TISSUE CULTURE STUDIES AND MOLECULAR CHARACTERIZATION OF PONGAMIA (*Pongamia pinnata* (L.) Pierre), AN OIL PRODUCING TREE LEGUME

A THESIS

SUBMITTED TO THE **UNIVERSITY OF PUNE**

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN **BIOTECHNOLOGY**

BY

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Dedicated

to my beloved

mother-in-law,

husband,

daughter

and my parents

CONTENTS

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(Mrs. K. Sujatha)

Date :
Place: Pune

CERTIFICATE OF THE GUIDE

CERTIFIED that the work incorporated in the thesis entitled **"Tissue Culture Studies and molecular characterization of Pongamia** (*Pongamia pinnata* (L.) Pierre)**, an oil producing tree legume"** submitted by Mrs. K. Sujatha 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:

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **"Tissue Culture Studies and molecular characterization of Pongamia** (*Pongamia pinnata* (L.) Pierre)**, an oil producing tree legume"** Submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me under the guidance of **Dr. (Mrs.) Sulekha Hazra** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

May 2007 Mrs. K. Sujatha.

Key to abbreviations

°C	Degree Celsius
v/v	Volume/volume (concentration)
w/v	Weight/ volume (concentration)
ANOVA	Analysis of variance
sd	Standard deviation
$HgCl_2$	Mercuric Chloride
MS	Murashige and Skoog medium (1962)
GR	Plant Growth Regulator
KN	Kinetin (6-furfuryl amino purine)
BA	6-Benzyl amino purine
Z	Zeatin
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
AdS	Adenine Sulphate
Taxim	Cefotaxime (antibiotic)
PPM	Plant Preservative Mixture
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)]

ABSTRACT

ABSTRACT

Pongamia pinnata (L) Pierre (Syn: Pongamia glabra. Vent), an oil producing tree legume, popularly known as Karanja, is the most widely available non-edible oil tree in India. This multipurpose tree species is well adapted for various agro climatic conditions and is one of the most extensively chemically investigated plants. Potential of the seed derived oil of *Pongamia* as a substitute for diesel is recognized and often identified as **"Bio-diesel"**. A large number of bioactive compounds have been isolated and characterized from various parts of this tree (flavonoids, furanoflavonoids), which has many industrial and medicinal uses. Pongam oil resembles groundnut oil in its fatty acid composition. However, due to presence of substances like Karanjin and Pongamol, the oil is non-edible.

Biotechnological approaches offer the potential to alter qualitative and quantitative improvement in the products obtained from trees. By subjecting to these approaches, *Pongamia* can serve as a source of high quality fuel and raw material for industries. Raising plantations of clonally propagated, high seed yielding trees will contribute to increased seed production. These plantations will also serve the purpose of seed orchard for production of superior quality seeds. *Pongamia* tree can also be exploited as an alternate source of edible oil by silencing the related genes, which cause production of undesirable substances.

From the existing literature it is apparent that there is a lot of scope for genetic modification of *Pongamia* for which there is need to develop a reproducible in vitro regeneration system. There is no literature on *in vitro* regeneration or tissue culture studies in this species. Keeping in view, the recalcitrant nature of leguminous tree species, standardization of protocols for *in vitro* morphogenesis remains a challenging proposition. To date, there is no literature available regarding the molecular scaffold of this tree. There is a need to undertake a preliminary study to assess the variability/ similarity among the individual tree species as well as the fidelity of the tissue culture raised plantlets.

The present research work entitled **"Tissue Culture Studies and Molecular Characterization of Pongamia (*Pongamia pinnata* (L.) Pierre), an oil producing tree legume"** is designed to address some of the limitations.

1. Optimization of conditions for propagation using juvenile tissues.
2. Optimization of conditions for clonal propagation using mature nodal buds.
3. Optimization of protocol for *de novo* morphogenesis (organogenesis and somatic embryogenesis).
4. Assessment of variability/similarity among the trees and fidelity of the *in vitro* raised clones using ISSR markers.

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

CHAPTER 1: General Introduction

This chapter covers the literature on the nature of *Pongamia*, chemical constituents and its industrial and chemical applications and importance of *Pongamia.* It also includes the literature on the current status of research on in vitro morphogenesis in tree legumes. A genomic approach in tree improvement program especially the role of molecular markers is emphasized. The significance and objectives of the study is included in this chapter.

CHAPTER 2: Materials And Methods

Common materials and methods for tissue culture and molecular studies implemented during the course of the work are described in this chapter. Materials and methods specific to individual experiment have been dealt with in respective chapters.

CHAPTER 3: Clonal Propagation using juvenile tissues

This chapter describes the protocol standardized for clonal propagation using explants from dry seed derived seedlings and semi mature green pod derived cotyledon node and meristems of the axes. Several parameters were tested to obtain microbe free seedlings. Increased germination frequency of dry mature seeds was obtained with addition of BA in MS medium. Cotton plugged culture vessels favored germination to obtain healthy seedlings. Optimum shoot proliferation from nodal explants and cotyledon nodes was achieved in MS medium supplemented with 8.88μM BA and 3% sucrose. Reculturing of

cotyledon node explants after removal of shoots, produced more shoots from same site. This process was repeated for 8 cycles and 4-8 shoots were obtained in each cycle. This process offers an alternative to produce clones and to avoid the steps of seed germination to obtain more seedlings. The shoots elongated and rooted (75%) in ½ MS basal medium supplemented with 0.22% activated charcoal. Plantlets survived on transfer to soil. The *in vitro* raised shoots rooted *extra vitrum* (67.5%) and hardened successfully.

To obtain more number of meristematic explants for *in vitro* regeneration studies the cotyledon node explants and the shoot tip of the embryo axes was isolated from the semi mature seeds obtained by dissecting the surface sterilized green pods (approximately 2.5cm). Thidiazuron is identified as an effective GR for shoot multiplication than aminopurines especially in woody species. To avoid duplication of the previous experiments in standardization of protocols with juvenile tissues, BA used in induction of multiples in nodal explants was substituted with varying concentrations of TDZ for induction of shoots from cotyledon nodes and apical meristems of the embryo axes. After 20 days pretreatment in TDZ multiple shoots proliferated from the meristems on transfer of the cotyledonary nodal explants and embryo axes to GR free basal media for several passages. There was increase in number of buds with increase in TDZ concentration. The optimum TDZ concentration was 11.4 μM. Buds induced in high TDZ (13.6 & 22.7μM) took longer time to differentiate, delaying the rooting step. The shoots, rooted in half strength MS basal media (80%) and survived (75%) in soil. In embryo axes in control medium radical emergence was noted after 7 days of culture. TDZ restricted rooting and induced swelling of plumule. The buds did not appear readily in GR free MS basal medium. Some buds dedifferentiated or were necrotized. Few shoots obtained were rooted in half strength MS basal media and hardened successfully.

CHAPTER 4: Clonal Propagation using mature nodal buds

This chapter deals with the standardization of highly reproducible protocol for clonal propagation from mature nodal buds. Parameters including basal media, pH and carbon source were optimized. Differential response was observed among the trees tested for sprouting. Effect of various concentrations of growth regulators like BA, Kinetin, zeatin and TDZ on sprouting was studied. No multiples were produced in MS supplemented with BA and Kinetin singly. Addition of Adenine sulfate (ADS) with BA promoted 2-3

multiples. Media with kinetin and ADS was ineffective. In TDZ, sprouting was completely suppressed and the meristems were swollen. Caulogenic buds appeared from the swelling on withdrawal of TDZ. Number of buds increased with increased concentration of TDZ. Optimum response was achieved in TDZ 0.44μM. Clusters of buds were isolated and transferred in GR free MS medium for elongation. Shoot cultures were maintained by subculturing in 0.45μM TDZ. Reculturing of primary explants after cropping the shoots produced more shoots. This procedure was followed for 6 cycles to obtain additional shoots in each cycle. Shoots elongated and rooted (70%) in GR free MS medium. Rooted shoots survived in green house (65%). Repeated proliferation of caulogenic buds from same origin may find application in production of true to type plants and also in development of transgenics.

CHAPTER 5: *De Novo* Morphogenesis

This chapter describes the development of adventitious shoots from deembryonated cotyledon and embryo axis explants excised from nearly mature green pods. These explants were precultured for 10 days and 20 days in varying concentrations of TDZ in MS basal media and transferred to GR free MS basal media. Explants were cultured adaxially and abaxially. Exposure of 20days in 11.35μM TDZ yielded more number of buds than 10d culture. Proximal segment was more responsive than other segments of cotyledon. Adaxial surface of cotyledon in contact of the media produced more buds. Buds differentiated and elongated on further passages in GR free medium. The nature and origin of the buds were determined histologically as *de novo* origin and without intervening callus. The shoots were rooted and plantlets recovered. In embryo axes, adventitious buds appeared near wounding region but did not differentiate further to shoots. Many of these dedifferentiated. Effect of various growth regulators at different concentrations was tested for induction of somatic embryos from immature zygotic embryos of varying sizes. Size dependent response in the formation of proembryogenic masses in the explants was noted. Cotyledon explants excised from nearly mature pods were also tested for induction of somatic embryos. Occasionally somatic embryo like structures appeared on the explants. Repeated attempts to induce viable somatic embryos remained futile.

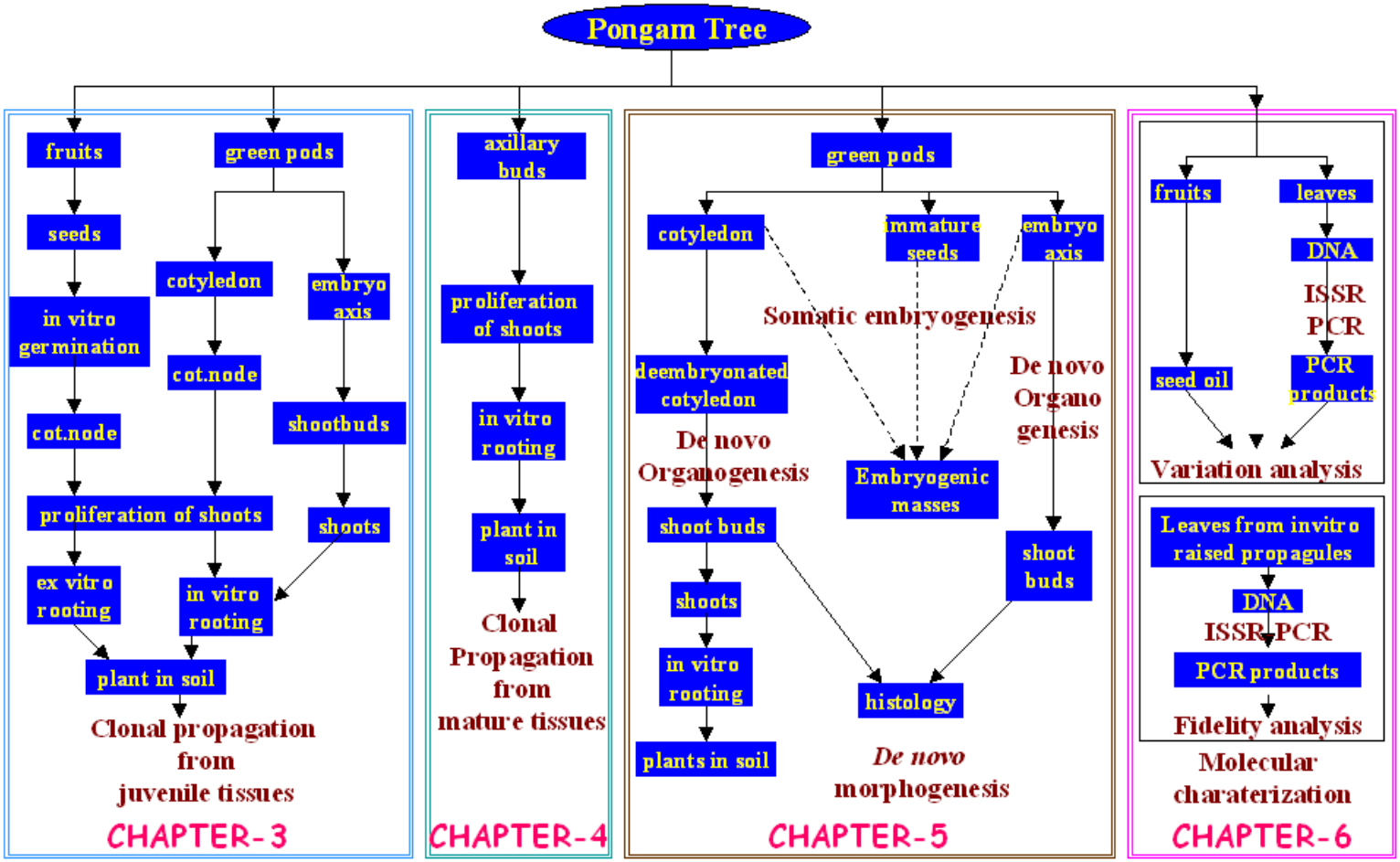
CHAPTER 6: Molecular Characterization

This chapter describes the experiments carried out to assess the variability/similarity among the selected trees and fidelity of the *in vitro* raised clones using ISSR markers. 100 primers were screened. Five primers were chosen for variability studies and 3 primers for fidelity studies. Band patterns obtained with ISSRs were analysed using Jaccard's coefficient to study the extent of similarity or dissimilarity among the selected trees. *In vitro* raised plantlets showed uniformity with the mother and no polymorphic bands were noted. Highly polymorphic band pattern were obtained within the selected trees. Attempts made to correlate the band pattern with some of the qualitative and quantitative characters such as oil content, pod size and shape, seed size, color etc. did not show definite correlation. The data indicates the level of variation existing in this species and emphasizes the use of tissue culture techniques in getting desired clones.

SUMMARY

The main findings of this research work conducted on *Pongamia* tissue culture and molecular studies are summarized in this section.

Schematic representation of the research work included in the thesis



CHAPTER 1:
GENERAL INTRODUCTION

1.1 INTRODUCTION

Trees are complex biological systems in themselves and interact in many ways with other biological systems and with the environment. The importance of trees in maintaining our ecosystem and their significance 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. Tree borne oil seeds are not only potential renewable energy source to supplement the increasing energy requirement, but also serves as an alternative source of oil to meet the increasing demand of edible oil globally. The plant polysaccharides are used for the production of fuel-grade ethanol. Vegetable oils of some tree species like *Pongamia*, *Jatropha*, Neem, Mahua, Neem, Polang, Simarouba, Sal, Kusum, Linseed, Castor, Baigaba etc., are in utilization for the commercial production of biofuels. Tree borne edible oil seeds are also used in cosmetic and Pharmaceutical industries. These examples suggest that product derived from trees may not remain just paper and timber. The essential and useful raw products derived from trees have potential to drive demand for greater tree productivity.

Leguminous tree species are well known for their utility to humanity. They are of special interest due to their economic and ecological importance. Legumes have been domesticated for the production of food, fuel, feed, forage, fiber, industrial and medicinal compounds, flowers etc. Understanding the molecular basis of nitrogen fixation and the unique metabolic pathways would be beneficial for the betterment of human race. Woody legumes play a considerable role in sustainable agriculture, especially in arid and tropical regions (Trigiano et al., 1992). These trees are particularly considered in afforestation and soil erosion control programs due to their ability to fix atmospheric nitrogen. Woody legumes offer unique qualities desirable in an agroforestry system. They are widely adaptable, often producing a sparse crown that allows light penetration to co-cultivated crops and providing additional soil nutrition through leaf litter and nitrogen fixation.

There has been relatively little effort expended towards crop improvement either through conventional or unconventional breeding efforts considering the potential usefulness demonstrated by woody legumes. Most tree improvement programs are based on management of genetic resources. This includes 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 agricultural crops, tree species have been left far behind (Trigiano, 1992).

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. Several superior hybrid trees with accelerated growth, altered form and environmental adaptations have been obtained through classical breeding. But, 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. For majority of trees, traditional breeding approaches are simply not a realistic means for achieving genetic improvement (Dean et al., 1997)

POTENTIALS OF BIOTECHNOLOGY

The potential of biotechnology for overcoming many of the limitations of classical breeding and for accelerating tree-breeding programs can be realized at several levels (Tzfira et al., 1998):

- 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.

Development of **tissue culture techniques** and molecular genetics allowed the exploration of new pathways for the improvement of trees that could offset some limitations of conventional breeding or add value to the breeder's work. A tissue culture technique allows the rapid multiplication of superior individuals. *In vitro* regeneration of organs, tissues, cells, protoplasts was not seen as an alternative to stem cutting's for commercial propagation, already efficient and cheap, but as the gateway for the application of new techniques such as *in vitro* selection of somaclonal variants, the rescue of embryos of difficult crosses, and development of homozygous lines (Confalonieri et al., 2003).

The most promising technology is **genetic transformation** of trees. This technology has two main advantages with respect to conventional breeding: (a) genes encoding specific proteins can be cut from virtually any living being, from viruses to higher plant to animals and 'pasted' into desired tree, thus broadening the range of genes available outside the current boundaries of the genus. (b) Individuals genotypes can be modified for one or a small number of well defined traits while preserving the rest of the genome intact; targeted modification of commercial cultivars could add value to them without disrupting the genome.

Advanced biotechnological methods for **genome analysis** and identification of novel **molecular markers** have been successfully applied to the model tree species, Poplar. Using DNA based markers, researchers can potentially access all of the variation contained within a given genome, thereby increasing their chances of finding a marker that segregates with the specific phenotype of interest. Thus, the main advantage of molecular markers is that they are based on the polymorphisms occurring naturally in the DNA of a given species, and as trees are among the most genetically variable organisms known, molecular markers useful for tree breeding programs should not be difficult to identify (Dean et al., 1997). In addition to constituting a larger pool of potential markers, DNA markers have advantage that they do not change in response to environmental factors or the developmental stage of a particular plant tissue. Molecular markers like RFLPs, RAPDs, AFLPs, STS and Microsatellites have been widely used to produce dense genetic maps of different populus species (Confalnieri et al., 2003). Production of genetic maps will facilitate the dissection of complex inherited traits and the identification of quantitative trait loci (QTL). A large-scale collection of Expressed Sequence Tags (ESTs) represents an invaluable tool for the identification of genes involved in the physiological process and for a more detailed analysis of functions.

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 essential for the successful management and improvement of tree species (Bonga, 1987).

POTENTIAL OF TISSUE CULTURE TECHNIQUES

Tissue culture is an important component of an integrated approach towards crop improvement. It offers great potential for rapid multiplication of superior individuals. This is important for many woody legumes species that have long maturation periods and are difficult to multiply through conventional vegetative propagation. It serves to enhance conventional breeding efforts by increasing

individuals from intra- or inter specific hybrids. Tissue culture also provides an alternative production of alkaloids and other secondary products.

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 germplasms using cryopreservation. Finally, *in vitro* culture provides the only route for generation of genetically engineered genotypes of trees. Gene transfer technologies including *Agrobacterium* mediated gene transfer, electroporation, microinjection, and micro projectile bombardment all depend on ability to culture cells *in vitro*. *In vitro* techniques, if they can be applied to trees, provide an even greater potential advantage than in other higher plants (Durzan DJ, 1985; Haissig BE, 1986).

The goal most often cited for *in vitro* culture of trees is propagation. The primary objective of *in vitro* propagation or micropropagation is to capture the total genetic superiority of the parent material, including both additive and non-additive genetic components. In addition, micropropagation systems allow the application of a very high selection differential, since new populations of plants can be cloned from just a few elite individuals.

Micropropagation of trees has several advantages over conventional propagation methods. These 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.

Micropropagation systems fall into three broad categories: Axillary bud proliferation, Organogenesis and Somatic Embryogenesis. Axillary shoot methods rely on multiplication of preformed structures,

while organogenesis or somatic embryogenesis relies on *de novo* generation of either plant organs or embryos respectively.

Axillary bud proliferation:

The principal advantage of axillary shoot methods for tree multipliation is that all propagules are derived from preformed buds, thereby enhancing the likelihood that propagules will be true to type. In this method, development and multiplication of the growing meristem is achieved. The economical and ecological importance of leguminous forest trees necessitates the application of this technique for their clonal propagation (Tomar and Gupta, 1988b). Initially juvenile tissues were used for the standardization of protocol in tree legumes like *Dalbergia, Acacia, Sesbania, Pterocarpus* etc. Recently, with concerted efforts, micropropagation protocol using pre existing meristem from mature tissues of many of the leguminous tree species has been achieved. Table 1.1 list the reports on propagation of leguminous tree species using juvenile and mature explants.

Table 1.1: Reports on clonal propagation in tree legumes

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Acacia albida	Root	Shoot regeneration	Plant in soil	Ahee & Duhoux, 1994
Acacia auriculiformis	Axillary bud	Shoot regeneration	Plant in soil	Mittal et al., 1989
Acacia catechu Willd.	Mature nodal segment	Shoot proliferation	Plant in soil	Kaur et al., 1998
	Shoot tip	Shoot proliferation	Plant in soil	Kaur & Kant, 2000
Acacia mangium	Seedling axillary buds	Shoot proliferation	Plantlet	Saito et al., 1993
	Juvenile and mature nodal explant	Shoot proliferation	Plant in soil	Monteuuis & Bon, 2000
	Juvenile and mature nodal explant	Shoot proliferation	Plant in soil	Monteuuis, 2004
A. mearnsii de Willd	Seedling shoot tip	Shoot proliferation	Plantlet	Huang et al, 1994
	In vitro and Ex vitro seedling explant	Shoot proliferation	Plant in soil	Beck et al., 1998a
	Coppice nodal explant	Shoot proliferation	Plant in soil	Beck et al., 1998b

Plant species	Explant	Pathway of morphogenesis	Status	Reference
A. mearnsii	Shoot tip	Shoot proliferation	Plant in soil	Beck et al., 2000
A. nilotica	Nodal buds	Shoot proliferation	Plant in soil	Singh et al., 1993
	Shoot tip	Shoot proliferation	Plant in soil	Beck et al., 2000
A senegal (L) Willd.	Seedling Juvenile tree Nodal explant	Shoot proliferation	Plant in soil	Badji et al., 1993
A.sinuata (Lour.) Merr.	Nodal segments	Shoot regeneration	Plant in soil	Vengadesan, 2003
A. tortilis subsp *raddiana* (Forsk) Hayne	Seedling cotyledonary node	Shoot buds, shoots	Plant in soil	Nandwani, 1995
Albizia julibrissin	Roots	Shoot regeneration	Plantlet	Sankhla et al, 1996
	Root segment	Shoot regeneration	Plant in soil	Hosseini-Nasr, & Rashid, 2002
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 & Dhar 1996
	Nodal segment of shoots from mature liana	Shoot proliferation	Plant in soil	Dhar & Upreti, 1999
	Seedling cotyledonary node	Shoot proliferation	Plant in soil	Bhatt & Dhar, 2000
B. variegata L.	Mature nodal explant (6-8 yrs.)	Axillary shoot proliferation	Plant in soil	Mathur & Kumar, 1992
Caesalpinia pulcherrima Sw.	Mature (20 yrs.) nodal bud	Shoot proliferation	Plant in soil	Rahman et al., 1993
Cassia fistula Linn.	Seedling explants	Callus	Plant in soil	Bajaj, 1989
Ceratonia siliqua L.	Seedling shoot tip, Mature nodal segment	Shoot proliferation	Plant in soil	Sebastian & Mc Comb, 1986
	Mature shoot tip, nodal stem segment	Shoot proliferation	Plantlet	Thomas & Mehta, 1983
Cercis yunnanensis	Seedling explants	Shoot regeneration	Plantlet	Cheong & Pooler, 2003
Ceropegia candelaebrum	---	Shoot regeneration	Plantlet	Beena et al., 2003

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Dalbergia sissoo Roxb.	Mature Axillary bud	Shoot proliferation	Plantlet	Datta et al., 1983a
	Mature nodal explant	Shoot proliferation	Plantlet	Datta & Datta, 1983b
	Mature Axillary bud	Shoot proliferation	Plant in soil	Dawra et al., 1984
	Cells of cambial origin of mature tree	Cell suspension derived callus regeneration	Plant in soil	Kumar et al., 1991
D.sissoo Roxb.	Seedling explant - Cotyledonary node	Shoot proliferation	Plant in soil	Pradhan et al., 1998
	Hypocotyl explants	Shoot proliferation	Plant in soil	Pattnaik et al., 2000
D. latifolia Roxb.	Mature nodal explants	Shoot proliferation	Plantlet	Mascarenhas et al., 1982
	Mature Shoot callus	Callus regeneration	Plant in soil	Ravishankar & Chandra,1988
	Seedling shoot tip, nodal segment from *in vitro* derived shoots	Shoot proliferation	Plant in soil	Ravishankar & Chandra, 1989
	Mature Nodal explant of 60-80 yr old tree	Shoot proliferation	Plant in soil	Raghava Swamy et al., 1992
Leucaena leucocephala	Axillary node 1-2yr. old plant	Shoot proliferation	Plantlet	Kulkarni et al., 1984
Leucaena leucocephala	Seedling nodal segment, Mature nodal segment	Shoot proliferation	Plant in soil	Dhawan, 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 et al., 1985
	Cotyledon	Shoot proliferation	Plantlet	Saffi & Borthakur, 2002
Mimosa tenuiflora (Willd.) Poiret	Axillary node	Shoot Proliferation	Plant in soil	Villarreal & Rojas, 1996
Ougeinia dalbergioides	Seedling nodal segment	Shoot proliferation	--	Dhawan, 1988

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Peltophorum pterocarpum (DC.) Backer ex K. Heyne	Shoot tip, nodal segment, cotyledonary node	Shoot proliferation	Plant in soil	Uddin et al., 2005
Poinciana regia (Boj.*)*	Seedling explants Excised maturing embryos with well-developed cotyledons	Callus Seedling formation	-- Plant in soil	Bajaj, 1989
Prosopis alba Griseb	Seedling shoot tip, nodal stem segment	Shoot proliferation	Plantlet	Jordan, 1987
P. cineraria (L.) Druce	Shoot tip or nodal stem segment	Shoot proliferation	Plantlet	Goyal, 1984
P. tumarugo F. Phil.	Seedling shoot tip or nodal stem segment	Shoot proliferation	Plantlet	Jordan, 1987
P. chilensis	Yound and mature nodal buds	Shoot proliferation	Plant in soil	Caro et al., 2002
Psoralea corylifolia L.	Cotyledonary node	Shoot proliferation	Plantlet	Jayakumar & Jayabalan, 2002
	Ex vitro seedling	Shoot proliferation	Plant in soil	Faisal et al., 2006
Pteroarpus marsupium	Cotyledonary node	Shoot proliferation	Plant in soil	Chand & Singh, 2004; Anis et al., 2005
Pterocarpus santalinus	Seedling explants	Shoot proliferation	Plantlet	Sarita Patri et al., 1988
P. angolensis	Seedling explants	Shoot proliferation	Plantlet	Chisha-Kasumu et al., 2006
Robinia pseudoacacia	Mature nodal stem segment	Shoot proliferation	Plantlet	Chalupa, 1983
	Mature nodal stem segment	Shoot proliferation	Plantlet	Barghchi, 1987
	Hypocotyl	Shoot proliferation	Plantlet	Hosseini-Nasr, 2003
Sophora toromiro	Seedling explants	Shoot proliferation	Plantlet	Jordan et al., 2001
Sesbania drummondii	Nodal explant from seedling	Shoot proliferation	Plant in soil	Cheepala et al., 2004

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Sesbania. rostrata	Seedling stem, leaves, roots of micropropaga-ted plant	Callus Caulogenesis	Plantlet	Pellengrineschi & Tepfer, 1993
Swainsona Formosa	Mature axillary node	Shoot proliferation	Plant in soil	Jusaitis, 1997
Swartzia madagascariensis (Desv.)	Seedling 1st node, 2nd node, shoot apex	Shoots Roots	Plantlet	Berger& Schaffiner, 1995
Tamarindus indica L.	Seedling Shoot tip	Shoot proliferation	Plant in soil	Kopp & Nataraja, 1990
	Seedling Cotyledonary node	Shoot proliferation	Plant in soil	Splittstoesser & Yasseen,1991
	Seedling Shoot tips, nodes of shoot regenerated from shoot tip	Shoot proliferation	Plant in soil	Jaiwal & Gulati,1992
	Seedling Excised nodal segment	Shoot proliferation	Plant in soil	Kopp & Nataraja, 1992

De novo Morphogenesis:

De novo Morphogenesis *in vitro* occurs in two different patterns i.e. organogenesis and embryogenesis.

Organogenesis: Organogenesis is the *de novo* production of plant organs (buds, shoots and roots) from organized tissues or callus. In contrast to axillary bud proliferation, organogenesis proceeds *de novo* via organization of meristems. It involves the induction of localized meristematic activity by treatment with phytohormones. This leads to the formation of primordium and eventually the formation of shoot. Like axillary bud proliferation, juvenile tissues have been reported as capable of regeneration via organogenesis in number of woody legume species (Table 1.2). Recently, with the advancement in molecular genetics, 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 and listed (Phillips, 2004) in the Table 1.2.

Table 1.2: Genes involved in shoot organogenesis

Gene	Putative function	References
CYCD3	Involved in acquisition of competence for organogenesis	Sugiyama, 1999; Fletcher, 2002
SRD3	Competence for shoot organogenesis	Sugiyama, 1999,2000
SRD1, SRD2	Competence for redifferentiation of shoots	Sugiyama, 1999,2000
ESR1	Enhances shoot regeneration, vegetative-to-organogenic transition	Zuo et al., 2002
CRE1	Cytokinin receptor	Zuo et al., 2002
CK11	Cytokinin perception	Fletcher, 2002; Zuo et al., 2002
CLV, WUS	Preserve stem cell identiy in shoot apical meristems	Fletcher, 2002
KN1, STM	Initiate ectopic shoot meristems, shoot apical meristems function	Fletcher,2002
SHO, MGO	Modifiers of the shoot apical meristems involved in leaf founder cell recruitment, lateral organ primordial	Fletcher, 2002

(Ref: Phillips, 2004)

Somatic embryogenesis: It is the *de novo* prodution of structures resembling zygotic embryos , either from organized tissues or from callus. Structures classified as somatic embryos are bipolar and have no vasular connection to the source tissue. Somatic embryogenesis has been cited by many authors as the *in vitro* regeneration system of choice for economial prodution of clonal populations of forest trees (Gupta et al., 1991). A major advantage is the potential for very high frequency regeneration, as well as their liquid cultures allows the production and handling of thousands of embryos at one time (bioreactors). The similarity of somatic embryo with zygotic embryo, led to the idea of somatic embryo encapsulation to produce synthetic seeds which would facilitate mechanical handling and automated planting of somatic embryos.

