures. This was followed by 14.1% and 12.5% in $1mg.l^{-1}$ picloram and 2,4,5-T, respectively. Data on frequency of response in various auxins at various concentrations was subjected to ANOVA and was found significant at 5% level. However between dicamba 5 $mg.l^{-1}$ (17.5%) and picloram (14.1%) or 2,4,5-T 1 $mg.l^{-1}$ (12.5%) the difference in response was not significant. Since, the response was highest in 5 $mg.l^{-1}$ dicamba and more number of embryos gave rise to multiple embryos, this concentration was used in the subsequent experiments to study the effect of various other factors. Embryogenesis was not induced in the IZEs cultured in TDZ. Even at a concentration of 1 $mg.l^{-1}$, callusing was noted in all the explants cultured (Fig.5.9B). The callus was yellowish green in color.

The embryogenic response was in two forms, single structure and multiple structures. In dicamba 5 $mg.l^{-1}$, out of 17.5% total response, 14.3 %, of the cultures were single structures of various shapes (Fig.5.10A,B,C,D,E) whereas 3.1 % of the cultures showed multiple somatic embryos (Fig. 5.11A,B,C, D). In 2,4-D 0.5 and 1 $mg.l^{-1}$, NAA 0.5 - 3 $mg.l^{-1}$, 2,4,5-T 0.5 and 1 $mg.l^{-1}$, multiple somatic embryo formation was noted at frequencies lower than that obtained in dicamba 5 $mg.l^{-1}$. Picloram inhibited the formation of multiple structured somatic embryos at all the concentrations tested.

Weaver and Trigiano (1991) also noted two types of somatic embryos in *Cladrastis lutea* where most of the embryos formed as fused clusters from the proximal ends of zygotic cotyledons and were morphologically and anatomically abnormal. However, in some cases they observed two or more somatic embryos developing singly. They rarely found formation of a single embryo with well-developed cotyledons.

Dicamba has been used for direct embryogenesis from mesophyll cells of orchard grass (Conger *et al*.,1982), indirect plant regeneration from immature embryos of maize (Duncan *et al*., 1985) and SE at a high frequency in rye (Zimny and Lorz, 1989). Furini and Jewell (1994) found that dicamba was significantly superior to 2,4-D, both for stimulating and maintaining embryogenic calli from immature and mature embryos of *Zea may* L. Dicamba along with Zeatin has been used for SE from leaf and root explant

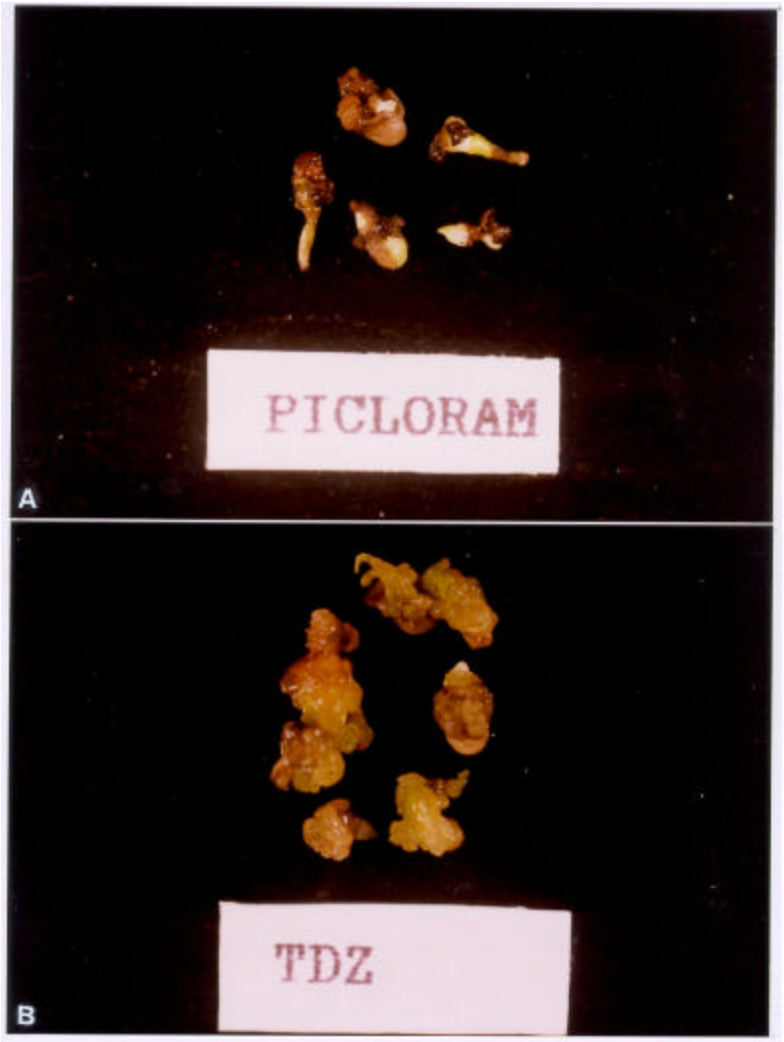

Fig. 5.9

Fig. 5.9 : (A) Immature zygotic embryos cultured on Picloram at 1 $mg.l^{-1}$ show formationof the single somatic embryo like structure in some of the concentrations and in
(B) TDZ at 1 $mg.l^{-1}$ the IZEs dedifferentiated to form callus.

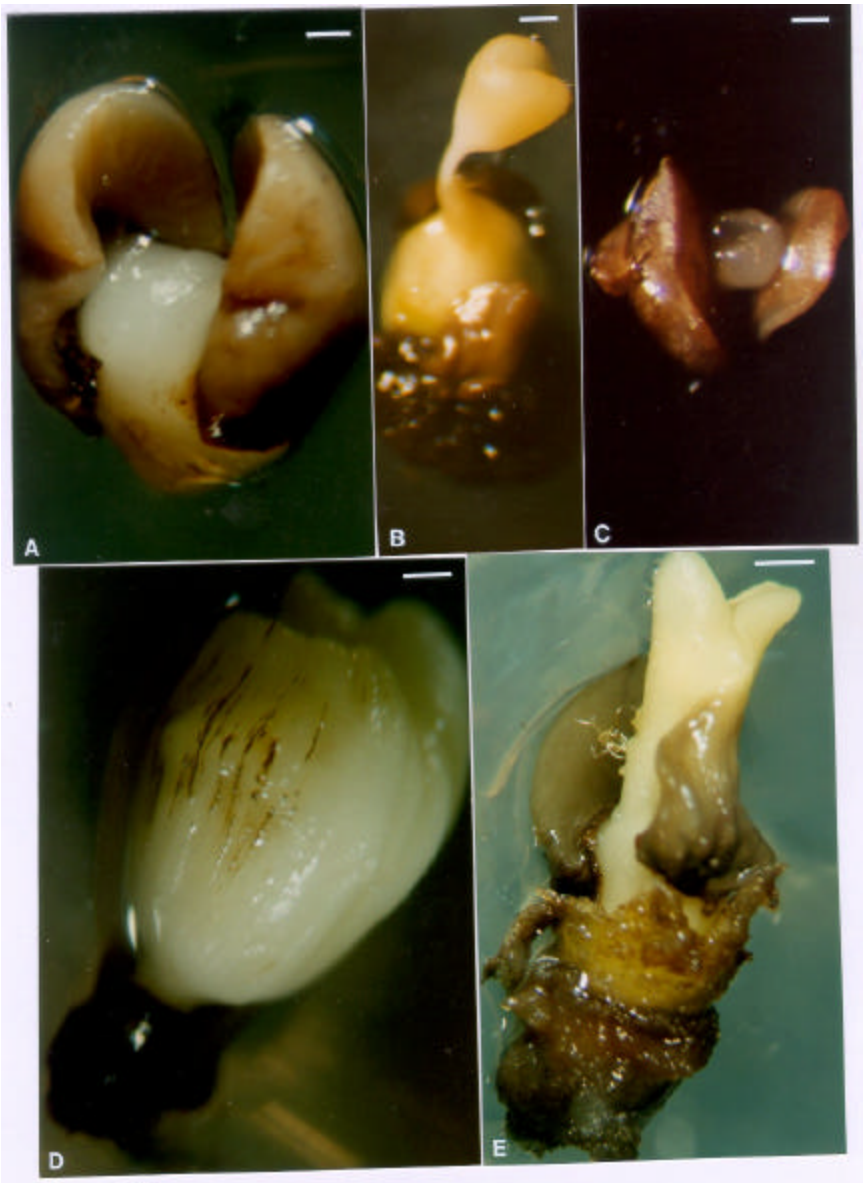

Fig. 5.10

Fig. 5.10 : (A – E) Somatic embryo like structures of different shapes and sizes developed on IZEs cultured in dicamba 5 mg.l^{-1}.
(A) bar = 500µm, (B) bar = 500µm, (C) bar = 500µm,
(D) bar = 500µm (E) bar = 588µm

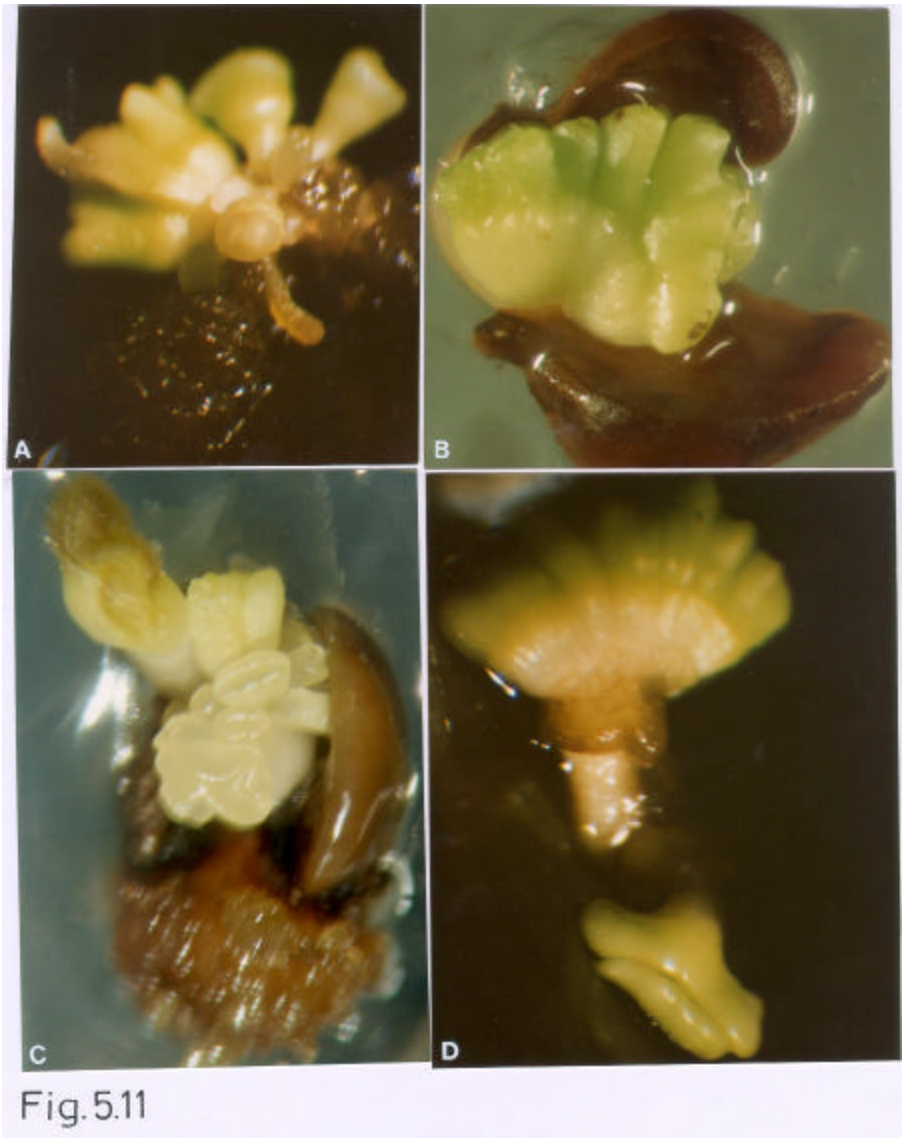

Fig. 5.11

Fig. 5.11 : (A – D) Multiple somatic embryos of different shapes and sizes developed on IZEs cultured in dicamba 5 $mg.l^{-1}$.
In (A) the somatic embryos are isolated structures whereas in (B) and (D) they are fused at the base. In (C) several embryos at various stages of development can be seen in the same cluster.

in suspension culture of Bananas (Novak *et al*., 1989). Brisibe *et al*. (1994) while investigating on somatic embryo differentiation in *Saccharum officinarum* L. conducted experiments to evaluate the influence of various medium components and culture conditions. These conditions could be manipulated to enhance long-term retention of embryogenic competence and high frequency initiation and development of somatic embryos in this species. Plazek *et al*. (1999) reported that in contrast to 2,4-D, Dicamba was more effective to induce callus in *Phleum pratense* L. from mature caryopses. Callus induced on medium containing Dicamba produced a high number of regenerants in subsequent passages and callus-derived cell suspension culture. Castillo *et al*. (1998) observed that the medium containing dicamba gave the best embryogenic callus induction, maintenance and regeneration from immature zygotic embryos of barley cultivars. Though dicamba has mostly been used in monocots to obtain embryogenic callus, it was used by Finer (1987) to induce somatic embryos from immature embryos of hybrid sunflower (*Helianthus annus* L.). The effect of dicamba on frequency of SE was also noted in a grain legume, *Cicer arietinum* L. (Eapen and George, 1994). From the five growth regulators tested, dicamba was found to be most effective in inducing embryogenic response in the IZEs of tamarind without involving a callus phase. To the best of our knowledge, dicamba was used for the first time for induction of direct embryogenesis in a woody legume. TDZ was not effective for induction of embryogenic response.

5.3.2.2 Effect of age/size of explant

Flowering in tamarind starts in the month of May. Pods become visible from the month of July. In the preliminary experiments, attempts were made to use very young pods. However, it was observed that till the end of August the IZE was jelly-like and the seed coat was also too tender to be isolated without damaging the IZE. IZE with the seed coat ceased to grow and turned brown within a few days on culturing them in any medium with or without growth regulator. Therefore for this experiment pods were taken from the first week of September to the first week of October. This means the age of the immature zygotic embryos used here are of 12-16 weeks. Secondly the period for availability of explant material was practically 4-5 weeks only.

The age of the immature zygotic embryos of tamarind was difficult to ascertain, therefore size of the immature embryo was taken as a criterion. Explants approximately ranging from 1mm to 6mm were found to be responsive while explants from 7mm size onwards produced lot of phenolics in culture medium and turned brown. Among the sizes 1-6mm, 3mm IZE showed the optimum response (18.7 ± 17.2 %) (Table 5.4). The frequency of response noted from using various sizes was highly significant when subjected to ANOVA. On performing 't' test it appeared that sizes 1, 2, 3, 4 were not significantly different in their frequency of response. Response in size 5 and 6 were significantly lower when compared with size 3. The embryos above size 6mm did not respond in culture even after the incubation period of 8-9 weeks. There was no callusing either. The embryos turned brown and necrotic.