All somatic embryogenic cultures of woody legumes have been initiated from seed or seedling tissues (Table 1.2). Studies employing immature embryo or seed explants established that developmental stage of the embryo explant was critical in determining embryogenic response (Trigiano, 1992). Auxins like 2,4-D, 2,4,5-T, picloram, NAA have been used for induction of somatic embryogenesis. There is need for reliable methods for *in vitro* embryogenesis in woody legumes to achieve high probability of success, high conversion frequency, high reproducibility and production of normal somatic embryos. To achieve this,better understanding of molecular basis of embryo development is needed. Currently, the trend is towards recognition of embryo development which is comprised of variety of different stages involving specific gene expression (Goldberg et al., 1989; Hughes and

Galau, 1989; Kermode, 1990). The general aspects about the specific genes involved in somatic embryogenesis are reviewed (Phillips et al., 2004). The discovered genes are listed in Table 1.3.

Table 1.3: Genes involved in Somatic Embryogenesis

Gene	Putative function	References
LEC2	Initiates ectopic somatic embryogenesis	Zuo et al., 2002
WUS (PGA6), SERK, LEC1	Involvedin the vegetative-to-embryognic transition	Harada, 1999; Zuo et al., 2002
SHR	Establishes ground tissue via asymmetric cell division	Von Arnold et al., 2002
CLV, WUS	Regulates stem cell fate	Fletcher, 2002; Von Arnold et al., 2002
CLV1,CLV3, STM	Regulate shoot apical meristem development	Fletcher, 2002; Von Arnold et al., 2002
LEC1,FUS3, ABI3	Regulates embryo maturation	Von Arnold et al., 2002

(Ref: Phillips, 2004)

De novo morphogenic regeneration systems via organogenesis and embryogenesis (both direct and indirect) have been developed for a number of leguminous tree species and are listed in Table 1.4.

Table 1.4 : Reports on morphogenetic studies in tree legumes

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Acacia arabica	Immature zygotic embryos	Indirect embryogenesis	Plant in soil	Nanda & Rout, 2003
Acacia auriculiformis A. Cunn. ex Benth.	Seedling hypocotyl	Indirect organogenesis	Plantlet	Ranga Rao & Prasad, 1991
Acacia catechu Willd.	Immature cotyledon	Indirect embryogenesis	Plant in soil	Rout et al., 1995
Acacia koa Gray	Seedling shoot tip	Indirect organogenesis Embryogenesis	Plant in soil --	Skolmen & Mapes, 1976; Skolmen, 1986
A. magnium	Cotyledon	Direct organogenesis	Plant in soil	Douglas & Mcnamara, 2000
A. mangium Willd.	Immature zygotic embryo explants, seedling	Indirect organogenesis	Plant in soil	Xie & Hong, 2001
A. nilotica (L.) Delile	Seedling stem	Direct organogenesis	---	Mathur & Chandra,1983
A. nilotica (Linn.) Willd.	Immature endosperm	Indirect embryogenesis	Plantlet	Garg et al., 1996
A. salicina		Indirect embryogenesis	Plantlet	Zhao et al., 1990

Plant species	Explant	Pathway of morphogenesis	Status	Reference
A. sinuata	Seedling hypocotyl	Indirect organogenesis	Plant in soil	Vengadesan et al., 2000
	Cotyledon	Indirect organogenesis	----	Vengadesan,, 2003
Acacia spp.		Indirect embryogenesis	Plantlet	Jones et al., 1990
Albizia falcataria	cotyledon	Direct organogenesis	Plantlet	Sinha & Mallick, 1993
Albizia lebbeck L.	Seedling hypocotyl Hypocotyl, root, leaves, cotyledon	Embryoids Caulogenesis	Plantlet Plantlet	Gharyal & Maheswari, 1981
	Anther	Haploid	Plantlet	Gharyal et al., 1983a
Albizia lebbeck L.	Seedling root, hypocotyl	Indirect organogenesis	Plantlet	Upadhyaya & Chandra, 1983
	Seedling hypocotyl, cotyledon, root, leaf rachis	Indirect Organogenesis	Plant in soil	Varghese & Kaur, 1988
	Mature Petiole, stem	Indirect organogenesis	Plant in soil	Gharyal & Maheswari, 1990
	Seedling, stem, hypocotyl, leaf,	Indirect organogenesis	Plant in soil	Lakshman Rao & De, 1987
Albizzia falcataria (L.) Forberg	Cotyledon pieces	Direct organogenesis	Plantlet	Sinha & 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. procera	Seedling leaflet	Direct organogenesis	Plant in soil	Sandeepkumar et al., 1998
A. richardiana King.	Seedling hypocotyl	Organogenesis Indirect embryogenesis	-- Plantlet	Tomar & Gupta 1988a
A. amara Boivin. *Albizzia lucida* Benth. *A. richardiana King.*	Seedling hypocotyl	Direct organogenesis	Plant in soil -- Plant in soil	Tomar & Gupta, 1988b
Alhagi camelorum Fitch.	Seedling cotyledon, leaf, root, stem	Direct and Indirect organogenesis	--	Bharal & Rashid, 1981
Bauhinia purpurea L.	Mature stem segments 15-18 yr. old tree	Callus regeneration	Plant in soil	Kumar, 1992

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Calliandra tweedii	Internodal and petiolar explants	Direct embryogenesis	Plant in soil	Shashi kumar etal., 2002
Cassia fistula Linn.	Seedling explants	Callus	--	Bajaj, 1989
Cassia fistula L. and *C. siamea* Lam.	Mature stem	Indirect organogenesis	Plant in soil Plantlet	Gharyal & Maheswari, 1990
Cercis canadensis L.	Developing zygotic embryos	Direct embryogenesis Rhizogenesis	Plant in soil	Geneve & Kester, 1990
Cercis canadensis L.	Immature embryos	Direct embryogenesis	Germinated, only one embryo formed shoot	Trigiano et al., 1988
Cladrastris lutea (Michx.) K. Koch.	Immature embryos	Direct embryogenesis	Plant in soil	Weaver & Trigiano, 1991
Dalbergia. latifolia Roxb.	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, & Nataraja, 1987a; 1987b
	Mature Shoot callus	Callus regeneration	Plant in soil	Ravishankar & Chandra, 1988
	Immature zygotic embryos	In direct embryogenesis	Plant in soil	Rao, 1986
	Seedling hypocotyl segment	Indirect regeneration from cell suspension derived callus	Plant in soil	Pradhan et al., 1998
D. lanceolaria Linn.	Seedling root, hypocotyl, cotyledon, stem, leaf	Indirect caulogenesis, rhizogenesis	Plantlet	Anand & Bir, 1984
Dalbergia sissoo Roxb.	Seedling Excised root	Direct organogenesis	Plantlet	Mukhopadhyay & Ram, 1981
	Seedling Hypocotyl	Indirect organogenesis	Plantlet	Sharma & Chandra, 1988
	Cells of cambial origin of mature tree	Cell suspension derived callus regeneration	Plant in soil	Kumar et al., 1991
	Semi mature zygotic embryos	Indirect embryogenesis	--	Das et al., 1997

Plant species	Explant	Pathway of morphogenesis	Status	Reference
	Semimature and mature cotyledons	Direct organogenesis	Plant in soil	Chand, 2002
Faidherbia= Acacia albida (Del.) A. Chev.	Seedling Roots	Direct organogenesis	Plantlet	Ahee 7 Duhoux, 1994
Hardwickia binata	Immature cotyledonary explant	Indirect Embryogenesis	---	Das et al., 1995
	Zygotic embryos	Direct embryogenesis	Plantlet	Chand & Singh 2001
Indigofera enneaphylla L.	Cotyledon	Indirect organogenesis	Plantlet	Bharal & Rashid 1979; 1981
Leucaena leucocephala (Lam.) de Wit	Cotyledon and epicotyl	Direct organogenesis	Plantlet	Nataraja & Sudhadevi,1984
	Cotyledon	Indirect organogenesis	Plantlet	Nagamani & Venkateswaran,1987
L. retusa Benth.	Cotyledon	Indirect organogenesis	--	Nagamani, 1987
Mimosa pudica L.	Seedling cotyledon, hypocotyl,leaf, shoot apex	Indirect organogenesis	Plantlet	Gharyal & Maheswari, 1982
M. tenuiflora (Willd.) Poiret	Root segment	Direct organogenesis	Plantlet	Kackar et al., 1992
	hypocotyl segments, excised cotyledons	Somatic embryogenesis and shoot buds		
P. cineraria (L.) *Druce*	Seedling hypocotyl	Indirect regeneration	Plant in soil	Goyal & Arya,1981
Parkia timoriana	cotyledon	Indirect somatic embryogenesis	--	Thangjam & Singh, 2006
Robinia pseudoacacia	Zygotic embryo	Embryogenesis	Plantlet	Merkle & Wiecko, 1989
	Immature seeds	Indirect embryogenesis	Plant in soil	Arrillaga et al., 1994
Sesbania aculeata (Pers)	Seedling hypocotyl	Adventitious Organogenesis	Plant in soil	Bansal & Pandey, 1993
Sesbania bispinosa (Jacq.) W.F. Wight	Seedling hypocotyl, cotyledon	Direct organogenesis	Plant in soil	Kapoor & Gupta, 1986
Sesbania grandiflora	Seedling hypocotyl, cotyledon	Indirect organogenesis	Plantlet	Khattar & Ram, 1983
Sesbania grandiflora	Seedling hypocotyl, cotyledon	Indirect organogenesis	Plantlet	Shankar & Ram, 1990

Plant species	Explant	Pathway of morphogenesis	Status	Reference
	Cotyledon pieces	Direct organogenesis	Plant in soil	Detrez et al., 1994
Sesbania rostrata	Seedling cotyledon, hypocotyl, zygotic embryo	Organogenesis	Plantlet	Vlachova et al., 1987
	Cotyledon	Direct embryogenesis	Plant in soil	Shahana & Gupta, 2002
Swainsona salsula.taubert	Cotyledon	Direct Organo genesis	Plant in soil	Yang et al., 2001
Tamarindus indica L.	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
	Seed/Seedling	*De novo* organogenesis	Plant in soil	Mehta et al., 2004; 2005

POTENTIAL OF GENETIC TRANSFORMATION

Most funtional genomic approaches require high throughput transformation system useful for developing gene identification strategies. Advancement of molecular genetics in legumes, eg. Gene overexpression, gene suppression, promotor analysis, T-DNA tagging and expression of genes for crop improvement, requires effiient transformation systems that produce low frequencies of tissue culture induced phenotypic abnormalities in the trangenic plants (Somers et al., 2003). Recently transformation methods have been developed for *Acacia mangium* (Xie and Hong, 2002) and *Robinia pseudoacacia* (Han et al., 1993; Igasaki et al., 2000), *Leucaena leucoephala*, *Sesbania sesban*, *Acacia* etc. Recent progress in legume transformation suggests that some systems will achieve transformation efficiencies required for functional genomics applications in the near future.

Agrobacterium based genetic transformation is the main approach used for developing transgenic trees (Giri et al., 2004) Choice of explants having competene for transformation and regeneration is a crucial factor. In addition to regeneration through organogenesis, somatic embryogenesis definitely offers the advantage of single cell regeneration and currently appears to be the most promising approach to introdue new genes into woody species. With biotechnological approaches, the genetic improvement of trees can be achieved with desired targets.

Targets for Tree Transformations: Trunk is the major harvested organ in trees and breeding programs are usually aimed directedly at improving trunk performance and wood quality. This includes biochemical modification of wood characteristics and trunk structure, increasing its growth

rate and altering its shape. Additional breeding targets include improving root-system and tree-canopy performance, pest resistane and tolerance to abiotic stresses. Worldwide upto the year 1999, a total of 68 trials with transgenic trees have been approved, out of which 51 are in poplars alone (Confalonieri et al., 2003).Most common trait specific genes introduced in tree species are:

Herbicide resistance *(aroA, bar*),
Insect resistance (*Bt, crylA, crylB,* trypsin proteinase inhibitor),
Lignin metabolism (COMT,*CAD,CCoAOMT*),
Disease resistance (*chsA, Bo,*)
Abiotic stress resistance (*GR, gshII*),
Flower development (*leafy, PTLF*),
Growth and wood quality (*rolC, phyA,GS*) and *merA* gene for phytoremediation.

In addition genes like Δ9 *desaturase* have been introduced and transformed in many oil seeds bearing plants like soya bean, rape seed etc.for improved nutritional oil content. It has been shown that seeds containing oil with varied modification in fatty acid composition can be utilized at different levels (Gunstone, 2002):

- Lowering the level of saturated acids on nutritional grounds
- Increasing the level of saturated acids to avoid hydrogenation and consequent formation of transacids
- Increasing mid chain saturated acids for oleochemical purposes
- Increasing levels of oleic acid on nutritional grounds
- Lowering the linolenic acid to avoid partial hydrogenation and extend shelf life

POTENTIAL OF MOLECULAR TECHNIQUES

Development of moleular techniques for genetic analysis has led to a great increase in our knowledge of tree legume genetics and our understanding of structure and behaviour of genomes which will be useful for improvement of leguminous tree species. These molecular techniques, in particular use of DNA markers have been used in monitoring DNA sequence variation in and among the species there by increasing their chances of finding a marker that segregates with the specific phenotype of interest. DNA markers have the advantage that they do not change in response to environmental factors or developmental stage of a particular plant tissue. DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analysed objectively.

With the advent of Recombinant DNA technology and PCR a new class of DNA profiling markers are introduced. This facilitated the development of marker based gene tags, map based cloning of agronomically important genes, variability studies, phylogenetic analysis, synteny mapping marker assisted selection of desirable genotypes etc.

As the tree species show variation, it is important to study the genetic composition of the germplasm of existing species (germplasm characterization) and related species. This will not only provide information on their relationship but will also indicate a chance of finding new and useful genes, as accessions with most distinct DNA profiles are likely to contain a greater number of novel alleles. DNA markers will provide information on genetic composition of the germplasm, amount of genetic variation existing among them and prvoide accessibility of novel alleles. RAPD markers have been used in investigation of distribution of variability in natural populations of *Eucalyptus globulus* and provided the foundation for effective breeding and gene conservation strategies (Nes bitt et al., 1995). They can be powerful tools for analysing population structure with respect to geneflow and paternity.

DNA markers have also been used for testing genetic fidelity during micropropagation/ *ex situ* conservation on one hand and for characterization of plant genetic resources (PGRs) on the other. Molecular markers have particularly been suggested to be useful for confirmation of genetic fidelity in micropropagated tree species, where life span is quite long and performance of micropropagated plant could only be asertained after their long juvenile stage in field conditions (Rani, 2000). A large number of RFLPs were recorded in some tree species like populus, eucalyptus etc., for studying the variation and genetic fidelity during micropropagation.

DNA markers are generally classified as hybridization based markers and polymerase chain reaction (PCR) based markers. Restriction Fragment Length Polymorphism (RFLPs), Random amplified polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphism (AFLPs), Simple Sequence Repeats (SSRs), Single Nuleotide Polymorphism (SNPs) etc., are some of the DNA markers in use. Relative advantages and disadvantages of some of these techniques are summarised in Table 1.5 (Korzan, 2000).

Inter simple sequence repeats (ISSR), a promising DNA marker, combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. Details regarding the nature and utility of this marker is dealt in detail in Chapter 6. Potential for integrating ISSR-PCR into programs of plant improvement is enormous. ISSRs offer greater potential to determine intra genomic and intergenomic diversity comparted to other arbitary primers (Zietkicwicz et al.,1994).

Table 1.5: Comparison of DNA markers

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (µg)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	High	High	Moderate	Moderate	High
PCR based	No	Yes	Yes	Yes	Yes
Number of polymorph loci analysed	1-3	1.5-50	20-100	1-3	1
Ease of use	Not easy	Easy	Easy	Easy	easy
Ameable to automation	Low	Moderate	Moderate	High	High
Reproduibility	High	Unreliable	moderate	High	High
Development Cost	Low	Low	Moderate	High	High
Cost per analysis	High	Low	Moderate	Low	Low

Several properties of microsatellites like high variability among the taxa, ubiquitous occurrence and high copy number in eukaryotic genomes (Weising et al., 1998) make ISSRs extremely useful markers. The evolutionary rate of change within mirosatellites is considerably higher than most other types of DNA, so the likelihood of polymorphism in these sequences is greater. It has been proved in species like C*amellia sinensis* (Devarumath et al., 2002), strawberry (Anita, 2004) that ISSRs found to yield more information in the fidelity analysis of tissue cultured derived plants than RAPD. Hence, ISSRs are found to be very useful especially in assessment of genetic uniformity, variability and characterization of germplasm.

PONGAMIA PINNATA

Pongamia (Indian Beech) is a genus having one species *Pongamia pinnata* L. [*Pongamia glabra*, Vent.; *Derris indica*, Lamk.]. It belongs to the family Leguminosae and sub-family *Papilionaceae*. The *Lineage* of this genus is as follows:

Cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Eudicotyledons; Core Eudicotyledons; Rosids; Eurosids I; Fabales; Fabaceae; Papilionoideae; Millettieae; Pongamia.

Pongamia, popularly known as Karanja, is an important shade tree of India. The Pongam tree is considered to be native to India (Western Ghats). It is also found in China, Florida, Malaysia,

Seyhelles, Philippines and Australia (Daniel, 1997). Pongam is capable of growing under a wide range of agroclimatic conditions and is a common sight around coastal areas, riverbanks, tidal forests and roadsides. It is medium sized glabrous, fast growing, evergreen tree (Fig. 1.1), attains generally 8–18 ft height (Anonymous, Wealth of India, 1998). The trunk is short with drooping branches. Bark is grayish brown covered with tubercles. Imparipinnate leaves with 5-7 ovate or elliptical leaflets. Presence of viral galls in leaves is a frequent incident (Fig.1.1A) Flowers pinkish (Fig. 1.1B) to white (Fig. 1.1 C& D).

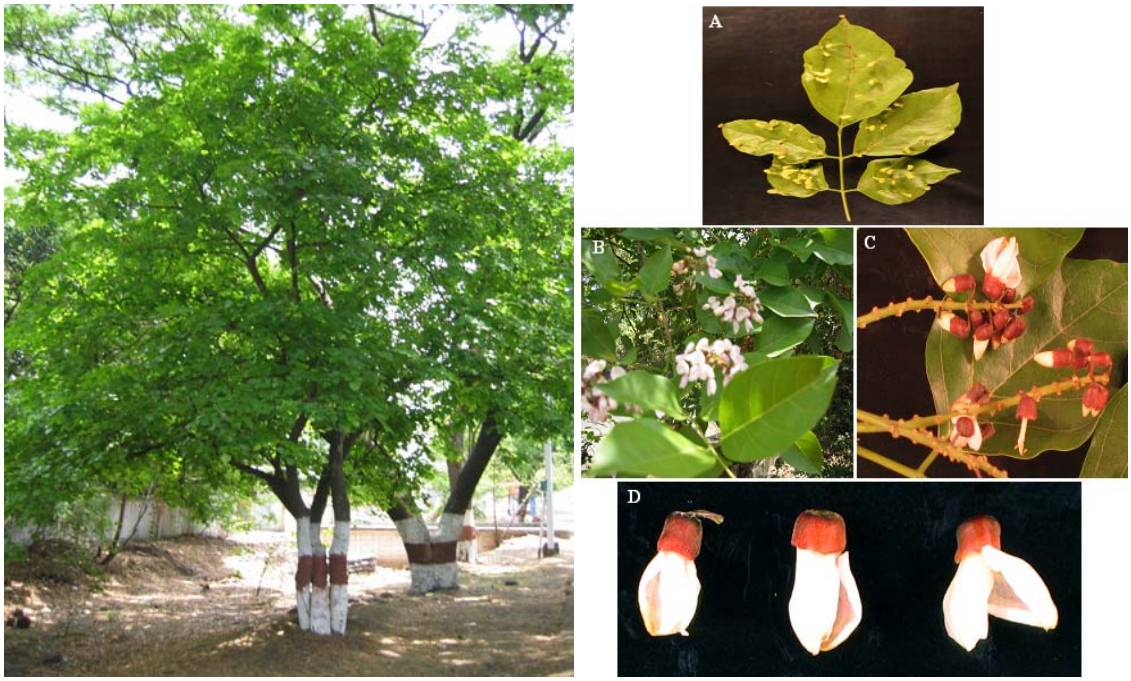


Fig. 1.1: *Pongamia* tree (A) Leaf galls in *Pongamia.*
(B) Inflorescence with pink colored flowers.
(C) Inflorescence with white coloured flowers
(D) Magnified view of flowers with indehiscent calyx.

The tree starts bearing at the age of 4-7 years. The harvest season extends in general from November to December to May-June. The yield of the seed is said to range from 9-90kg per tree, indicating a yield potential of 900-9000 kg/seed/hectare. Different stages of pod formation are seen in the Fig.1.2. Fruit in a pod is thick, woody, compressed, and elliptic to obliquely oblong with a short curved beak. Seeds usually one or two, are thick, reniform, broad wrinkled with reddish brown leathery testa (Fig. 1.2 E). The seeds are said to be viable for a year. Natural reproduction is through seed or by root suckers. The germplasm (2n=22) is reported to tolerate drought, frost, heat, limestone, salinity, sand and shade (Duke, 1983).

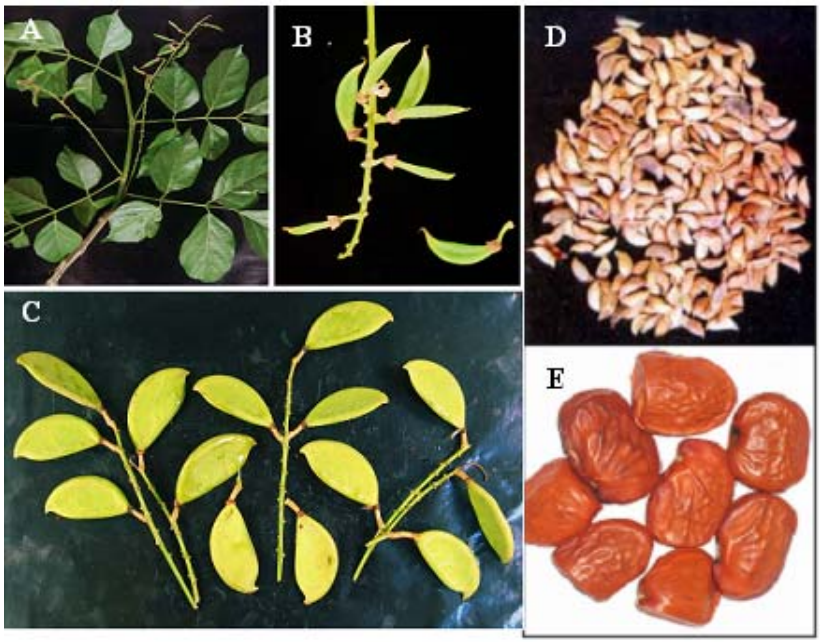


Fig. 1.2: Different stages of immature pods of *Pongamia* (A to.D). (E)Mature seeds of *Pongamia*.

Seeds are mainly valued for the oil obtained from them that has many industrial and medicinal applications. In general, Indian mills extracts 24-27% oil. Pongam oil is yellow orange to brown in color, which darkens on storage. It has a disagreeable odor and bitter taste due to the presence of biogenetically active compounds like Karanjin and Pongamol. Pongam oil resembles Groundnut oil in its fatty acid composition (Table 1.6).

Table 1.6: Comparison of fatty acid composition

Fatty acid	Pongam oil	Groundnut oil
Palmitic acid	3.7 - 7.9	9.0 - 24.9
Stearic acid	2.4 - 8.9	0.0 - 5.5
Arachidic acid	2.2 - 4.7	2.0 - 10.2
Behenic acid	4.2 - 5.3	0.7 - 3.9
Lignoceric acid	1.1 - 3.5	0.0 - 2.8
Oleic acid	44.5 - 71.3	38.7 - 56.2
Linoleic acid	10.8 - 18.3	6.2 - 38.4
Eicosenoic acid	9.5 - 12.4	0.7 - 2.3

Pongamia or Karanj is the one of the most extensively chemically investigated plant. A large number of biogenetically related compounds have been isolated and characterized. *Pongamia* is a rich source of flavonoids (Table 1.7), Chalcones (Table 1.8), Steroids and Terpenoids (Table 1.9) and miscellaneous compounds (Table 1.10) like diketones, esters and amides and others (Meera et al., 2003). The following tables (Ref: Meera et al., 2003) will list the compounds isolated from various parts of the plant with their appropriate reference.

Table1.7: Flavonoids from *Pongamia pinnata*

Compound	Part	Reference
Demethoxykanugin	Root bark	Mukerjee, 1969
	Seed oil	Satam, 1973
	Seed shells	Roy, 1979
	Heartwood	Subramanyam, 1977
	Stem bark	Garg, 1978
	Flowers	Talapatra, 1982
3,7-Dimethoxyflavone	Root bark	Tanaka, 1992
Gamatin	Flowers	Garg, 1983
	Seeds	Pathak, 1983e
Glabone	Flowers	Kanungo, 1987
Glabra II	Stem bark	Garg, 1978
Isoglabrachromene	Stem bark	Saha, 1991
Isolonchoarpin	Seed oil	Satam, 1973
	Flowers	Talapatra, 1980
Isopongachromene	Seeds	Pathak, 1983a
	Root bark	Tanaka, 1992
Isopongaglabol	Flowers	Talapatra, 1982
Kamepferol	Flowers	Garg, 1983
Kaempferol-7-O-gluoside	Flowers	Garg, 1983
Kanjone	Leaves	Malik, 1977
	Seeds	Aneja, 1963
	Flowers	Garg, 1983
	Seed shells	Roy, 1977
Kanugin	Root bark	Mukerjee, 1969
	Seed shells	Roy, 1977
	Heartwood	Subramanyam, 1977
	Stem bark	Garg, 1978
	Leaves	Talapatra, 1985
	Flowers	Talapatra, 1980
Karanjachromene	Seed oil	Satam, 1973
Karanjin	Flowers	Garg, 1983; Talapatra, 1980
	Leaves	Malik, 1977
	Seed shells	Roy, 1977
	Stem bark	Garg, 1978
Lanceolatin B	Seeds	Roy, 1979
	Flowers	Talapatra, 1985
2'-Methoxyfurano (2",3":7,8) flavone	Seeds	Pathak, 1983e
4'-Methoxyfurano (2",3":7,8) flavone	Leaves	Rastogi, 1993
5'-Methoxyfurano (2",3":7,8) flavone	Stem bark seeds	Garg, 1978 Pathak, 1983e
	Flowers, Leaves	Talapatra, 1982; 1985
7'- Methoxyfurano (4",5":6,5) flavone	Leaves	Malik, 1977
8'- Methoxyfurano (4",5":6,7) flavone	Leaves	Malik, 1977

Table 1.8: Chalcones from *Pongamia pinnata*.

Compound	Part	References
Dihydromilletenonemethylether	Root bark	Tanaka, 1992
Glabrachalcone	Seeds	Pathak, 1983b
Glabrachromene	Heartwood	Subramanyam, 1977
	Seeds	Pathak, 1983e
Glabrachromene II	Heartwood	Subramanyam, 1977
	Seeds	Saini, 1983
2-hydroxy-3,4,4',6'-tetramethoxychalone	Root bark	Tanaka, 1992
Lonhocarpin	Heart wood	Subramanyam, 1977
Ovalitnin B	Root bark	Tanaka, 1992
Pongachalcone I or Obovatohalcone	Heartwood	Subramanyam, 1973
Pongachalcone II	Heartwood	Subramanyam, 1977
Pongagallone A	Leaf galls	Gandhidasan, 1987
Pongagallone B	Leaf galls	Gandhidasan, 1987
Ponganone I	Root bark	Tanaka, 1991
Ponganone II	Root bark	Tanaka, 1991

Table 1.9: Steroids and terpenoids from *Pongamia pinnata*.

Compound	Part	Reference
Betulinic acid	Stem bark	Saha, 1991
Cycloart-23-ene-3β,25-diol	Flowers	Talapatra, 1992
	Leaves	Talapatra, 1985
Friedelin	Flowers	Talapatra, 1982
	Leaves	Talapatra, 1985
Lupenone	Leaves	Talapatra, 1985
Lupeol	Leaves	Talapatra, 1985
β-Sitosterol	Leaves	Talapatra, 1985
β-Sitosterol-β-D-glucoside	Flowers	Talapatra, 1982
γ-Sitosterol	Flowers	Khanna, 1965

Table 1.10: Miscellaneous compounds from *Pongamia pinnata*.

Compound	Part	References
Acrylamide	Leaf galls	Gandhidasan, 1987
Aurantiamide acetate	Flowers	Talapatra, 1980
Glbra I or Ovalitenone	Seeds	Sharma, 1977
	Stem bark	Garg, 1978

	Flowers	Talapatra, 1980
	Seeds	Pathak, 1983e
Glabrin	Leaf galls	Gandhidasan, 1987
Hexacosyl caffeate	Stem bark	Saha, 1991
Pongamol or Lanolatin C	Immature seed shells	Roy, 1997
	Flowers	Talapatra, 1980
	Seeds	Pathak, 1983a
Pongapinone A	Bark	Kitagawa, 1992
Triacontyl caffeate	Stem bark	Saha, 1991

This multipurpose tree has lot of industrial and medicinal applications. Utility of the seed oil as "**Bio diesel**" (Srivinisa U, 1997; Vivek and Gupta, 2004) is well recognized. In India, this oil was traditionally used as a fuel for cooking and in lamps, as a kerosene substitute. This oil has been tried as a fuel in diesel engines, showing a good thermal efficiency (CSIR, 1948-76). De (1999) studied the fuel characteristics and found Karanja oil can be used as cheap raw material for synthesis of Biodiesel. SUTRA (Sustainable Transformation of Rural areas), a group of scientist from IISC Bangalore, revealed that karanj oil could be used as an alternative to diesel. The fuel characteristics of this oil are compared with diesel (Table 1.9). The oil used in 7.5 KVA gensets has electrified three villages in Adilabad district of Andhra Pradesh and in six villages in Jadol area of Rajasthan (The Indian Express dated 2/6/2002).

Table 1.9: Bio diesel Specifications of Pongam oil

Parameters	**Pongam oil (un filtered)**	**Diesel**
Saponification value	178.47	nil
Iodine value	115.8	38.3
Acid value	2.29	0.06
Color in 1 inch cell (Y+5R)	50	102.5
Specific gravity	0.92	0.84
Refractive index	1.481	1.472
Moisture & Volatile matter	0.05%	24.66%
Viscocity at 25ºC	84.9 CST	8.5 CST
Cloud Point	15ºC	13ºC
Pour Point	-1ºC	1ºC

A study by RANWA (Research and action in Natural Wealth Administration) says the Pune Municipal Transport could run its 800-bus fleet on karanja oil if the tree is planted on 68.6 sq. km. (The Indian Express dated 2/6/2002). Recently, The Maharashtra Energy Development Agency (MEDA) has setup Biodiesel Park. This park will comprise a research laboratory as well as a plantation area for different species of oil producing plants like *Jatropha, Pongamia* and *Castor*. (The Indian Express, dated 12/12/2005). Mint Biofuels, a firm launched country's first commercial production plant of biodiesel from Karanja seeds in India. This bio diesel has been successfully used in running motors in cars like Skoda Activa, TATA 407 and in Kirloskar gensets (The Indian Express dated 12/12/05).