Table 5.4 : Influence of the stage of development of IZE on induction of somatic embryogenesis in 5 mg.l^{-1} Dicamba

Size of explants (mm)	Total number of explants cultured	Total number of explants responded	Frequency of response (%) Mean ± s.d.
1	19	3	10.4 ± 15.3
2	143	26	15.6 ± 15.3
3	333	58	18.7 ± 18.2
4	368	43	12.6 ± 9.2
5	312	12	6.3 ± 10.4
6	242	1	0.6 ± 2.1
7	143	0	0.0 ± 0.0
8	86	0	0.0 ± 0.0
9	15	0	0.0 ± 0.0
10	4	0	0.0 ± 0.0

ANOVA	S 1%

The developmental stage of the explant is critical for the expression of embryogenesis. According to Wetzstein *et al.* (1989), embryogenic potential was influenced by the time of embryo explanting and developmental stage. A 'restricted window' or sampling period has similarly been described in other embryogenic systems in woody species.

Trigiano *et al.* (1988) demonstrated that 96 and 110 post anthesis zygotic embryos of Red bud (*Cercis canadensis*) produced somatic embryos at highest frequency. Number of somatic embryos produced per explant was also highest. Tulecke and McGranahan (1985) described an optimum sampling time for induction of SE, of between 6 and 11 weeks after pollination in *Juglans* spp. and *Pterocarya*. In studies with peach, the characteristic of callus obtained from immature embryos were highly dependent on the stage of embryo development; the regeneration of embryo-like structure and plantlets was induced as a specific callus type (white, compact and nodular) (Hammerschlag *et al.*, 1985). Similarly, the initiation of embryogenic callus in Norway Spruce was significantly higher during a 4-week period (from June30th to July 21st) than earlier or later collections (Becwar*et. al.*, 1988).

Researcher working in tree legumes noted similar observation. Weaver and Trigiano (1991) reported that the greatest number of somatic embryos in *Cladrastis lutea* were formed from zygotic embryos explanted from 6-8 weeks post anthesis. Explants harvested prior to this developmental time period produced little callus and usually became necrotic. Zygotic embryos harvested from 10 weeks post-anthesis ovules did not form somatic embryos, however, immature cotyledons expanded and formed copious amounts of yellow friable callus which could be maintained for over a year, but never produced somatic embryos.

In *Dalbergia sissoo* (Das *et al.*, 1997), the callus derived from 40-day-old zygotic embryo explants consisted of yellowish, friable, non-embryogenic sectors and yellowish-green, globular, embryogenic sectors. The calli derived from 30 and 50-day-old zygotic embryos did not become embryogenic on any of the media tested, even after prolonged maintenance and subculture. Rao and Lakshmi Sita (1996) observed the time taken for the induction of SE increased with the age of the immature cotyledons. Maximum number of somatic embryos per explant was observed from 90 days old cotyledons,

where somatic embryos stated emerging within 7-10 days after transfer to second phase media; whereas from 120 days old explants the induction required 20-25 days after transfer. Moreover callus growth was also enhanced the age of the explant. Callus started growing from the periphery of older cotyledons and continued to grow allover the explant. On the other hand, 90 days old explants produced less callus, and white pearl like somatic embryos could be clearly seen arising from the surface on the expanded cotyledons. Fully mature cotyledons, excised from dry seeds, did not show any embryogenesis but callused profusely regardless of the media composition. Immature seeds of 60 days age did not respond to treatment and turned brown afterwards.

Arrillaga *et al.* (1994) reported that in *Robinia pseudoacacia* the seeds collected 2-3 weeks post anthesis (2-2.5mm in length) provided the highest embryogenic percentage. Efficiency of the immature zygotic embryos at particular age/stage, to produce somatic embryos was attributed to their favourable physiological make up at that stage of development.

White Spruce had a developmental window in which optimal initiation of embryogenic callus was obtained using cotyledon embryos of about 1.5-2.0mm (Becwar, 1995). Although the timing of zygotic embryo explanting has been reported to have a critical influence on obtaining successful SE, few studies have evaluated or defined zygotic embryo developmental stages. Wetzstein *et al.* (1989) achieved highest embryogenic frequencies in Pecan when embryos were actively growing and cotyledons rapidly expanding. Embryogenesis decreased when cotyledons were fully elongated, and was obtained when cotyledons began filling. Variation in cotyledon length was also observed within a single sampling date. Defining optimal sampling time based on developmental characteristics is thus a useful index for explanting time.

Geneve and Kester (1990) reported that in *Cersis canadensis*, number of days post anthesis was an inadequate measure of developmental age of the zygotic embryo. A nearly 30 day variance in time to first flower and seasonal environmental differences may have contributed to the large difference in the number of days post anthesis indicating a similar stage of development.

5.3.2.3 Effect of carbohydrate

In the randomly designed preliminary experiments it was observed earlier that compared to 2% sucrose, 4-6% sucrose concentration in the medium was more effective in inducing embryogenic response. The experiment with various sucrose concentration was conducted to determine the optimum concentration of sucrose required for induction of embryogenic response and formation of multiple embryos. In the present investigations, sucrose at 2% could induce formation of single embryogenic structure at very low frequency. Multiple embryos were not seen in any explant even after incubating the cultures for 8 weeks. In 4% sucrose the embryogenic response was 8.8% and in 2.5% cultures multiple embryos were visible. Sucrose at 6% (Fig. 5.12A) induced 16.3±15.1% total response (Table 5.5). In 2.5±3.2% of the cultures embryos were formed in multiple. The data in Table 5.5 was not significant. The high S.D. values of the data may be due to the various sizes of embryos (1-6mm) with different embryogenic potential (Table 5.4). Higher sucrose concentration (8%) was inhibitory for embryogenic response and the few structures formed therein showed browning and deteriation in the form of dedifferentiation of the radicle end (Fig. 5.12B).

Table 5.5 : Effect of sucrose concentration on induction of somatic embryogenesis

Concentration of sucrose (w/v)	Total number of explants cultured	Total number of explants responded	Frequency of total response (%) Mean ± s.d.	Frequency of response in form of multiple structure (%) Mean ± s.d.
2%	100	2	1.4 ± 2.4	0.0 ± 0.0
4%	100	9	8.8 ± 6.3	2.5 ± 5.0
6%	172	34	16.3 ± 15.1	2.5 ± 3.2
8%	100	8	10.0 ± 9.1	0.0 ± 0.0
ANOVA			NS	NS

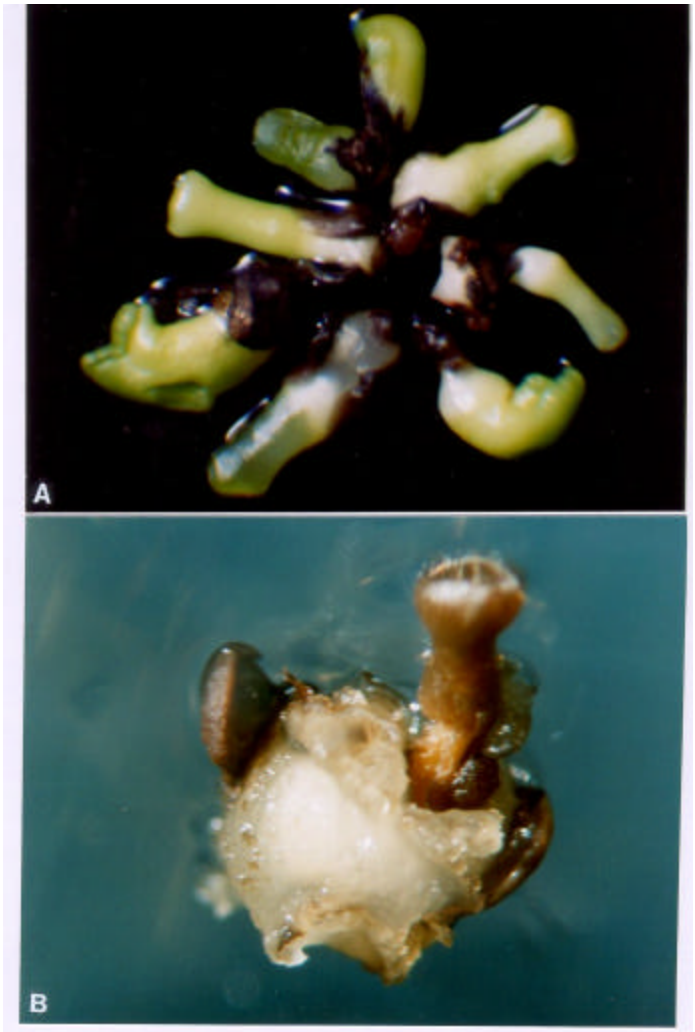

Fig. 5.12

Fig. 5.12 (A) Somatic embryos / emnbryogenic structure formed on 5 $mg.l^{-1}$ dicamba supplemented with6% sucrose

(B) Somatic embryos formed on 5 $mg.l^{-1}$ dicamba supplemented with 8% sucrose. Browning of the structure and dedifferentiation of the radicle was noted.

The primary role attributed to the supplied carbohydrate is that of carbon source. Sucrose has been identified as one of the factors, influencing induction of embryogenesis (Thorpe, 1988). Sucrose plays dual role in plant tissue culture. It is incorporated in the medium at 2-3% concentration as a carbohydrate source. Suppression of embryogenesis at higher concentrations, may be due to the increased osmolarity (Pence, 1981). Inhibition of embryogenesis in peanut, in sucrose concentration above 7% is reported earlier (Eapen and George, 1993). It has been demonstrated that merely by altering the sucrose concentration, the morphogenetic pathway can be changed from organogenesis to embryogenesis and vice versa (Jeannin *et al.*, 1995). Tomar and Gupta (1988a) observed in *Albizzia richardiana,* that 4% sucrose was optimal for organogenesis and 2%sucrose was optimal for embryogenesis. Higher levels of sucrose (6-10%) caused browning of green calli and also inhibited differentiation of the calli into embryos. Rao and Lakshmi Sita (1996) also maintain that in addition to plant growth regulators, 10% sucrose (w/v) favoured direct SE in Rosewood and there was no induction of embryogenesis using 2% sucrose. Eapen and George (1993) demonstrated that sucrose was the best carbon source tested for peanut and 6% sucrose gave the highest frequency of responding cultures and average number of somatic embryos per explant. High sucrose has been used in forage legumes such as *Medicago sativa* and *Trifolium* sp. (Maheswaran and Williams, 1984), grain legumes like groundnut (Hazra *et al.*, 1989) and pea (Jacques *et al.*, 1995) to induce SE. In several non-legumes, high sucrose has also been used successfully (Finer, 1987).

Carbohydrates other than sucrose, incorporated in the medium are known to influence embryogenic response. Galactose yielding sugars like lactose and raffinose stimulated embryogenesis in citrus even in callus lines (Kochba *et al.*, 1978). Chengalrayan (1997) found that other than sucrose, only maltose and glucose were effective in inducing embryogenic mass formation in the mature zygotic embryo derived leaflets of peanut. While testing the influence of carbon sources on callus induction in leaf tissues of peach (*Prunus persica* L. Batsch), it was observed that in presence of glucose green and compact callus development was at a higher frequency than those grown on either fructose or sucrose (Declerck and Korban, 1996).

Keeping the literature in view, two other carbohydrates including glucose and fructose were tested for further improvement of embryogenic efficiency. Fructose at all concentrations (2-8%) inhibited SE response in light and dark (Table 5.6), glucose at 8% in light showed 9.3% response and in dark 3.1% response was noted. Sucrose at 6% showed 19.2% response in dark and 9% response in light. The two carbohydrates tested did not show any improvement in response over and above 6% sucrose. Therefore the data was not subjected to statistical analysis.

Table 5.6 : Effect of glucose and fructose as carbohydrate source on induction of somatic embryogenesis

Carbohydrate Type	Concentration used (w/v)	LIGHT			DARK		
		Total no. of explants used	No. of explant responded	% of response	Total no. of explants used	No. of explant responded	% of response
Glucose	2%	28	1	3.5	20	1	5.0
Glucose	4%	36	0	0.0	20	0	0.0
Glucose	6%	36	0	0.0	28	2	7.1
Glucose	8%	32	3	9.3	32	1	3.1
Fructose	2%	28	0	0.0	8	0	0.0
Fructose	4%	28	0	0.0	20	0	0.0
Fructose	6%	28	0	0.0	20	0	0.0
Fructose	8%	24	0	0.0	12	0	0.0
Sucrose	6%	88	8	9.0	52	10	19.2

5.3.2.4. Influence of culture conditions (gelling agents, light, dark, culture vessel)

Physical and environmental conditions are critical to the process of embryogenesis in some systems (Tulecke,1987), light being one of them. For example prolonged culture in the dark was required for embryogenesis in *Coffea* (Sondahl and Sharp, 1979) but light was better for *Theobroma* (Kong and Rao, (1981).

Axillary shoot multiplication in tamarind showed better growth at 24h light conditions compared to 16h photoperiod (Section 3.3B.2.4). Therefore comparative effect of varying photoperiod was studied for induction of somatic embryogenesis in culture. It was found that IZE cultures incubated in 16h photoperiod showed 19.4±2.1 % response which was significantly higher than 5.3±4.6% response, in 24h photoperiod (Table 5.7).

This indicates that longer photoperiod is inhibitory for induction of embryogenesis in tamarind.

Table 5.7 : Effect of photoperiod on induction of somatic embryogenesis

Photoperiod	Total number of explants cultured	Total number of explants responded	Frequency of response (%) Mean ± s.d.	Frequency of response in form of multiple embryos (%) Mean ± s.d.
16 h	192	36	19.4 ± 2.1	3.8 ± 1.8
24 h	176	11	5.3 ± 4.6	2.0 ± 2.6
ANOVA			S 1%	NS

There has been no report on the effect of culture vessel on induction of SE in tree legumes. We observed that, given the similar conditions, the frequency of response in tube was higher (Table 5.6) than in petridishes. None of the explants formed multiple somatic embryos in petridishes. The higher response in test tube may be attributed to the chemical inertness of the glass surface and lack of moisture accumulation in the culture vessel. This observation is consistent with earlier findings noted in regeneration rates from leaf explants of soybean (Wright *et al.*, 1987) in test tube and petridishes for shoot growth of five woody species (Mc Clelland and Smith, 1990) and for cotton (Agrawal *et al.*, 1997 ; Hazra *et al.*, 2000).

Responses observed in the culture are the results of an integrated effect of the nature of the explant, chemical factors in form of nutrients and growth regulators and physical factors such as microenvironment of the culture, temperature, light, gelling agent etc. Results of the present investigation shows that irrespective of the culture vessel or gelling agent the embryogenic response is higher in the cultures incubated in the dark. When compared between the tubes and the petridishes the response is always higher in the tubes compared to petridishes. When compared between phytagel and agar, the response is higher in phytagel. Thus it appears that optimum embryogenic response can be induced in IZE of tamarind when the explants are cultured in medium in tube, gelled with phytagel.

When cultures were incubated in dark they showed the highest 26.7% response out of which 21.7% were single structure somatic embryos and 5% were multiple structure somatic embryos (Table 5.8).

Table 5.8 : Influence of culture conditions (culture vessel, gelling agent, light and dark) on induction of somatic embryogenesis

Different treatments			Total number of explants cultured	Total number of explants responded	Frequency of response (%) Mean ± s.d.	Frequency of response in form of multiple embryos (%) Mean ± s.d.
Tube	Agar	Light16h	60	4	6.7±11.6	0.0±0.0
``	Phytagel	``	340	46	13.4± 6.8	2.7±1.9
Petridish	Agar	``	54	1	1.9± 3.2	0.0±0.0
``	Phytagel	``	66	4	4.4± 7.7	0.0±0.0
``	Agar	Dark	48	9	17.6±13.7	0.0±0.0
``	Phytagel	``	54	10	19.0± 7.9	0.0±0.0
Tube	Agar	``	60	15	25.0±13.2	1.7±2.9
``	Phytagel	``	60	16	26.7±18.9	5.0±8.7

ANOVA Table

Source	df	SS	MSS	Fcal	Ftab	
Rep	3	708.65	236.22	2.24	3.41	ns
Light	1	1389.028	1389.03	13.19	4.67	*
GEL	1	67.32	67.32	0.64	4.67	ns
Ves	1	334.80	334.80	3.18	4.67	ns
LXG	1	19.91284	19.91	0.19	4.67	ns
LXV	1	0.035012	0.04	0.00	4.67	ns
GXV	1	11.08	11.08	0.11	4.67	ns
LXGXV	1	8.93	8.93	0.08	4.67	ns
Error	13	1368.58	105.28			
Total	23	3908.32	169.93			

5.3.3 Effect of explants from different trees

Varying embryogenic capacities of different plants, have been noted by Weaver and Trigiano (1991) in *Cladrastis lutea* (yellow wood). Arrillaga *et al.* (1994) noted that seeds from some mother trees had a higher potential to produce embryogenic culture than seeds of other trees in black locust (*Robinia pseudoacacia* L.). Trigiano *et al.* (1988) observed direct induction of SE in explants of two trees while explant from other trees produced only callus. Thus the establishment and maintenance of morphogenic cultures for leguminous species is often genotype dependent (Oelck and Scheider, 1983). In our studies, we observed that explants from all the plants responded in culture at different frequencies. Plant number II was most responsive while plant number III remained the least reactive one. The statistical analysis (ANOVA) shows that the data is significant (Table 5.9). This confirms the earlier observations that embryogenic response is genotype dependent.

Table 5.9: Effect of genotype on embryogenic response in Immature zygotic Embryos

Genotype	Number of explants cultured	Total number of explants responded	Frequency of response (%) Mean ± s.d.
I	171	32	21.6± 7.0
II	280	60	28.0± 13.3
III	1083	52	4.3 ± 3.3
IV	232	31	14.2± 6.5
V	248	19	6.3 ± 1.1

ANOVA	S1%

In some cases somatic embryos were noted to be forming from the plumular region (Fig.5.13A,B) or from the epicotyl region (Fig.5.14A,B) of the embryogenic structure. In course of time the growth and differentiation of these masses with or without somatic embryos is restricted. Instead of further differentiation, the somatic embryos also

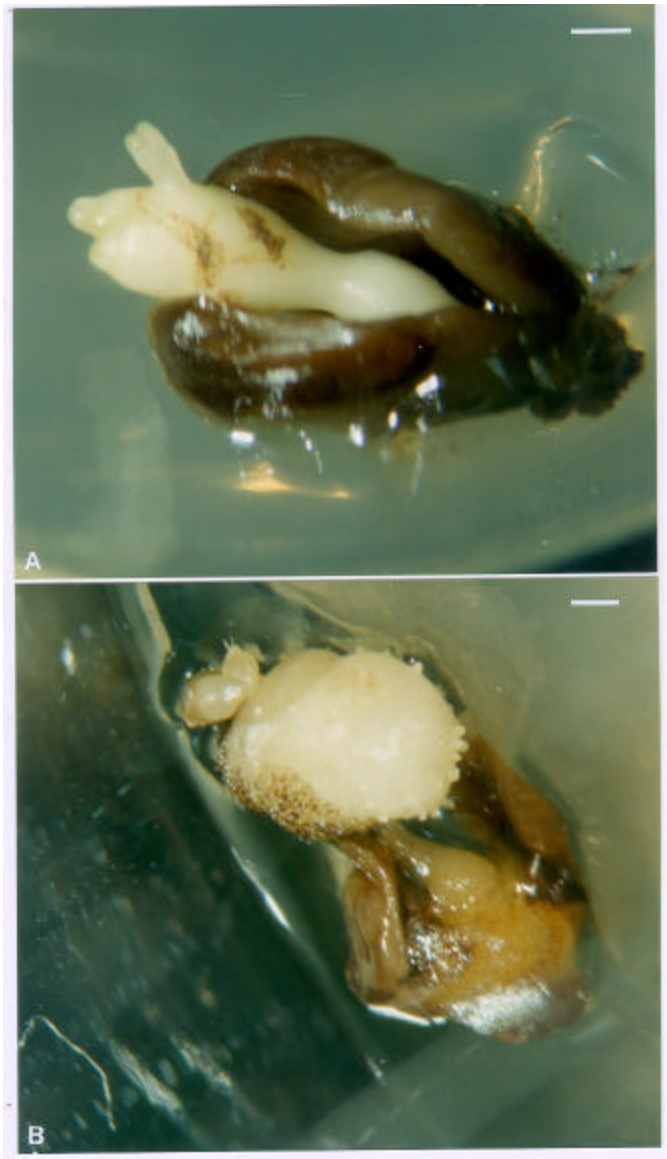

Fig. 5.13

Fig. 5.13 (A,B) Primary somatic embryogenesis from the plumular region of the embryogenic structure in 5 mg.l^{-1} dicamba (A) bar = 588µm. (B) bar = 500µm

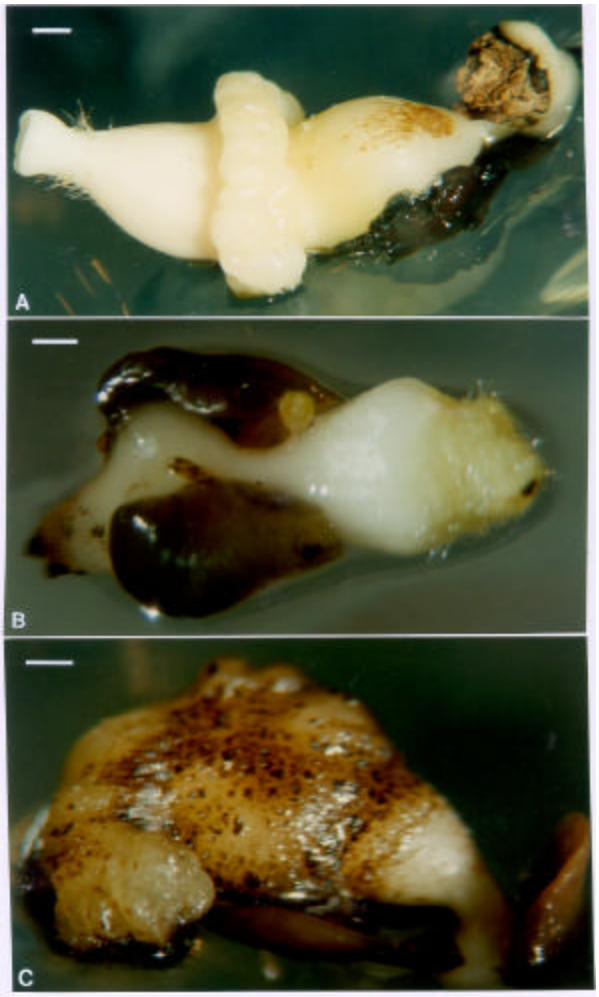

Fig. 5.14

Fig. 5.14 : (A – C) Primary somatic embryogenesis from the epicotyl region of the Embryogenic structure in 5 $mg.l^{-1}$ dicamba. (A,B,C) bar =500 µm

turn into glistening masses. Subsequently new embryos develop from these structures (Fig. 5.14C).

5.3.4 Secondary /Repetitive SE

Somatic embryos have shown to be excellent source for secondary embryos (Raemakers *et al.*, 1995). It is associated with loss of integrated group control of cells organized in the somatic embryo. Some cells break away from control and initiate new somatic embryos (Williams and Maheswaran, 1986). In most cases somatic embryo develop upto globular embryos, without differentiation into organs, before they are subjected to secondary somatic embryogenesis (SSE). Such cultures are difficult to synchronize and it is difficult or impossible to distinguish between different cycles of SSE. In other cases embryos develop upto maturity (Raemakers *et al.*, 1995). In these cases it is often more easy to distinguish between different cycles of SSE. In most of these cases the embryos originated from epidermal and/or subepidermal cells of older somatic embryos; either from single cells (Polito *et al.*, 1989); from multiple cells (Maheswaran and Williams, 1986) or from both (Liu *et al.*, 1992).

In *Acanthopanax senticosus* (Gui *et al.*, 1991), *Manihot estculanta* (Szabados *et al.*, 1987) and *Trifolium* (Maheswaran and Williams, 1986) mature embryos gave rise to SSE. However in *Trifolium repens* (Maheswaran and Williams, 1986) higher concentrations of BAP and in *Arachis hypogaea* (Durham and Parrott, 1992) lower concentration of 2, 4-D were required. In *Helianthus annus* (Finer, 1987) dicamba was ineffective in inducing primary embryogenesis but was effective in triggering SSE.

Secondary somatic embryogenesis has been reported in some tree species such as *Hardwickia binata* (Chand and Singh, 2001), *Eucalyptus citriodora* (Murlidharan *et al.*, 1989), *Picea abies* (Mo *et al.*,1989), *Hevea brasiliensis* (Cailloux *et al*., 1996) and *Acer palmatum* (Vlasinova and Havel, 1999). Long term maintenance of embryogenic cultures of *Hardwickia binata* (for a period over 4 years) has been reported (Chand and Singh, 2001), while maintenance of embryogenic capacity for more than two years by repeated embryogenesis has been reported in *Eucalyptus citriodora* (Murlidharan *et al.*, 1989), *Picea glauca* (Kong and Yeung, 1992) and *Picea glauca* X *P.engelmannii* (Eastman *et al.*

1991). The production of embryos is determined by the number of responding explants and the number of embryos produced per responding explant. In *Arachis hypogaea* (Baker and Wetzstein, 1992; Durham and Parrott, 1992; Sellars *et al.*, 1990), *Beta vulgaris* (Tenning *et al.*, 1992) and *Glycine max* (Sellars *et al.*, 1990; Liu *et al.*, 1992) less than 50% of the primary explants produced embryos whereas more than 75% of the SE formed secondary embryos. In *Medicago sativa* (Parrott and Bailey, 1993) only 5 of the 300 zygotic embryos produced primary embryos, whereas all the somatic embryo explants initiated secondary embryos. In *Glycine max* (Liu *et al.*, 1992) between 0.2 to 0.3 primary embryos per explant are formed and between 8 and 30 secondary embryos per explant. In other species like *Trifolium repens* (Maheswaran and Williams, 1986), *Eucalyptus citriodora* (Murlidharan and Mascarenhas, 1987) and *Arachis hypogaea* (Sellars *et al.*, 1990) the production of embryos in primary embryogenesis was lower than in SSE.

Continuous proliferation of somatic embryos via repetitive embryogenesis (RE) has several possible applications.

(i) Large quantities of somatic embryos can be produced by repetitive embryogenesis from preserved embryogenic lines. This is of particular importance for rapid clonal propagation from somatic embryos directly or from synthetic seeds prepared with the somatic embryos (Wann, 1988).

(ii) Mature trees are often recalcitrant for propagation. For propagation purposes mature trees can be rejuvenized by repetitive embryogenesis (Wann, 1988).

(iii) Immature embryos of interspecific plants from incompatible crosses may be rescued by culturing them for secondary SE and subsequently multiplied (Merkle *et al.*, 1990).

(iv) Transformation combined with repetitive, direct embryogenesis may be an efficient method by which transgenic plants can be obtained.

(v) Development of chimeric transformants may be avoided by inducing repetitive embryogenesis from the transformed sectors of primary embryos. As embryos formed during repetitive embryogenesis can originate from single cells (Haccius, 1977; Polito *et al.*, 1989), the number of transformed cells in the original embryo need not be high.

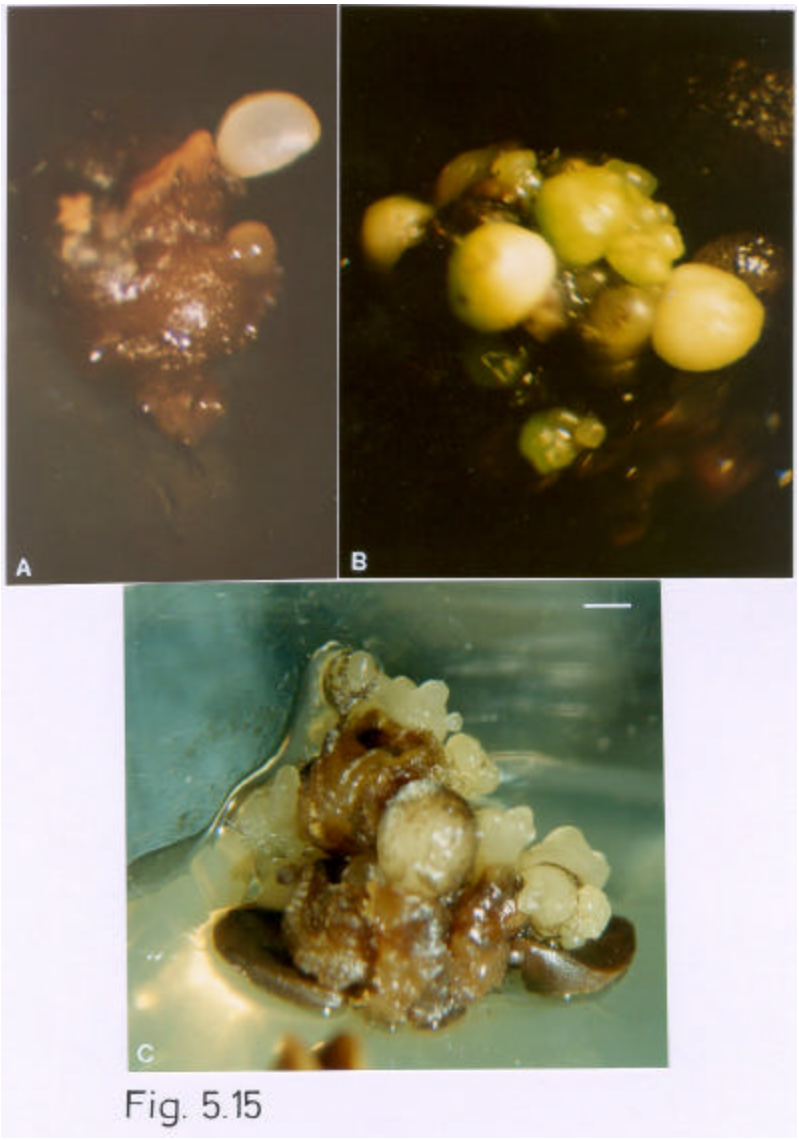

Fig. 5.15 : (A-C) Repetitive / Secondary somatic embryogenesis in the cultures maintained by repeated subcultures. (C) Due to slow proliferationof the secondary embryos the cotyledons which turned brown in the initial stage of culture could still be seen with the cluster of embryos after 1 year in culture. (bar = 1000µm).