Apart from biodiesel utility, pongam oil is used in tanning industry for dressing of EI leathers. In leather industry it is used for making fat liquors. After neutralization, the oil is used as lubricant for heavy lathes, chains, bearings of small gas engines, enclosed gears and heavy engines.

Karanj and its isolates have been shown to have piscidal and insecticidal activities. Karanj cake is against the nematode *Meloidogyne javanica* (Parmar et al., 1976), which reduces incidence of root knot of tomato. Karanj oil has been reported to synergise endrin against houseflies. It is reported that it can usefully incorporated in various malathion formulations (Parmar, 1987).

Being nitrogen fixing tree legume, the seed cake is utilized in improving soil fertility. It has been shown that the seed cake has nitrification inhibition properties.

Various parts of *Pongamia* are highly esteemed for its medicinal applications.

- Oil is used as liniment for rheumatism.
- Leaves are active against *Micrococcus*; their juice is used for colds, coughs, diarrhea, dyspepsia, flatulence, gonorrhea and leprosy.
- Roots are used leaning gums, teeth and ulcers.
- Bark is used internally for bleeding piles.
- Juices of the plant and oil are antiseptic against itch, herpes and *Pityriaris vesicolor*.
- Powdered seeds are valued as febrifuge, tonic and in bronchitis and whooping cough.
- Seeds find use as an external application in skin diseases.

Above all, the wood is considered a quality timber. The wood is used for cabinet making, cart wheels, pests, agricultural implements, tool handles and combs. It is a shade bearer and considered to be a good tree for planting in pastures. The tree is used for afforestation especially in watersheds.

There is a very little information available regarding the genetic makeup of this plant. Recently, Lavin et al., (1998) partially sequenced the PHYA (Phytochrome A gene) and PHYA-like genes from *Pongamia.* Partial sequence of a small subunit ribosomal RNA gene and internal transcribed spacer 1, complete sequence of 5.8S ribosomal RNA gene and internal transcribed spacer 2, and partial sequence of large subunit ribosomal RNA gene were reported from *Pongamia* by Hu et al., 2002. Shi et al., (2005) sequenced ribulose-1, 5-biphosphate carboxylase/oxygenease large subunit gene, maturase, a mitochondrial gene and 18S ribosomal RNA gene from this species. No report regarding the genomic DNA is available.

There is scope for improvement of tree species using biotechnological approaches. These approaches offer the potential to alter qualitative and quantitative improvement in the products obtained from *Pongamia* trees. By subjecting to these techniques, this oil borne tree species can serve as a source of high quality fuel and raw material for industries. Non-edible oil borne tree species can also be exploited as an alternate source of edible oil by silencing the related genes, which cause production of undesirable substances. *In vitro* culture provides the only route for generation of genetically engineered genotypes of trees.

With the present state of knowledge, it is apparent that no literature on tissue culture studies has been done in this multipurpose, leguminous tree species. Thus, there is need to develop reliable tissue culture protocols for *in vitro* regeneration of *Pongamia pinnata.* Protocols developed for clonal propagation can then be used for rapid propagation of identified elites and genetically modified plants. Raising plantations of clonally propagated, high seed yielding trees will contribute to increased seed and oil production. These plantations will also serve the purpose of seed orchard for production of superior quality seeds. 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 oil composition and biochemicals.

Pongamia species, which represent a substantial variability in phenotype as well as chemo type properties for the oil and its components, need to be documented for establishing phylogenetic relationships and unique marker profiles at DNA level. These molecular relationship and markers can be useful for designing strategies for gene introgression and breeding programs to produce desired recombinant hybrid genotypes with both oil quality and yield. To date, there is no literature available regarding the molecular scaffold of this tree. There is need to assess the variability/ similarity among the individual tree species. PCR based molecular marker like, ISSRs can be used for this purpose.

Detection of subtle genetic variation at the phenotypic, cytological, biochemical and DNA sequence levels among the micropropagated plants raised from organized meristems (somatic embryogenesis, enhanced axillary branching methods) have exploded the view that they retain complete genetic fidelity (Rani and Raina, 2000). Thus, there is need to assess the genetic fidelity of tissue culture raised *Pongamia* plants using molecular markers. Among the molecular markers, presently ISSRs are preferred as it provides more information on germplasm, PCR based and ease to perform.

OBJECTIVES OF THE PRESENT STUDY

Dearth of literature on *in vitro* regeneration of *P.pinnata*, demands development of reliable protocols for studies on *in vitro* morphogenesis. Experiments on standardization may be initiated with juvenile tissues and can then be extended to mature tissues. Resultant tissue culture derived clones may be assessed for uniformity using ISSR markers. Variability studies on germplasm of *Pongamia* can provide insight for its characterization.

Thus, the present study on *Pongamia* was initiated with the following objectives:

1. Optimization of conditions for clonal propagation using juvenile tissues.
2. Optimization of conditions for clonal propagation using mature nodal buds.
3. Optimization of protocol for *de novo* morphogenesis (organogenesis and somatic embryogenesis).
4. Assessment of variability/similarity among the trees and fidelity of the *in vitro* raised clones using ISSR markers (Molecular characterization).

CHAPTER 2:
MATERIALS AND METHODS

INTRODUCTION

This chapter describes the materials and general techniques routinely practiced in plant tissue culture (A), and in isolation and amplification of DNA (B). The materials and methods, specific to particular experiment, are dealt in details in respective chapters.

2.1 PLANT TISSUE CULTURE

A. MATERIALS

Most of the consumables and chemicals were procured from various local suppliers. Few chemicals including the growth regulators and Phytagel were imported (Sigma chemicals,USA).

Glasswares and Plastic wares:

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. Autoclavable, screw cap bottles (100, 250 & 500ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (60 ml capacity), slides (Blue Star, India) and cover slips (Micro-Aid, India).

Plasticwares including sterile disposable plastic petriplates of 55 & 85 mm diameter were procured from Tarson, Pune. Klin wrap, used for sealing the petriplates. Micropipette of different precision measurements (1000ml, 200ml, 100ml, 20ml, 10ml & 2ml) and microtips were procured from Gilson and Tarson respectively.

Chemicals:

Chemicals used for surface sterilization procedures were Bavistin® (BASF, India), Savlon (Johnson and Johnson Limited, USA) and Mercuric chloride (Qualigens Fine Chemicals, India). A media additive sterilant, Plant Preservative Mixture (PPM) was procured from Sameer Science Laboratory, India. An antibiotic, Cefotaxime (Alkem, India) was often used for controlling the growth of the contaminating bacteria in plant tissue culture medium.

Inorganic salts and vitamins used for preparation of culture media and for other experiments were of Analar grade (BDH, Hi-Media and Qualigens Fine Chemicals, India). Sucrose, fructose, glucose, maltose were procured from Hi-Media (India) and Qualigens Fine Chemicals (India). Agar agar

(bacteriological grade), used as gelling agent in the semisolid culture medium was procured from Hi-Media (India). Gelling agent Phytagel™ (Gelrite) used in one of the experiments was procured from Sigma Chemicals Co. (U.S.A.). Activated charcoal was procured from Sarabhai M Chemicals, India.

Growth regulators including Kinetin (KN), 6-benzyl adenine (BA), Zeatin (Z), N-phenyl-N'-1, 2,3-thidiazol-5-ylurea (Thidiazuron, TDZ), α-naphthalene acetic acid (NAA) and 3,6-dichloro-o-anisic acid (Dicamba) 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 (tertiary butyl alcohol), iron alum (S.D.fine chemicals, India); paraffin wax (m.p.58-60°C) (E. Merck, India Ltd.). Haematoxylin and Eosin stain were 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.

The major equipments used include:

pH meter: pH is the negative logarithm of hydrogen ion concentration. Measurement of pH in pH meter (Thermo Orion) is based on ion exchange in between hydrated layers formed on glass surface. Change in ion exchange results in e.m.f or voltage difference causing current flow. The current intensity gives the value of pH.

Electronic Balance: A manual top loading balance (Contech) used for quick weighing and for analytical purposes. This is a single pan balance capacity 100-200gm, sensitivity 0.1mg operating on 230 V 50 H_2 AC mains. Precision of ± 0.005g, weighing range 0-1, 200g, digital read out was used for making stock solutions of growth regulators and for other fine weighing.

Autoclave: The autoclave (Nat Steel Equipment Private Limited Bombay) was used for sterilization of media, glassware, water, dissecting instruments etc and for decontamination of contaminated cultures in culture vessels. It is based on application of steam under pressure. Autoclaving was carried out at 121°C temperature under 15lb/in^2 pressure. Except culture media, all other materials were autoclaved for one hour. The culture media were autoclaved for 15 min.

Laminar airflow ultra clean unit: All aseptic manipulations were carried out on this unit. In laminar (Klenzoids/ Microfilt, India), with the help of air pump air is passed through HEPA filters of pore

size, 0.22 micron. Due to positive pressure, the entry of any contaminant is restricted from the open side of the bench. The instrument is fitted with UV tubes in addition to the fluorescent tubes.

Apart from these, instruments like Magnetic stirrer (Remi, India), Steamer (Ultradent, India), Temperature controlled oven (Pathak Electricals, India), Light microscope (Carl-Zeiss Jena), Microtome (Reichert Jung), Camera (Nikon/Zeiss), membrane filter sterilizing unit (Laxbro, Pune) and Pipetman (Gilson/Tarson) were used. With the exception of pipetman, microtome, microscopes and camera, all other equipments used in course of this study are fabricated by different companies in India.

Source of Explants:

Seeds of *Pongamia* were obtained from mature fruits extracted from the trees growing locally. These mature seeds were used for developing seedlings *in vitro.* Actively growing twigs were collected from mature trees. Nodal explants dissected from these twigs were used as explant. Cotyledon and Zygotic embryo axis explants were excised from nearly mature pods extracted from the trees growing locally. Immature seeds of different sizes, excised from young fruits were also used as explants.

B. METHODS

Preparation of glassware and instruments:

Glassware used in our studies was 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 and 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 (Safe surgical industries, Beawar, India). All dissecting instruments were either wrapped singly or were put in closed aluminum 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. Blue and yellow tips used for aseptic addition by micropipets were arranged in cases meant for their size, wrapped with brown paper and autoclaved. Sterilization of the glassware and instruments was carried out by autoclaving at 121°C for 1 h in 15 lbs/(inch)^2.

Preparation of media:

Success of a tissue culture protocol depends on the appropriate composition of the medium. Several basal formulations like Murashige and Skoog's media, B5 Gamborg media, Shrenk and Hildebrandt media, Woody plant media etc. are now available. Concentrations of the macro and microelements, salts and organic constituents of the MS (Murashige and Skoog, 1962) basal medium are listed in Table 2.1 Stock solutions of the media ingredients were prepared by dissolving weighed amounts of these salts as per Table 2.1 in double distilled water. Appropriate aliquots of these solutions were mixed to prepare the media.

Table 2.1: Composition of macro and microelement salts and vitamins in Murashige and Skoog basal medium

Ingredients	Amount ($mg.l^{-1}$)	Stock solution
Macronutrients		**(20 X) in 500ml**
KNO_3	1900	19 g
NH_4NO_3	1650	16.5 g
$CaCl_2.2H_2O$	440	4.4 g
$MgSO_4.7H_2O$	370	3.7 g
KH_2PO_4	170	1.7 g
Micro-nutrients		**(100 X) in 100 ml**
$MnSO_4.4H_2O$	22.3	62 mg
$ZnSO_4.7H_2O$	8.6	223 mg
H_3BO_3	6.2	86 mg
KI	0.83	2.5 mg
$CuSO_4.5H_2O$	0.025	0.25 mg
$Na_2MoO_4.2H_2O$	0.25	8.3 mg
$CoCl_2.6H_2O$	0.025	0.25 mg
$FeSO_4.7H_2O$	27.8	278 mg
$Na_2EDTA.2H_2O$	37.3	373 mg
Vitamins		**(100 X) in 100 ml**
Myo-inositol	100	1 g
Thiamine-HCl	0.1	5 mg
Nicotinic acid	0.5	20 mg
Pyridoxine-HCl	0.5	1 mg
Glycine	2	5 mg

Stock solutions of growth regulators (GR) were prepared by adding few drops of solvent in the required amount of growth regulator to dissolve. After dissolution, the required concentration was made by addition of double distilled water and stored in refrigerator in sterilized bottles. Table 2.2 describes the list of solvent and diluents, stock concentration of growth regulators used.

Table 2.2: Preparation and Concentration of Growth Regulators

Growth Regulator	Molecular weight	Solvent	Diluent	Stock Concentration
BA	225.3	1N NaOH	H_2O	10 mg/100ml
KN	215.2	1N NaOH	H_2O	10 mg/100ml
TDZ	220.2	DMSO	H_2O	10 mg/100ml
2,4-D	221	1N NaOH	H_2O	10 mg/100ml
Picloram	241.5	EtOH	H_2O	10 mg/100ml
2,4,5-T	255.5	EtOH	H_2O	10 mg/100ml
Dicamba	221	EtOH	H_2O	10 mg/100ml
NAA	186.2	1N NaOH	H_2O	10 mg/100ml
Ads	184.2	H_2O	H_2O	20mg/100ml

For media preparation a calculated amount of aliquots were added from these stock solutions. Carbohydrate (Sucrose, Fructose, Glucose) was weighed and added in required quantity (2%, 3%, 6%) 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 double 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 (20ml of media), flasks (100ml of media) or bottles (80ml of media) after thorough mixing. 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. For culturing in petridishes, molten media was poured in sterile dishes before gelling. Media additive sterilants like PPM (Plant Preservative Mixture) and antibiotic like taxim (Cefotaxime) in required concentration were added aseptically to the autoclaved semisolid medium before it gelled. In case of charcoal containing medium individual tube was shaken after autoclaving and before setting of medium for uniform distribution of charcoal.

Preparation of explants:

Mature seeds, green pods or mature tree derived nodal buds were washed thoroughly in running tap water. These were treated with 1% Bavistin (Carbendazim 50%WP, BASF, India) containing 4-5 drops of detergent (Labolene, Qualigens, India) for an hour, on a gyratory shaker followed by rinsing three times with sterile distilled water to remove Bavistin and detergent. Hereafter the tissues were manipulated under aseptic condition. These were treated with 4% savlon (Chlohexidine Gluconate solution I.P. 1.5% v/v and Cetrimide I.P. 3% w/v), Johnson and Johnson, USA) for 10 min. Washings with sterile distilled water to eliminate savlon followed this. This was followed by 0.1% mercuric chloride treatment for specified time depending on the nature of the explant (10min. for mature seeds and green pods and 8 min. for mature buds). Adhering mercuric chloride was removed by washing the explants repeatedly with sterile double distilled water. The explants like cotyledon and embryoaxis were isolated from the surface sterilized green pods.

Initiation of cultures:

Cultures were initiated from the surface sterilized explants in laminar airflow cabinets. All the dissections were carried out on sterile filter papers. The instruments used for aseptic dissection or transfer of tissues was presterilized. During aseptic operations, these were sterilized intermittently by dipping in rectified spirit and flaming. After transferring the tissues, the petriplates were sealed with klinwrap. All cultures were labeled appropriately before incubation.

Culture conditions:

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. During hardening procedures, the cultures were incubated in continuous light (24h), other conditions remaining unchanged.

Histological Techniques:

Sections were prepared for histological studies following the methods described by Sharma and Sharma, 1980. The tissues were cut into small pieces (approx 3 x 4 mm) and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48-h at room temperature. Tissues were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (mp 58-60°C). Serial sections of 10 μM were cut using a rotary microtome (Reichert-Jung 2050, Germany). Sections were double stained with haematoxylin-eosin and mounted with DPX *(Loba Chemie,* Mumbai, India) for studies under microscope.

Statistical Procedures:

Statistical methods were used for comparison of treatment means during optimization of the parameters for micropropagation using both seedling and mature explants, and for morphogenic 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 differences among the treatment means were tested using Duncan multiple range test (DMRT) at a 5% probability level ($P<0.05$), wherever applied. The data was analyzed using Microsoft Excel package.

Microscopy and Photography:

The morphogenic response in various explants was evaluated under stereomicroscope and was photographed. Histological slides were studied under microscope and photographed. Magnifications of the photomicrographs were noted.

2.2 MOLECULAR TECHNIQUES

A. MATERIALS

Glasswares and Plasticwares:

Micropipette of different precision measurements (1000, 200, 100, 20, 10 & 2µl) was procured from Gilson medical Electronics, France. Micro tips, appendorffs (0.2,0.5,1.5 & 2ml) and microtip-boxes were procured from Axygen scientific Pvt. Ltd. (India).

Reagents and Chemicals:

- Tris-HCl pH 8.0 (1M); EDTA pH 8.0 (0.5M); NaCl (5M); CTAB (20%); Chloroform:Iso-amylalchol (24:1 v/v); Polyvinyl pyrrolidone; β-mercaptoethanol; cold isopropanol and ethanol (70%)
- Extraction buffer: 100mM Tris-HCl (pH 8.0), 25mM EDTA, 1.5M NaCl, 2.5% CTAB, 0.2% β-mercaptoethanol (v/v) (added immediately before use) and 1% PVP (w/v) (added immediately before use).
- High salt TE buffer: 1M NaCl, 10mM Tris-Cl (pH 8.0) and 1mM EDTA.
- Agarose (sigma, India)
- Electrophoresis buffer: Tris-acetate-EDTA (0.5x)
- Loading buffer: Bromophenol blue (0.25%) and glycerol (30%)
- Fluorescent dye: Ethidium bromide (10µg/mL)
- Marker: Low range DNA ladder (3 Kb) (Genei, India)
- Enzymes: RNAase A (10mg/mL) and Taq DNA Polymerase (Genei, India)

- Buffers: *Taq* DNA Polymerase buffer with $MgCl_2$ (Genei, India)
- Nucleotides: dNTPs (G, A, T, C) (Genei, India)
- PCR additives: Spermidine (20mM)
- Primers: ISSR Primer Set #9 (801…900) obtained from UBC, British Columbia (Table 2.1)

Table 2.1: List of ISSR Primers tested with DNA of *Pongamia* for amplification by PCR

UBC Primer Set #9 (Microsatellite)
3 nanomoles/tube

801	ATA TAT ATA TAT ATA TT	851	GTG TGT GTG TGT GTG TYG
802	ATA TAT ATA TAT ATA TG	852	TCT CTC TCT CTC TCT CRA
803	ATA TAT ATA TAT ATA TC	853	TCT CTC TCT CTC TCT CRT
804	TAT ATA TAT ATA TAT AA	854	TCT CTC TCT CTC TCT CRG
805	TAT ATA TAT ATA TAT AC	855	ACA CAC ACA CAC ACA CYT
806	TAT ATA TAT ATA TAT AG	856	ACA CAC ACA CAC ACA CYA
807	AGA GAG AGA GAG AGA GT	857	ACA CAC ACA CAC ACA CYG
808	AGA GAG AGA GAG AGA GC	858	TGT GTG TGT GTG TGT GRT
809	AGA GAG AGA GAG AGA GG	859	TGT GTG TGT GTG TGT GRC
810	GAG AGA GAG AGA GAG AT	860	TGT GTG TGT GTG TGT GRA
811	GAG AGA GAG AGA GAG AC	861	ACC ACC ACC ACC ACC ACC
812	GAG AGA GAG AGA GAG AA	862	AGC AGC AGC AGC AGC AGC
813	CTC TCT CTC TCT CTC TT	863	AGT AGT AGT AGT AGT AGT
814	CTC TCT CTC TCT CTC TA	864	ATG ATG ATG ATG ATG ATG
815	CTC TCT CTC TCT CTC TG	865	CCG CCG CCG CCG CCG CCG
816	CAC ACA CAC ACA CAC AT	866	CTC CTC CTC CTC CTC CTC
817	CAC ACA CAC ACA CAC AA	867	GGC GGC GGC GGC GGC GGC
818	CAC ACA CAC ACA CAC AG	868	GAA GAA GAA GAA GAA GAA
819	GTG TGT GTG TGT GTG TA	869	GTT GTT GTT GTT GTT GTT
820	GTG TGT GTG TGT GTG TC	870	TGC TGC TGC TGC TGC TGC
821	GTG TGT GTG TGT GTG TT	871	TAT TAT TAT TAT TAT TAT
822	TCT CTC TCT CTC TCT CA	872	GAT AGA TAG ATA GAT A
823	TCT CTC TCT CTC TCT CC	873	GAC AGA CAG ACA GAC A
824	TCT CTC TCT CTC TCT CG	874	CCC TCC CTC CCT CCC T
825	ACA CAC ACA CAC ACA CT	875	CTA GCT AGC TAG CTA G
826	ACA CAC ACA CAC ACA CC	876	GAT AGA TAG ACA GAC A
827	ACA CAC ACA CAC ACA CG	877	TGC ATG CAT GCA TGC A
828	TGT GTG TGT GTG TGT GA	878	GGA TGG ATG GAT GGA T
829	TGT GTG TGT GTG TGT GC	879	CTT CAC TTC ACT TCA
830	TGT GTG TGT GTG TGT GG	880	GGA GAG GAG AGG AGA
831	ATA TAT ATA TAT ATA TYA	881	GGG TGG GGT GGG GTG
832	ATA TAT ATA TAT ATA TYC	882	VBV ATA TAT ATA TAT AT
833	ATA TAT ATA TAT ATA TYG	883	BVB TAT ATA TAT ATA TA
834	AGA GAG AGA GAG AGA GYT	884	HBH AGA GAG AGA GAG AG
835	AGA GAG AGA GAG AGA GYC	885	BHB GAG AGA GAG AGA GA
836	AGA GAG AGA GAG AGA GYA	886	VDV CTC TCT CTC TCT CT
837	TAT ATA TAT ATA TAT ART	887	DVD TCT CTC TCT CTC TC
838	TAT ATA TAT ATA TAT ARC	888	BDB CAC ACA CAC ACA CA
839	TAT ATA TAT ATA TAT ARG	889	DBD ACA CAC ACA CAC AC
840	GAG AGA GAG AGA GAG AYT	890	VHV GTG TGT GTG TGT GT
841	GAG AGA GAG AGA GAG AYC	891	HVH TGT GTG TGT GTG TG
842	GAG AGA GAG AGA GAG AYG	892	TAG ATC TGA TAT CTG AAT TCC C
843	CTC TCT CTC TCT CTC TRA	893	NNN NNN NNN NNN NNN
844	CTC TCT CTC TCT CTC TRC	894	TGG TAG CTC TTG ATC ANN NNN
845	CTC TCT CTC TCT CTC TRG	895	AGA GTT GGT AGC TCT TGA TC
846	CAC ACA CAC ACA CAC ART	896	AGG TCG CGG CCG CNN NNN NAT G
847	CAC ACA CAC ACA CAC ARC	897	CCG ACT CGA GNN NNN NAT GTG G
848	CAC ACA CAC ACA CAC ARG	898	GAT CAA GCT TNN NNN NAT GTG G
849	GTG TGT GTG TGT GTG TYA	899	CAT GGT GTT GGT CAT TGT TCC A
850	GTG TGT GTG TGT GTG TYC	900	ACT TCC CCA CAG GTT AAC ACA

The major equipments used include:

Milli-RO water system: The instrument (Millipore, USA) is used for obtaining deionized water. There are series of ion exchanging columns. When ordinary water is passed through these columns, it is made free of salts and minerals that are naturally present in tap water. It is important to use deionized water for preparation of reagents as the presence of minerals and salts may alter the final composition.

Horizontal electrophoresis unit (Tarson, India): The basic principle of electrophoresis is, charged ions or molecules migrate when placed in an electric field. The rate of migration of a substance depends on its net charge, size, shape and the applied current. It consists of a power pack and electrophoresis unit. The power pack supplies a stabilized current at controlled or required voltage and current output. The electrophoresis unit contains the electrodes, buffer reservoirs and gel casting assembly.

Gel Documentation System (Biorad): It is a powerful, flexible package including the hardware and the software for imaging and analyzing 1-D electrophoresis gels, dot blots arrays and colonies. The lane-based functions can be used in determination of molecular weights and other values.

Water bath (Julabo): It is used for maintaining the constant temperature, in which, temperature setting, temperature indicator, and cooling effect are also available.

SpinWin (Tarson): It is a mini centrifuge equipped with continuously variable electronic speed control, speed indicator, Amp meter, timer, dynamic break, zero starting switch and fuse safety device for 230 V 50 Hz AC mains.

Spectrophotometer (Perkin & Elmer): For quantitating DNA in a solution. Reading is taken at wavelengths of 260 and 280nm and ratio between them provide an estimate of purity of sample DNA ((Maniatis, 1989).

PCR Robocyler (Stratagene, USA): It is microprocessor controlled for block laboratory instrument utilizing a robotic arm to quickly move from one temperature block to another based on user defined program. This system has four separate anodized aluminum temperature blocks (3-heating block &1 cold blocks) containing 96 precision cut wells that remain at set temperature, where thermal cyclers have a single block that changes temperature during each cycle. In the Robocycler tubes are moved from one block to next by the robotic arm. The four-block design decreases cycling time by up to 30% and achieve a well to well temperature uniformity of ± 0.1 °C for amplification process.

Source of plant material:

Ten trees growing locally were selected. Both Pods and fresh leaf materials were collected from these trees. Collected Seeds were used for extraction and quantification oil. Leaf materials were used for DNA isolation. Leaf material of the mothers and their respective *in vitro* raised plantlets were used for DNA isolation.

B. METHODS

DNA isolation protocol: The DNA isolation was carried out based on the Khanuja's isolation protocol (Khanuja et al., 1999). The protocol is as follows:

- Ground the plant material in liquid nitrogen (200mg of fresh leaf tissue).
- Transferred the material to 2ml microfuge tube and added 1ml of freshly prepared extraction buffer, Mixed by inversion to a slurry.
- Incubated at 60 ºC in a shaking water bath (100 rpm) for 1-2 h.
- Added 1ml of chloroform: isoamyl alchol (24:1) and mixed by inversion for 15 min.
- Spinned at 8000 rpm for 10 min at 25-30 ºC.
- Carefully transferred the upper clear aqueous layer to another 2ml microfuge tube.
- Added 200µl of 5M NaCl and mixed properly (do not vertex).
- Added 500µl of cold Isopropanol and by careful mixing produced fibrous nucleic acid that can be scooped and transferred to 2ml microfuge tube and centrifuged. Alternatively, the samples were centrifuged at 10,000 rpm for 10 min at 25-30ºC after mixing of isopropanol.
- Discarded the supernatant and washed the pellet with 70% ethanol.
- Dried the pellet in a vacuum for 15 min and dissolved it in 300µl high salt TE buffer.
- Added 5 µl of RNAase A and incubated at 37ºC.
- Extracted with equal volume of chloroform: isoamyl alchol (24:1).
- Transferred the aqueous layer to a fresh 1.5ml microfuge tube and added equal volumes of isopropanol.
- Spinned at 10,000rpm for 10 min at 25-30ºC.
- Washed the pellet with 70% ethanol. Dried the pellet in a vacuum and dissolved in 200µl of sterile milliQ water and then stored at 4 ºC until required.
- DNA concentrations were determined either by running aliquots of DNA samples on a 0.8% agarose gel electrophoresis or by taking the absorbance at 260 nm. The ratio between 260 and 280nm provides an estimate the purity of the sample DNA. DNA samples with a ratio of approximately 1.8 under spectrophotometer and producing an intact single band without smear on 0.8% Agarose gel electrophoresis were considered as good quality DNA.

PCR protocol using ISSR primers:

- The PCR protocol described by Mullis (1986) was followed and carried out in a total volume of 25µl containing following components: 4µl of genomic DNA (20ng); 2.5µl of 10X *Taq* buffer with 1.5mM $MgCl_2$; 2.5µl of dNTPs (1mM/µl); 0.16µl of Taq Polymerase (3u/µl); 0.5µl of Primer (1.5pm/µl); 1µl of Spermidine (20mM) and 14.32µl of sterile milliQ water.
- PCR amplifications were performed on a *Stratagene RoboCycler* under the following conditions (Fig. 2.1).
- Negative controls, containing all PCR components except DNA were also set up and run with each set of reactions.
- A low range DNA ladder (3 Kb) was used for band sizing.
- The ISSR products were loaded on 1.5% agarose gel stained with ethidium bromide for electrophoresis in 0.5X TAE at a constant current 60mA, < 150V for 2 h. Visualization of gel was undertaken in Gel Documentation system.

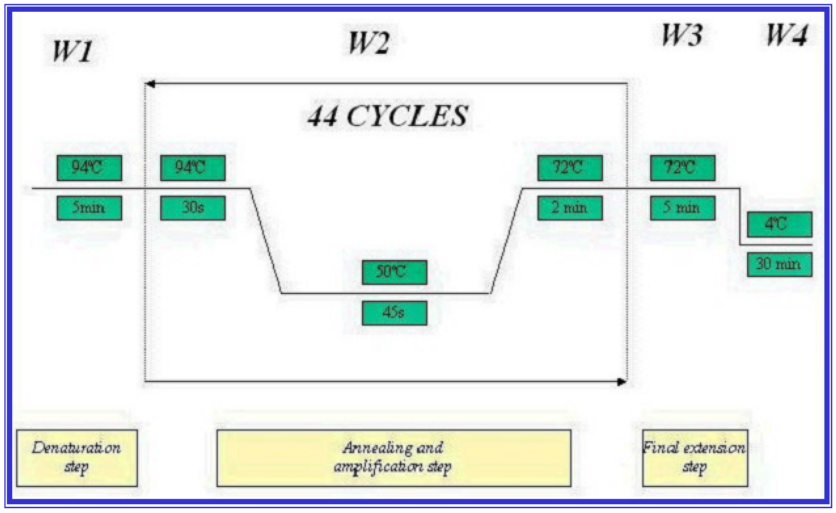


Fig. 2.1: Schematic diagram of PCR process

Data Analysis:

Presence/absence of each scorable fragment was recorded in a binary data matrix and the frequency of each band in sample was determined. Wind Dist software program in NTSYS format was used to produce similarity matrix. Cluster analysis was performed in TAB format using Win Boot software package. Pair wise genetic similarities were calculated by both softwares from the data matrix using Jaccard's coefficient, $F= M_{xy} /(M_t - M_{xy0})$, where M_{xy} represents the number of fragments shared between the two samples, M_t the total number of bands in the data matrix and M_{xy0} the number of bands in the data matrix that were not evident in either of these samples.

CHAPTER: 3
CLONAL PROPAGATION USING JUVENILE TISSUES

3.1 INTRODUCTION

Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques. It can broadly be divided in four stages:

Stage I - Establishment of culture:

The purpose of stage-I is to initiate axenic culture. This stage begins with the excision of meristem tissue from an identified stock plant. This explant is treated with anti microbial chemicals to remove contaminating organisms. Using aseptic techniques the explants are cultured into appropriate nutrient medium. A rapidly proliferating culture is established under optimum levels of light and temperature.

The major constraints in establishing sterile cultures of woody tissues are microbial contamination and interference of phenolic exudates. Use of fungicides and antibiotics limits the microbial infection to some extent. Use of antioxidants like ascorbic acid, PVP and charcoal helps in eliminating the interfering phenolic exudates. Contamination in tissue culture can originate from two sources through carry over of microorganisms on the surface and/or in the tissues of explants or through faulty procedures in the laboratory.

For most micropropagation work the explant of choice is an apical or axillary bud. The developmental stage of the explant is an important factor. The explant must be physiologically competent to survive the initial culture and elicit the appropriate response. In general, younger tissues such as terminal or axillary shoot buds regenerate better than older and mature tissues of the same stem. The age of the stock plant, physiological age of the explant and its developmental stage as well as its size can determine the success of a procedure (Franclet, 1987).

In general the ingredients of culture medium in this stage are determined by the kind of response desired e.g. axillary shoot formation or adventitious shoot formation or callusing etc., Supplementation of cytokinins like BA, TDZ in media is desired for axillary shoot formation whereas auxins like NAA, 2,4-D is preferred for callusing. 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. Most of the earlier studies strongly suggested the use of sucrose as carbon source. Depending on the mineral requirements of different plant species, several media compositions have been formulated. The most commonly used basal medium is Murashige and Skoog's medium. Details of this media composition are included in chapter 2.

Stage II - Proliferation of shoots in culture:

In this stage, shoots are proliferated in culture, which then serves as the source of shoots for subsequent propagation as well as the material that is required to maintain the stock. Multiplication of shoots is achieved by sub culturing the shoot cultures at regular intervals in appropriate medium. Variant plants may arise from the cultures maintained *in vitro* for a long period. For the purpose of micropropagation, usage of shoots from the cultures maintained for several passages and having higher subculture numbers is avoided to ensure genetic fidelity.

Stage III – Rooting:

In this stage the shoots obtained from multiplication media are separated and shifted to conditions that favor root initiation and shoot elongation. Rooting in shoots can be induced either *in vitro* or extra vitrum. For rooting *in vitro*, shoots are cultured either in growth regulator free medium or subjected to pulse treatment of auxin prior to transferring to an auxin free medium for root induction.

For ex vitro rooting, shoots are excised as small cuttings (micro cuttings), treated with commercial rooting mixture and planted in soil. These are then placed in a high humidity chamber for rooting. Rooting *ex vitro* has several advantages (Debergh and Read, 1991):

- It is easier to stick a cutting in soil 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 appropriate conditions for ex *vitro* rooting.

Stage IV – Hardening of tissue culture raised plants:

This stage involves transfer of plantlets from aseptic condition to green house and ultimately to the final location (environment). Plantlets develop within culture vessel under aseptic condition, on a medium containing sugar and nutrients to allow heterotrophic growth and in an atmosphere with high relative humidity and low levels of light, these all contribute to a phenotype that cannot survive the environmental conditions when directly placed in green house or field. Thus it is necessary to acclimatize plantlets gradually to ensure survival until they develop new leaves that are more adapted to ambient conditions under which plants are normally grown. High relative humidity has to be maintained during hardening process to protect the plants from desiccation and enable them to initiate new roots and shoots.

Juvenile tissues as source of explant

Developmental stage of an explant is an important factor for initiation of cultures for propagation. Younger the tissues better the *in vitro* response. Age of stock plant, physiological age of the explant and its developmental stage, as well as its size can determine the success of a procedure. Mature plant derived explants reported to be highly recalcitrant *in vitro*. Moreover, high degree of contamination in mature tissues poses problem in the establishment of culture. Juvenile explants such as cotyledons, hypocotyls, epicotyls, embryo axis or buds from seedlings are more responsive in culture than the tissues like bud explants or leaves from mature trees (Ahuja MR, 1993). Semi mature green pods derived explants like cotyledon, embryo axis are juvenile in nature, and can be used for micropropagation. Juvenile explants have been extensively employed for clonal propagation of woody plants. Frequency of sprouting is always higher in the buds taken from juvenile tissues than from mature plant during its vegetative phase.

Micropropagation from juvenile explants is useful for differentiation studies. Propagation using juvenile tissues can serve as a model system for standardization of protocol with mature tissues (Pierik, 1987). Propagation system using juvenile tissues provides a better understanding on the requirement of the plant for its growth and maturity.

Reports of successful micropropagation of various leguminous species are reviewed extensively (Trigiano et al., 1992; Parrott et al., 1992). In forest tree species, the success has been largely restricted to seedling materials (Rodriguez & Vendrame (2003). *In vitro* protocols, using seedling explants, have been employed for rapid propagation and manipulation of woody legumes, such as *Acacia mangium* (Monteuuis, 2004); *Acacia mearnsii* (Beck et al., 1998a); *Acacia tortilis* subsp. *raddiana* (Nandwani, 1995); *Albizzia* (Gharyal and Maheshwari, 1981); *Bauhinia* vahlii (Upreti & Dhar, 1996, Bhatt & Dhar, 2000); *Cercis yunnanensis* (Cheong, 2003); *Ceropegia candelaebrum* (Beena et al., 2003); *Dalbergia latifolia* (Sudhadevi and Nataraja, 1987a,b); *Dalbergia sissoo* (Pradhan et al., 1998); *Leucaena leucocephala* (Saffi & Borthakur, 2002); *Mimosa tenuiflora* (Villarreal and Rojas, 1996); *Pterocarpus marsupium* (Chand & Singh, 2004; Anis et al., 2005); *Peltophorum pterocarpum* (Uddin et al., 2005); *Sesbania drummondii* (Cheepala et al., 2004) and *Swartzia madagascariensis* (Berger and Schaffner, 1995).

In the present investigation, studies were conducted with the juvenile explants of *P.pinnata*. This was primarily to generate information on the nature of responses exhibited *in vitro* by the tissues of this

plant. This information can then be extended to studies with mature explants. Two types of juvenile tissues were studied for the establishment of *in vitro* regeneration protocol.

(A) *In vitro germinated seedling derived explants:*
This method is routinely followed in many leguminous tree species for establishment of propagation protocol. In this method, seeds are germinated *in vitro*. After the seedling attain appropriate size, the nodal explants and cotyledon nodes are excised from seedlings and are used for establishment of culture. Factors that influence establishment of culture like seedcoat, microbial interference, growth regulators, organic supplements, culture vessel, culture conditions for seedling of appropriate size and sucrose concentration are studied during optimization.

(B) *Semi mature greed pod derived juvenile explants like cotyledon nodes:*
Usage of green pod derived juvenile explants like cotyledon node has certain advantages. Constraints encountered in establishment of seedlings *in vitro* like seed viability and seed borne contamination can be eliminated using these explants. Using these the process of optimization is hastened, as no incubation period is required for seed germination and seedling growth.

3.2 EXPERIMENTAL PROTOCOL

(A) Establishment of sterile seedling cultures:

Mature dry pods of *Pongamia* were extracted from the trees prior to their natural abscission. Seeds extracted from these pods were surface sterilized using the procedure described in chapter 2. Seeds with or without seed coat were cultured in full and half strength MS basal media with varying concentrations of four growth regulators (GRs) including Gibberlic acid (GA_3) 0.29, 2.89, 5.77µM, 6-Benzyl Adenine (BA) 0.44, 4.44, 8.88µM, Thidiazuron (TDZ) 0.45, 4.54, 9.0µM, and Adenine Sulphate (Ads) 0.54, 5.43, 10.86µM. Seed coats were removed aseptically from surface sterilized seeds using forceps and scalpel and cultured in plastic capped glass bottles having 50ml medium. The experiment was carried out with 7 seeds per treatment and was repeated twice with two seeds per bottle. To control microbial contamination, antibiotic Cefotaxime, (Taxim, Alkem India Pvt. Ltd) was incorporated (250mg/l) aseptically. Nature of contamination and germination frequency was noted after 4 weeks of incubation. The data on microbial contamination was scored independent of the effect of growth regulator and the observations were grouped as follows:

- *Media without Cefotaxime: seeds with seed coat.* (**C**)
- *Media without Cefotaxime; seed without seed coat.* (**c)**
- *Media supplemented with 250mg/L of Cefotaxime; seeds with seed coat.* (**C**T)
- *Media supplemented with 250mg/L of Cefotaxime; seeds without seed coat.* (**c**T)

Germination frequency was noted in the groups mentioned above, taken into account the effect of various growth regulators and effect of seed coat.

Optimization of BA concentration and selection of culture vessel:

The seeds with seed coat were cultured in MS media without or with various concentrations of BA (2.22, 4.44, 6.66 and 8.88µM) to determine the concentration for optimum germination frequency and seedling growth. To identify the appropriate culture vessel both cotton plugged culture tubes and plastic capped jam bottles were used in this experiment. Tubes and bottles containing 20 and 50ml medium respectively were used. Single seed was cultured per vessel. For each medium formulation 20 tubes and 15 bottles were used. The experiment was repeated twice. Frequency of germination, length of shoots and number of shoot multiples were noted after 4 weeks of incubation.

Establishment of shoot culture from seedling derived axillary buds:

Shoots were isolated from seedlings germinated in MS media supplemented with 0.44µM BA. Nodal segments, 2 cm in length having one or two node/s were excised from decapitated shoot and were cultured in cotton plugged boiling tubes in 20ml MS media without or with various concentrations of BA (2.22, 4.44, 6.66, 8.88µM). Frequency of sprouting and number of multiples produced in axillary buds were scored after 4 weeks of incubation in light. The experiment was conducted with 10 replicates in each concentration and was repeated thrice. Shoots obtained from axillary nodes were subcultured for one passage in the medium of similar composition for further growth. The shoots that attained the length of 2-3cm were isolated and tested for elongation and rooting. Shorter shoots were sub cultured for further growth and multiplication.

Recycling of cotyledon node explants:

On isolation of shoot from the seedling, remaining part was the cotyledon node with pair of cotyledons and root. Roots were eliminated and nodal explants, with attached cotyledons were re-cultured in MS media supplemented with 0.44µM BA in cotton plugged 250ml Erlenmeyer's flasks. Number of shoots produced in each culture and lengths of these shoots were noted after 4 weeks of incubation in light. Following this shoots were excised and either sub cultured for further proliferation or transferred to rooting medium. After excision of shoots remaining part of the explant (stump) with

attached cotyledons was re-cultured in medium of same composition to induce second crop of shoots. This process of removing the shoots and reculturing the initial explant was repeated for eight cycles.

Optimization of Sucrose concentration on growth and multiplication of *in vitro* raised shoots:

To study the effect of sucrose, different concentrations of sucrose (3%, 4%, & 5%) were incorporated in MS media supplemented with 8.88µM BA. Medium with 2% sucrose was treated as control. Seedling derived nodal explants were cultured. Observation on sprouting, growth and multiplication were scored after 4 weeks of culture. Experiment was replicated thrice with 10 replicates in each concentration.

Rooting of shoots:

The shoots 2-3 cm in length, obtained from above experiments, were isolated and cultured in half strength MS media supplemented with 0.22% charcoal. Number of shoots rooted was scored after 4 – 6 weeks of incubation. This experiment was repeated 4 times with 10 to 12 shoots in each batch.

Transfer of propagules to soil:

The rooted shoots were removed from culture and washed gently under running tap water to eliminate agar and sucrose from the roots and were dipped in 1% Bavistin solution for 5 min prior to planting in autoclaved sand soil (1:1) mixture in plastic cups. These were covered with transparent plastic sheets and were hardened at 25± 2 ºC in 24 h photoperiod for 4 weeks. Hardened plants were transferred to bigger pots in greenhouse.

Ex vitro rooting of shoots:

In vitro raised shoots of *Pongamia* were also tested for ex vitro rooting. The shoot base was treated with a commercially available rooting mixture, Seradix B (May & Baker (India) Ltd.) and planted in sterilized soil: sand mixture (1:1) in plastic cups. These were covered with plastic bags to minimize loss of moisture and maintain high humidity and incubated in 24h photoperiod at 25±2ºC for four weeks. The experiment was carried three times with 10 shoots each. The stabilized plants after 4 weeks were transferred to greenhouse.

(B) Establishment of green pod derived cotyledon node cultures:

Green pods were surface sterilized as described in chapter 2. Seeds were isolated aseptically from green pods and were cut along the cotyledon junction. Two cotyledons were separated with half embryo axis (longitudinal) in each. Often, the halved embryo axis got detached from cotyledon or

degenerated due to damage after 3 to 4 days of culture. However each cotyledon carried small section of the cotyledon node.

The cotyledon carrying small section of nodal tissue were cultured in 85mm petriplates containing MS media supplemented with different concentrations of Thidiazuron (0, 0.05, 0.02, 0.45, 2.27, 4.54, 6.81, 9.08, 11.4, 13.6 & 22.7μM) for 20 days. MS medium without growth regulator served as a control. Cotyledons were cultured with their abaxial side touching the media. Six seeds were used per treatment (i.e. 12 cotyledon explants per concentration) and the experiment was repeated thrice.

After 20 days of incubation in TDZ, all explants were transferred to GR free MS media for 30 days. For larger and swollen cotyledon explants, responsive proximal portion having nodal region with shoot buds was cultured in tubes or bottles for differentiation of buds. Transfer into fresh media was carried out for three passages of 30 days each. Data on frequency of response and number of shoot buds per explant were scored after 30 days of culture. Number of shoot buds and shoot length were recorded after 60 and 90 days of culture respectively.

Elongated shoots were separated and cultured for 30 days in half strength MS media supplemented with 0.22% charcoal for induction and differentiation of roots. Rooted shoots were hardened, following the method described in the previous section. Hardened plants were transferred to bigger pots in greenhouse.

The MS media used in all the experiments were supplemented with 2% sucrose unless otherwise mentioned and all the data obtained were subjected to statistical analysis (chapter 2).

3.3 RESULTS AND DISCUSSION

Many of the difficulties encountered during micropropagation of woody legumes are common to woody plants in general. Major constraint encountered is extent of contamination, exudation of phenolics associated with culture initiation and the recalcitrant nature of shoot or root formation related to the ontogenetic stage of maturity inherent in a perennial woody crop (Trigiano et al., 1992). Success of shoot or root formation *in vitro* is often related to the maturity of donor plant (Hackett, 1985). Explants obtained from seedlings or plants in juvenile phase of growth have greater potential for organ formation. There is no report on tissue culture of *P. pinnata*. Therefore, for standardization of micropropagation protocol in this species, initially seedling explants were used.

Germination of *P. pinnata* seeds *in vivo* is asynchronous thereby restricting the availability of similar explants for experiments on optimization. Secondly germination behavior of this plant is hypogeal in nature in which the pair of cotyledons with the nodes remains under soil and is infested by microorganisms by the time the seedling grows to a certain size for obtaining nodal explants. To avoid these constraints attempts were made to standardize the conditions to obtain sterile seedlings of similar age and size by germinating the seeds in vitro.

Initial attempts were made to raise seedlings *in vitro* from seeds extracted from dry pods that dropped naturally from trees on maturation. In spite of the pods being tightly sealed and seeds being covered with hard coat, all cultures developed heavy fungal and bacterial contamination preceded by leaching of phenolics. To overcome the heavy contamination, which could be due to adhering of spores in the pods that remained in contact of the ground for several days prior to collection, mature pods of *Pongamia* were collected from trees manually and tested for germination *in vitro*. In spite of using elaborate method of surface sterilization 50% and 20% of the seeds developed fungal and bacterial contamination (Fig. 3.1A & B) respectively. Some seeds had both. Closer examination of the seeds under microscope revealed innumerable grooves and ridges in the seed coat (Fig.1.1G). To eliminate microbes harbored in these undulations of the seed coat, seeds with and without coat were tested as explants. Cefotaxime have been found effective in controlling bacterial growth in plant tissue cultures of Chrysanthemum (Jaime et al., 2003), Lycopersicon (Raj et al., 2005), Cucumber (Sapountzakis et al., 2002) etc. Thus this antibiotic was tested to establish microbe free cultures of *P. pinnata*.

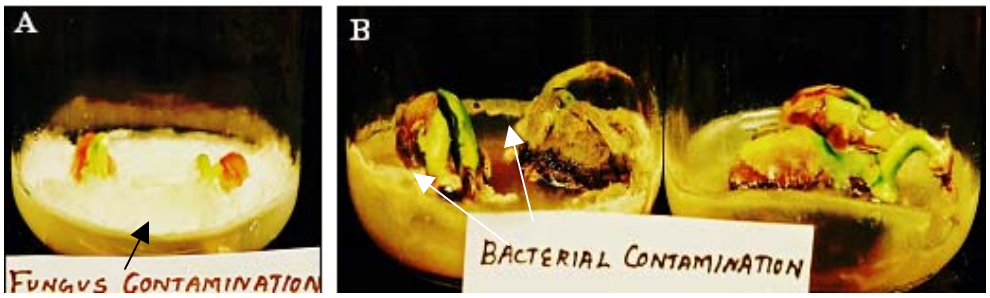


Fig. 3.1: (A) Fungal contamination during seed germination in culture.
(B) Bacterial contamination during seed germination in culture.

Influence of cefotaxime, seed coat and growth regulators on germination:

With an objective to hasten the process of standardization and minimize number of experiments, influence of five factors including basal medium concentration, an antibiotic, presence and absence of seed coat and 4 GRs were tested in single experiment. In these cultures, there was increase in bacterial

growth and reduction in fungal contamination on removal of seed coat from explant (Fig. 3.2). In view of this result, possible **association of seed coat** directly or indirectly in microbial growth in *Pongamia* cultures cannot be ruled out. The possibilities are that (i) the fungal spores were harbored in seed coat or (ii) there may be factors in seed coat conducive for growth of fungus. Removal of seed coat thus helped in reducing fungal contamination. On the contrary, (iii) there may be factors in seed coat, which prevents bacterial growth. Thus frequency of bacterial contamination was high in cultures without seed coat. Pongam oil and tissue extracts are known for their antifungal and antibacterial activity (Anonymous 1988; Meera et al., 2003). However there is no specific information on production of antibacterial compound in the seed coat.

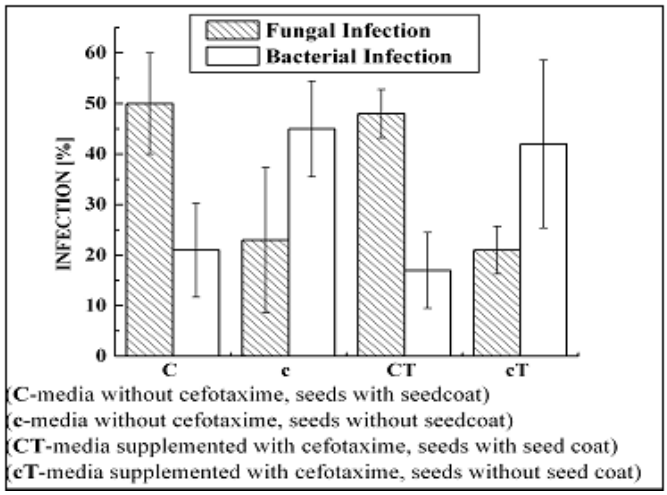


Fig. 3.2: Influence of seed coat and cefotaxime on the extent of fungal and bacterial growth in the seedling cultures of *Pongamia*.

The bacterial growth in the *Pongamia* cultures could not be controlled by incorporation of **Cefotaxime**. Several gram positive/negative bacteria including *Bacillus subtilis*, *B. typhosa*, *B. paratypii* A&B, *Micrococcus pyogenes Var. aureus*, *Micrococcus pyogenes Var. albus, Micrococcus pyogenes Var. citreus, E. coli, Mycobacterium tuberculosis* etc., are sensitive to Cefotaxime. Addition of this antibiotic failed to control the infection indicating resistance of the bacteria to Cefotaxime.

Overall the frequency of *Pongamia* seed germination in **full strength MS media** was higher compared to half strength medium (Table 3.1). There was no definite pattern noted in response of the seeds with or without seed coat in media with or without Cefotaxime. Similarly, frequency of germination in various concentrations of **growth regulators** like GA, TDZ and Ads was distributed widely. Thus the response in each instance between the concentration of GR, seeds with or without seed coat and in presence or in absence of Cefotaxime, could not be correlated. This wide variation in

germination frequency in presence of various GRs could be the results of intricacy created by variation in maturity status of the seeds and the multifaceted experimental condition in which seeds were exposed. Seeds were extracted from the tree on visual assessment of maturity.

Table 3.1: Influence of seed coat, Cefotaxime and different growth regulators on Germination of *Pongamia* seeds

Media*	Germination Frequency (%) Mean ± sd.			
	C	**c**	**CT**	**cT**
1/2 MS	8.3 ± 11.7	27.1 ± 14.8	12.5 ± 17.7	8.3 ± 11.7
MS	18.8 ± 26.5	22.9 ± 14.7	35.4 ± 3.0	20.8 ± 5.9
0.29 GA_3	29.2 ± 5.9	35.4 ± 3.0	50.0 ± 0.0	6.3 ± 8.8
2.89 GA_3	35.4 ± 3.0	14.6 ± 2.9	20.8 ± 5.9	29.2 ± 5.9
5.77 GA_3	8.3 ± 11.7	6.3 ± 8.8	22.9 ± 14.7	20.8 ± 5.9
0.44 BA	58.3 ± 11.7	50.0 ± 0.0	35.4 ± 3.0	56.3 ± 8.8
4.44 BA	52.1 ± 20.6	31.3 ±26.5	50.0 ± 0.0	41.7 ± 11.8
8.88 BA	22.9 ± 14.7	45.8 ± 29.4	35.4 ± 3.0	58.3 ± 11.7
0.45 TDZ	20.8 ± 5.9	20.8 ± 5.9	27.1 ± 14.8	6.3 ± 8.8
4.54 TDZ	0.0 ± 0.0	0.0 ± 0.0	20.8 ± 5.9	0.0 ± 0.0
9.0 TDZ	6.25 ± 8.8	6.25 ±8.8	0.0 ± 0.0	0.0 ± 0.0
0.54 Ads	27.1 ± 14.8	20.8 ± 5.9	20.8 ± 5.9	12.5 ± 17.7
5.43 Ads	16.7 ± 23.5	20.8 ± 5.9	20.8 ± 5.9	22.9 ± 14.7
10.9 Ads	29.2 ± 5.9	0.0 ± 0.0	0.0 ± 0.0	22.9 ± 14.7
Anova	**S 5%**	**S5%**	**S1%**	**S 1 %**

*****Concentration of GR in µM

C- media without Cefotaxime, seeds with seedcoat;
c- media without Cefotaxime, seeds without seedcoat;
CT- media supplemented with Cefotaxime, seeds with seedcoat;
cT- media supplemented with Cefotaxime, seeds without seedcoat.

At all concentrations of BA, germination frequencies were enhanced irrespective of other condition of the seed or media. Optimum response (58.3 ± 11.7%) was noted in seeds with coat in MS basal medium supplemented with BA 0.44µM and in seeds without seed coat in MS basal medium supplemented with BA 8.88µM and Cefotaxime (Fig. 3.3). Thus from the various GRs tested for germination of *Pongamia* seeds it is apparent that BA was more potent in accentuating *Pongamia* seed germination. Removal of seed coat or incorporation of Cefotaxime did not demonstrate any obvious effect on frequency of germination. Therefore, following experiment to determine the BA concentration for optimum seedling germination and growth was conducted with seeds with coat in medium and without Cefotaxime. Tree derived tissues grow slow in culture; seeds at the right stage of maturation is available only for a limited period annually. In the present investigation, by combining five parameters in single experiment, need for more number of explants was reduced and the process of standardization was hastened.

Fig. 3.3: Seeds cultured with and without seed coat for *in vitro* germination

Optimum BA concentration: Keeping in view the effect of BA on *Pongamia* seed germination in the above experiment and also the literature on supportive role of BA in seed germination (Sandeepkumar et al., 1998; Jain et al., 2000; Jordan et al., 2001; Shende et al., 2005), the seeds were cultured in MS media with various concentrations of BA (2.22, 4.44, 6.66 and 8.88µM) to determine the concentration optimum for germination frequency and seedling growth. **This experiment was coupled with the experiment for selection of appropriate culture vessel.**

Frequency of germination (Table 3.2) in all concentrations of BA was more than control, irrespective of the culture vessel. This indicates the supportive role of BA on *Pongamia* seed germination. High ($P<1\%$) frequency of response in tubes than in bottles in all concentrations of BA, suggests that culture vessel with cotton plug provides more supportive microenvironment for growth of *Pongamia* cultures. In bottles, the leaves were small and only partially opened (Fig. 3.4A). On the contrary the leaves were fully opened and green in the seedlings germinated in tubes (Fig. 3.4B). The medium with 0.44µM BA was optimum for germination in either culture vessel. In bottles, the germination frequency in medium without GR was 27 ± 8.4 %. It increased significantly (53 ± 3.9) on supplementing the medium with 0.44µM BA.

On further increase of this GR (2.22, 4.44, 6.66, 8.88µM) there was no significant change. The response varied within 40–53%. In tubes the germination frequency was 43% in medium without GR and it increased to 77±9.4 % on supplementing the medium with 0.44µM BA. At higher concentrations (2.22, 4.44, 6.66, 8.88µM), there was no further increase in response. Instead there was marginal decrease. Thus optimum germination frequency (77±9.4 %) was attained in MS medium with 0.44µM BA in cotton-plugged tubes.

Table 3.2: Influence of BA and Culture Vessel on germination frequency of *Pongamia* seeds

Conc. of BA (μM)	Germination Frequency / radical emergence (%)		Average length of shoot (cm) mean ± sd.		Average number of shoot multiples mean ± sd.	
	Bottles	Tubes	Bottles	Tubes	Bottles	Tubes
Control	27 ± 8.4	43 ± 0.0	2.80 ± 0.8 (05)	3.39 ± 0.9 (10)	1.00 ± 0.0 (05)	1.00 ± 0.0 (10)
0.44	53 ± 3.9	77 ± 9.4	2.78 ± 0.9 (15)	3.41 ± 1.1 (35)	1.25 ± 0.5 (12)	1.67 ± 0.9 (21)
2.22	48 ± 5.7	75 ± 0.0	2.75 ± 0.9 (15)	3.37 ± 1.6 (31)	1.15 ± 0.4 (13)	1.55 ± 0.8 (20)
4.44	40 ± 4.6	73 ± 7.1	2.64 ± 0.7 (09)	3.24 ± 0.9 (16)	1.13 ± 0.34 (8)	1.23 ± 0.4 (13)
6.66	42 ± 11.8	72 ± 7.8	2.52 ± 0.9 (09)	3.16 ± 1.4 (20)	1.00 ± 0.0 (9)	1.11 ± 0.3 (18)
8.88	50 ± 14.1	67 ± 18.0	2.11 ± 0.9 (15)	3.16 ± 0.8 (15)	1.00 ± 0.0 (15)	1.0 ± 0.0 2.0 (15)
Two way Anova	**(t X b) S1%**		**(t X b) S5%**		**(t X b) S1%**	

(Figures in parenthesis indicate number of replicates)

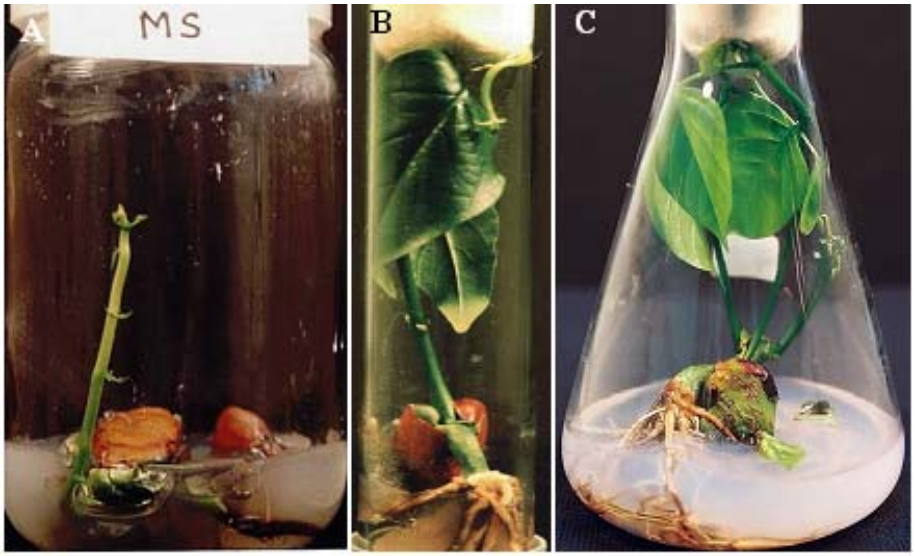


Fig. 3.4: (A) Germination of *Pongamia* seed in plastic capped bottle. Seedling with diminutive leaves.
(B) Germination of *Pongamia* seed in cotton plugged tube in MS media. Seedling with healthy root, shoot and fully opened leaves.
(C) Germination of *Pongamia* seed in cotton plugged flask containing 4.44μM BA. Seedling with multiple shoots and fully opened broad leaves

Compared to bottles elongation of shoot was significantly higher (P<5%) in tubes at all concentrations of BA tested (Table 3.2). In media with or without BA in bottles, average lengths of shoots ranged between 2.11 ± 0.88 to 2.80 ± 0.75 cm whereas in tubes it ranged between 3.16 ± 0.80 to 3.41 ± 1.05cm. Thus optimum elongation of shoot (3.41cm) was achieved in medium with 0.44µM BA in tube. Within the cultures in various concentration of BA either in bottle or in tube there was no significant difference in average shoot length. This suggests that 0.44µM of BA was optimum for shoot elongation.

Seedlings cultured in medium devoid of GR possessed single shoot whereas in media with BA it was often more than one, irrespective of culture vessel. Number of multiples was significantly higher in tubes (P<1%) than in bottles and optimum number of shoots (1.67 ± 0.86) was obtained in MS basal medium with 0.44µM BA in tube. BA is often used for multiplication of shoots in culture (Jain et al., 2000; Fracaro et al., 2001). From the data (Table 3.2) it is apparent that at the concentration of 0.44µM BA in tube, optimum germination frequency, shoot elongation and shoot multiplication was achieved.

Cotton plugs in tubes permit exchange of gases and the moisture produced from evaporation of medium escapes through plugs creating a drier microenvironment for the tissue. On the contrary in bottles the moisture is trapped and the microenvironment of the culture remains saturated with moisture. This is reflected in condensation of moisture on the inner surface of the bottle. Seeds on germination in the flask, produced multiples with open leaves as in tubes (Fig. 3.4C). This study further confirms the requirement of drier environment for the growth of this tissue. Influence of culture vessels on growth and development of culture has been emphasized in woody species (McClelland et al., 1990) and in cotton (Hazra et al., 2000) cultures.

Multiplication of *in vitro* raised shoots with BA:

Information generated on the positive influence of BA on seed germination, seedling growth and development of multiples, was extended to *in vitro* raised seedling derived nodal explants to optimize concentration of BA for induction of multiple shoots. The seedling tip was eliminated and nodal buds were used for this experiment to maintain uniformity in explants. In medium devoid of GR, 97% of the seedling derived explants responded to sprout (Table 3.3) and produced one or two shoots whereas all explants (100%) responded in cultures supplemented with BA and produced 2 – 4 shoots.