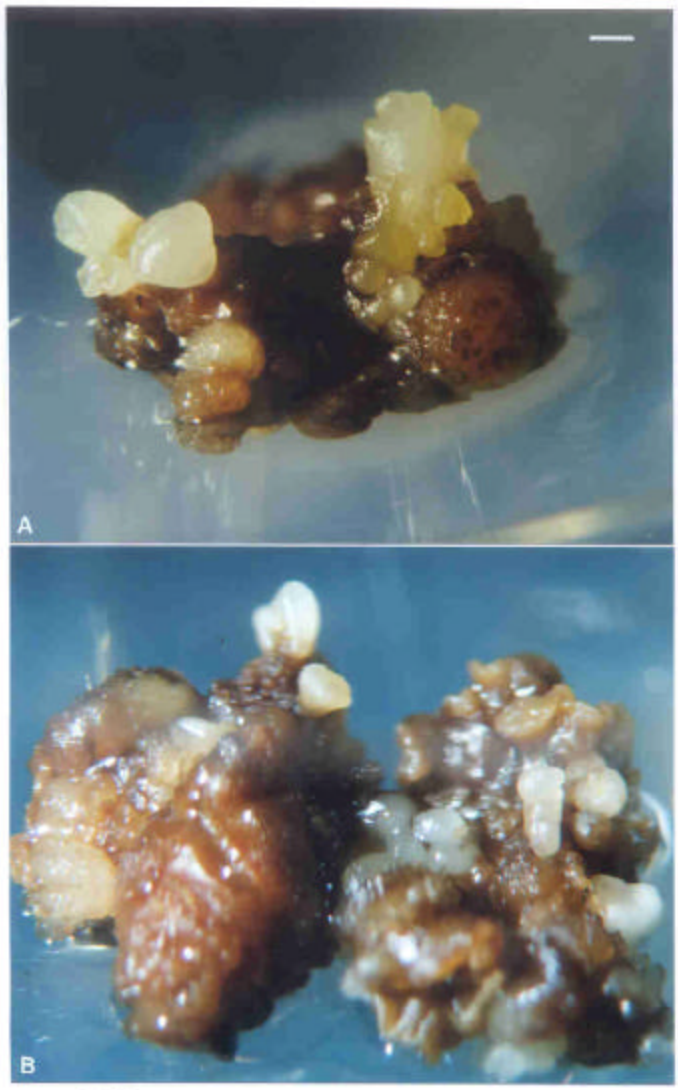

Fig.5.16

Fig. 5.16 : (A,B) Proliferation of somatic embryos maintained in culture for two years.
Development of embryos restricted after reaching the globular stage. Embryos turn brown and new embryos develop from these. Advanced stages of somatic embryos were not seen in these cultures. In (A) bar = 500µm

(vi) Finally, repetitive embryogenesis can be used for the production of somatic embryos of species in which the zygotic embryos contain commercially important metabolites (Litz, 1988; Southworth and Kwiatkiowski, 1991).

Immature zygotic embryos when cultured on dicamba 5 $mg.l^{-1}$ with 6% sucrose and 0.22% phytagel developed embryogenic structure that produce somatic embryos from the upper region of the embryogenic structure. Their growth and development is asynchronous. Cultures showing multiple structures along with the original explant when shifted and later subcultured showed proliferation of somatic embryos. The proliferation of culture producing secondary somatic embryos (Fig.5.15, 5.16) in Tamarind has now been maintained for nearly two years though the rate of proliferation is very slow. Xie and Hong (2001) reported that in *Acacia mangium* extended incubation of the heart shaped embryos on the same medium induced secondary embryogenesis.

5.3.5 Histological studies of somatic embryos

Explant IZEs, on histological examination (Fig.5.17A) show two cotyledons attached to the embryo axis. The vascular strand diverting into the two cotyledons. The plumule and the radical of the embryo axis show highly concentrated meristematic cells. On exposing the explants in medium containing dicamba 5 $mg.l^{-1}$, the differentiation of both plumule and radicle was restricted. The apical meristem of the IZE, which was determined to form the shoot primordia, changes the course and proliferate to form a elongated, fleshy, glistening, white, cellular structure. These fleshy structures often appeared like torpedo stage or cotyledonary stage somatic embryo. This gave the impression that somatic embryo was formed from the plumule of the IZE. In chickpea (Suhasini *et al.* 1996) somatic embryos of similar morphology were described as "horn-shaped embryo with a broad hypocotyl". On fixing the IZE derived structures in fixative (FAA) the cotyledons, which were brown and brittle, broke off. In the section of torpedo shaped IZE axis (Fig.5.17B) it is apparent that the cells of the apical meristem proliferated to form a fleshy embryogenic structure with small, compact, meristematic cells with dense cytoplasm and prominently stained nuclei.

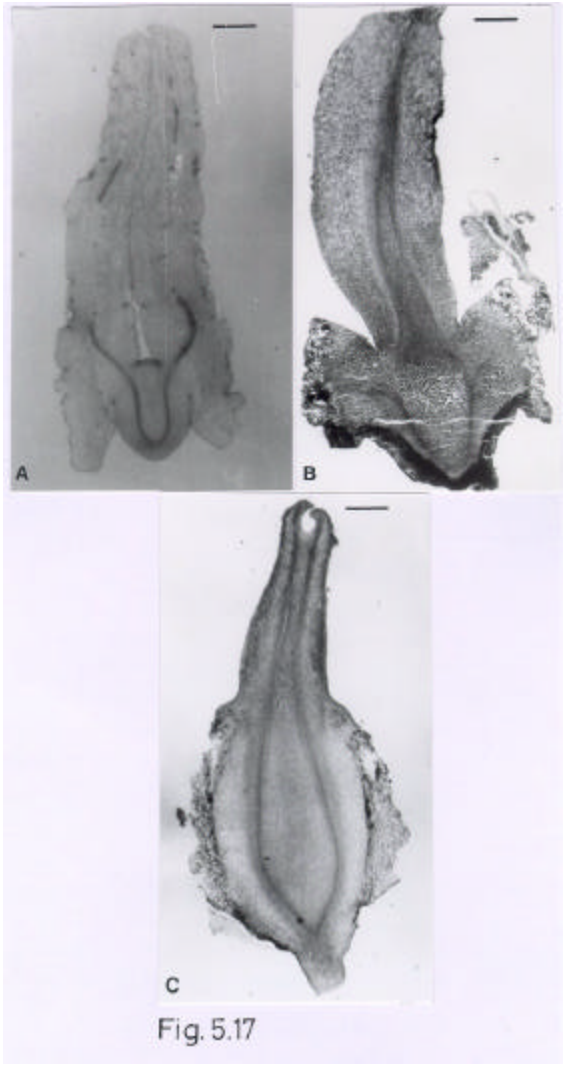

Fig. 5.17

Fig. 5.17 : (A) Explant IZE on histological examination show two cotyledons (c) attached to the embryo axis (a). The embryo axis shows the vascular strand (v) diverting into the two cotyledons. The plumule (p) and the radical (r) of the embryo axis shows highly concentrated meristematic cells. (bar = 500µm)

(B) Histology of somatic embryo like fleshy embryogenic structures with dense cytoplasm and prominently stained nuclei. Strand of vasculature (v) seen along the length of the structure. (bar = 385µm)

(C) Torpedo stage embryo/ embryogenic structure with cup shaped tip (t). The vascular connection seen from the tip of the structure upto the radicle end (r). (bar = 435µm)

Structures (B) and (C) developed on the IZE in presence of dicamba 5mg.l^{-1}

However the differentiation of vasculature was maintained. Therefore the strand of vasculature was seen passing through this embryogenic structure. On examination of the funnel shaped structures (Fig.5.17C) it appeared that the tip developed in form of a cup. On examining the sections at different levels it also shows that the vascular connection is maintained from the tip of the structure till the radicle end. The cotyledonary nodal region or the portion of epicotyl immediately above the node was swollen due to the proliferation of the cells in the subepidermal layers and in the central part of the IZE.

In some explants somatic embryos developed from the cotyledonary node junction. Transverse section of these explants (Fig.5.18A) confirms that these somatic embryos are multicellular in origin and somatic embryogenesis was asynchronous. Embryos at various stages of development were noted in the same section (Fig.5.18B). No vascular connection was seen between the IZE and the somatic embryos developed from these IZE.

In some of the IZE axis derived embryogenic structures primary somatic embryos developed from the apical region (Fig.5.19A). Histological studies on structures with multiple embryos confirm that the somatic embryos develop from both apical region and cotyledonary node region (Fig.5.19B,C). The embryos developed from these two regions could be at different stages of development. The somatic embryos appear to originate directly from epidermal and subepidermal layers of the explant with no visible callus, suggesting that the cells of the meristematic region may be preembryonically determined cells (PEDCs) (Sharp *et al*., 1982). It also confirms that the somatic embryos are multicellular in origin. Formation of secondary embryo from the primary embryo was also confirmed by the histological studies (Fig. 5.19C).

5.3.6 Maturation / germination / conversion of somatic embryos

Somatic embryos developed from the IZE explants were subjected to various treatments for further morphogenetic development to achieve maturation and conversion of embryos. The treatments included, shifting on plain basal medium with or without charcoal, basal medium with 10% sucrose, medium with reduced dicamba concentration, media supplemented with different cytokinins like KN, BAP, Zeatin, TDZ alone

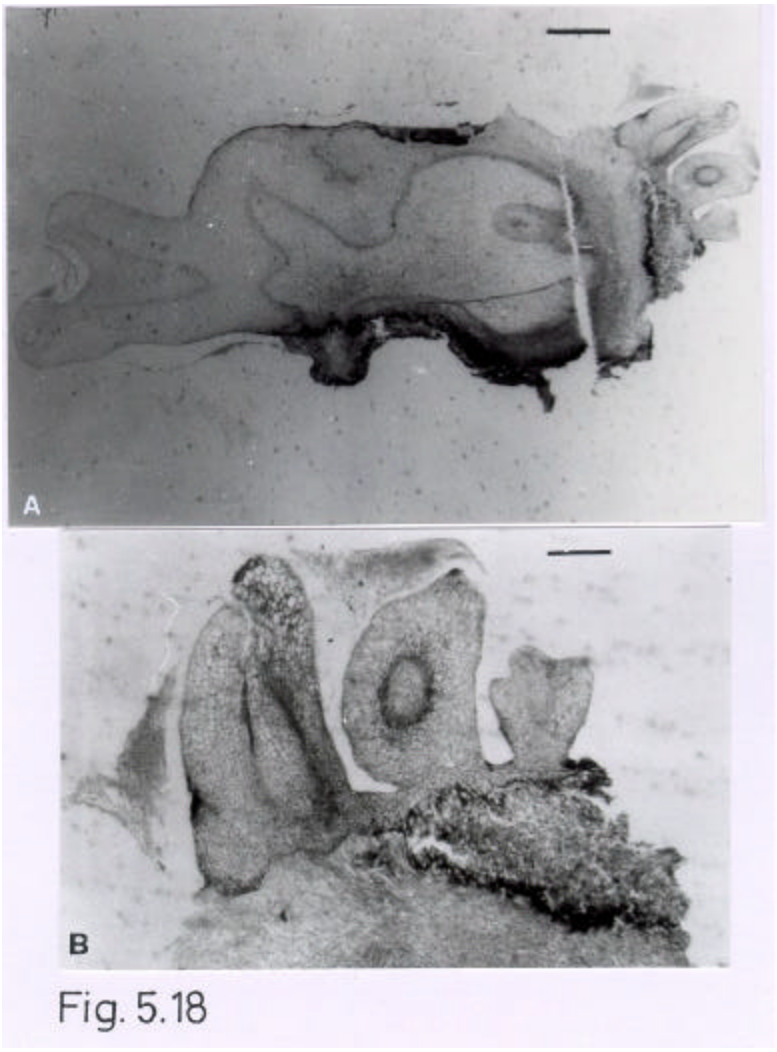

Fig. 5.18

Fig. 5.18 : (A) Transverse section passing through the cotyledonary node junction show embryo formation from both the cotyledonary nodal region. (bar = 435μm)

(B) Asynchronous development of somatic embryos which are multicellular in origin. No vascular connection between the somatic embryos and the IZE (bar = 227μm)

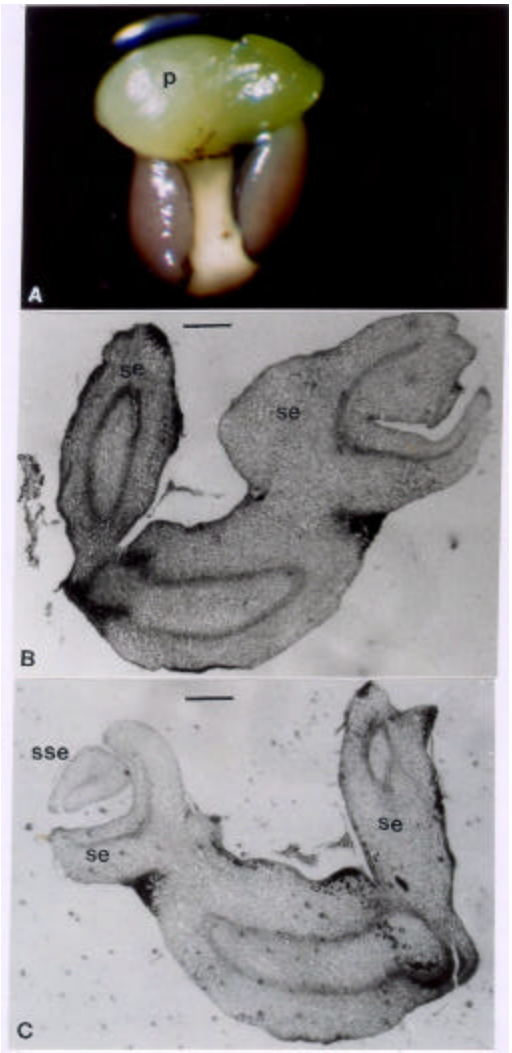

Fig.5.19

Fig. 5.19 : (A) Primary somatic embryos (p) developed from the apical region of the embryo / embryogenic structure.