Table 3.3: Influence of BA on shoot multiplication of *in vitro* raised seedling derived shoot explants of *Pongamia*

Conc. of BA (µM)	Freq. of response (%) Mean ± sd.*	No of shoot multiples Mean ± sd.*
Control	96.7 ± 5.8	1.70 ± 1.60
2.22	100 ± 0.0	2.03 ± 2.05
4.44	100 ± 0.0	2.40 ± 1.71
6.66	100 ± 0.0	3.40 ± 1.81
8.88	100 ± 0.0	3.77 ± 4.96
ANOVA	**NS**	**S 5%**

There was increase in number of shoots from each explant with increase in concentration of BA. It increased from 1.7±1.6 in GR free medium to 3.77 ±4.96 in medium with 8.88µM BA (Fig. 3.5A).

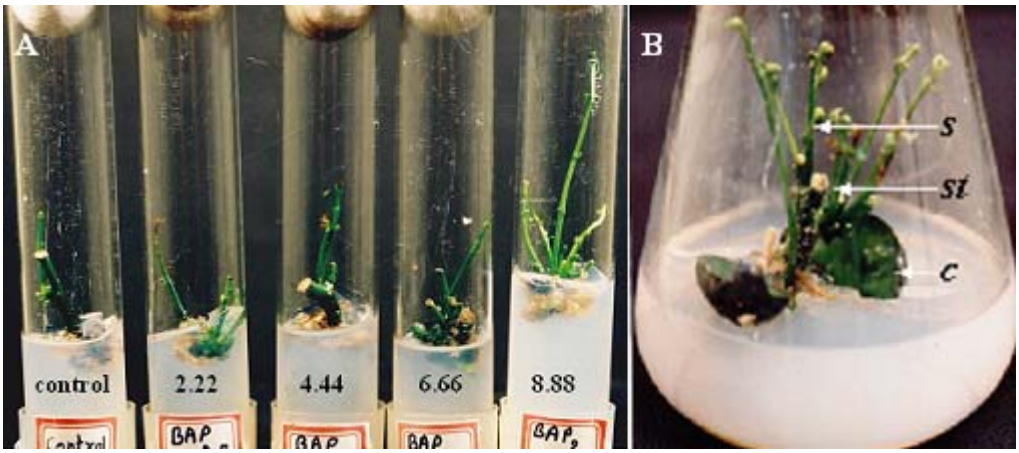


Fig. 3.5: (A) Effect of BA on shoot multiplication on *in vitro* raised seedling derived nodal explants. Increased number of multiples in higher concentrations (µM) of BA.
(B) Cotyledon node with attached cotyledons (*c*) in the first cycle. Multiple shoots(*s*) formed from the nodal axil on removal of seedling stem (*st*) and reculturing the explant**.**

Effect of BA on proliferation of multiple shoot was significant (P>5%) and was optimum in medium with BA 8.88µM. Shoot proliferation in higher concentrations was associated with callusing. To restrict dedifferentiation of the newly formed shoots, use of BA higher than 8.88µM was avoided. Shoot buds developed in clusters. Clusters of buds produced more buds on sub culture in the medium of similar composition and some of the shoots elongated to 2-3 cm.

Cotyledon node recycling:

The shoots and shoot buds were removed from the cotyledon node explant for rooting, elongation and further propagation. The cotyledon node was treated separately. Culturing of the cotyledon node explant with attached cotyledons for the second time in MS media supplemented with 0.44µM BA,

produced more caulogenic buds from the same axil. These buds differentiated to form shoots (Fig. 3.5B). Three to four shoots/seed were produced with an average length of 3.2 ± 0.98 cm. This process of removing the shoots and reculturing the explants was repeated for 8 cycles.

Recycling of cotyledon node explants was achieved in cultures of *Albizia falcata* (Sinha & Mallik, 1993), *Acacia tortilis* subsp. *raddiana* (Nandwani, 1995), *Bauhinia vahili* (Upreti and Dhar, 1996), *Dalbergia sissoo* (Pradhan et al., 1998). Residual epicotyl explants of *Syzygium cuminii* retained its organogenic response for five passages of recycling and the explants turned brown thereafter (Jain & Babbar, 2000). In *Pongamia*, the cotyledonary node retained its organogenic response even after eight cycles followed.

Cycling of original explant provides continual supply of shoots that could either be directed for rooting or for further shoot proliferation. Shoots and shoot buds obtained from axillary nodes and cotyledon node, proliferated and developed clusters of shoots/buds on subculturing in BA (8.88μM) containing media. The shoots regenerated by cycling the original explant rooted and produced plants following the method described in the previous section.

Sucrose concentration optimized for the multiplication of *in vitro* raised shoots:

Significant interactions of Sucrose concentration with cytokinin were observed for shoot height, bud number, vigor and rooting in the plant species like *Swainsona Formosa* (Jusaitis M, 1997) *Chrysanthemum coronarium* (Chae et al., 2004) *Acacia mangium* (Monteuuis, 2004).

Table 3.4 shows the effect of different sucrose concentration on the growth and multiplication of Pongamia shoots. Shoots were well differentiated and elongated in the media with 3 % sucrose. The shoot differentiation was not enhanced in media with sucrose more than 3%. With increase in sucrose

Table 3.4: Effect of sucrose concentration on shoot elongation and multiplication of seedling raised shoots of *Pongamia*

Concentration of sucrose (%)	Initial shoot length (cm) mean ± sd	Final shoot length (cm) mean ± sd	Difference in shoot length (cm) mean ± sd	No. of Shoot Multiples mean ± sd
S 2	2.75 ± 0.35	3.14 ± 0.45	0.39 ± 0.31	3.09 ± 0.51
S 3	2.55 ± 0.09	3.79 ± 0.89	1.39 ± 1.20	4.62 ± 0.47
S 4	2.40 ± 0.27	3.05 ± 0.45	0.61 ± 0.51	5.09 ± 1.52
S 5	2.33 ± 0.19	2.89 ± 0.49	0.56 ± 0.63	5.80 ± 1.50
ANOVA	**NS**	**NS**	**NS**	**NS**

concentration above 3%, there was increase in multiples with reduction in shoot height. However the data was not significant statistically. Further studies will determine if shoots are directed towards proliferation rather than differentiation (Fig. 3.6), with increase in sucrose concentration. Sucrose plays more than one role in plant tissue culture. It is incorporated in the mediun as carbon source but at higher concentrations it may influence the cultures as an osmoticum.

Fig. 3.6: Effect of sucrose on shoot multiplication on seedling derived nodal buds.

In *Swainsona formosa* (Jusaitis, 1997), the shoot number increased linearly with sucrose concentration. Sucrose alone had no effect on bud proliferation, but BA stimulated proliferation was sucrose dependent, reaching an optimum at 2-3 % sucrose. Similarly in *Pongamia*, a sucrose dependent stimulated proliferation of shoot in BA (8.88µM) was observed. While shoot numbers were highest on media with 0.88µM BA and 5% sucrose, but the shoots did not grow vigorously.

Rooting *in vitro*:

Rooting in shoot of *Pongamia* could be achieved (75%) in MS full and half strength media with 2% sucrose. Incorporation of activated charcoal (0.22%) was beneficial to obtain healthier root system with side roots. Isolated shoots rooted in, half strength MS basal medium supplemented with 0.22% activated charcoal. Several reports suggest use of low salt concentration to promote rooting (Pierik, 1987). Half strength MS media was used for root induction in *in vitro* raised shoots of *Pongamia*.

Activated charcoal (0.22%) was supplemented in the rooting media for its beneficiary role in rooting in vitro. It reduces the light intensity at the base of the shoots providing an environment conducive to the accumulation of auxins or cofactors or both, thus providing a dark environment for the growth of the roots (Sanchez, 1996). It absorbs substances such as inhibitory phenolics and any excess auxin or cytokinin carried over from previous media (McCown 1988; Bonga and Aderkas, 1992). It enhances

lateral root formation and it is often effective in arresting intervening callus formation, thereby helping in establishment of vascular connection between roots and shoots (Dawra et al., 1984). Incorporation of Charcoal in the rooting media supported high rooting response and healthy root system in *Pongamia*.

Out of 48 shoots tested for rooting, 36 shoots rooted in 4 weeks (Fig. 3.7A) giving the rooting frequency of 75%. Development of adventitious roots from the pongam shoots required no supplementation of auxins in the rooting media.

Fig. 3.7: (A) Differentiation of leaves, elongation and rooting of shoots cultured in half strength MS media with 0.22% charcoal**.**
(B) *In vitro* regenerated *Pongamia* plants hardened for transfer to green house.

Transfer of Propagules to soil:

Rooted shoots were dipped in Bavistin solution to provide a shield from fungal infection till it establishes anchorage in soil (7-10days). After 20 days, small leaves in the rooted shoots broadened and turned dark green indicating robust functioning of the photosynthetic system. Usage of transparent plastic sheets allowed the light to penetrate while maintaining high humidity internally. From the 36 plants transferred to sand: soil (1:1) mixture, 30 plants survived and hardened in 4 weeks (Fig. 3.7B). All hardened plants survived on transfer to pots in greenhouse.

Shoots rooted ex vitro:

To reduce the steps in micropropagation process, ex vitro rooting of shoots is more desirable. Commercial rooting compounds are either applied at the cut end of shoot base or rooting substrates

are fortified with these compounds. In aspen and poplar, shoots could be directly rooted in peat perlite substrates, which helped in shortening of multistep procedure. Direct rooting has been achieved in different species of *Eucalyptus*, eastern white Cedar and *Salvadora persia* using commercial rooting compounds and different rooting substrates (Mascarenhas et al., 1993). In teak commercial hormone mixture in combination with a fungicide and talc powder have been successful (Mascarenhas et al., 1993). Major advantage of this technique is omission of *in vitro* rooting step, which result in considerable saving of time, money, chemicals and establishment costs.

Rooting of *Pongamia* shoots *in vitro* was rather easy. Adventitious roots appeared from the base of the stem explants on culturing in GR free MS media during sprouting experiment (Fig. 3.8A). This prompted us to attempt ex vitro rooting using a commercial rooting compound Seradix B. Reduced shade and high humidity were critical in this stage since the rooting and hardening phases were carried out together. Out of thirty shoots tested roots developed in 20 shoots to form plants and stabilize in soil (Fig. 3.8B) in four weeks. Thus the ex vitro rooting efficiency was 66.6%.

Fig. 3.8: (A) Adventitious rooting from the shoot base of *in vitro* raised seedling segment cultured in MS media while sprouting.
(B) *Ex vitro* rooted *Pongamia* plants hardened for transfer to green house

B. Explants from Green Pods:

Contamination posed major problem in germination of mature seeds *in vitro* to obtain sterile seedlings. Moreover, it took 4 weeks time for seedling to attain the appropriate size. For optimization of protocol for clonal propagation, primary explants required are the meristems, which could be triggered to produce multiple shoots. Semi mature green pod (approximately 2.5cm; Fig. 3.9A) derived explants were promising as source of meristem for establishment of shoot cultures. Preformed meristem in the cotyledonary nodes can be targeted for induction and proliferation of shoots. Use of

these explants is advantageous, as it hastens the micropropagation protocol by evading incubation period of seedling growth. Moreover seed borne microbial contamination is often nil.

Thidiazuron (TDZ), a substituted phenyl urea induces high rate of regeneration and axillary shoot proliferation in plant species like *Rubus* (Fiola et al., 1990), *Pisum sativum* and *Lens culinaris* (Malik and Saxena, 1992), *Calendula officianalis* (Çöçü et al., 2004), *Rauvolfia tetraphylla* (Faisal et al., 2005), *Psoralea corylifolia* (Faisal et al., 2006). Its mode of action may be attributed to its ability to induce cytokinin accumilation (Victor et al., 1999) and or to enhance the accumulation and translocation of auxin (Murch and Saxena, 2001). Keeping in view the efficacy of TDZ to promote shoot multiples, and to avoid duplication of the previous experiments in standardization of protocols with seedling derived explants, in which BA was used in induction of multiples in nodal explants, in this experiment BA was substituted with varying concentrations of TDZ for induction of shoots.

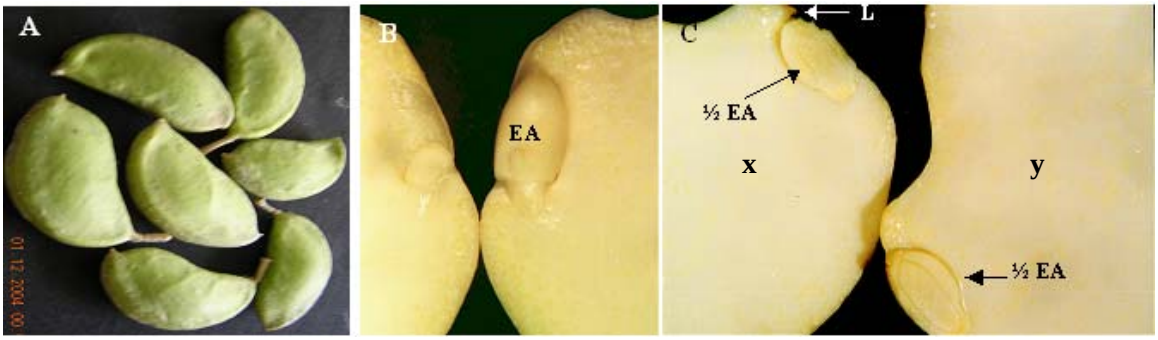


Fig. 3.9: (A) Green pods. Source of cotyledon node explants.
(B) Cut opened seed. The embryo axis (EA) attached to one side of cotyledon node.
(C) Cotyledons with splitted embryo axis (1/2E). The explants tested for shoot regeneration. The cotyledon (x) with loosely attached embryoaxis (L) responded

In semi-mature *Pongamia* seed, it was observed that the embryoaxis is attached strongly to one cotyledon. Thus when the cotyledons are separated, the embryoaxes remains attached to one of the cotyledons (Fig. 3.9B). Therefore after surface sterilization of the pods the seeds were isolated aseptically and were cut into two through the axes such that the two cotyledons separated with approximately half portion of embryo axis attached to each cotyledon (Fig. 3.9C). This way it was ensured that each cotyledon carries one cotyledon node meristem. However often after separation, the portion of the embryo axis, got detached from one of the cotyledons even before transferring the explant into media. Hence, only the cotyledon with node (x) was inoculated into media. The other cotyledon (y) in which a portion of embryoaxis attached firmly was cultured in to the media. But, the embryoaxis portion degenerated after 3 to 4 days of culture. By making the excision through the

embryo axes it was ensured that the cotyledon nodes remained undamaged. The 50% response (Table 3.5) in the cotyledon node explant suggests that only one of the cotyledons responded in culture.

It is reported that an optimum exposure of explants in TDZ supplemented medium followed by the withdrawal of GR effectively triggers shoot multiplication in species like *Psoralea corylifolia (*Faisal et al., 2006), *Tamarindus indica* (Mehta, 2005), *Saintpaulia ionantha* (Mithila et al., 2003), Medicago *truncatula* Gaertn. cv. Jemalong and *Medicago truncatula* ssp. Narbonensis (Neves et al., 2001), *Hibiscus cannabinus* (Srivatanakul et al., 2000). On the contrary, cultures continuously grown on TDZ containing media resulted in formation of fasciated and distorted shoots. Deleterious effect of continued presence of TDZ was reported on growth and multiplication of *Cicer arietinum* (Murthy et al., 1996), *Pisum sativum* (Bohmer et al., 1995), *Anoectohilus formosanus* (Ket et al., 2004) and *Rauvolfia tetraphylla* (Faisal et al., 2005). Keeping these observations in view *Pongamia* tissue was exposed in TDZ for 20 days followed by the withdrawal of GR.

In the present investigation cotyledon node without (x) or with (y) half portion of embryoaxis were cultured in different concentrations of TDZ (0, 0.05, 0.02, 0.45, 2.27, 4.54, 6.81, 9.08, 11.4, 13.6 & 22.7μM) containing media for 20 days and then transferred to GR free MS media for 30 days. Axenic cultures could be established with ease, as microbial interference was very low in this tissue. After 30 days of culture, overall sprouting frequency was approximately 50% (Table 3.5).

Table 3.5: Effect of TDZ on Cotyledon node from green pods after 30 days of culture

Conc. of TDZ (μM)	Total number of explants	Frequency of Response (%) Mean ± sd	Average number of shoot buds Mean ± sd.
0.00	36	50.0 ± 0.0	1.00 ± 0.0 (17)
0.05	36	52.8 ± 4.8	1.00 ± 0.0 (17)
0.02	36	53.3 ± 5.8	1.00 ± 0.0 (18)
0.45	36	52.8 ± 4.8	1.00 ± 0.0 (18)
2.27	36	47.8 ± 19.7	1.53 ± 0.53 (15)
4.54	36	41.7 ± 14.4	2.40 ± 0.53 (15)
6.81	36	46.7 ± 5.8	3.67 ± 1.27 (15)
9.08	36	47.2 ± 4.8	4.31 ± 1.15 (17)
11.4	36	50.0 ± 0.0	5.18 ± 0.90 (17)
13.6	36	30.0 ± 8.7	3.90 ± 1.27 (10)
22.7	36	38.9 ± 12.7	2.50 ± 0.95 (10)
Anova		NS	S 1%

(Number of parentheses indicates the number of replicates)

Shoot regeneration response occurred in most cases from one of the two cotyledons, but occasionally, both cotyledons (x & y) produced shoots. The cotyledon without embryo axis portion (x) responded well in culture. It may be presumed that cotyledon node of y portion got degenerated along with halved embryo axis. Mante et al., (1989) reported similar observation in Prunus species (*P. persica, P cerasus and P.domestica*), in which only one cotyledon from seed produced shoots. Shoot development was completely inhibited in cotyledon with attached embryo axis. The frequency of response explants for formation of shoot was ranged between 30 and 53% (Table 3.5).In control, only single shoot was produced (Fig. 3.10A). No multiples were observed in the explants in lower concentrations of TDZ (0.05 to 0.45) media. The cotyledon nodes produced multiple buds in the media containing 2.27µM TDZ (Fig. 3.10B) and above. Maximum number of shoot multiples (5.18 ± 0.90) were produced in the media containing 11.4 µM TDZ (Fig. 3.10C).

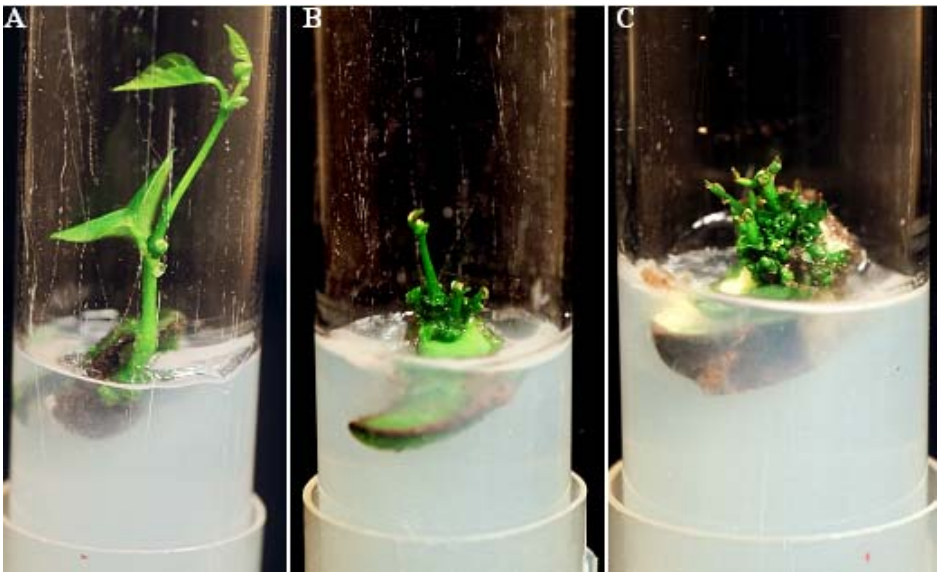


Fig. 3.10: (A) Single shoot derived from the cotyledon node in GR free MS medium
(B) Differentiation of multiple shoots in GR free medium after 60 days of culture in explants pretreated with TDZ (2.27µM) .
(C) Differentiation of multiple shoots in GR free medium after 60 days of culture in explants pre-cultured for 20 days in TDZ (11.4µM).

There was a definite pattern of response noticed in number of shoot buds produced. In lower concentrations (0.05 to 0.45 µM), TDZ was ineffective in inducing multiples. Thereafter the number of multiples per explants increased with concentration to reach the optimum at 11.4µM. Above 11.4µM, there was decrease in the number of shoots buds produced. Thus, 11.4µM of TDZ was optimum for induction of shoot regeneration in the cotyledon node explant. Explants were transferred

to GR free MS media for two passages of 30 days each. Data on average number of shoot buds per cotyledon produced in 60 and 90 days culture in GR free media is depicted in the graph (Fig. 3.11).

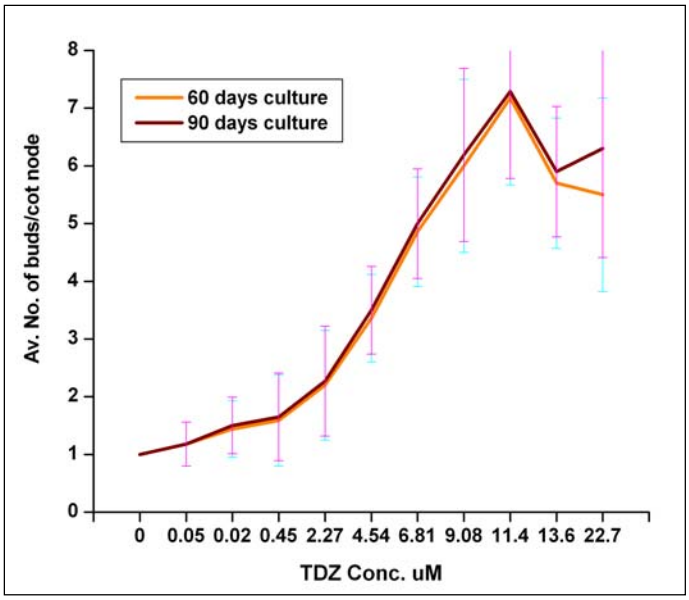


Fig. 3.11: Average number of buds in GR free MS media following incubation with various concentrations of TDZ in cotyledon node explants after 60 and 90 days of culture.

The explants cultured in 0.05, 0.02 and 0.45µM TDZ produced multiples in second passage. After 60 days of culture (ie. in 2nd Passage of MS), the number of shoot buds increased in all concentrations of TDZ tested. The increase in the number of buds may be due to the differentiation of buds from meristems on withdrawal of TDZ and a decline in carry over concentration of TDZ. Maximum number of buds (7.18±1.51 in 60 days; 7.29 ± 1.51 in 90 days) was produced in the cultures in incubated 11.4µM of TDZ.But in 90 days of culture, over all there is a marginal increase in the number of buds produced compared to 60 days of culture. Like in many other species, in *Pongamia* meristems triggered by TDZ, failed to differentiate in presence of TDZ. On withdrawal of TDZ the triggered meristems differentiated. In the second passage, additional buds were differentiated confirming the inhibitory influence of TDZ (Fig. 3.12A).

Transfer of explants to fresh GR free MS medium for every 30 days supported elongation by gradual reduction in inhibition.The inhibitory response of TDZ on shoot differentiation, and the mechanism involved in reduction of the inhibitory response on repeated cultures in GR free medium is not understood. On transfers of the control and TDZ exposed explants to MS media, the elongation of the

shoots progressed to an average length of 1.19 ± 0.57 cm in 60 days and 2.89 ± 0.52 cm in 90 days in control (Table 3.6).

Table 3.6: Elongation of shoots in GR free MS media following the incubation with various concentrations of TDZ in cotyledon node explants

Conc. of TDZ in MS medium (µM)	Shoot Length after 60d of culture (cm) mean ± sd	Shoot Length after 90d of culture (cm) mean ± sd
0.00	1.19 ± 0.57 (17)	2.89 ± 0.52 (17)
0.05	1.09 ± 0.45 (17)	2.66 ± 0.60 (20)
0.02	1.04 ± 0.48 (18)	2.31 ± 0.44 (22)
0.45	1.03 ± 0.54 (21)	1.83 ± 1.28 (25)
2.27	1.01 ± 0.53 (22)	1.79 ± 0.54 (28)
4.54	0.95 ± 0.30 (20)	1.76 ± 1.20 (25)
6.81	0.88 ± 0.57 (23)	1.67 ± 0.75 (28)
9.08	0.85 ± 0.57 (24)	1.59 ± 1.26 (30)
11.4	0.84 ± 0.38 (25)	1.53 ± 1.00 (33)
13.6	0.74 ± 0.23 (11)	1.23 ± 0.96 (21)
22.7	0.68 ± 0.25 (10)	0.83 ± 0.54 (18)
Anova	**S 5%**	**S 1%**

(Number in parentheses indicates the number of replicates)

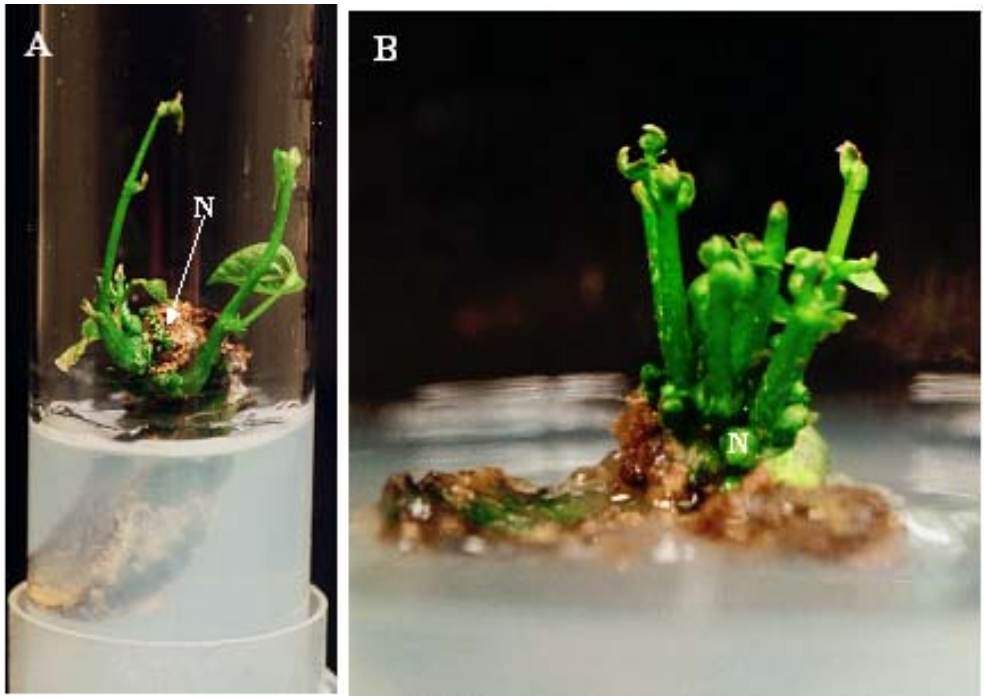


Fig. 3.12: (A) Differentiated shoots with new buds (N) in second passage of MS from the meristem pre-incubated with TDZ (2.27 µM)
(B) Differentiated multiple shoots from the meristem induced by 11.4µM TDZ, after 90 days of culture, in GR free MS media.

The shoots developed from the buds pre-cultured in lower concentrations of TDZ (0.05µM and 0.02 µM TDZ) started elongating faster (2.66 ± 0.60 cm) like the shoots in control. Elongation of shoots in GR free media was slower with the increase in concentration of TDZ in pre-culture medium. The mean shoot height increased every 4 weeks on diminution of carry over TDZ in the explants. The explants precultured in 11.4 µM TDZ containing medium produced optimum number of buds (7.29 ± 1.51). The average length of shoots was 1.53 ± 1.00 in 90 days (Fig. 3.12B). Thus the optimum concentration of TDZ for bud induction and multiplication in the cotyledon node explant is 11.4 µM.

Shoots, which attained the height of 2-3 cm after 90 days in MS medium, rooted in half strength MS media supplemented with (0.22%) charcoal. These cotyledon explants with smaller shoots and shoot buds produced more shoots on transfer to fresh MS basal medium. Buds induced in high TDZ (13.6 & 22.7µM) took longer time and several passages in GR free medium to differentiate thereby delaying the rooting step. Similar results were noted in *Medicago trunculata*. In *Medicago* cultures, serial sub culturing in growth regulator free medium improved shoot elongation and rooting efficiency and suggested that carry over effect of TDZ used for shoot induction from cotyledons disappears upon subculture (Neves et al., 2001). Half strength MS basal media with 0.22% charcoal which was effective for rooting of seedling explant derived shoots was effective for rooting in cotyledon node derived shoots. Out of 20 shoots tested for rooting, 16 rooted in 4 weeks (Fig. 3.13A). The rooting frequency was 80%. Rooted shoots transferred to the autoclaved sand soil mixture survived and hardened (Fig. 3.13B). All hardened plants survived on transfer to pots in green house.

Fig. 3.13: (A) Rooted shoot in half strength MS basal media supplemented with charcoal (0.22%).
(B) Hardened shoot in sand soil mixture in plastic cups.

3.4 CONCLUSION

Experiment was conducted with an objective to generate information on *P.pinnata* tissues for establishment of sterile shoot cultures, nutritional requirements for sprouting *in vitro* and for proliferation of multiple shoots, incubation conditions for optimum response, rooting behavior and survival in soil etc. Considering the various advantages in using immature tissues, seedling derived meristems were tested. We encountered serious limitations in germination of seeds due to microbial contaminations from seed borne microbes. In order to hasten germination and minimize number of experiments we combined several factors like the concentration of basal medium, a broad spectrum antibiotic Cefotaxime, removal or seed coat, four growth regulators etc in single experiment. The experiment for refinement of BA concentration on germination was coupled with testing of culture vessel. An agar gelled medium composed of full strength MS with BA 0.44µM and sucrose 2% was optimum for germination of *Pongamia* seeds. PPM was incorporated in medium to control fungus growth. Cotton plugged tube and flasks are appropriate to obtain healthy seedlings with fully opened leaves. Axillary node and cotyledon node of the seedlings were isolated and tested for establishment of shoot cultures. An agar gelled medium composed of MS with BA 8.88µM and sucrose 3% was optimum for proliferation, elongation and maintenance of shoots. The cotyledon node re-cultured after removal of shoots produced additional shoots. This recycling of cotyledon node was followed for 8 cycles. Rooting was achieved in these shoots both *in vitro* and extra vitrum.

To simplify the surface sterilization procedure, semi-mature seeds of *P.pinnata* were tested as source of explants, assuming that contaminants are harbored in the undulations of mature dry seed. While separating the cotyledons it was ensured that each cotyledon carries one node. One of the two nodes with attached cotyledon responded in MS medium containing 11.4µM TDZ and produced shoot buds. Shoots differentiated and rooted in GR free medium for several passages. Using semi-mature green pod as source of explant was fast and convenient. Approach of sterile seedling culture method could be avoided. To obtain meristems for establishment of culture or genetic transformation, this explant may be considered. This concept can be extended to other species where establishment of sterile seedling is a constraint or where more number of meristems is needed within a limited period.

A part of this work has been published

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CHAPTER: 4
CLONAL PROPAGATION USING MATURE NODAL BUDS

4.1 INTRODUCTION

Possibility of mass propagation of elite, endangered, or difficult-to-propagate genotypes, in relatively small area and period is the most important characteristic of clonal propagation. *In vitro* multiplication of tree species using mature nodal buds has many advantages including the possibility of clonal mass propagation, reversion to juvenility and uniformity of stands.

PROPAGATION FROM MATURE TREES

In tree species, juvenile explants have been extensively employed for *in vitro* propagation. However, cloning from mature trees is generally preferred over cloning of embryos or seedlings due to the following reasons (Bonga, 1987):

- Majority of the tree species are out breeders and have been propagated by seed. Due to recalcitrant nature, many tree species can presently be propagated only from juvenile tissues like embryos and seedlings. Considering the genetic heterozygosity of the most of the tree species, cloning of mature trees is the only method that permits immediate and total capturing of genetic gains (Franclet et al., 1987) and to obtain true-to-type large-scale clonal propagules.

- Cloning of mature trees is generally preferred over cloning of embryos or young seedlings because often it is not possible to determine if these embryos or seedlings have the genetic potential to develop the desired qualities later in their life cycle.

- Preferential cloning of mature trees is desired when establishing plantations of dioecius species. For example, in some dioecius species only the female trees have economic value. Since the gender of these trees is not known before maturity, they are cloned preferentially from sexually mature trees. Examples of such preferential cloning are *Carica candamarensis* (Jordan et al., 1983), *Phoenix dactilifera* (Reynolds, 1982) *and Simmondsia chinensis* (Rost and Hinhee, 1980).

Obtaining juvenile material from mature trees is of considerable importance for clonal propagation of woody plants, because *in vitro* cloning is a function of the degree of juvenility of the explanted tissues. Two options are available before excising the explant. Selection of most juvenile tissues with the mature tree and rejuvenate parts of the donor tree by special treatments and excise explants from these parts.

A. Selection of juvenile tissues within mature tree:

For most clonal propagation work, the explant of choice is an apical or axillary bud. Meristems in these buds are composed of fast dividing meristematic cells. Explants like leaf pieces and flower heads were also used, but it is risky as the chances of obtaining off-type plants would increase. It has been known that cuttings from lower branches especially near the trunk are more juvenile than branches in other parts of the tree (Rouland, 1973). Stump sprouts were considered to be more juvenile and have been used in cloning mature eucalyptus (Durand-resswell et al., 1982). For the species that do not produce stump sprouts like the pines, a degree of juvenility was achieved by repeated pruning of the parent trees.

B. Rejuvenation by special treatments:

In conifers, juvenile sprouts are not naturally available for micropropagation. With some trees, this problem has been overcome by special rejuvenating treatment before or during micropropagation. These pretreatments include BA spraying, serial grafting on juvenile rootstock or serial rooting of cuttings and serial subculture *in vitro*.

Successful clonal propagation of tree species is no more a difficult phenomenon. Several woody species such as poplars, wild cherry, eucalyptus, redwood, radiata pine, teak, sandalwood, birch, loblolly pine, *Shorea* and *Dalbergia* are being standardized for mass multiplication and are commercially micropropagated (Rodriguez and Vendrame, 2003). A list of leguminous tree species in which clonal propagation has been achieved is tabulated (chapter 1,Table 1.1).

Pongamia pinnata has generated a lot of scientific interest (Chapter 1) not only as a source of raw material for biodiesel, but also for its industrial and medicinal uses. From literature, it is apparent that there is a lot of scope for genetic modification of *Pongamia*. For this there is need to develop reproducible *in vitro* regeneration systems. Secondly raising plantations of clonally propagated, high seed yielding trees will contribute to increased seed production. These plantations will also serve the purpose of seed orchard for production of superior quality seeds. In addition, *in vitro* culture currently provides the only route for generation of genetically modified genotypes of trees. Keeping in view the recalcitrant nature of leguminous tree species, standardization of protocols for *in vitro* morphogenesis remains a challenging proposition.

Out breeding species generally bound to have high variation in them. *Pongamia* is highly out breeding species, in which cross-pollination takes place primarily by insects and air. This leads to

high variations in characters like pod length and shape, number of seeds per pod, oil content etc. These trees might have differential response in culture too. This necessitates standardization of a protocol and test its reliability for its effective application by extending it to several genotypes.

Attempts were made to standardize a protocol for clonal propagation of mature trees of *Pongamia* using tree derived axillary buds. Various parameters including basal media, growth regulators, carbon source and pH were optimized. The data generated from the experiments are included in this chapter. The standardized protocol was extended to other pongam trees growing locally to test its reliability.

4.2 EXPERIMENTAL PROTOCOL

Twigs of *Pongamia* were collected during vegetative season (March-May) from mature trees growing locally. Healthy and sturdy twigs of 6-8 mm thickness and 15-30cm length were excised from the mature tree, as source of explant (Fig. 4.1A). The leaves were cut off with a sharp blade or scissors leaving a small portion (about 5-6 mm) of petiole attached to the twig (Fig. 4.1B) 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 were used. Twigs were cut into 2 cm (approximately) pieces (depending on the internodal distance). Each piece had one axillary node and a portion of inter nodal segment. These nodal bud explants were surface sterilized following the procedure described (Chapter 2).

Fig. 4.1: (A) A vegetative mature twig of *Pongamia*.
(B) A portion of twig showing prominent vegetative bud at nodal region. Explants used were with single node.

All media used had 2% sucrose unless specified. The methodology followed in media preparation, conditions used for incubation of the cultures and the methods used in statistical analysis of the data are included in chapter 2. Test tubes were used as culture vessel in the experiments with mature nodal explants.

Effect of Basal medium on sprouting response of mature nodal explants:

Nodal explants were cultured in four basal media formulations without any growth regulator: (1) MS medium (Murashige and Skoog, 1962); (2) Woody plant medium (WPM) (Lloyd and McCown, 1980); (3) B5 medium (Gamborg et al., 1968); (4) SH medium (Schenk and Hildebrandt, 1972). Plant preservative mixture (PPM) 2 ml l^{-1} (Sameer Science Lab, India) and Cefotaxime 250 mg l^{-1} (ALKEM, India) were added aseptically to the primary culture media. Cefotaxime 250 mg l^{-1} was incorporated in the medium for two passages. Sprouting frequency and length of the sprouts were noted after four weeks of culture. Experiment was repeated thrice with ten replicates in each medium.

Effect of plant growth regulators on sprouting and shoot multiplication:

Mature tree derived nodal explants were cultured in MS medium supplemented with plant growth regulators including 6-benzyladenine (BA), kinetin (KN), zeatin (Z) and TDZ at concentrations of 0.45, 4.54, 9.08, or 13.62 μM. Sprouting frequency and number of shoots produced per explant were noted after 4 weeks of culture. Seven explants were used per treatment and the experiment was repeated five times.

Hereafter the cultures in BA, KN and Z and the cultures in TDZ were treated differently. Cultures in TDZ were transferred to GR free MS medium every two weeks for six passages. Number of shoots produced per explant was noted after every two passages. Lengths of all the shoots were measured.

Cytokinins (BA, KN, Z), singly were ineffective in induction of multiple shoots. Therefore in another experiment Adenine sulphate (AdS) 13.76 μM was incoprporated in MS media containing different concentrations of BA (0.4, 2.2, 4.4, 6.6, 8.8, 13.3, 22.2 & 31.1μM) or Kinetin (0.4, 2.2, 4.6, 7.9, 9.3, 13.9, 23.2 & 32.5μM). Mature tree derived nodal explants were cultured in these media. MS basal medium with AdS was considered as control. Eight replicates per treatment were used and the experiment was repeated three times. Cultures were incubated in light for 4 weeks. Thereafter the explants were shifted to corresponding fresh medium of same composition and concentration for further growth and differentiation. Data on frequency of response, number of shoot buds per explant and length of the shoots were scored after second passage. From explants cultured in KN

supplemented media (0.46 to 8 μM), shoots obtained were subcultured in their respective media. From the shoots obtained from subcultures, twelve were cultured in rooting media (Chapter 3). The rooted shoots were transferred to soil and hardened.

In an attempt to determine the **optimum concentration of TDZ**, the nodal buds were cultured for 4 weeks on MS basal media supplemented with TDZ at 0.45, 2.27, 4.54, 6.81, 9.08, or 11.35μM. Eight explants were used per treatment and the experiment was repeated four times. Buds cultured on TDZ were transferred to GR-free MS basal media, for six passages of 2 weeks each. Frequency of response, number of shoots produced per explant, and their length were noted every two passages. For shoot length, all the shoots produced from an explant were measured. Shoot primordial less than 0.2 cm were scored using a stereomicroscope.

For further refinement of the protocol, parameters like **source of carbon** and **pH** were tested. Sucrose (2%) was substituted with other carbon sources including fructose and glucose at the same concentration. Experiment was carried out with 15 replicates per treatment and was repeated twice. To optimize sucrose concentration, nodal explants were cultured in MS medium with 0.45μM TDZ and different concentrations (2%, 3%, 4% , 5%) of sucrose. Experiment was carried out thrice with treatments having 10 replicates each. Explants were cultured in MS basal medium with 0.45μM TDZ at different pHs (5, 5.8, 6.5). Media pH was adjusted before autoclaving. The experiment was repeated twice using 10 replicates per treatment.

Primary nodal explant after harvesting of shoots or meristematic buds was identified as '**stump'**. Stumps were recultured on medium containing 0.45 μM TDZ for 4 weeks, followed by four passages of 2 weeks each in GR free basal media. This process of removal of shoots and reculturing of stumps was repeated for six cycles. Elongated shoots were isolated from the clusters developed in the axillary nodes and cultured individually for two passages of 15 days each for elongation. Clusters of shoots were made into smaller clusters and were recultured in MS medium with 0.45μM TDZ for further proliferation and maintenance of shoot cultures.

Elongated shoots (3-4 cm) isolated from the clusters were cultured on GR free half-strength MS medium supplemented with 0.25% activated charcoal for **rooting**. This experiment was repeated 4 times with 15 to 20 shoots. Rooted shoots were transferred to autoclaved sand soil mixture (1:1) and **hardened** following the method described (Chapter 3). Forty-six propagules thus raised were transferred to soil in two batches. Hardened plants were transferred to bigger pots in greenhouse.

Extension of the protocol to other trees:

To determine the reliability of the protocol, nodal buds were collected from ten different mature trees and were cultured in MS medium supplemented with 0.45µM TDZ for four weeks, followed by a passage of GR free MS media for four weeks. Ten explants from each tree were tested and the experiment was repeated thrice.

4.3 RESULTS AND DISCUSSION

Pongamia pinnata plants harbor several microbes including bacteria and fungi. The microbial growth often appeared at the cut end of the explants in contact of medium after several days in culture. This poses a serious limitation in establishment of sterile cultures from vegetative buds of mature trees. Media additives, Plant preservative mixture (PPM) 2 ml l^{-1} (2%) and Cefotaxime 250 mg l^{-1} were effective in controlling the growth of the contaminants to a limited extent. Niedz reported (1998) that PPM at 0.2% concentration could prevent post-culture contamination in plant culture vessels. Babaoglu and Yorgancilar (2000) reported similar observation in regeneration of salad burnet. In our studies, PPM was used only in the primary culture. Cefotaxime, added in the media is a broad spectrum antibiotic and is often used for elimination of contaminant bacteria during establishment of sterile cultures and in transformation studies.

Mature tree derived nodal buds were collected at different seasons of the year and tested for *in vitro* sprouting. Sprouting behavior and contamination frequency varied with season (data not included). Degree of fungal and bacterial contamination was high during June to September. Sprouting frequency was least during November to January. Seasonal dependent *in vitro* response is reported in many tree species like *Ceratonia siliqua* (Romano et al., 2002), Scots pine (Hohtola, 1988), mulberry (Pattnaik and Chand, 1997), *Eucalyptus* (Sharma and Ramamurthy, 2000). In *Ceratonia*, establishment of culture was not possible year round since significant seasonal fluctuation responses affected the explant viability. March-April was the best season for culture initiation. In *Pongamia*, the vegetative buds collected during March-May responded well for the establishment of cultures.

Effect of Basal Media:

In our previous study (Chapter 3), MS basal medium was used for clonal propagation of *Pongamia* using the seedling derived axillary meristems. Other basal media were not tested. Mature tree derived nodal buds are physiologically and morphologically different from the seedling derived buds and the

requirement of the basal medium for optimum response may vary. Moreover, there is no literature on tissue culture of *Pongamia*. Thus, four basal media formulations were tested to identify the appropriate one. After four weeks of culture in light single shoot sprouted from each explant in the mature tree derived nodal buds cultured in different basal media (MS, WPM, B5 & SH) (Table 4.1).

Table 4.1: Effect of different basal medium on the sprouting of mature tree derived nodal buds

Basal media	Sprouting Frequency (%) mean ± sd	Average length of shoot (cm) mean ± sd
MS	56.7 ± 5.8	1.12 ± 0.3 (17)
WPM	50.0 ± 0.0	1.01 ± 0.3 (15)
B5	43.3 ± 5.8	1.10 ± 0.2 (13)
SH	43.3 ± 5.8	0.98 ± 0.2 (13)
Anova	**S 5%**	**NS**

(Number in the parentheses represents number of replicates)

From the four basal media formulations tested, optimum sprouting was achieved in MS (57%). Length of the shoots did not vary significantly in the buds cultured in different basal media. Shoots obtained in MS medium were marginally longer compared to the shoots noted in other basal media (Fig. 4.2). Thus MS basal medium was used in experiments conducted in *Pongamia*.

Fig. 4.2: Sprouting pattern of mature nodal buds in different basal media

MS medium has been used successfully for shoot induction in tree tissue culture (Rodriguez and Vendrame, 2003; Dunston and Thorpe, 1986). Superiority of MS over other salt formulations has been demonstrated in several other leguminous trees. A few to mention are *Prosopis cineraria* (Shekawat et al., 1993), *Swartzia madagascarensis* (Berger and Schaffner, 1995) and *Bauhinia vahlii* (Upreti and Dhar, 1996).

Effect of Plant Growth Regulators:

Like several other tree species *Pongamia* is also recalcitrant in culture and sprouting of buds and differentiation of shoots takes long period involving one or more transfers. On culturing the nodal buds in various GRs like BA, KN, Z and TDZ to obtain optimum sprouting and to induce multiple shoot development, bud break was initiated after 15 days of culture except in TDZ containing media (Table 4.2).

Table 4.2: Effect of growth regulators on mature nodal bud explants of *Pongamia*

GR conc. in μM	Frequency of Response (%) mean ± s.d.	Av. No. of shoots mean ± sd	Av. Ht. of shoots (cm) mean ± s.d.
Control	64 ± 30	1 ± 0	1.03 ± 0.46 (28)
BA (0.44)	72 ± 20	1 ± 0	1.07 ± 0.53 (20)
BA (4.44)	58 ± 23	1 ± 0	1.38 ± 0.53 (28)
BA (8.88)	41 ± 10	1 ± 0	0.65 ± 0.22 (22)
BA (13.32)	43 ± 28	1 ± 0	0.83 ± 0.42 (25)
KN (0.46)	55 ± 32	1 ± 0	1.85 ± 0.58 (26)
KN (4.65)	77 ± 18	1 ± 0	1.24 ± 0.64 (17)
KN (9.29)	66 ± 21	1 ± 0	1.38 ± 0.38 (22)
KN (13.94)	40 ± 24	1 ± 0	1.21 ± 0.17 (27)
Z (0.46)	54 ± 12	1 ± 0	1.32 ± 1.10 (25)
Z (4.56)	53 ± 28	1 ± 0	0.88 ± 0.41 (26)
Z (9.12)	48 ± 32	1 ± 0	1.10 ± 0.38 (26)
Z (13.68)	68 ± 32	1 ± 0	0.90 ± 0.32 (23)
TDZ (0.45 - 13.6)	---	---	--- (23---29)
Anova	**NS**	**NS**	**NS**

(Number in the parentheses indicate number of shoots considered)

There was no significant variation in the sprouting frequency of the buds in media with BA, KN and Z as compared to control. The frequency of sprouting ranged between 40 and 77%. There was no significant variation in the length of the shoots produced by the buds cultured in media with BA, KN and Z as compared to control. Average length of the shoots ranged from 0.65 to 1.85 cm.

The aminopurines (BA, KN & Z) tested were ineffective in triggering multiple shoot production and produced a single shoot as in control (Fig. 4.3A). On the contrary no bud break or sprouting was noted in the buds cultured in TDZ containing media. Only swelling was noted (Fig. 4.3B).

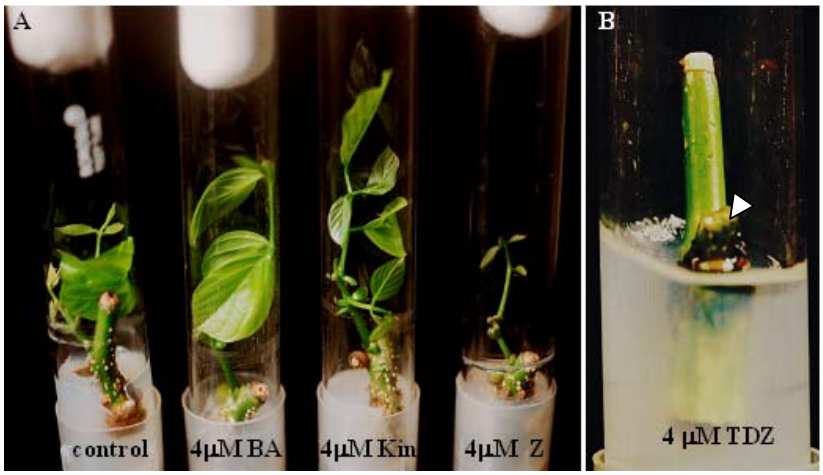

Fig. 4.3: (A) Sprouting of nodal buds in MS with different aminopurines after 4 weeks of culture.
(B) Swelling of meristem in the axillary node of the explant cultured in 4µM TDZ for 4 weeks.

In *Pongamia*, each nodal bud (Fig. 4.4A) has one meristematic dome in the axil. This dome is composed of a set of three meristems. The central meristem is more developed. This meristem differentiated into a single shoot in four weeks in media with or without GRs except in TDZ. In media with TDZ, none of the buds differentiated in 4 weeks and the meristems were swollen. The swelling was more pronounced in higher concentrations.

Fig. 4.4: (A) Nodal bud of *Pongamia* with three meristematic domes (m). Central meristem (c) in between the side buds (s). Cut end of stem (n).
(B) Swollen meristem on removal of bud cover after 4 weeks of TDZ exposure. Pair of meristematic masses (mm) developed from central meristem (c).
(C) Sprouting and elongation of axillary meristem in medium without GR.

In *Pongamia*, shoot primordia appeared from the swollen, dome shaped meristem after two passages of 2 weeks each in GR-free medium. Keeping in mind the influence of TDZ on proliferation of meristematic cells in tamarind (Mehta et al., 2004, 2005) it is presumed that the swelling of the axillary bud in *Pongamia* was the result of proliferation of the meristematic cells inside the dome cover. Removal of the dome cover exposed a pair of swollen structures (Fig. 4.4B) from the central meristem. Caulogenic buds and shoot primordia differentiated from these meristematic masses upon withdrawal of TDZ for 4 weeks. Thus, the frequency of sprouting, number of shoots produced from the nodal bud and the length of shoots produced could be determined after 8 weeks from the initiation of culture in TDZ (Table 4.3).

Table 4.3: Differentiation of shoot primordia in TDZ pre-treated nodal buds of *P. pinnata* after two passages of 15 days each in GR free medium

Conc. of TDZ (µM)	Elongation Medium (GR-free MS medium)						
	Freq. of response (%) mean ± sd	Average number of shoots per explant* mean ± sd			Average length of shoot (cm)** mean ± sd.		
	4w	4w	8w	12w	4w	8w	12w
Control	61.8± 5.2	1± 0 (28)	1± 0 (26)	1 ± 0 (22)	1.03± 0.4 (29)	1.60± 0.3 (42)	1.9 ± 0.4 (42)
0.45	63.4± 6.1	2.2± 0.3 (28)	2.8± 0.2 (28)	3.2± 0.3 (27)	1.65± 0.3 (62)	1.84± 0.4 (78)	2.21± 0.1 (86)
4.54	59.2± 4.4	2.6± 0.4 (26)	2.8± 0.3 (24)	3.0± 0.5 (23)	1.06± 0.3 (68)	1.65± 0.3 (67)	1.97± 0.2 (69)
9.08	54.6± 21.4	6.0± 0.7 (29)	6.4± 0.8 (25)	6.5± 0.4 (25)	0.74± 0.1 (154)	0.96± 0.1 (160)	1.06± 0.1 (160)
13.6	46.2± 13.9	7.0± 0.7 (24)	7.2± 0.8 (24)	7.3± 0.3 (22)	0.43± 0.0 (168)	0.53± 0.1 (173)	0.70± 0.1 (155)
Anova	**NS**	**S1%**	**S1%**	**S1%**	**S1%**	**S1%**	**S1%**

Each passage is of 15days ie. 4 wk=two passages, 8 wk = four passages, 12 wk = six passages;
*Figures in parenthesis indicate the number of explants producing shoots.
** Figures in parenthesis indicate the number of shoots measured.

The frequency of sprouting noted after four weeks on MS medium with TDZ pretreated explants did not increase after 12 weeks. The concentrations of TDZ tested, failed to induce sprouting in the remaining non-responding buds. After 4 weeks on MS medium the sprouting noted in the TDZ

pretreated buds were 59, 55 and 46% for 4.54, 9.08 or 13.62 µM TDZ respectively versus 62% for the control medium (Table 4.3). This repression in sprouting was not reversible upon withdrawal of TDZ. In *Bauhinia vahlii* (Upreti and Dhar, 1996), 1.0 µM TDZ was optimum for induction of sprouting in 96% of cultures that did not sprout in medium without growth regulator. *Pongamia* buds (62%) cultured in medium devoid of GR sprouted and elongated (Fig. 4.4C), but did not produce multiple shoots. Multiple shoot primordial appeared from the bud upon first transfer of TDZ containing medium to GR free medium. These primordial differentiated into shoots after repeated transfer to medium devoid of GR.

From this phenomenon, it is apparent that TDZ, at the concentrations tested, induces proliferation of meristematic cells but does not support shoot differentiation in this plant. Proliferation of meristematic cells in response to TDZ exposure was reported in peanut (Joshi et al., 2003) and cotyledon node meristem of tamarind (Mehta et al., 2005). Withdrawal of TDZ reduced the negative influence on the process of shoot differentiation resulting in appearance of shoot primordia from the dome shaped meristem. Larger domes in higher concentrations of TDZ suggest increased morphogenic cell proliferation leading to emergence of more number of shoots in explants pre-cultured in higher concentrations (Table 4.3). Faster differentiation of shoot buds in explants pretreated in lower concentrations of TDZ was possibly due to reduced inhibitory influence on the shoot differentiation.

In tamarind, presence of TDZ in the seed germination medium, suppressed differentiation of the apical meristem, and innumerable meristematic domes appeared in the form of undulations circling the cotyledon node (Mehta et al., 2004). These undulations differentiated into caulogenic buds and subsequently into shoots upon withdrawal of TDZ. In *Pongamia*, no such undulations were noted in the axillary meristem after 4 weeks of culture in TDZ containing medium, but the swelling in the meristem was obvious. This difference in the pattern of response between tamarind and *Pongamia*, in the initial stages of meristem culture in TDZ, could be due to variation in species or explant. In tamarind, the meristem was in the cotyledonary node of the intact seedling (juvenile tissue) whereas in *Pongamia* the meristem was in the axil of the mature tree derived nodal segment. In tamarind, histological studies revealed multiple layers of meristematic cells on and around the meristem indicating proliferation the meristematic cells. Caulogenic buds originated from these cells upon withdrawal of TDZ (Mehta et al., 2005).

From the above observations, it is apparent that mature tree derived nodal meristems of *Pongamia* respond differently in TDZ and in aminopurine cytokinins. In BA and KN, the meristem sprouted to form single shoot primordia but in TDZ, differentiation of the existing meristem was totally suppressed. The distinctly different responses in the nodal meristems, in cytokinins and in TDZ prompted us to design the following experiments.

As the sprouting of *Pongamia* bud and single shoot formation was achieved in cytokinin media, the attempt was to induce multiple shoot formation by further supplementing these media with Adenine sulphate. On the other hand TDZ incorporated at the tested concentrations induced proliferation of cells but suppressed differentiation of shoots therefore attempts were made to optimize the TDZ concentration to obtain both shoot proliferation and elongation.

A. Effect of AdS supplementation in BA or KN media on shoot multiplication:

Adenine in form of adenine sulphate is a commonly added additive in tissue culture media to stimulate cell growth and enhance shoot formation (Pierik, 1987). It is reported that metabolites like adenine alter the level of endogenous phytohormones and results in regeneration (Thorpe, 1994). It provides an available source of nitrogen to the cell and can be taken up more rapidly than inorganic nitrogen (Thom et al., 1981).

Combination of cytokinin and Adenine is favourable for shoot formation (Skoog and Miller, 1957; Pierik, 1987). AdS-BA combination media was effective in multiple shoot regeneration in many species. Addition of AdS (7.8-271.4μM) in the shoot regeneration media was reported in *A. nilotica*, *A. senegal*, *A. mangium* (Venkadesan et al., 2002). Kaur et al., (1998) achieved optimum shoot multiplication (8.66 ±1.71) in *Acacia catechu* on MS medium fortified with BAP 4 $mg.l^{-1}$, NAA 0.5 $mg.l^{-1}$ and AdS 25mg l^{-1}. In *Bauhinia vahlii,* maximum shoot multiplication was achieved in MS medium supplemented with 2.5 μM KN + 100 $mg.l^{-1}$ AdS. (Dhar and Upreti, 1999).

In contrast to the observations reported earlier, mature tree derived nodal explants of *Pongamia* when cultured in different concentrations of BA-AdS, did not show bud break after 15 days of culture as in the previous experiment with BA singly (Table 4.2). Whereas, in the explants cultured in KN-AdS media, bud break was initiated after 15 days of culture but the shoots did not elongate. These observations prompted us to transfer all the cultures to fresh medium of same composition for further growth and differentiation for one more passage of four weeks. Thus the data on frequency of

response, number of shoot buds per explant and length of the shoots could be scored after second passage (Table 4.4)

Table 4.4: Effect of BA-AdS or KN-AdS combination on the shoot multiplication in the mature nodal buds of *Pongamia.*

MS +AdS Medium (GR conc. in µM)	Frequency of response (%) mean ± sd	Av. no. of shoots/ explant mean ± sd	Av. Ht. of shoots (cm) mean ± sd
Control	54 ± 26	1 ±0 ^{e}	1.18 ± 0.34^{bc}
BA (0.44)	67 ± 14	1.06 ± 0.10^{de}	1.07 ± 0.19^{bcd}
BA (2.22)	42 ± 26	1.33 ± 0.58^{bcde}	0.92 ± 0.12^{cde}
BA (4.44)	67 ± 19	1.48 ± 0.50^{bc}	1.19 ± 0.20^{bc}
BA (6.66)	75 ± 22	1.86 ± 0.14^{a}	1.26 ± 0.07^{abc}
BA (8.88)	42 ± 14	1.44 ± 0.10^{bc}	0.89 ± 0.17^{cde}
BA (13.32)	46 ± 26	1.35 ± 0.13^{bcd}	0.99 ± 0.34^{bcde}
BA (22.20)	59 ± 26	1.54 ± 0.16^{ab}	1.12 ± 0.08^{bcd}
BA (31.08)	42 ± 26	1.17 ± 0.29^{cde}	1.06 ± 0.39^{bcd}
Kin (0.46)	63 ± 05	1±0^{e}	1.59 ± 0.08^{a}
Kin (2.28)	50 ± 33	1±0^{e}	1.25 ± 0.25^{abc}
Kin (4.65)	50 ± 13	1±0^{e}	1.24 ± 0.24^{abc}
Kin (7.93)	42 ± 19	1±0^{e}	1.33 ± 0.30 ^{ab}
Kin (9.29)	25 ± 05	1±0^{e}	1.09 ± 0.09^{bcd}
Kin (13.94)	29 ± 19	1±0^{e}	0.67 ± 0.29^{e}
Kin (23.23)	43 ± 28	1±0^{e}	0.86 ± 0.13^{de}
Kin (32.52)	13 ± 05	1±0^{e}	0.75 ± 0.25^{de}
Anova	**NS**	B x K- **S1%** BA- **S 5%** Kin- **NS**	B x K - **S1%** BA- **NS** Kin- **S1%**

Sprouting frequency of the buds ranged from 13 to 75%. It produced single shoot in control medium (Fig. 4.5A). No definite pattern of response was observed in BA-AdS media whereas a definite pattern of sprouting response was noted in explants cultured in KN-AdS. Sprouting response decreased with increase in concentration of KN. Combination of AdS with BA induced the nodal meristem to produce shoot multiples. Maximum multiples (1.86 ± 0.14 shoots/explant) were induced in the nodal meristem in media containing 6.66µM BA. With increase in concentration of BA up to 6.66µM, there was a significant (P>0.05) increase in the number of shoot multiples, beyond which the response declined (Table 4.4). The morphology of the shoot produced in BA-AdS combination was different from the shoots produced in BA singly. With AdS, BA produced thick small shoots (Fig. 4.5B). The leaves remained unopened. Beyond 6.66µM BA, the sprouts were thick and with shortened internode (Fig. 4.5C).

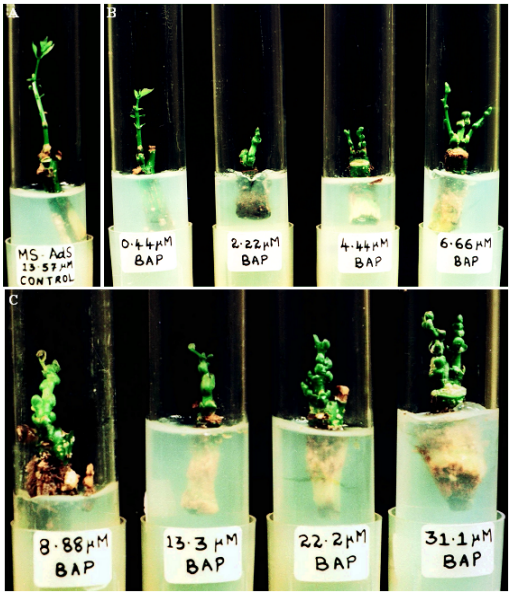

Fig. 4.5: (A) Single sprout produced in the control media.
(B) Shoot multiples produced from axillary node meristem cultured in different concentrations of BA (0.44 to 6.66µM).
(C) Sprouting pattern in the higher concentrations of BA (8.88 to 31mM)

In different concentrations of KN-AdS the explants did not produce multiples and morphologically shoots were similar to the shoots produced by KN (Fig. 4.6A). At higher concentrations of KN-AdS, (9.3 to 32µM) shoots were abnormal with thick, stunted and unopened leaves (Fig. 4.6B).