(B) Somatic embryo developed from both apical meristem and cotyledonary nodal junction. In this cluster embryos developed from the nodal region was torpedo shaped whereas the one developed from the tip was at the cotyledonary stage. These embryos appear to originate directly from the epidermal and subepidermal layers of the embryo/embryogenic structure. The embryos are multicellular in origin. (bar = 250µm)

(C) Formation of secondary embryo (sse) from the tip of the primary somatic embryos. (bar = 250µm)

or in combinations directly or after giving ABA treatment. Abscisic acid has been described as an effective plant growth regulator leading to normal maturation of somatic embryos (Ammirato, 1974; Roberts *et al*., 1990). Rooting was observed in the embryogenic structures sporadically, once in each of the following media:-

(i) Half strength MS basal medium ® Half strength MS basal medium supplemented with 0.25% charcoal

(ii) MS basal medium supplemented with 10% sucrose (Fig.5.20A)

(iii) MS basal medium supplemented with ABA 0.1 $mg.l^{-1}$ (Fig.5.20B)

(iv) MS basal medium supplemented with phytagel 0.3% (Fig.5.20C)

(v) MS basal medium supplemented with 10mg/l NAA ® Half strength MS basal medium (Fig.5.20D)

However, the shoot did not differentiate and the root also was thick and stunted, which later deteriorated.

There are several reports describing apparently well formed somatic embryos that failed to produce plantlets (Ammirato, 1987). The failure in conversion of somatic embryos has been attributed to malformation of epicotyl pole (Kerns *et al*., 1986) or abnormal shoot apex development (Trigiano *et al.*, 1988). Weaver and Trigiano (1991) achieved low conversion of *Cladrastris lutea* somatic embryos. The failure in conversion of embryos was attributed to physiological immaturity or anatomical abnormalities. Das *et al*. (1997) reported that the rooted somatic embryos could not be converted to plantlets in *Dalbergia sissoo*. Germination of the somatic embryos of *Hardwickia binata,* also could not be achieved (Das *et al.*, 1995).

The efficiency of plant recovery from somatic embryos of all species continues to be unacceptably low because of our lack of understanding of basic developmental processes that underlie zygotic and somatic embryogeny. Utilization of plant cell regeneration systems in biotechnology ultimately must depend on a deeper understanding of developmental control mechanism (Litz and Gray, 1992).

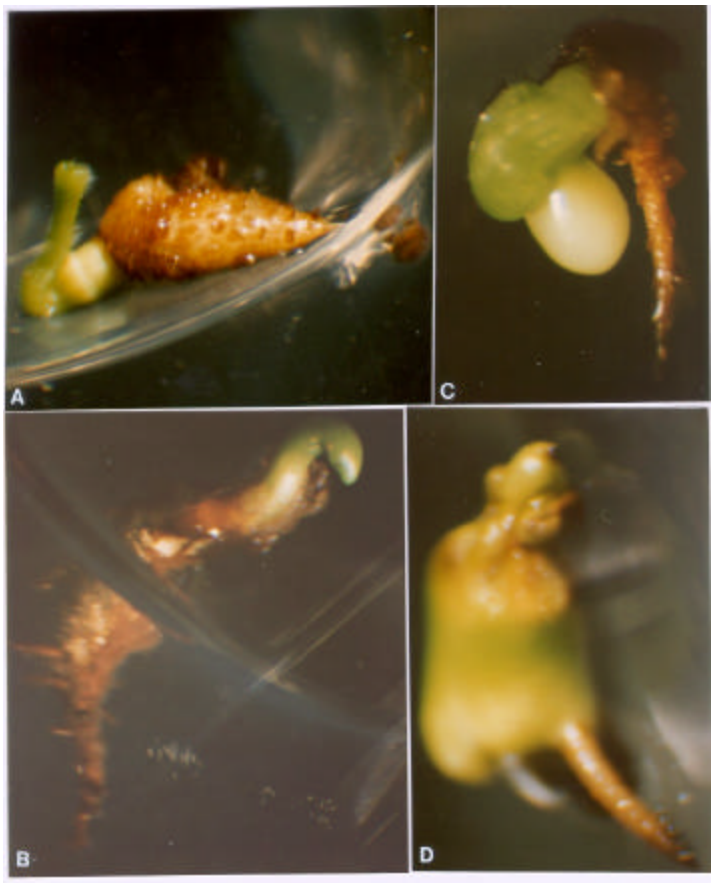

Fig. 5.20

Fig. 5.20 Germination of somatic embryo in :

(A) MS medium supplemented with 10% sucrose

(B) MS basal medium supplemented with ABA (0.1 $mg.l^{-1}$) for 15 days and shifting to MS medium

(C) MS basal medium supplemented with 0.3% phytagel

(D) MS basal medium supplemented with NAA 10 $mg.l^{-1}$ and then transferred to half strength MS medium

Low frequency embryo production, poor germination and conversion of somatic embryos into plantlets and somaclonal variation are the major impediments limiting the utility of SE for biotechnological applications in legumes (Lakshmanan and Taji, 2000). These limitations however may be considerably reduced in the near future as more newly developed growth regulators with specific morphogenic targets become available for experimentation. Efforts should be given to develop repetitive embryogenic systems, since such systems will find immediate application in mass propagation and other crop improvement programs. As understanding of various morphogenic processes, including growth and differentiation of zygotic embryos, is fast expanding, it is conceivable that development of highly efficient somatic embryogenic systems with practical application can be anticipated at least for the important leguminous crops, in the foreseeable future.

5.4 CONCLUSIONS

Developing zygotic embryo explants possess a high potential for SE. Unfortunately, the limitations involving these explants are that of their limited period of availability on an annual basis, difficulty in obtaining the plant material at the correct developmental stage and potential seasonal variations. Somatic embryogenesis in tree legumes has mostly been reported from immature zygotic embryos and immature cotyledonary explants.

In this study, we demonstrated development of somatic embryos in tamarind for the first time. Somatic embryos could be induced from embryogenic structure raised from the immature zygotic embryo explants of size 1-6mm on MS medium supplemented with Dicamba 5 $mg.l^{-1}$, 6% sucrose and 0.22% phytagel at frequency of about 20% out of which about 2-3% are in the form of multiple structures. Multiple somatic embryos could be induced in test-tubes only and not in petridishes. The embryo development was asynchronous. This morphogenetic response was observed in the IZE explants collected from various plants, at different frequencies. This suggested the influence of genotype on embryo induction. However, the maturation, germination and conversion of the somatic embryos into plantlets could not be achieved.

Formation of secondary somatic embryos could be observed from the apical region of the primary somatic embryos. Their growth was asynchronous. Cultures slowly proliferating by repetitive SE are being maintained for nearly two years. This is an attempt to establish SSE/RE and maintenance of embryogenic line.

Histological studies revealed that the apical meristem of the IZE explant elongated into an embryogenic structure with high meristematic activity. Somatic embryos originated from the apical and cotyledonary nodal region. Primary somatic embryos appeared directly from the epidermal and subepidermal tissue of these embryogenic structures.

CHAPTER 6: MORPHOGENETIC RESPONSE IN SEEDLINGS GERMINATED IN PRESENCE OF THIDIAZURON

6.1 INTRODUCTION

Thidiazuron (N-phenyl-N'1,2,3-thiadiazol-5-ylurea), a substituted phenylurea compound developed for mechanized harvesting of cotton boll, has now emerged as a highly efficacious bioregulant of morphogenesis in the plant tissue culture. Application of thidiazuron (TDZ) induces a diverse array of culture responses ranging from induction of callus to formation of somatic embryos. Despite the structural dissimilarity, TDZ exihibits the unique property of modulating both auxin and cytokinin effect on growth and differentiation of cultured explants. Cytokinins can be classified into atleast two broad groups, viz. adenine and phenylurea derivatives, according to their chemical structure (Mok *et al*., 1987). In TDZ molecule, the replacement of either of two functional group i.e. phenyl and thidiazole, with other ring structures results in the reduction in its activity (Mok *et al*., 1982). The activity of TDZ varies widely depending on its concentration, exposure time, cultured explant and species. A number of physiological and biochemical events in cells are likely to be influenced by TDZ which are responsible for the ultimate result of morphogenic responses.

In 1982, TDZ was reported to show high activity in promoting growth of cytokinin-dependent callus cultures of Phaseolus lunatus CV Kingston (Mok *et al*.,1982). Cytokinin activity of TDZ was the highest of several thiadiazolylurea derivatives tested and exceeded that of zeatin. The ability of TDZ to stimulate cell division has been demonstrated in soybean callus (Thomas and Katterman, 1986). This PGR induces adventitious shoot formation from tobacco leaf discs and stimulate radish cotyledon expansion (Thomas and Katterman, 1986). Morphogenetic effects of TDZ has been evaluated by several researchers (Huetteman and Preece, 1993; Mohamed *et al*., 1992; Malik and Saxena, 1992a; Mok *et al*. 1987; Saxena *et al*. 1992; Visser *et al*., 1992).

The mechanism by which TDZ induces cytokinin-like responses is not understood. One theory is that TDZ promotes conversion of cytokinin ribonucleotides to biologically more active ribonucleotides (Capelle *et al*., 1983). Other researchers suggest that TDZ supports synthesis of endogenous purine cytokinins or inhibits their degradation (Thomas and Katterman, 1986; Mok *et al*., 1987). Laloue and Fox (1989) demonstrated that phenylureas inhibit cytokinin oxidase activity. TDZ can substitute auxin/cytokinin requirement for induction of somatic embryogenesis in geranium and in inducing

embryogenesis in peanut, which depends upon the presence of auxins in the culture medium. Thus it is suggested that TDZ may modulate endogenous auxin levels (Saxena *et al*., 1992; Visser *et al*., 1992).

There are reports on induction of adventitious shoot formation in a number of species, especially woody plants (Lu, 1993; Huetteman and Preece, 1993). A vast amount of literature generated on TDZ-induced physiological and morphological responses in a wide variety of plant species is reviewed by Murthy *et al*. (1998). Upreti and Dhar (1996) achieved shoot proliferation (96.20%) and multiplication (5.55 shoots/explant) from cotyledonary node explant of Bauhinia vahlii Wight and Arnott- a leguminous liana, cultured in MS basal medium containing 1µM TDZ. Wilhelm (1999) noted best proliferation of adventitious shoot formation when hypocotyl and plumule sections of the zygotic embryo of sycamore maple (*Acer pseudoplatanus*) were cultured in MS basal medium with 0.04µM TDZ and 1.0µM BAP. Effect of TDZ on adventitious bud and shoot formation from hypocotyl segment of sweetgum (*Liquidamber styraciflua* L.) a hardwood tree, was tested singly and in combination with 2,4-D, by Kim *et al*. (1997). The combination of 1 $mg.l^{-1}$ TDZ with 0.01 $mg.l^{-1}$ 2,4-D, resulted in highest frequency of bud production. Sankhla *et al*. (1996) demonstrated that TDZ was highly effective in stimulating shoot formation from the root segments (1cm long), excised from seedlings of silk tree (*Albizzia julibrissin*). At 0.05µM TDZ, 95% of the explants produced shoots and about 10 shoots were formed per explants.

In a review on somatic embryogenesis, Lakshmanan and Taji (2000), have identified TDZ as one of the new generation growth regulators, emerging as successful alternatives for high-frequency direct regeneration of somatic embryos, even from well differentiated explant tissues. Somatic embryo-like structures have been observed on immature cotyledons of walnut cultured on woody plant medium containing TDZ and 2,4-D (Neuman *et al*.,1988). SE in presence of TDZ was reported in watermelon (Compton and Gray, 1992) and muskmelon (Gray *et al*.,1992). In geranium, direct SE was obtained from hypocotyls on medium containing 0.22 $mg.l^{-1}$ TDZ (Qureshi and Saxena, 1992). Somatic embryos were observed on 80-90% of peanut seedlings cultured on medium with 0.22 $mg.l^{-1}$ TDZ. All seedlings produced somatic embryos when TDZ at 1.1-8.8 $mg.l^{-1}$ was included in the medium and no significant differences in the numbers

of somatic embryos per seedling were observed at these concentrations (Saxena *et al.*, 1992).

Merkle *et al.* (1997) reported that mature sweetgum trees could be regenerated via somatic embryogenesis at a low frequency by culturing parts of staminate inflorescence from mature trees on TDZ medium. It was further demonstrated (Merkle *et al.* 1998) that on TDZ medium embryogenesis can be induced from multiple genotypes, using seeds or inflorescence tissues as explants.

Sunnichan *et al.* (1998) observed that the nodular callus produced from hypocotyl explants of a medium sized tree, gum karaya (*Sterculia urens* Roxb.) cultured on MS media supplemented with 2,4-D and BAP gave rise to somatic embryos, on transfer to MS media containing 0.45µM TDZ. Direct and indirect somatic embryogenesis in neem, was achieved by Murthy and Saxena (1998) on culturing mature seeds in medium supplemented with a wide range of TDZ concentrations (1-50µM). According to Xie and Hong (2001) the medium containing 2mg/l TDZ and 0.25 $mg.l^{-1}$ IAA was most effective (16.41%) for inducing embryogenic calli from IME of *Acacia magnium*.

Morphogenetic response of tamarind tissues in culture is very poor. Explants taken either from seedling or from mature plant dedifferentiate very often irrespective of culture conditions. When the explants are cultured in medium, phenolics is often produced from the wounded part in contact with the medium and dedifferentiation starts from the injured tissue. This poses a serious limitation on studies on morphogenesis in tamarind. Our efforts to culture various seedling explants, mature buds and IZE in 1 and 5 $mg.l^{-1}$ TDZ containing medium to induce morphogenesis led to profuse callusing from the wounded part of the tissue. Keeping in view, the literature on effect of TDZ on intact seedling and to avoid the wound in the explant, an attempt was made to seek the morphogenetic effect of TDZ in tamarind seedlings germinated in medium containing TDZ. This procedure based on culturing intact seedlings, rather than excised plant parts in presence of TDZ was tested earlier in soybean (Wright *et al.*, 1986; Hinchee *et al.*, 1988). It avoids manipulations required with the use of explant optimization of many factors such as physiological age of the explant, and its orientation, culture vessel, all of which have been shown to be important for regeneration of legumes from explants (Hammatt *et*

al., 1986; Lazzeri *et al*., 1987). As direct morphogenesis from intact seedlings offers several advantages as on experimental system (Malik and Saxena, 1992), the current investigation was initiated to test the feasibility of using this simple approach for inducing regeneration in tamarind.