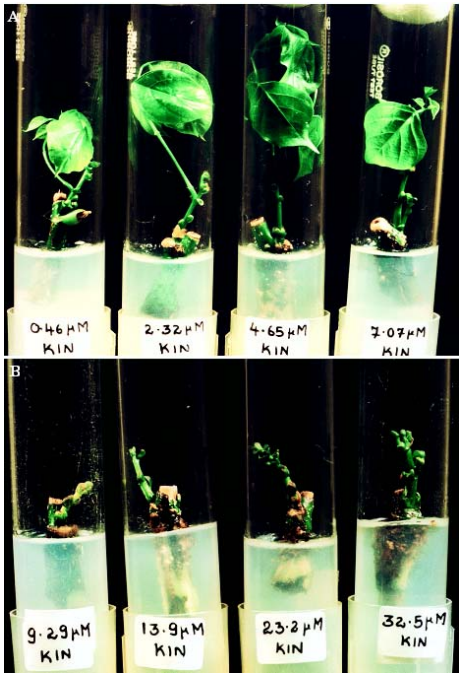

Fig. 4.6: (A) Shoot multiples produced from axillary nodal meristem cultured in different concentrations of KN (0.4 to 7µM).
(B) Sprouting pattern in the higher concentrations of KN (9 to 32 µM)

In the previous experiment, when BA and KN were used singly, there was no significant difference in shoot lengths. On addition of AdS with BA shoot multiplication was promoted, but the shoots did not elongate. In KN containing media, elongation was not affected by addition of AdS but shoot elongation was limited in higher concentrations of KN. From this experiment, it may be concluded that AdS supplement with BA promoted shoot multiplication in *Pongamia* whereas this supplement in KN containing medium was ineffective towards production of multiple shoots.

The shoots so obtained from BA-AdS combinations were transferred either to fresh medium of same combination for further proliferation. No further growth was noticed. Lower concentrations of BA (0.45μM) or AdS (13μM) were tested for *in vitro* multiplication but failed to induce proliferation of the shoots in growth in the shoots. Similarly, shoots obtained at higher concentrations of KN (above 9μM) did not respond in culture. Possibly the disturbances in hormonal equilibrium in the tissue due to incorporation of AdS, resulted in negative signaling on growth. Efforts taken to multiply and grow these shoots was unsuccessful.

Single shoots obtained in lower concentrations of KN (0.46 to 8μM) elongated on transfer to media of same composition. These shoots rooted in half strength MS medium with charcoal.

B. Experiments with TDZ treated nodal bud cultures:

The experiment with narrower range of TDZ (0.45, 2.27, 4.54, 6.81, 9.08, 11.35μM) confirmed the inhibitory effect of TDZ on sprouting and augmenting effect on caulogenesis. In the medium without GR, 62.5 ± 14.4 % of the buds sprouted after 4 weeks, whereas the buds on TDZ containing media did not sprout and developed the characteristic swelling at the node. The frequency of sprouting response was scored on appearance of caulogenic buds after 4 weeks on MS medium. Compared to 62.5% sprouting on TDZ-free medium, sprouting was 53.5% on 11.35 μM TDZ. The number of responding explants did not increase with additional passages on GR-free medium (Table 4.5) confirming the non-reversible inhibition in some of the buds. In the responding explants, numerous shoot buds appeared from the swollen node (Table 4.5).

The total number of shoot primordia formed on each explant (Table 4.5) was higher in all the concentrations of TDZ compared to the number of buds formed per explant in similar concentration in the previous experiment (Table 4.3). This difference in response of the buds in two similar experiments could be due to more accurate scoring of buds under the microscope in the second experiment, and a result of the physiological status of the initial explant collected at different times

of the vegetative phase. In the second experiment, numbers of shoot primordia and shoots per explant were significantly higher in explants pre-cultured in higher concentrations of TDZ (Table 4.5). Total number of caulogenic buds per explant indicates an increase in the number of multiples in higher TDZ treated explants.

Table 4.5: Effect of TDZ on sprouting of meristems, multiplication and elongation of shoots in the mature nodal bud explants of *Pongamia*

Conc. of TDZ (μM)	Sprouting Frequency (%) mean ± sd 4w	Av. no. of shoots/ explant mean ± sd 4w	Av. No. of caulogenic buds per explant mean ± sd 4w		Av. length of shoots (cm) mean± sd		
			<0.2cm	>0.2cm	4w	8w	12w
Control	62.5 ± 14.4	1.0^{e} ± 0 (19)	0.0 ± 0.0	1.0 ± 0.0	1.06^{ab} ± 0.24 (20)	1.78^{ab} ± 0.27	1.86^{ab} ± 0.19
0.45	62.8 ± 17.4	4.5^{d} ± 1.43 (20)	2.5 ± 1.3	2.0 ± 0.3	1.18^{a} ± 0.28 (32)	1.93^{a} ± 0.22	2.21^{a} ± 0.22
2.27	59.8 ± 25.6	7.3^{cd} ± 2.32 (18)	4.5 ± 1.7	2.8 ± 0.6	0.91^{bc} ± 0.04 (44)	1.53^{bc} ± 0.22	1.76^{b} ± 0.25
4.54	66.0 ± 35.8	10.7^{bc} ± 2.55 (21)	8.0 ± 1.4	2.7 ± 1.2	0.83^{cd} ± 0.12 (44)	1.25^{cd} ± 0.21	1.55^{bc} ± 0.23
6.81	38.5 ± 17.3	16.2^{ab} ± 2.95 (12)	10.8 ± 2.8	5.4 ± 0.3	0.67^{de} ± 0.02 (81)	1.09^{de} ± 0.26	1.37^{cd} ± 0.21
9.08	53.3 ± 06.5	17.9^{ab} ± 2.67 (17)	11.5 ± 2.5	6.4 ± 0.8	0.62^{de} ± 0.04 (102)	0.99^{de} ± 0.25	1.28^{cd} ± 0.23
11.35	53.5 ± 18.6	22.0^{a} ± 2.97 (17)	14.8 ± 2.5	7.2 ± 1.2	0.55^{e} ± 0.07 (115)	0.83^{e} ± 0.13	0.99^{d} ± 0.25
Anova	**NS**	**S 1%**	**S 1%**	**S 1%**	**S 1%**	**S 1%**	**S 1%**

[a-e] Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at P≤ 0.05.
Figures in parenthesis indicate number of replicates

Appearance of the buds following 4 weeks culture on GR-free medium confirms differentiation of meristematic cells after withdrawal of the inhibitory influence of TDZ. Transfer of explants to fresh GR free medium every 15 days supported further elongation by reduction in inhibition (Fig. 4.7A). Persistence of TDZ in plant tissues and suppression of shoot elongation have been described (Huetteman and Preece, 1993, Lu, 1993). The mean shoot height of *Pongamia* increased every 4 weeks (Table 4.5) but the number of caulogenic buds scored initially after 4 weeks on GR free medium remained constant. Number of buds was more in explants pre-cultured on higher concentrations of TDZ, but the rate of shoot elongation was less. Length of shoot did not catch up with the control or with the shoots from 0.45μM TDZ after six passages of 15 days each.

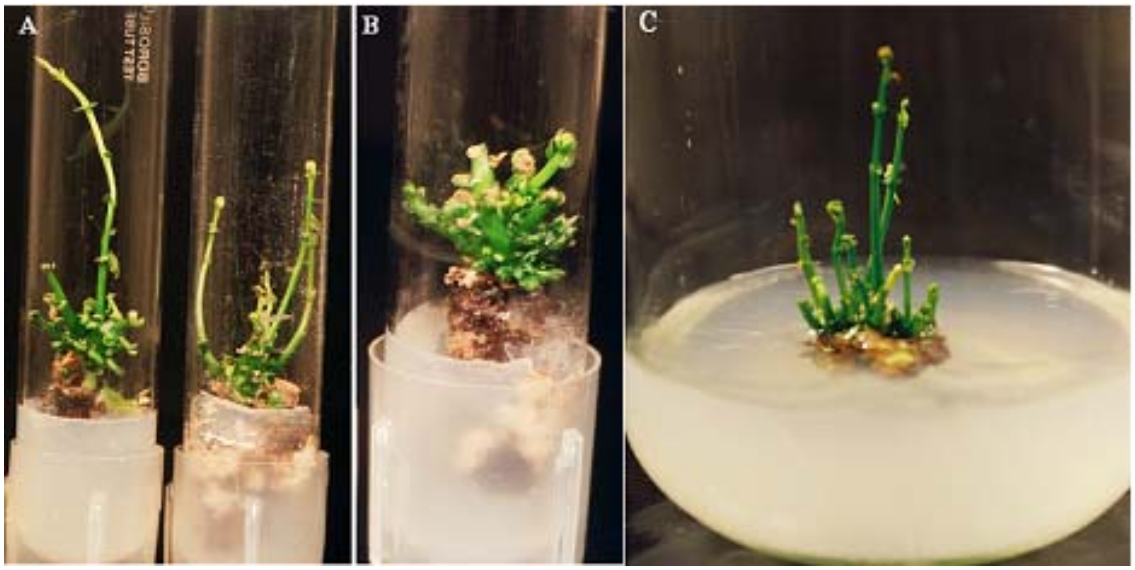

Fig. 4.7: (A) Differentiation and elongation of shoots in medium without PGR from explants pre-cultured in TDZ (0.45µM).
(B) Differentiation and limited elongation of shoots in GR free medium in explants pre-cultured in higher concentration of TDZ (11.35µM). Shoots are stunted but more in number.
(C) Elongation of shoots in GR free MS medium transferred from cluster of buds developed in the stump.

At higher concentrations (9.08 µM or 11.35 µM), the number of shoot primordia was more and the differentiated shoots were thick and stunted (Fig. 4.7B). The shoots were close to each other, and appeared fasciated. Formation of stunted shoots or the fasciation of shoots on TDZ containing medium was reported in other species (Chalupa, 1988, Pradhan et al., 1998). After 12 weeks on MS medium the buds did not attain the desired height of 2-3 cm for root induction. Thus, the cluster of buds was isolated from the initial explants and was subcultured as smaller clusters to medium for two more passages of 4 weeks each to attain the desired length (Fig. 4.7C).

In order to refine the protocol for optimum sprouting and shoot multiplication, parameters like carbon source, pH was tested. Among the three **carbohydrates** (Glucose, Sucrose and Fructose) tested, sucrose was more supportive resulting in high sprouting frequency, number of bud induction and elongation of the sprout (Table 4.6). In the media containing fructose, excessive callusing was observed at the base of the explant. The sprout produced in glucose containing media showed blackening of tissues (Fig. 4.8A). Hence, sucrose has been chosen as the source of carbon in *Pongamia*.

Table 4.6: Effect of different carbon source on the sprouting of mature nodal buds

Carbon Source	Sprouting Frequency (%) mean ± sd	Average number of buds mean ± sd	Length of the shoot (cm) mean ± sd	Callusing at base
Glucose (2%)	44.00 ± 2.83	1.85 ± 0.70 (13)	1.01 ± 0.56	+
Sucrose (2%)	65.00 ± 1.41	3.80 ± 1.11 (20)	1.34 ± 0.48	+
Fructose (2%)	51.00 ± 2.83	1.44 ± 0.63 (16)	1.16 ± 0.51	++
Anova	**S 1%**	**S 1%**	**NS**	

(Number in parentheses represents number of replicates)

Keeping in mind the influence of sucrose on proliferation and growth of shoot cultures in seedling derived explants (Chapter 3), an experiment was designed to optimize the sucrose concentrations for the protocol in which the seedling derived buds are substituted with mature plant derived nodal buds.

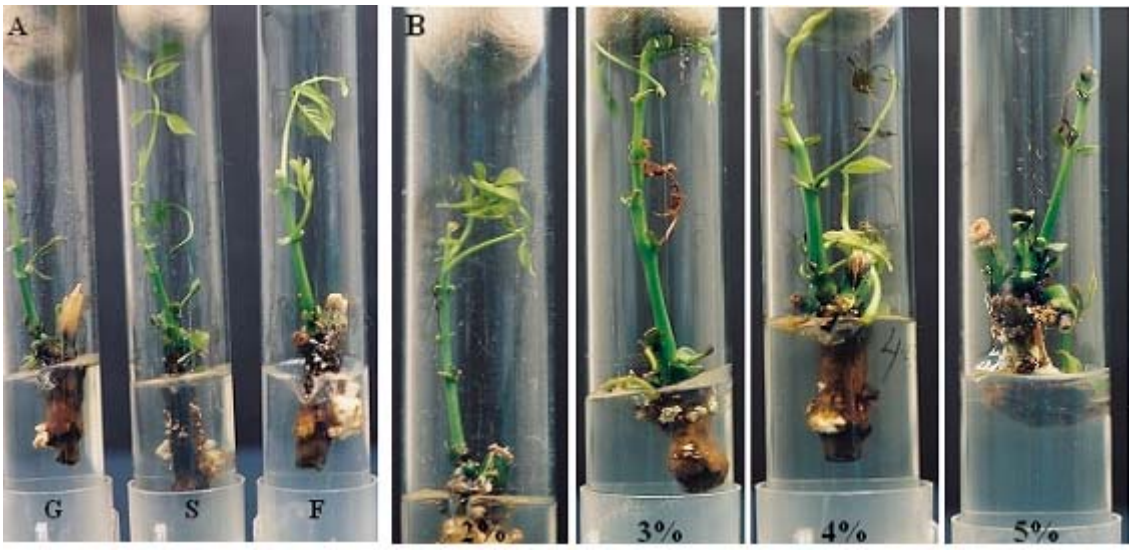

Fig. 4.8: (A) Effect of different carbohydrates. G-Glucose, S-sucrose and F- Fructose. (B) Effect of different concentrations of Sucrose (2,3,4 and 5%). Note the increasing number of multiples with concentration and elongation was restricted with increasing sucrose concentration.

Table 4.7: Effect of varying concentrations of Sucrose on sprouting of mature tree derived nodal buds.

Sucrose conc. (%)	Sprouting Frequency (%) mean ± sd	Average number of buds mean ± sd	Length of the shoot (cm) mean ± sd
2	53.3 ± 15.3	3.88 ± 1.07	1.24 ± 0.22
3	56.7 ± 5.8	3.88 ± 0.82	1.29 ± 0.22
4	56.7 ± 5.8	4.35 ± 0.79	1.41 ± 0.33
5	50.0 ± 10	4.80 ± 0.76	1.06 ± 0.28
Anova	**NS**	**S 5%**	**S 5%**

Table 4.7 shows the effect of different sucrose concentration on sprouting and multiplication of shoots from mature nodal bud of Pongamia. Sprouting frequency did not vary significantly. However, the number of buds induced per explant increased significantly with increase in sucrose concentration in media. In seedling explants, sucrose dependent shoot proliferation in BA (8.88µM) supplemented media was observed (chapter 3). Similarly, the shoot number increased linearly with increase in sucrose concentration. A sucrose dependent stimulated proliferation of TDZ (0.45µM) in mature nodal explants was observed. Shoot height was optimum at 4% sucrose concentration (Fig. 4.8B). In presence of TDZ, shoot height and bud proliferation were optimized on media containing 4% sucrose, presenting a suitable compromise for healthy shoot proliferation and growth.

The pH of a plant tissue cultue medium has pronounced effect on growth of tissues *in vitro* and it influences some plant developmental process (Owen et al., 1991). It was reported that low pH's (lower than 4.5) and high pHs (higher than 7.0) generally stop growth and development *in vitro* (Pierik, 1987). Hence, three pHs (5, 5.8 & 6.5) were tested for optimum *in vitro* growth of *Pongamia* buds in culture. The data (Table 4.8) confirmed the significant effect of pH on sprouting and elongation of the mature tree derived nodal buds.

Table 4.8: Effect of pH on sprouting of mature tree derived nodal buds

pH	Sprouting Frequency (%) mean ± sd	Average number of buds mean ± sd	Length of the shoot (cm) mean ± sd
5	40 ± 0.0	2.38 ± 0.98	1.08 ± 0.1
5.8	55 ± 7.1	3.27 ± 0.76	1.27 ± 0.3
6.5	30 ± 0.0	3.67 ± 0.82	0.97 ± 0.2
Anova	**S 5%**	**NS**	**S 5%**

At pH 5.8, maximum sprouting frequency and elongation was noted. Hence, pH–5.8 was chosen for *in vitro* multiplication and growth of shoots using mature nodal buds (Fig. 4.8C).

Fig.4.8C: Effect of pH on mature nodal buds of *Pongamia*.

Shoot maintenance and Stump culture:

The buds induced on high TDZ took longer and required several passages on GR free medium to elongate. Thus the rooting step was delayed. This period was reduced by subculturing the buds on medium with low concentration of TDZ (0.45µM) for maintenance of the shoot cultures. The strategy of alternate culture in medium with 0.45µM TDZ followed by passages on GR free medium was used for maintenance of the shoots.

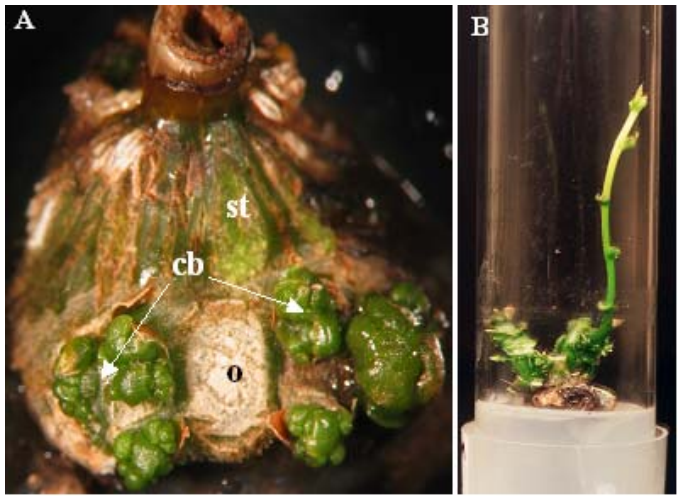

Fig. 4.9: (A) Induction of caulogenic buds (cb) from the side buds on reculturing the stump (st) in TDZ (0.45µM). Note the site of removed shoots (o).
(B) Elongation of shoots from the side buds on GR free MS media.

The 'stumps' when recultured on medium with 0.45µM TDZ for 4 weeks, produced more caulogenic buds from the pair of side buds (Fig. 4.9A). After transfer to GR free medium, these buds differentiated into shoots. The cluster of buds once again isolated from the 'stumps', elongated to produce shoots 2–3 cm in length on medium, after six passages of 15 days each, and the 'stump' produced more buds after reculturing on 0.45µM TDZ. This process of excision of shoot buds, reculturing of the stumps on 0.45 µM TDZ was repeated for six cycles to produce 25-30 shoots from each nodal bud. This strategy was used for micropropagation of *Bauhinia vahlii* (Upreti and Dhar, 1996) and *Dalbergia sissoo* (Pradhan et al., 1998), but the origin of the shoots was not defined in these protocols. In *Pongamia,* the first and second crop of shoots isolated from explants was from the central and side meristems respectively (Fig. 4.9B). The origin of the shoots obtained from the 'stump' in the following five cycles could be from the TDZ induced proliferated meristematic cells (Mehta et al., 2005), which differentiated with each passage in GR-free medium.

Production of more shoots for several cycles from mature tree derived original explants is intriguing. This strategy may find application in establishment of shoot cultures of recalcitrant

woody species and of endangered species. In woody species establishment of culture is often limited due to availability of vegetative buds at right stage of development. Secondly, elimination of microbial contaminants from the buds is laborious and involves several manipulations thereby delaying the process of establishment of cultures. For endangered species, repeated extraction of the buds is an additional limitation that can be overcome by reusing the same explant repeatedly to obtain the shoots, which can then be used as starting material for clonal propagation.

Shoots Rooted and propagules transferred: *In vitro* rooting is a common phenomenon in *Pongamia* tissues. Adventitious root formation was noticed from the base of the nodal explant cultured, after first transfer to GR-free MS media (Fig. 4.10A). *In vitro* raised *Pongamia* shoots of 2-3 cm in length rooted (70%; Fig. 4.10B) and elongated on half-strength with activated charcoal medium in 4 weeks. TDZ does not seem to inhibit adventitious root formation of the micro shoots of *Pongamia*. Similar results were reported in apple and silver maple tissues ((Huetteman and Preece, 1993).

Fig. 4.10: (A) Adventitious roots formation from the basal portion of explants after eight weeks of culture apart from sprouting of nodal bud.
(B) Rooted shoot in MS medium.
(C) Hardened plantlets in green house.

The rooted shoots were transferred to sand: soil mixture and cultured for four weeks. After 10 days of transfer, leaf fall was noticed in some plantlets but was followed by new leaf formation. After 4 weeks of hardening, all forty-six propagules were transferred greenhouse successfully (Fig. 4.11A).

Field Trials:

Field studies were also undertaken to assess the performance of the *in vitro* raised plantlets. About twenty plants were planted in Mhaske Farms for field trial (Fig. 4.11B). Till date, all the plants are growing well without any phenotypic abnormalities. Moreover, these plants have more vigor compared to the seedlings grown *ex vitro*.

Fig. 4.11: *In vitro* raised plantlets growing in Mhaske farms.

Reliability of the Protocol:

Out breeding species generally exhibit high variation in them at high frequency. *Pongamia* is highly out breeding species, in which cross-pollination takes place primarily by insects and air. High variations in characters like pod length, pod shape, number of seeds per pod, oil content etc. were observed (chapter 6). It is expected that these trees might have differential response in culture too. This necessitates the testing of a standardized protocol in other genotypes for its effective application. Hence, the protocol standardized using identified tree (tree-2) was tested in other trees for its reliability.

In the experiment conducted to determine the reproducibility of the protocol, the nodal buds were collected from ten different mature trees were cultured in 0.45 µM TDZ for four weeks followed by the transfer to GR free MS media for four weeks. The nodal explants excised from tree 2 were considered as control. Though all other parameters except the explant source were maintained the same, significant variation (1%) was noted in the parameters like frequency of sprouting, number of shoots produced and their length among the trees tested after eight weeks of culture (Table 4.9).

Table 4.9: Response of mature nodal buds excised from ten different trees

Trees	Sprouting Frequency (%) mean ± sd	Average number of buds mean ± sd	Length of the shoot (cm) mean ± sd	callusing	Phenolic exudation in media	Leaf opening
T1	26.7 ± 5.8	1.1 ± 0.38	0.55 ± 0.28	+	-	--
T2	60.0 ± 10	3.4 ± 0.76	1.27 ± 0.15	-	*	opened
T3	30.0 ± 10	1.6 ± 0.79	0.81 ± 0.22	+	-	partial
T4	40.0 ± 10	1.8 ± 0.69	1.03 ± 0.25	-	*	partial
T5	30.0 ± 10	1.6 ± 0.49	0.72 ± 0.32	++	-	-
T6	56.7 ± 5.8	1.9 ± 0.58	1.26 ± 0.36	-	*	opened
T7	33.3 ± 5.8	1.1 ± 0.38	0.79 ± 0.22	+	*	-
T8	33.3 ± 5.8	1.3 ± 0.49	0.66 ± 0.20	++	-	-
T9	60.0 ± 10	3.1 ± 0.90	1.32 ± 0.36	++	-	opened
T10	56.7 ± 5.8	2.8 ± 1.07	1.18 ± 0.35	+	-	opened
Anova	**S 1%**	**S 1%**	**S 1%**			

Fig. 4.12: (A&B) Effect of explant source from ten different trees cultured in MS media pre conditioned with TDZ (0.45µM),on *in vitro* sprouting. Note the differential response of explants in culture.

Trees T2, T6, T9 and T10 showed normal sprouting with two or three multiple shoots, producing sprouts with an average length of 1.2 cm. Sprouts with average length of 1.03 cm with multiple shoots was obtained from the nodal explants of tree 4 ,but with phenolic exudation (Fig. 4.12A). Less than 35% frequency was obtained from the explants excised from the trees T1, T7 and T8. Moreover, these trees produced only one sprout with short shoots. Phenolic exudation was prominent in the media having the explants excised from tree T7 and so the resultant sprouts were dark. Extensive callusing was noted from the explants of T8 (Fig. 4.12B). The nodal explants from the trees T3 and T5 sprouted at a lesser frequency but produced two shoots per meristem. Leaf opening was absent in the explants excised from the tree T5; instead, extensive callusing was noted at the base. The above protocol was reproduced effectively in the five selected tree species and hence may be applied commercially.

As expected, difference in responses among the selected *Pongamia* trees was observed. Differential response in similar media combination among the tree species was well documented. It has been suggested that the differences in the number of shoots and roots produced among the salix genotypes during *in vitro* culture is mediated by the differences in the endogenous balance of auxin and cytokinin (Gronroos et al., 1989). In *Pongamia*, the differential response in similar media combination may be due to variation in developmental or physiological status and or the genotypic variation in this species.

4.4 CONCLUSION

A method for clonal propagation of *P. pinnata* from mature tree derived axillary meristem is described. MS basal media was superior to other formulations for sprouting and shoot multiplication of *Pongamia.* BA, KN, and Z are ineffective in inducing multiple shoots, and TDZ in equimolar concentration influences the meristem differently. Adenine sulphate supplementation in BA containing media was effective in inducing multiple shoots whereas this compound in combination with KN remained ineffective in inducing of multiple shoots from the nodal meristems. At the tested concentrations of TDZ, sprouting of the axillary buds is suppressed and meristematic domes are formed. Shoot primordia develop from the dome on withdrawal of TDZ and differentiate into shoots after repeated culture on medium devoid of GR. Number of caulogenic buds that appear after the first two passages on GR-free medium remained constant even after six passages. For the buds pre-cultured on higher concentrations of TDZ, number of shoot primordia and shoots are significantly higher. The number of shoot primordia formed, follows a pattern with increase or decrease in TDZ.

Differentiation and elongation is slower in the buds induced on higher TDZ concentration. Concentration of 0.45μM TDZ is optimum for proliferation and maintenance of *Pongamia* shoot cultures. Sucrose at 4% and pH–5.8 was found to be optimum for shoot proliferation and growth. Reculturing of original explants yielded shoots. Resprouted axillary shoots being harvested in each cycle. These shoots were used as cuttings for propagation.

Differential *in vitro* response was observed among the explants excised from selected trees. The above protocol was reproduced effectively in the five selected tree species and hence may be applied commercially

This report demonstrates the dual role of TDZ in *Pongamia* tissue culture. It selectively accentuates the meristematic cells to produce more number of cells determined to form caulogenic buds after withdrawal of the GR but suppresses differentiation of these cells and meristems into shoots. It also indicates that the suppression is more pronounced in the higher concentrations when the proliferation of meristematic cells is faster. Concentration of TDZ chosen for optimum proliferation and maintenance of shoot culture is 0.45μM. Original explants resprouted on reculture up to six cycles. This study not only describes a simple protocol for clonal propagation of an important tree species "*Pongamia pinnata"* for the first time, but also a system to study some of the processes of TDZ induced morphogenetic activity. The described protocol will be useful as a part of isolation stage in establishing the standard shoot culture protocol. Repeated proliferation of caulogenic buds from the same origin may find application in rescue of endangered germplasm.

A part of this work has been accepted for publication

Micropropagation of mature *Pongamia pinnata*. Pierre.

In Vitro Cell. Dev. Biol. Plant. 2007 (in press)

CHAPTER 5:
DE NOVO MORPHOGENESIS

5.1 INTRODUCTION

De novo morphogenesis is 'the *de novo* production of plant organs (buds, shoots, roots) or structures resembling zygotic embryos from organized tissues or callus'. Capacity of cultured plant tissues and cells to undergo *de novo* morphogenesis, resulting in formation of discrete organs or whole plants, has provided opportunities for numerous applications of *in vitro* biology, in studies of basic botany, biochemistry, propagation, breeding and development of transgenic crops (Phillips, 2004).

De novo morphogenesis *in vitro* occurs in two different pathways i.e. organogenesis and embryogenesis. It can occur directly on explant or indirectly via callus. Primary advantage of *de novo* morphogenic methods over axillary shoot methods is a potential for **higher frequency plantlet production** in a shorter period. Direct or indirect *de novo* morphogenesis via organogenesis or embryogenesis is more desirable for genetic transformation studies. Advantage of these systems over the axillary shoot methods lies in the **amenability to different gene delivery techniques**. *Agrobacterium*-mediated transformation of preexisting meristem is often unsuccessful or inefficient, which is perhaps due to the low number of attachment sites for *Agrobacterium* in the cell walls of meristematic cells (Mattysse and Gurlitz, 1982).

Unlike axillary shoot methods, *de novo* morphogenic methods especially those requiring intermediate callus, may be associated with the production of significant amounts of somaclonal variation in the regenerated plantlets, which are often heritable. It is recognized that somaclonal variations could be useful for improvement of plants and might be a valuable source for introduction of new traits. Thus, *de novo* morphogenic methods have promising application in studies on **isolation of somaclonal variants**. Frequency of variation in cells could be increased, by treating them with mutagenic agents. Hence, this system provides platform for **induction of mutants** and studies on mutagenesis. Morphogenic regeneration systems via organogenesis and embryogenesis (both direct and indirect) have been developed for a number of leguminous tree species.(Chapter 1, Table 1.2).

A. *DE NOVO* ORGANOGENESIS

***De novo* organogenesis** is the process by which cells and tissues are manipulated to undergo changes, which lead to *de novo* production of unipolar structure namely a shoot or root primordium, whose vascular system is often connected to the parent tissues. It can occur directly on explant or indirectly via callus (Thorpe, 1994).

The distinction between ***de novo*** organogenesis and shoot proliferation from preexisting meristems is that *de novo* organogenesis requires a redetermination of existing genetic programs expressed within a cell. The application of these processes differs. Mature tree derived nodal explants are often used for rapid propagation of elite plants whereas direct or indirect *de novo* morphogenesis is more desirable for genetic transformation. Like micropropagation from preexisting meristem, this procedure also involves four stages. It involves the induction of localized meristematic activity leading to formation of primordium and the shoot (Thorpe and Patel, 1984). In contrast to the axillary bud proliferation of woody legumes, initiation of adventitious shoots is usually limited to the explants obtained from juvenile material.There are only few reports of shoot organogenesis from the explants of mature trees like cambial explants in *D.sissoo* (Kumar et al., 1992), internodes from new branch growth on mature tree of *D.latifolia* (Lakshmi Sita et al., 1986), petioles from newly emerged leaves of *A.lebbeck, Cassia fistula,and C. siamea* (Gharyal and Maheswari, 1990).