6.2 EXPERIMENTAL PROTOCOL

6.2.1 Preparation of explant /Culture Induction.

Tamarind seeds were collected from the mature pods of locally grown trees. The seeds were washed thoroughly and surface sterilized (as in section 3.2A.1). These were then cultured on MS basal medium supplemented with varying concentrations (1, 2, 3, 4 $mg.l^{-1}$) of TDZ and 2% sucrose. Media were solidified using $7gm.l^{-1}$ agar. The pH of the medium was adjusted to 5.6-5.8 prior to addition of agar. Cultured seeds were incubated in dark or light at $25\pm2^{0}C$ for 6 weeks.

On completion of 6 weeks, the cracked seed coat was removed and seedlings were transferred on agar-gelled MS basal medium devoid of TDZ and supplemented with 2% sucrose. Number of cultures showing epicotyl, hypocotyl, root differentiation and elongation after 6 weeks was scored. The height of epicotyl and lengths of hypocotyl and root were measured. These measurements were taken from outside the tube. The number of seedlings showing proliferation of existing nodal meristem or induction of de novo organogenesis was scored and the frequency of response was determined. All the cultures whether initiated in dark or in light, were incubated in light at $25\pm2^{0}C$ from this step onwards. Shifting of seedlings on fresh MS medium was repeated three times at an interval of 15 days. After the third transfer cultures were incubated for 6 weeks and similar measurements were taken. The data was subjected to statistical analysis. These cultures were incubated for an extended period of 4 weeks for further elongation of shoots. At the end of this period, number of shoots or shoot buds in each seedling showing differentiation and elongation were noted. The average number of shoots or shoot buds showing differentiation per seedling was determined.

6.2.2 Micrografting of TDZ-induced shoots

The elongated axillary and adventitious shoots were excised aseptically from the TDZ-exposed seedling and used as scion for the *in vitro* and *ex vitro* micrografting. After removing the elongated shoots the seedlings with numerous shoot buds were transferred to fresh medium of same composition for elongation of more shoots. Decapitated *in vitro* grown seedlings were used as the rootstock for in vitro micrografts, while the decapitated seedlings germinated in soil in polybags/pots were used as rootstock for *ex vitro* micrografts. The procedure used for micrografting is described in section 3.2C.

6.2.3 Histological studies

The cotyledonary nodal segments measuring ~1cm. in length was excised from the seedlings grown in TDZ medium for 6 weeks and from those grown in MS basal medium for 6 weeks. The excised segment of TDZ treated seedling was cut longitudinally into two halves. Each piece was cut once more into two halves, so that 4 quarter segments ~1cm in length, were formed. These were used for histological studies. Following 6 weeks of exposure in TDZ the seedlings were cultured in MS medium for 6 weeks for differentiation of the buds. Cotyledonary nodal segments from cultures after 6 weeks of incubation in MS medium were also used for histological studies. Serial sections, 10 µm, were cut using a Reichert - Jung 2050 rotary microtome. Sections double stained with haematoxylin - eosin and mounted with DPX, were studied under microscope. To study the effect of TDZ in the hypocotyl region of the seedling ~1cm piece from just below the cotyledonary nodal segment was cut and fixed in FAA and used for histological preparation.

6.3 RESULTS AND DISCUSSION

6.3.1 Influence of TDZ on germination of seed and differentiation of seedling

Radicle emergence was noted in the seeds after 20 days of culture in MS medium and also in medium supplemented with TDZ. Presence of TDZ in medium did not affect germination. However, the growth of the seedling, was negatively influenced, irrespective of the light conditions. The seedling that developed in presence of TDZ (Fig.6.1A) exhibited reduced elongation of root, epicotyl and hypocotyl (Table 6.1). The roots of the TDZ grown

seedlings were short and thick and the development of secondary roots was arrested. Similar effect on germination has been noted in *Albizzia julibrissin* (Sankhla *et al*.,1994) in presence of 0.1-10 µM TDZ and *Phaseolus acutifolius* A., *P. aureus* (L.) Wilczek, *P. coccineus* L. and *P. wrightii* L.(Malik and Saxena, 1992a) in presence of 50-80 µM BAP. Tamarind tissues produce phenolics in culture, which in turn inhibits growth. In the seedling culture, seed coat is a significant source of phenolics In our study, the medium turned brown and the growth of the seedling was arrested not only due to the presence of TDZ, but also accumulation of phenolics released from the seed coat. Therefore the seed coat was removed prior to transfering the seeds in agar gelled MS basal medium devoid of growth regulator and supplemented with 2% sucrose (Fig. 6.1B).

Table 6.1 : Effect of TDZ on growth and differentiation of tamarind seedlings

Conc. (mg. l^{-1})	Parameter	Response after 6 weeks in TDZ		6 weeks in TDZ + 6 weeks in light in MS basal medium	
		Light	Dark	Cultures from Light	Cultures from Dark
0	cultures showing epicotyl differentiation % Mean±S.D.	75.0±21.2 (20)	26.7±30.6 (30)	65.0±35.4 (20)	66.7±40.7 (30)
1	″	30.0±26.5 (30)	26.7±46.2 (30)	40.0±10.0 (30)	66.7±15.3 (30)
2	″	16.7±28.9 (30)	23.3±40.4 (30)	30.0±10.0 (30)	43.3±11.6 (30)
3	″	10.0±17.3 (30)	6.7±11.6 (30)	40.0±20.0 (30)	46.7±15.3 (30)
4	″	16.7±28.9 (30)	10.0±14.1 (20)	36.7±11.6 (30)	35.0± 7.1 (20)
ANOVA		**NS**	**NS**	**NS**	**NS**

Conc. (mg. l^{-1})	Parameter	Response after 6 weeks in TDZ		6 weeks in TDZ + 6 weeks in light in MS basal medium	
		Light	Dark	Cultures from Light	Cultures from Dark
0	Ht.of epicotyl (cm) Mean±S.D	3.9±0.1 (20)	2.6±2.3 (30)	3.6±0.9 (20)	5.7±0.3 (30)
1	″	0.9±1.0 (30)	0.2±0.3 (30)	3.3±1.0 (30)	2.0±1.1 (30)
2	″	0.2±0.3 (30)	0.1±0.1 (30)	1.3±0.7 (30)	0.8±0.4 (30)
3	″	0.1±0.2 (30)	0.1±0.2 (30)	1.8±1.1 (30)	1.0±0.6 (30)
4	″	0.1±0.2 (30)	0.3±0.4 (20)	2.0±1.9 (30)	1.2±0.1 (20)
ANOVA		**S**	**NS**	**NS**	**S**
0	cultures showing hypocotyl differentiation % Mean±S.D	80.0±14.1 (20)	61.5±7.8 (30)	70.0±28.3 (20)	77.0±15.7 (30)
1	″	83.3± 5.8 (30)	56.7±41.6 (30)	76.7±11.6 (30)	93.3± 5.8 (30)
2	″	80.0±10.0 (30)	63.3±37.9 (30)	76.7±11.6 (30)	96.7± 5.8 (30)
3	″	83.3± 5.8 (30)	66.7±35.1 (30)	86.7± 5.8 (30)	96.7± 5.8 (30)
4	″	86.7±15.3 (30)	60.0±42.4 (20)	86.7± 5.8 (30)	75.0±21.2 (20)
ANOVA		**NS**	**NS**	**NS**	**NS**
0	Length of hypocotyl (cm) Mean±S.D	7.7±0.5 (20)	5.5±4.5 (30)	7.7±0.2 (20)	7.8±1.3 (30)
1	″	2.6±0.4 (30)	1.9±0.8 (30)	3.7±0.2 (30)	4.0±1.4 (30)
2	″	2.1±0.6 (30)	1.8±0.2 (30)	3.0±0.6 (30)	2.8±0.2 (30)
3	″	1.8±0.5 (30)	1.8± 0.6 (30)	3.1±0.2 (30)	2.9±0.1 (30)
4	″	2.2±0.6 (30)	1.6±0.4 (20)	2.9±0.2 (30)	3.1±1.3 (20)
ANOVA		**S**	**NS**	**S**	**S**
0	cultures showing root differentiation % Mean±S.D	80.0±14.1 (20)	61.5± 7.8 (30)	75.0±21.2 (20)	77.0±15.7 (30)
1	″	83.3± 5.8 (30)	56.7±41.6 (30)	76.7±11.6 (30)	93.3± 5.8 (30)
2	″	80.0±10.0 (30)	63.3±37.9 (30)	76.7±11.6 (30)	93.3± 5.8 (30)
3	″	80.0±10.0 (30)	66.7±37.1 (30)	86.7± 5.8 (30)	93.3± 5.8 (30)
4	″	86.7±15.3 (30)	50.0±28.3 (20)	90.0± 0.0 (30)	70.0±14.1 (20)
ANOVA		**NS**	**NS**	**NS**	**S**

Conc. (mg. l^{-1})	Parameter	Response after 6 weeks in TDZ		6 weeks in TDZ + 6 weeks in light in MS basal medium	
		Light	Dark	Cultures from Light	Cultures from Dark
0	Length of root (cm) Mean±S.D	4.4±3.2 (20)	2.7±1.9 (30)	3.0±1.9 (20)	4.6±2.4 (30)
1	″	0.9±0.1 (30)	1.1±0.5 (30)	1.2±0.3 (30)	1.3±0.2 (30)
2	″	0.9±0.1 (30)	0.8±0.2 (30)	1.2±0.2 (30)	1.0±0.1 (30)
3	″	0.8±0.1 (30)	0.8±0.1 (30)	1.1±0.2 (30)	0.9±0.1 (30)
4	″	0.9±0.3 (30)	1.0±0.0 (20)	1.3±0.1 (30)	1.1±0.0 (20)
ANOVA		**S**	**NS**	**S**	**S**

Figures in parenthesis indicate number of replicates

Tamarind seedlings in MS basal medium with 2% sucrose and without PGR grew faster than that grown in TDZ enriched media and the apical meristem of these plants grew and several leaves appeared. The plants developed healthy roots with several secondary roots (Fig.6.2 A). The axillary buds in the cotyledonary node, occasionally showed presence of two three shoot primordia. These primordia were developed from existing meristem and did not elongate probably due to apical dominance. In contrast to the MS medium growth of the primary shoot apices of most seedlings raised on media containing TDZ ceased (Fig.6.2 B). Axillary shoots did not grow, but the areas adjacent to the cotyledonary node became meristematic and produced numerous shoot buds (Fig.6.2 C, 6.3). The cultures growing in TDZ medium for 6 weeks and incubated in light showed higher frequency of organogenic response than those incubated in dark (Table 6.2).

Table 6.2: Effect of TDZ concentration on frequency of cultures showing organogenesis in intact seedling of tamarind.

Conc. of TDZ (mg. l^{-1})	After 6 weeks in TDZ Frequency of response %		6 weeks in TDZ + 6 weeks in light in MS Frequency of response %	
	Light	Dark	Cultures from Light	Cultures from Dark
0	0.0± 0.0 (20)	0.0 ± 0.0 (30)	0.0± 0.0 (20)	0.0± 0.0 (30)
1	46.7±41.6 (30)	26.7±46.2(30)	70.0±10.0 (30)	66.7±15.3 (30)
2	26.7±23.1 (30)	26.7±37.9 (30)	53.3±15.3 (30)	83.3±11.6 (30)
3	33.3±30.6 (30)	23.3±40.4 (30)	63.3±25.2 (30)	83.3±11.6 (30)
4	40.0±45.8 (30)	30.0±42.4 (20)	76.7±23.1 (30)	65.0± 7.1 (20)
ANOVA	**NS**	**NS**	**S**	**S**

Figures in parenthesis indicate number of replicates

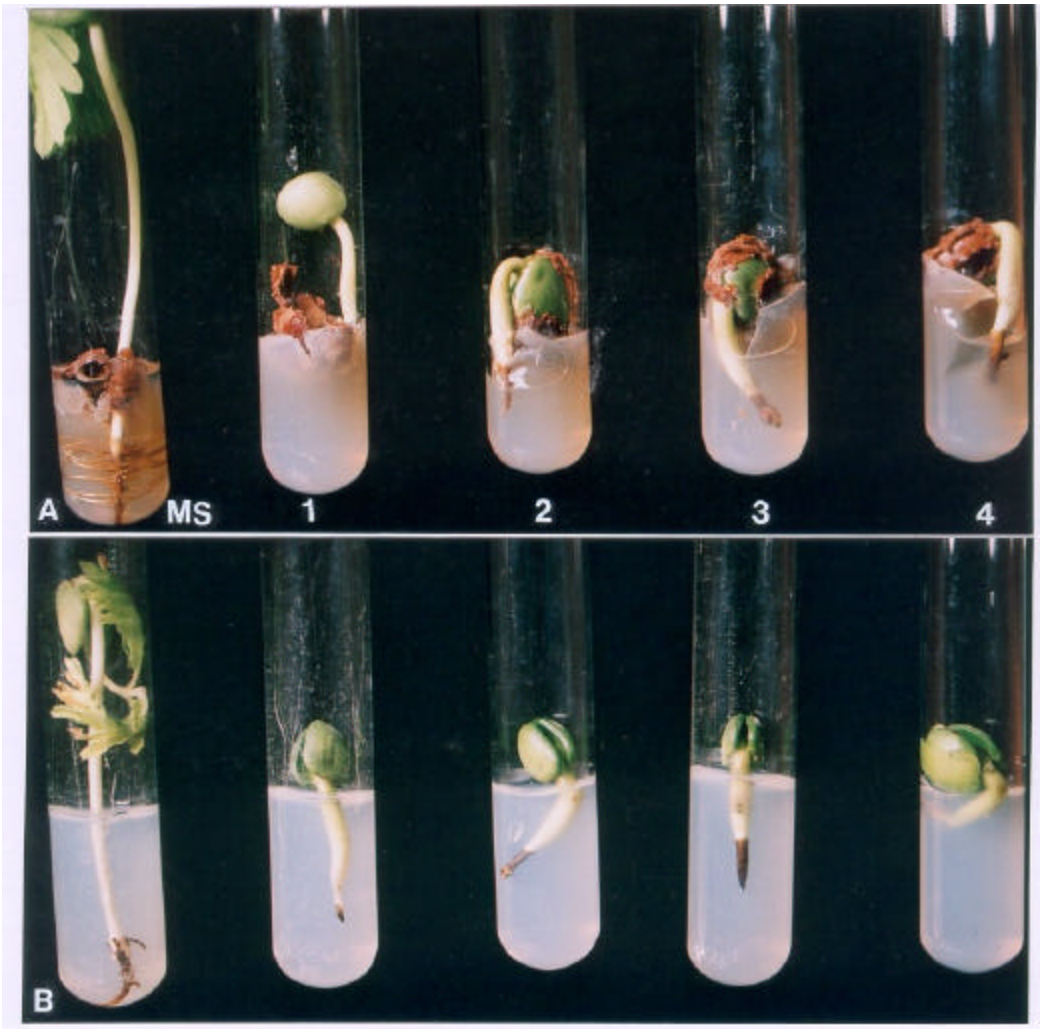

Fig. 6.1

Fig. 6.1A : Tamarind seedlings growing on MS basal medium devoid of TDZ and with various concentrations of TDZ (1,2,3,4 $mg.l^{-1}$). Gradual reduction of epicotyl, hypocotyl and root elongation with increase in TDZ was noted.

B : Germinated seed shifted to MS basal medium after 6 weeks exposure to TDZ and after removal of seed coat

Fig. 6.2

Fig. 6.2 : A : Seedling growing in medium without TDZ showed healthy roots

B : Seedling raised in presence of TDZ (in majority of the cultures) showed reduced growth of primary shoot apex

C : High meristematic activity in the cotyledonary node as well as in the region between the nodes in seedling grown in TDZ medium

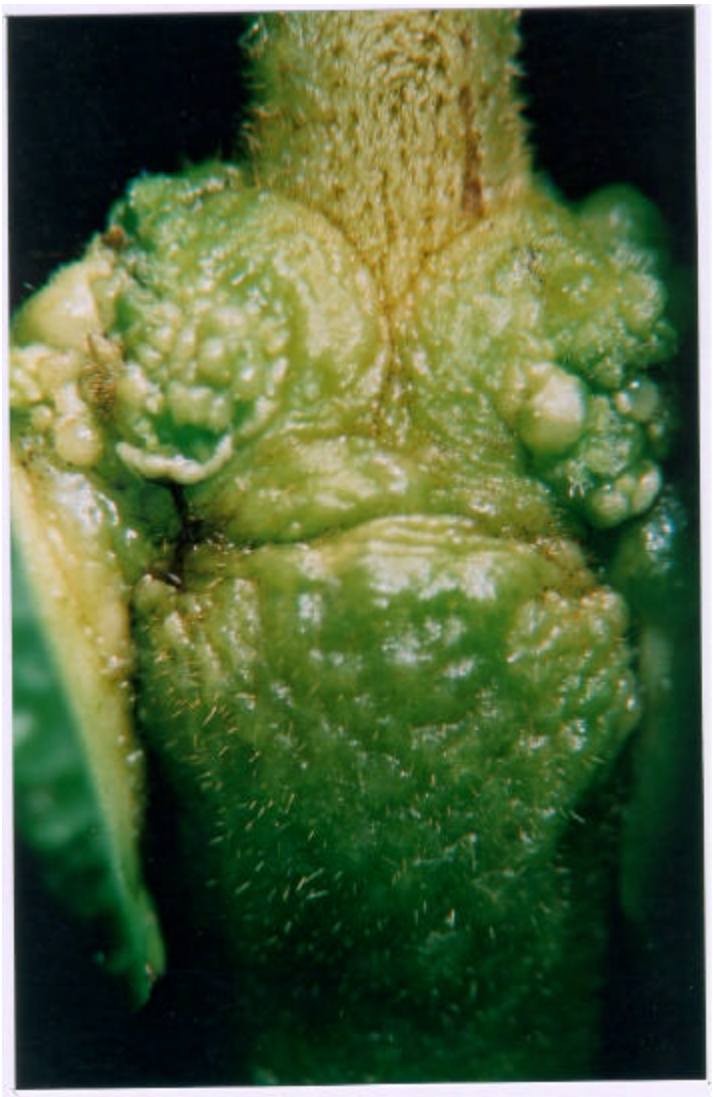

Fig. 6.3

Fig.6.3 : A close view of the localized meristematic activity in the cotyledonary node as well as in the region between the nodes in seedling grown in TDZ medium.

Organogenesis has been induced on intact seedlings of several herbaceous legume species grown on media supplemented with cytokinins (Malik and Saxena, 1992; Malik and Saxena, 1992a; Saxena *et al*., 1992). Two types of morphogenic responses have been observed: (1) The induction of prolific shoot formation from the cotyledonary node and areas surrounding the shoot apex (Malik and Saxena, 1992) (2) in planta differentiation of somatic embryos in the apical region and on the surface of cotyledons and hypocotyls. In the presence of relatively high levels of benzyl adenine (50-80µM), both somatic embryogenesis and shoot regeneration were observed with intact seedlings of *Phaseolus coccineus* (Malik and Saxena, 1992a). The researchers attributed this high efficiency regeneration response to the high level of BAP during seed culture without disturbing the morphological integrity of the seedling. However, the seedlings of *Albizzia julibrissin* (Sankhla *et al*., (1994) were distinctive in that they neither produced prolific shoot or somatic embryo from shoot tissue. Instead, shoots were regenerated from roots of intact seedlings.

TDZ, a potent cytokinin for woody plant tissue culture has been used for axillary shoot proliferation and adventitious regeneration. Although adventitious shoot production is generally undesirable for clonal propagation, it can represent an excellent opportunity to regenerate plants from tissues manipulated through biotechnology (Huetteman and Preece, 1993). The dose of TDZ is critical in regeneration studies. Lower concentration, stimulated only axillary shoot proliferation whereas in higher, adventitious shoot production was observed (Chalupa, 1988; van Nieuwkerk *et al*.1986; Yusnita *et al*.1990) in *Quercus robur*, *Malus domestica* and *Cercis canadensis*.

There are reports describing TDZ induced SE in intact seedlings of peanut. It is suggested that SE is induced by influencing endogenous levels of both auxins and cytokinins (Murthy *et al*., 1995). The embryogenic potential of seedlings was limited in explants without cotyledons and they did not respond to increasing levels of TDZ. In contrast, retention of atleast one or both cotyledons resulted in increased response to TDZ. Apart from a nutritional role, cotyledons may also have a regulatory role (Murthy *et al*., 1995). Somatic embryogenesis in intact seedling provide an excellent system to study the role of different metabolic pathways in regeneration due to the maintenance of

complete structural integrity and a physiological continuum of seedlings as compared to the culture of excised tissues.

The organogenic response appeared in more number of tamarind seedlings on shifting the cultures thrice (every fortnightly) on MS basal medium and incubating in light. Thus, it was observed that there was an increase in the frequency of cultures showing organogenic response, irrespective of whether the cultures were initiated in dark or in light. Possibly the response initiated at the cellular level during TDZ exposure became visible only after prolonged culture in medium devoid of growth regulator. The light-initiated TDZ-exposed cultures on shifting thrice to medium devoid of growth regulator in light showed an increase of 1.5 to 2 times in organogenic response, whereas cultures initiated in dark showed 2 to 3 times increase (Table 6.2).

Cytokinins like BAP, at higher concentrations, commonly stimulate shoot proliferation and inhibit their elongation. Therefore, inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity and should not be considered a toxic effect. The problem of shoot elongation can be overcome by transfer of shoot cultures to a secondary medium often lacking TDZ or with a different balance of plant growth regulators (Huetteman and Preece, 1993). Use of primary and secondary media has been successful with apple (Fasolo *et al*., 1989), pear (Singha and Bhatia, 1988), Populus (Russell and McCown, 1986), and Rhododendron (Preece and Imel, 1991). In Rhododendron a primary medium was used to maximize shoot proliferation, and the shoot or bud masses were transferred to secondary medium with other combinations of plant growth regulators. Preece and Imel (1991) reported that the majority of the shoots (induced on leaf explant) regenerated on TDZ medium, were short, but elongated after transfer to medium containing TDZ and IBA. Distabanjong and Geneve (1997) observed that in eastern redbud (*Cercis canadensis* L.) greatest number of shoots were observed in cotyledonary node explants in medium with the combination of 10 µM BAP and 0.5 or 1.0 µM TDZ cultured for 20 days prior to transfer to a medium containing BAP singly.

In the present studies, shifting the seedling to medium without TDZ, resulted in differentiation of the shoot buds (Fig.6.4A,) induced in presence of TDZ. Changing the medium three times was helpful to reduce the carry over TDZ in the seedlings. The

process of differentiation of the buds and formation of elongated shoot was asynchronous. Therefore elongated shoots and partially differentiated buds (shoot primordia) were seen in the same culture (Fig6.4B).

Number of shoot and shoot primordia seen in each explant were scored under the microscope. However, these are approximate figures as all the buds could not be counted (Table 6.3). Excision of the elongated shoots and reculturing of the seedling in growth regulator free medium triggered elongation of a second crop of shoots, which elongated and could be used for micrografting after excision. In many cases, numerous meristematic buds were observed in the axils of the first leaves and shoot meristem (Fig. 6.4 C).

Table 6.3 : Number of shoot or shoot bud differentiation in each seedling

Conc. of TDZ ($mg.l^{-1}$)	Frequency of seedlings showing shoot or shoot bud differentiation		Average number of shoots or shoot buds showing differentiation / seedling	
	Cultures from Light	Cultures from Dark	Cultures from Light	Cultures from Dark
0	100.0± 0.0 (8)	100.0± 0.0 (14)	3.4±1.5 (8)	3.6±1.3 (14)
1	83.0±20.7 (20)	96.3± 6.4 (28)	7.6±1.8 (17)	13.6±4.7 (27)
2	95.2± 8.3 (19)	77.0± 6.7 (25)	7.5±1.7 (15)	6.0±3.0 (19)
3	80.4±22.7 (24)	68.3±10.4 (26)	11.4±1.5 (19)	7.6±6.7 (18)
4	91.5± 7.5 (24)	75.0±35.4 (13)	12.1±0.7 (22)	7.7±8.0 (10)
ANOVA	**NS**	**NS**	**S**	**NS**

Figures in parenthesis indicate number of replicates

On extended incubation of 4 weeks after the third transfer in MS medium without growth regulators, it was observed that the shoot buds and shoots, differentiated from the buds in the cotyledonary nodes as well as in the region in between the cotyledonary nodes (Fig.6.5A). The seedling grown in the medium

Fig.6.4

Fig. 6.4 : (A, B) Differentiation of TDZ induced shoot buds in MS basal medium devoid of growth regulator. Asynchronous differentiation of shoots indicate asynchronous induction of buds

(C) Numerous meristematic buds (Arrow) induced in the axil of the first leaves

Fig. 6.5

Fig.6.5 : (A) Numerous shoots elongated from the TDZ induced buds in the cotyledonary segment of the tamarind seedling after three transfers in MS basal medium devoid of growth regulator. Shoots were also seen in the region between the nodes suggesting *de novo* origin of the shoots. Elongation of the seedling was limited.

(B) Seedling grown in medium without TDZ does not show multiple shoots / buds. Normal elongation of seedling was noted.

devoid of TDZ does not show proliferation of multiple shoots from the cotyledonary nodal region (Fig.6.5B).

Lu (1993) emphasized that long exposure to TDZ is not recommended as this may cause hyperhydriticity, abnormal short shoot and difficulty in rooting. Difficulty in elongation and rooting of TDZ regenerated shoots was reported in *Celtis occidentalis* (Meyer and Kerns, 1986) and *Ixia flexuosa* (Meyer and van Staden, 1988). Rooting of excised microshoots may be restricted due to 'carry over' effect of cytokinins used in the shoot proliferation medium. However, with most species TDZ does not seem to inhibit adventitious root formation once shoots are excised (Fasolo *et al*. 1989; Yusnita *et al*. 1990; Preece *et al*. 1991). Exceptions include inhibited rooting of *Vitis rotundifolia* (muscadine grape) (Gray and Benton, 1991), and Rhododendron (Preece and Imel, 1991). In muscadine grape only 8% of the shoots induced in TDZ, form roots compared to 34% rooting in shoots developed in BAP medium (Gray and Benton, 1991). Rooting frequency of leaf derived adventitious shoots of Rhododendron developed in medium with 0.1-10µm TDZ was 0-73% as against 74-100% in shoots grown in 25.75µm 2iP containing shoot induction medium (Preece and Imel, 1991). The reduced rooting capacity was attributed to persistence of TDZ in the shoot. Sankhla *et al*. (1994) noted delay in rooting of TDZ-induced shoot from intact seedlings of *Albizzia julibrissin*. In MS medium with or without 4.9µM IBA, the shoots developed in KN or BAP rooted 4-7 days earlier than those developed in TDZ containing medium. However all shoots developed into normal plantlets within 3 weeks.

Tamarind tissues in culture produce callus on contact of the wounded part to the medium. Moreover rooting in tamarind shoot is poor and requires auxin treatment (Section 3.3 A.3). Often the rooting is associated with callusing at the cut end of the shoot. This results in poor survival of tissue culture raised plantlets of tamarind in soil. Keeping in view the possibility of low rooting frequency due to carry over TDZ and callusing at the cut end in contact of medium and poor rooting and survival, the approach of micrografting was adopted for the TDZ-induced shoots. Micrografting

Fig. 6.6

Fig.6.6 : (A) TDZ induced shoot micrografted on *in vitro* raised rootstock.
(B) TDZ induced shoots micrografted on *in vitro* raised rootstock survived in soil.

studies in section 3.3C.1 showed a 70% success. Hence the TDZ derived, elongated shoots were excised and used for *ex vitro* and *in vitro* grafting.

6.3.3 Micrografting of TDZ-induced shoots

The shoots when micrografted in *in vitro* conditions showed union in 68 % of cultures (Fig.6.6A). These grafts on transfer to soil showed 50 % survival (Fig.6.6B). The shoots grafted *ex vitro*, however, showed only 12 % union and survival of the graft. Similar response was also observed in *ex vitro* grafting of the shoots induced by cytokinins such as KN, BAP etc. (section 3.3C.3).

Although the seed culture method for regeneration has been applied to a number of crop/tree species, the mechanism of the induction and expression of morphogenesis from intact seedlings remains unresolved. Since the differentiation occurs on intact seedling, the number of manipulations required to induce regeneration is reduced to one in comparison to several in procedures involving culture of seedling explants. Moreover, the use of mature seeds avoids labor-intensive optimization of factors associated with explant culture. In addition, direct morphogenic differentiation offers many advantages as an experimental system, for example, in the study of biochemical and molecular events in organ determination and development and the mechanism of action of growth hormones involved. The rapidity and high frequency of direct morphogenesis routinely obtainable in seed cultures are expected to facilitate stable Agrobacterium-mediated or direct, genetic transformation studies.

6.3.4 Histology

On examining the section taken from the cotyledonary segment of the seedling grown in medium devoid of growth regulators only two meristems of the two cotyledonary nodes could be seen (Fig.6.7A). The apical meristem differentiated to shoot. After culturing the seedling in TDZ containing medium for 6 weeks the cotyledonary nodal segment was swollen and the growth and development of meristematic buds was noted all around the nodal segment. For convenience of histological preparation the segment was cut vertically into 4 quarters. Thus the section (Fig.6.7B) shows the histology of nodal segment partially. Distabanjong and Geneve (1997) studied multiple shoot formation from cotyledonary node segments of eastern redbud in presence of TDZ.

These researchers confirmed the *de novo* origin of shoots histologically. In concurrence with their observation in tamarind, shoot bud development was noted at the axillary bud region at the cotyledonary node (Fig.6.7B). A newly formed meristematic layer developed on the cotyledonary node segment all around (in the axil of the cotyledonary node, as well as the region between the two cotyledonary nodal axils). Adventitious shoot primordia initiated as a result of organized directional growth of cells from meristematic cells. Apparently these shoots formed from epidermal and subepidermal cell layers. After 6 weeks of incubation in medium devoid of PGR, the buds differentiated further (Fig.6.7C). This became more evident in the transverse section (Fig.6.7D). Several meristematic buds with shoot apex and leaf primodia (Fig.6.7E) could be seen in the sections. These meristematic buds and shoot primordia were seen in all the four quarters of the cotyledonary node studied. Since there was no vascular connection from the buds, it is apparent that the shoot formation is via *de novo* organogenesis. As suggested by Distabanjong and Geneve (1997) it is presumed that the formation of the meristematic zone on the surface of the cotyledonary segment could be due to proliferation of the meristematic cells at the cotyledonary nodal axil. Similar observations were made by Malik and Saxena (1992a). The differentiation of shoots in cultured seedlings occurred in all cases directly and from evidently subepidermal tissue, without any callus formation. On examining the hypocotyl region in the same sections under the microscope it was apparent that the meristematic activity was not restricted to the cotyledonary segments but also spread to the hypocotyl region (Fig.6.8A). Although the shoots were not visible at the time of harvesting the culture after 6 weeks in TDZ, but meristematic activity could be detected at the subepidermal cells of the hypocotyl region. In the hypocotyl segment (~1cm) below the cotyledonary nodal segment, taken from the seedling after growing in MS medium for 6 weeks following exposure in TDZ, meristematic centers were observed in the subepidermal layers. Differentiation of a subepidermal zone with meristematic centers composed of small, isodiametric, actively dividing cells having dense cytoplasm and large nuclei were noted.

Histology of these type of shoot bud primordia has been shown by Distabanjong and Geneve (1997), which they achieved from excised cotyledonary node segment of Eastern redbud (*Cercis canadensis* L.).

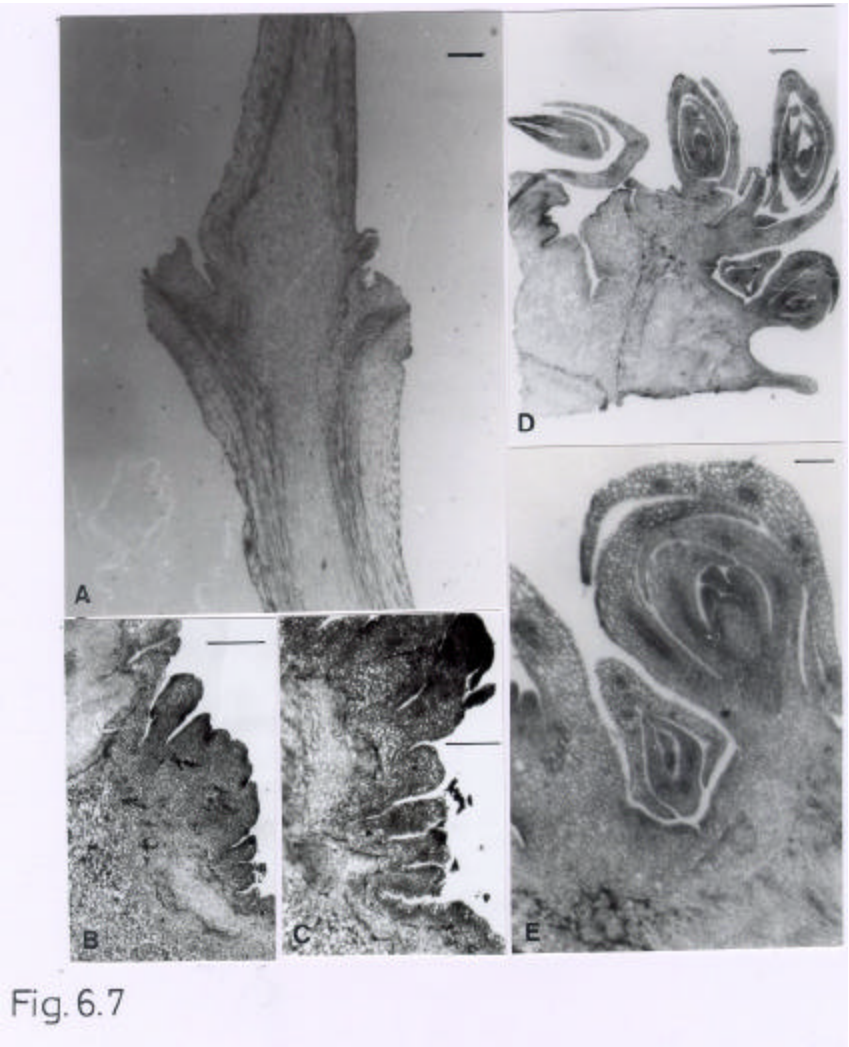

Fig. 6.7

Fig 6.7 : (A) Section of the cotyledonary segment of seedling grown in medium devoid of PGR showed two meristems of the two cotyledonary nodes. Apical meristem differentiated into shoot. (bar = 500µm)

(B) LS of the cotyledonary nodal junction shows newly formed meristematic layers develoved on the cotyledonary nodal segment. Induction of several meritematic buds from the epidermal and subepidermal layers is seen. (bar = 200µm)

(C) LS of the cotyledonary nodal junction from the cultures grown in MS basal medium for 6 weeks following TDZ exzposure shows, more developed meristematic buds. (bar = 200µm)

(D) TS of the quarter portion of the cotyledonary node shows several differentiated shoot primordia. (bar = 250µm)

(E) Well developed shoot primordia with severa leaf primordia was seen at higher magnification. (bar =125µm)

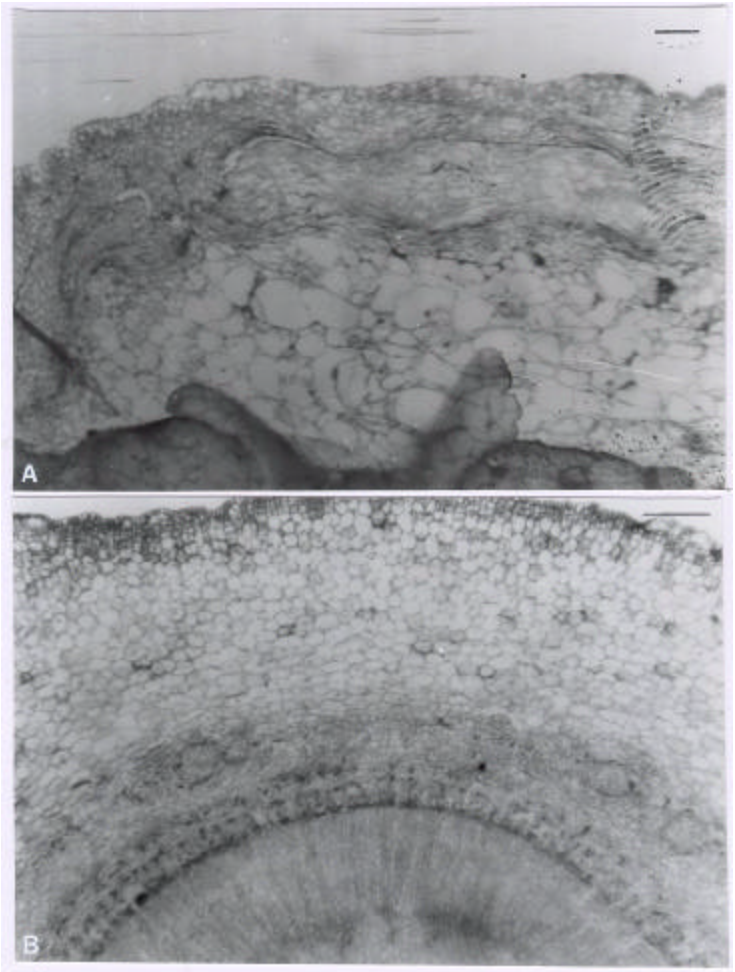

Fig. 6.8

Fig. 6.8 : (A) LS of the cotyledonary node segment showc meristematic activity in the subepidermal regior of the hypocotyl just below the node. (bar = 125µm)

(B) Transverse section of hypocotyl region ~ 1cm below the node showed meristematic centres outside the vascular ring. (bar = 250µm)

6.4 CONCLUSION

Thidiazuron displays typical cytokynin-like properties. Induction of direct morphogenesis in presence of TDZ has been reported earlier. Direct morphogenesis in intact seedlings offers several advantages in application of biotechnological approaches for improvement of plant and also as an experimental system. The present investigation was initiated to test the feasibility of using this simple approach for inducing regeneration in a woody species like *Tamarindus indica*.

Tamarind seeds collected from the locally grown trees were cultured in medium containing TDZ. TDZ did not affect germination, but did influence the subsequent growth of the seedling. The seedling, in presence of TDZ, showed reduced root, epicotyl and hypocotyl elongation. The formation of secondary roots was arrested. The hypocotyl was thick and stout, while the leaves of the shoot meristem did not open. The axillary shoot buds proliferated and the cotyledonary nodal segment also developed several meristematic humps in the localized area, indicating high morphogenetic activity, which was confirmed histologically. These buds, however, did not elongate to form shoots in TDZ medium. Therefore these seedlings with the buds were cultured in medium devoid of growth regulator for prolonged period. The medium was changed frequently to dilute the effect of carry over TDZ. The shoot buds differentiated into shoot primordia on incubation for 6 weeks in medium devoid of growth regulator. Elongated shoots developed from these shoot primordia after the extended incubation of four weeks. The seedling derived shoots were micrografted *in vitro* and *extra vitrum*. The plants developed from the TDZ induced seedling derived micrografted shoots, survived in soil.

SUMMARY

Tamarind (*Tamarindus indica* L.), a tree legume is a multipurpose monotypic genera. Apart from being economically important, due to its hardy nature and wide adaptability to various agroclimatic conditions, it is a suitable candidate for afforestation and soil reclamation / phytoremediation programs.

Very little work has been done in *in vitro* regeneration of tamarind and to study the basic processes involved in morphogenesis. The present study was directed towards optimization of conditions for clonal propagation. Seedling explants - shoot tips and cotyledonary nodes were tested for clonal propagation. These explants were cultured in agar gelled MS basal medium supplemented with 2% sucrose and different cytokinins at various concentrations and combinations. The effect of different carbohydrates was also studied. It was found that BAP 1 $mg.l^{-1}$ supported multiplication and elongation but the leaves of the shoots were not opened. Lower concentration of BAP (0.02-0.5 $mg.l^{-1}$) were beneficial for elongation of shoots and also showed a higher frequency of shoots with opened leaves. Frequency of elongation and height of the shoots, reduced with the increase in the concentration of BAP. Among the two explants tested, cotyledonary node gave higher response in all the parameters studied.

These elongated shoots were rooted after auxin treatment and plantlets were transferred to soil. However, the survival of plants was 21.4%. This method was extended to the mature tree derived axillary nodal explants. Studies using various factors on different parameters revealed that, BAP at 0.1-0.5 $mg.l^{-1}$ supported sprouting and elongation of these buds into shoot. Lower concentration of BAP, yielded more shoots with opened leaves. However, our efforts to root these shoots remained futile.

To improve upon the survival rates of *in vitro* raised tamarind plants in soil, the shoots were micrografted on decapitated seedlings. *In vitro* micrografting was successful in 70% of the grafts when shoot tips (measuring at least 3 cm) of the axenic cultures were used as scions. Histological studies confirmed the graft – union. From the *Ex vitro* micrografted plants graft union was achieved, only in 10% of the grafts. This method on refinement can be supplemented for the raising of tissue culture plant from the elite varieties.

On observing the high variation / fluctuation in the response of seedling and mature tree derived explants simple experiment was conducted to understand the source

of the variability noted in the different batches in our experiment. Simple exp eriment of germination of seeds in different batches and obtaining of seedlings of a particular stage (elongated hypocotyl with first pair of leaves) showed staggered pattern of germination of tamarind seeds over a period of 60days. This variation among the seeds may be due to heterozygosity among the seeds. Many seedlings at the required developmental stage could not be achieved at one time. Therefore our experiments were conducted with the seedlings on different days when it reached the particular stage of development. This may be one of the reasons for the variation in our data.

To understand the process of morphogenesis, different explants of tamarind seeds were cultured on various combinations of auxins and / or cytokinins. Longitudinal section of embryo axis attached to cotyledon (LSEC) was found to be effective in adaxial position in contact of the media to induce caulogenic buds in NAA 0.5 $mg.l^{-1}$ and BAP 10 $mg.l^{-1}$ with 4% sucrose. The direct *de novo* origin of these buds was confirmed histologically. These shoots on elongation could be rooted to form plants. The plants survived, on transfer to soil. This type of regeneration protocol for bud formation associated with the wounding site is often used for *Agrobacterium* - mediated transformation to generate modified plants with desired characteristics.

Somatic embryogenesis was achieved from the embryogenic structure raised from immature zygotic embryos of 1-6mm size when these were cultured on $5mg.l^{-1}$ dicamba supplemented with 6% sucrose and gelled with 0.22% phytagel. Cultures in tubes showed better response than petridishes and dark incubation was beneficial than light incubation for induction of somatic embryogenesis. The cotyledons of the immature zygotic embryos turned brown and necrotic and somatic embryos developed from the axes. However, the development of somatic embryos was restricted to the bipolar and cotyledonary stage. Repeated attempts to differentiate the embryos remained unsuccessful although rooting was noted sporadically on a few embryos. Embryogenic response varied when the immature zygotic embryos were collected from different trees.

Histological studies confirmed the direct and multicellular origin of the somatic embryos from epidermal subepidermal tissue of the apical region of the embryogenic structures. Somatic embryos proliferated on subculture through repetitive embryogenesis.

Cultures are being maintained for nearly two years. Embryogenic response was very slow and at a very low frequency.

Thidiazuron, a highly potent cytokinin has now been identified for induction of morphogenesis in woody plant species. Tamarind seeds were germinated in presence of TDZ. TDZ did not affect the germination of seeds, though it did restrict the elongation and morphology of the seedlings. It triggered morphogenetic activity in the cotyledonary nodal segment of the intact seedling. A large number of shoot buds developed on the suface of the explant via *de novo* organogenesis. TDZ induced morphological changes were also noted in the section of hypocotyl. Hypocotyl tissue showed swellings which was found to have meristematic centres in the subepidermal layers and also around the vascular ring. The shoots formed from the cotyledonary nodal segment, elongated on the intact seedling after 3 passages in medium devoid of PGR. These shoots on excision were micrografted on the tamarind seedlings. Both *in vitro* and *ex vitro* micrografting resulted in plant formation, at a frequency of 68% and 12%. These grafts survived in soil.

Today, with the growth of cities and suburbs, lands are no longer available for agriculture, let alone forestry. Thus forests will probably have to be planted on lands that are less than optimal with regard to fertility and water availability. In these cases, we will need trees that are specifically adapted for growth on sites where trees available today would be unable to survive. An extreme case might be the need to produce populations of trees adapted to growing on sites which are unsuitable for any other use, such as those contaminated by heavy metal residues.

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