Expression of organogenic potential is greatly influenced by the source, age, physiological status, size, type, orientation, genotype of explants and the culture conditions. Most frequently used explants that have led to successful regeneration have been the seed or seedling parts including cotyledons, hypocotyl, epicotyl and embryonic axis. Cotyledons were used as explant in many species like tamarind (Mehta et al., 2004) *Lens culinaris* (Khawar et al., 2004), *Dalbergia sissoo* (Chand et al., 2002) *Sesbania grandiflora* (Detrez et al., 1994), *Albizzia falcataria* (Sinha and Mallick, 1993), *Sesbania bispinosa* (Kapoor and Gupta, 1986) etc. Cotyledons excised from two-day-old seedlings of *Vigna radiata* were more regenerative and those older than 3 days lacked this potential (Gulati and Jaiswal, 1990). Morphogenic response within the cotyledon explant varies. Proximal portion of the cotyledon (portion close to node) is generally more responsive than the remaining parts of cotyledon in many species. Ainsley et al. (2001) and Chand et al., (2002) demonstrated high regeneration capacity of the proximal segment in *Prunus and Dalbergia* respectively. Response in explant varies with its orientation on medium. In *Vigna unguiculata,* Kulothungan (1997) reported organogenic differentiation in proximal end of cotyledon when the distal ends were in contact with medium. Explants with adaxial surface in contact with medium were more responsive in species like *Prunus* (Ainsley et al., 2001), mung bean (Tivarekar and Eapen, 2001), *Dalbergia* (Chand et al., 2002) etc.

Embryo axis explants are known to have high regenerative potential and has been used for morphogenetic studies in several species. Ozcan et al., 1996 used embryo axis explants for exploited rganogenesis in *Onlbrychis viciaefolia.* Non meristematic regions of the zygotic embryo axis were for the induction of *de novo* organogenesis in tamarind (Mehta et al., 2000).

The growth regulators most commonly used for induction of shoot primordia are cytokinins. Among the GRs, TDZ has proved to be potential in elucidating *de novo* organogenic response in various woody species (Mehta, 2005; Chenong and Pooler, 2003; Murthy et al., 1998; Huetteman and Preece, 1993; Lu, 1993). Effectiveness of TDZ in inducing organogenic response is described (Chapter 3 & 4). This GR has been reported to induce *de novo* organogenic response in species like *Lens culinaris* (Khawar et al., 2004), *Cajanus cajan* (Singh et al., 2003), *Saintpaulia ionantha* (Mithila et al., 2003), *Tamarindus indica* (Mehta et al., 2000 & 2004), *Acacia mangium* (Xie &Yang, 2001), *Swainsona salsula* (Yang et al., 2001), chestnut (San-jose et al., 2001) etc. Reports on successful regeneration via de novo organogenesis (both direct and indirect) in leguminous tree species are listed (Chapter 1, table 1.2).

B. SOMATIC EMBRYOGENESIS

Somatic embryogenesis (SE) is a process by which cells and tissues are manipulated to undergo changes, which leads to production of bipolar structures containing a root/shoot axis with an independent vascular system. This can occur directly on explant or indirectly via callus. In general, development of somatic embryos parallels that of zygotic embryos. This phenomenon was first described by Steward (1958) in carrot four decades ago.

Unlike organogenesis, which may either *de novo* or from existing meristem, somatic embryogenesis is always *de novo* in origin. It is the unique expression of totipotency of plant cells. Juvenile tissues like cotyledon, embryo axis and immature seeds appear to be more suitable for induction of somatic embryos (Tulecke, 1987). Cotyledons from semi mature green pods produced somatic embryos in woody legumes like *Dalbergia sissoo* (Singh and Chand, 2003), *Sesbania sesban* (Shanana and Gupta, 2002). The developing zygotic embryo explant generally shows a high potential for somatic embryogenesis (Williams and Maheswaran, 1986). In tree legumes, most reports of SE are from zygotic embryo (*A. arabica*, Nanda and Rout, 2003; *A. mangium*, Xie and Hong, 2001; *Hardwickia* binata, Chand et al., 2001; *D. latifolia*, Muralidhar Rao and Lakshmi Sita, 1996).

Developmental stage of explant is critical for expression of embryogenesis. According to Wetzstein *et al.*, embryogenic potential is influenced by the time of embryo explanting and developmental stage (1989). Trigiano *et al.*demonstrated that 96 and 110 days post anthesis zygotic embryos of Red bud (*Cercis canadensis*) produced somatic embryos at optimum frequency (1988). It is difficult to ascertain the age of immature zygotic embryos of many woody species since the time of anthesis is not known. Instead, size of the immature embryo can be taken as a criterion (Mehta, 2001).

The process of somatic embryogenesis normally takes place in two stages. First, induction of cells with **embryogenic competence** in presence of high concentrations of auxin. Second, development of embryos in absence or in presence of a lowered concentration of auxin. Embryogenesis was mostly induced by use of auxins like 2,4-D, NAA, picloram, dicamba, 2,4,5-T, etc. For SE induction from immature zygotic embryos of 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. Another herbicide with auxin-like properties, picloram, has proven to be very effective for maintenance of embryogenic calli and plant regeneration of peanut (Joshi, 2003). Dicamba, another herbicide with auxin like property induced somatic embryos in tamarind (Mehta, 2001).

Although a large number of trees have been regenerated via somatic embryogenesis, reports on woody legumes are limited (Lakshmi Sita, 1999). The reports on successful regeneration via somatic embryogenesis (both direct and indirect) in leguminous tree species are listed (Chapter1, Table 1.2).

MORPHOGENESIS IN *PONGAMIA*

Until recently, there was no literature on the morphogenetic studies in this species. Clonal propagation of this species from juvenile (Sujatha and Hazra, 2006) and mature tree derived existing meristems (Sujatha and Hazra, 2007) is reported recently. However, till date, there is no report on *de novo* morphogenesis in this species. Thus, experiments were designed and carried out to establish morphogenic systems via *de novo* organogenesis and embryogenesis. The experiments and the results are divided into two parts. Part 'A' includes the work on *de novo* organogenesis using cotyledon and embryo axis explants. Part 'B' describes the studies conducted to standardize a protocol for somatic embryogenesis.

5.2 EXPERIMENTAL PROTOCOL

A. De novo Organogenesis

(a) Cotyledon:

Semi mature green pods were collected from the trees growing locally. These were surface sterilized using the method described (Chapter 2). With the help of sterile secator and scalpel, the surface sterilized pods were opened aseptically and the seeds were isolated. Seed coat was eliminated and the cotyledons, separated and deembryonated. These were cultured in TDZ supplemented (0, 0.45, 2.27,

4.54, 6.71, 9.08, 11.35, 13.12, and 22.71 μM) agar gelled MS medium (Murashige and Skoog, 1962). Medium without TDZ was used for control. One cotyledon was cultured adaxially whereas the other one was cultured abaxially. After 10 days in TDZ, 50% of the cultures were transferred to MS basal medium and the remaining 50% were transferred after 20 days. Orientation of the explants were maintained in the corresponding medium.

Frequency of organogenic response in tested **concentrations of TDZ** was noted after 10 days and 20 days of culture in TDZ medium. Total number of buds appeared were scored cotyledon wise using microscope. This experiment was repeated thrice with 24 replicates in each repeat. In addition, the experiment with 20 days **TDZ exposure** was further repeated twice with 45 replicates in each repeat.

In the next experiment, **differential response in the cotyledon segments** was studied. Each cotyledon was cut into three segments. The one attached to the embryo axis was identified as proximal (p) while the other two as middle (m) and distal (d) respectively. These were cultured either adaxially (AD) or abaxially (AB) in 90-mm petriplates containing 30ml MS medium supplemented with various concentrations of TDZ (0, 0.45, 2.27, 4.54, 6.71, 9.08, 11.35, 13.12, and 22.71 μM). The cultures were incubated for 20 days. Number of responding explants was scored segment wise and the frequency of response was determined. This experiment was repeated thrice with 24 replicates.

The experiment was repeated to determine the differential response in individual section of cotyledon in accordance with **orientation** of the explant. Numbers of buds induced on adaxial and abaxial surfaces of each segment in 20 days of TDZ exposure were noted from the enlarged image on computer monitor connected to the microscope. Experiment was repeated thrice with 24 replicates of each segment.

TDZ exposed explants were transferred to MS basal medium for 8-12 passages of 15 days each for differentiation of shoots. From these 32 shoots of 2-3cm in length were isolated and cultured in rooting media following the method described (Chapter 3). Explants with buds and shorter shoots were recultured in GR free MS medium, for further **differentiation**. After 4 weeks of incubation in **rooting** media, 28 shoots were rooted. (Chapter 3). Twenty-eight propagules were transferred to soil and **hardened** for four weeks. Hardened plants were transferred to bigger pots in greenhouse.

All media used in this study were supplemented with 2% sucrose, methods for media preparation and culture conditions are described in chapter 2. The data obtained were subjected to ANOVA.

Histological studies:

Histological studies were carried out with the tissues taken from the proximal segments cultured in TDZ for 20 days and followed by 10 days and 30 days in GR free MS media. These explants were cut into small (approx 3x4mm) pieces and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90,v/v) for 48 h at room temperature. Histological methods are described. (Chapter 2).

(b) Embryoaxis:

Semi mature green pods collected from the local trees were surface sterilized using the method described (Chapter 2). Pods were opened aseptically and the seeds were isolated. Cotyledons were separated into two such that the embryo axes separated without any damage. The axes were cultured on agar gelled MS media supplemented with TDZ (0, 0.45, 2.27, 4.54, 6.81, 9.08, 11.4, 13.6 & 22.7μM). Medium without TDZ was used for control. After 10 days exposure in TDZ, 50% of the cultures were transferred to MS medium and the remaining explants were transferred after 20 days. The embryo axes were cultured in 55 mm petriplates containing 15 ml of agar gelled medium. The experiment was repeated five times with 12 replicates.

The explants cultured in TDZ for 10 and 20 days were transferred to GR free MS basal media for four passages of 15 days each. Data on the frequency of response and numbers of shoot buds per explant obtained were noted after four passages. Thereafter the explants with meristematic buds were cultured in MS media with 0.45μM TDZ, or 4.45μM BA, or 0.45μM GA_3 for bud differentiation. The methods for media preparation, culture conditions and statistical analysis are described in chapter 2.

Histological studies:

Histological studies were carried out in embryo axis tissues cultured initially in different concentrations of TDZ (4.54. 11.35 & 13.6μM) for 20 days and followed by 30 days in GR-free MS media. These explants were cut into small (approx 3 x 4 mm) pieces and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48 h at room temperature. Histological procedure followed is described in Chapter 2.

B. Somatic Embryogenesis:

Cotyledon and embryoaxis explants derived from semi mature green pods were tested for induction of somatic embryos. Immature seeds of different sizes excised from immature pods were also tested. Agar gelled MS media with 6% sucrose supplemented with the concentration of auxin to be tested was used in all embryogenesis experiments, unless otherwise mentioned.

(i) Cotyledon:

Semi mature green pods were surface sterilized and the seeds were isolated (Chapter 2). Seed coat was eliminated aseptically using forceps and scalpel and the cotyledons were cultured on MS media supplemented with different concentrations of various auxins like 2,4-D, NAA, Picloram, Dicamba and 2,4,5 T at the concentrations ranging from 0.45 to 55 μM. Explants were cultured in 90mm petriplates having 30 ml media and were incubated for four weeks. Experiment was repeated six times with six replicates. Following this, the explants were tested in following three media for embryo differentiation: -

(a) transferring to hormone free MS basal medium
(b) transferring to the respective media, whose auxin concentration is lower than the induction media. (Explants precultured in the concentration above 13μM were transferred to 13μM and the explants precultured below 13μM were transferred to the media having 0.45μM of the respective auxin in MS media).
(c) Transferring to fresh media of similar composition for four weeks.

(ii) Embryo axis:

The embryo axis explants were isolated aseptically from surface sterilized semi mature green pods (Chapter 2). These were cultured in 2ml of liquid MS media supplemented with different concentrations of various auxins like 2,4-D, NAA, Picloram, Dicamba and 2,4,5-T with the concentrations ranging from 0.45 to 55 μM, on a shaker for overnight and then transferred to respective agar gelled media for four weeks. Test tubes were used as culture vessel for initial liquid culture and 55mm petriplates for agar gelled media. Experiment was repeated eight times with five replicates in each concentration.

(iii) Immature Seeds:

Young, immature pods at different stages of development were extracted from the trees. Similar to explants mentioned above the young immature pods were surface sterilized and immature seeds were excised. These were classified into 6 stages based on the size (Table 5.1). As the time of anthesis is not known to determine the age, the size of the immature seed has been taken as a criterion in *Pongamia.*Immature seeds from size 1-6 were used for the experiment on induction of somatic embryogenesis. Seeds with coat were cultured for four weeks in MS medium supplemented with different auxins including 2,4-D, NAA, Picloram, Dicamba and 2,4,5-T at the concentrations ranging from 0.45 to 55 μM. Explants, which produced mass like structures were scored as responding

explant and the frequency of response was calculated. The experiment was repeated 8 times with five replicates in each repeat.

Table 5.1: Classification of immature seeds based on their size

Size classified	Length (mm)	Width (mm)
1	2-3	1
2	4	2
3	5	3
4	5	4
5	5	5
6	6	6

5.3 RESULTS AND DISCUSSION

A. De novo Organogenesis

(a) Cotyledon:

The responding cotyledon explants swelled and turned dark green whereas the non-responding explants faded to light green and subsequently to light brown. Regeneration commenced after 25 days of culture in the GR free media on the surface (Fig. 5.1A) of the explants. Meristematic bud formation became evident after 30 days (Fig. 5.1B & C), with clusters of dark green; protuberances (b) appearing from the surface and edges of the explants cultured in TDZ containing media.

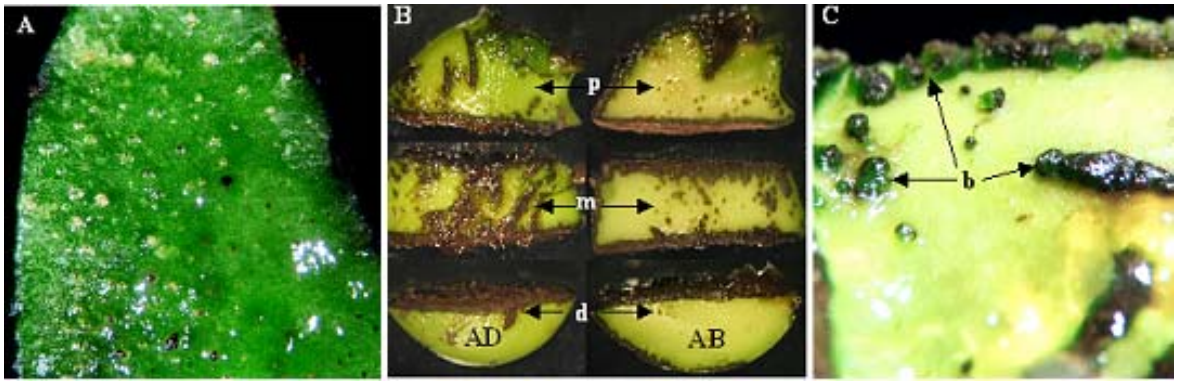

Fig. 5.1: (A) Initiation of buds in the form of protrusions on the surface of cotyledon.
(B) Cotyledonary segments proximal (p); middle (m) and distal (d) cultured on a petriplate in both orientations abaxially (AB) and adaxially (AD).
(C) Buds (b) on the adaxial surface and cut margins of cotyledon segment

These clusters appeared on both adaxial and abaxial surfaces and along the margins of the cotyledons. Neither swelling nor morphogenic response was induced in the cotyledons cultured on to MS medium devoid of growth regulators.

Data generated (Table 5.2) from the first experiment conducted with intact cotyledon to **optimize the concentration and exposure** of TDZ demonstrate that there was no bud formation in media without TDZ. Whereas buds were induced in all the cotyledon pre treated in various concentrations of TDZ for 10 days and 20 days. Frequency of response increased with concentration and exposure. Optimum of 51.8±11% at 13.12 µM in 10 days treated and 66.9±16.7% at 11.35 µM in 20 days exposed explants were noted. Thus at a lower concentration of TDZ (11.35 µM), a higher 66.9±16.7% frequency of response could be achieved when the exposure was extended from 10 days to 20 days. At concentrations higher than the optimum the caulogenic response declined in both 10 days and 20 days exposed culture. The results of these experiments suggest that either concentration or exposure in TDZ can be manipulated to obtain optimum response.

Table 5.2: Effect of TDZ concentration and exposure on *de novo* response in the immature cotyledon explants of *Pongamia*

Conc. of TDZ (µ M)	Frequency of response mean ± sd		Average no. of buds mean ± sd	
	10days	20days	10days	20days
Control	0.0 ± 0.0 (54)	0.0 ± 0.0 (144)	0.0± 0.0	0.0 ±0.0
0.45	9.0 ± 3.1 (54)	09.6 ± 14.2 (138)	74 ± 5.3	71 ± 11
2.27	9.9 ± 3.0 (48)	20.4 ± 21.3 (111)	89 ± 5.5	101 ± 15
4.54	11.2 ± 1.3 (54)	25.9 ± 26.5 (129)	97 ± 12	136 ± 13
6.71	19.4 ± 12. (54)	37.4 ± 17.9 (138)	106 ± 13	149 ± 16
9.08	30.8 ± 2.2 (70)	58.2 ± 18.0 (144)	139 ± 7.9	178 ± 8.8
11.35	44.4 ± 9.6 (70)	66.9 ± 16.7 (132)	175 ± 14	205 ± 14
13.12	51.8 ± 11 (42)	60.2 ± 22.2 (147)	162 ± 8.7	202 ± 21
22.71	33.3 ± 8.3 (48)	57.0 ± 29.9 (135)	148 ±15	190 ± 8.5
Anova	**S 1%**	**S 1%**	**S 1%**	**S 1%**

(Number in the parentheses represents the number of replicates)

Numbers of buds scored under microscope are approximate figures, as some buds were indistinguishable due to close origins and uniform dark green color of buds and explant. Number of buds produced per explant, ranged from 70-205. At both exposures (10 days and 20 days), there was decline in number of caulogenic buds produced in TDZ concentrations beyond the optimum of 13.12 and 11.3µM respectively. Many buds from cotyledons precultured in 13.32µM TDZ and above, failed to differentiate and turned necrotic or callused on transfer to MS media.

Concentration of TDZ is known to be critical in shoot organogenesis (Chalupa, 1988; Lu, 1993). For shoot production in woody legumes concentrations of TDZ ranging between 0.0091-0.363 µM are often used (Lu, 1993; Huetteman et al., 1993). However there are reports where it was used at higher concentrations. In white ash (Bates et al., 1992) adventitious shoots were regenerated using 10 µM TDZ. In *Prunus domestica* (plum), *P.erasus* (sour cherry) and *P. persica* L. (peach) 5-12.5 µM TDZ was effective (Huetteman and Preece, 1993). Excessive callus formation and cessation of shoot growth in lower concentration range of 0.45-4.45µM was reported in silver maple (Preece and Imel, 1991).

In P. pinnata, the shoots differentiated from lower concentration range of 0.45-4.45µM without intervening callus formation in TDZ 13.12µM and 11.35µM on 10 days and 20 days exposure respectively. At the higher concentrations (22.71µM), the frequency of response was reduced, and the explants turned brown and necrotic. Similar observation was noted in flax and apple in which TDZ (22.7-45.5µM) caused necrosis of tissues, hyperhydricity and abnormal leaf morphology (Kim et al., 1997). In *P. pinnata* cotyledons, large numbers of buds were formed in the TDZ concentrations above 11.3µM (Fig. 5.2A). However, several of these buds failed to differentiate and turned necrotic on transfer to MS medium and some buds dedifferentiated to callus (Fig. 5.2B). Hence, 11.3 µM TDZ containing MS media with exposure duration of 20 days was chosen for further experimentation on refinement for optimum bud induction and shoot formation.

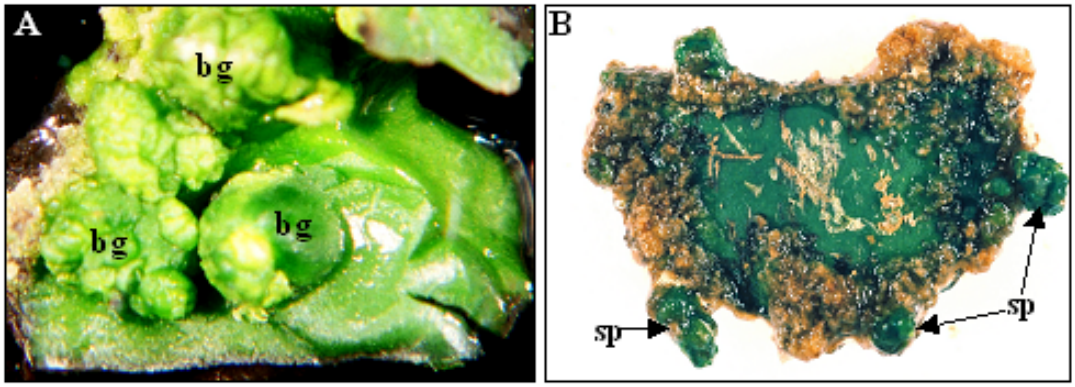

Fig. 5.2: (A) Caulogenic buldges (bg) visible on the adaxial surface of the cotyledon segment precultured in 13.12 µM TDZ for 20 days, in GR free MS media.
(B) Shoot buds (sp) were differentiated in the explant cultured in the second passage of GR free MS media. Note: many buds were dedifferentiated to callus.

In the second experiment, all three segments proximal (p), middle (m) and distal (d) responded to TDZ in culture. The proximal segment was more responsive (Table 5.3) followed by the middle and distal.

Table 5.3: TDZ induced morphogenic response in cotyledon segments of *P.pinnata* after 20 days of TDZ exposure.

Conc. of TDZ (μM)	Frequency of response		
	Proximal (p)	Middle (m)	Distal (d)
Control	0.0 ± 0.0 (48)	0.0 ± 0.0	0.0 ± 0.0
0.45	26.8 ± 32.3 (46)	12.5 ± 21.7	10.6 ± 13.7
2.27	31.4 ± 31.1 (37)	18.3 ± 20.5	18.2 ± 27.8
4.54	33.3 ± 19.3 (33)	26.5 ± 33.4	17.8 ± 27.9
6.71	53.3 ± 22.4 (32)	38.5 ± 17.3	23.0 ± 28.4
9.08	63.9 ± 12.9 (38)	62.5 ± 17.7	46.0 ± 28.0
11.35	80.7 ± 12.4 (34)	70.5 ± 15.7	54.6 ± 21.7
13.12	78.6 ± 13.9 (39)	59.5 ± 16.9	45.3 ± 38.5
22.71	70.1 ± 14.4 (35)	46.8 ± 35.6	43.1 ± 32.4
Anova	**S 1 %**	**S 1%**	**S 1%**

Segment wise there is distinct difference in response with concentration of TDZ. In all three segments the frequency of response was optimum in 11.3 μM TDZ supplemented MS media. This data (Table 5.3) confirms the concentration dependent response noted in the previous experiment (Table 5.2). High morphogenic potential of proximal end of deembryonated cotyledon is reported in plant species like *Prunus* (Ainsley et al., 2001), *Dalbergia* (Chand et al., 2002), etc. It is suggested that proximal region of the cotyledons might be the source of high regenerative cells (Murthy et al., 1995). Regeneration occurs in these cells, which were not predetermined for either organogenesis or embryogenesis and will not develop into meristematic zones *in vivo* normally. TDZ might be activating such cells to acquire morphogenic competence in cotyledon cultures.

Importance of orientation of explants in morphogenic response is described in species like Almond (Ainsley et al., 2001), *Sesbania grandiflora* (Detrez, 1994), Mung bean (Tivarekar and Eapen, 2001). In *Pongamia*, the caulogenic buds appeared from both adaxial and abaxial surfaces and from the edges and the wounded side of the explants. It was observed that the number of buds was more in the explants cultured with the abaxial side in contact of medium (Table 5.4).

At optimum concentration of TDZ (11.3 μM), average numbers of buds induced were 130±12 in the explant with abaxial side in contact of medium, whereas, it was 118±8.0 in the explants cultured with adaxial side in contact of medium. Scoring of buds from the enlarged image on computer monitor generated a more accurate data on the number of buds. With reference to influence of TDZ concentration, the pattern of response was similar in all three experiments. With increase in concentration, there was increase in number of caulogenic buds reaching the optimum at 11.3 μM.

Number of buds produced in all three segments (p, m & d) was optimum at 11.3 μM TDZ. Irrespective of the orientation, the number of buds produced were more in proximal segment in all tested concentrations of TDZ.

Table 5.4: Differential response in abaxial and adaxial surface of cotyledon segments on number of buds produced in the segments following 20 days TDZ exposure

Conc. of TDZ (μM)	Average number of buds (Mean ± sd)							
	Abaxial in contact of medium				Adaxial in contact of medium			
	p	m	d	Av/explant	p	m	d	Av/explant
Control	0 ± 0	0±0	0±0	0 ± 0 (34)	0± 0	0±0	0±0	0.0 ± 0.0 (34)
0.45	92 ± 14	71 ±16	48 ± 16	69.6 ±12 (31)	52 ± 22	36 ± 14	25 ± 8	37 ± 7.9 (32)
2.27	106 ± 20	83 ± 22	54 ± 25	80.8 ± 9.9 (28)	78 ± 26	51 ± 18	36 ± 12	55 ± 6.8 (28)
4.54	114 ± 14	88 ±18	64 ± 18	87.8 ± 6.9 (22)	104 ± 15	68 ± 06	48 ± 12	74 ± 8.2 (26)
6.71	128 ± 08	94 ± 14	72 ± 22	97.6 ± 7.7 (32)	106 ± 11	67 ± 23	42 ± 16	72 ± 2.3 (32)
9.08	144 ± 13	108 ± 12	89 ± 14	113 ± 12 (26)	138 ± 12	88 ± 12	64 ± 14	95 ± 7.5 (28)
11.35	173 ± 10	124 ± 13	97 ± 08	130 ± 12 (32)	163 ± 17	112 ± 24	80 ± 18	118 ± 8.0 (32)
13.12	166 ± 13	122 ± 25	95 ± 16	128 ± 13 (28)	158 ± 8	98 ± 15	72 ± 22	108 ± 10 (26)
22.71	156 ± 18	118 ± 15	93 ± 14	121 ± 9.2 (20)	141 ± 16	89 ± 26	63 ± 16	97 ± 6.4 (23)
Anova	S1%	S1%	S1%	S 1%	S1%	S1%	S1%	S 1%

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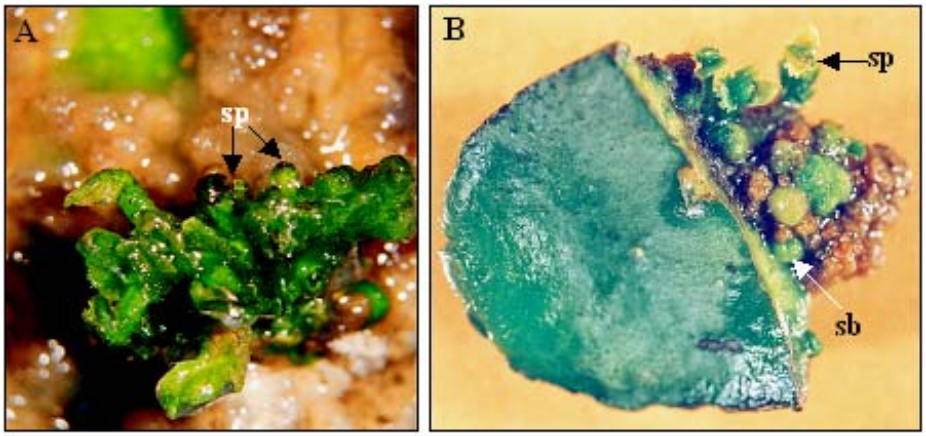

Fig. 5.3: (A) Differentiation of buds into shoot (sp) in GR free MS medium after four passages. Note. Many buds were dedifferentiated to callus.
(B) Few buds were differentiated to shoots (sp) from distal segment and many young buds (sb) were also seen. Note: buds originated from cut end of distal segment.

Differentiation of TDZ induced caulogenic buds into shoots on elimination of the growth regulator from the medium is reported (Yang et al., 2001; Mehta et al., 2004). It is proposed (Mehta et al., 2004) that TDZ induce proliferation of the existing meristem but suppress differentiation of the buds to shoot. Similarly in the present study TDZ induced buds in the cotyledon tissues did not differentiate. Repeated transfers for 8-12 passages in MS medium eliminated TDZ from the explant thereby supporting differentiation of the caulogenic buds to shoot primordia (Fig. 5.3A) Although TDZ induces large number of buds but all do not differentiate simultaneously. Few shoots differentiated, and the other young buds remained suppressed (Fig. 5.3B). Excision of developing shoots from the clusters hastened elongation of the remaining shoot from different segments of cotyledon (Fig. 5.4 A, B & C). Ainsley et al. (2001) reported similar result in almond.

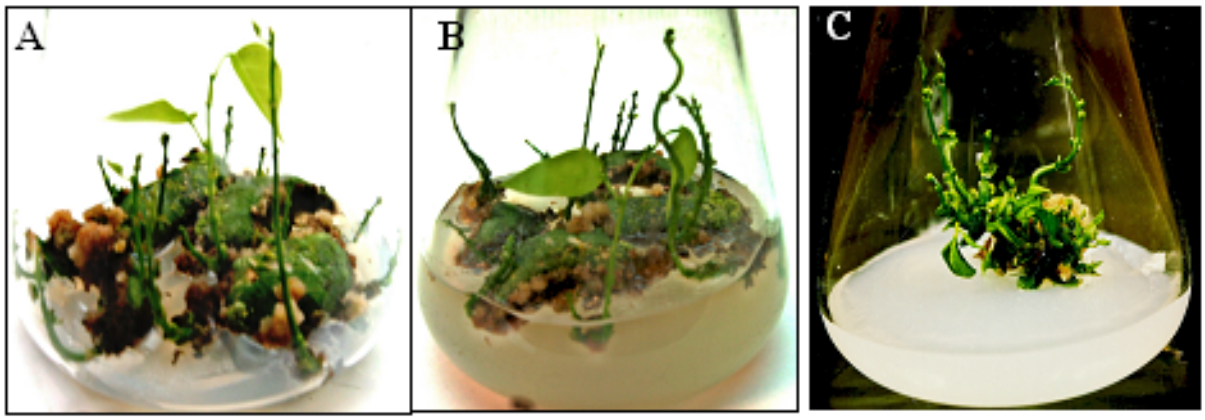

Fig. 5.4: Differentiated and elongated shoot clusters from proximal (A), middle (B) and distal (C) segment after eight passages in MS medium.

Reduction in the strength of MS media to half and supplementing it with 0.25% charcoal promoted **rooting** in the differentiated shoots in four weeks. The method for differentiation and rooting of shoots is described in the previous chapters. From large number of shoots obtained, 32 shoots were tested for rooting in this experiment. Out of these 32 shoots, 28 rooted (87.5%) in four weeks of incubation in MS-charcoal media (Fig. 5.5A). To accelerate the rooting further, elongated shoots were cultured in half strength liquid MS media with filter paper bridge.

The elongated shoots, rooted in two weeks in culture (Fig. 5.4B). In many species like *Tamarindus indica* (Mehta et al., 2004*), Vitis rotunifolia* (Gray et al., 1991) *Rhododendron* (Preece and Imel, 1991) low rooting frequency was attributed to carry over effect of TDZ. In *P.pinnata* several transfers in MS medium following TDZ exposure reduced this carry over effect resulting in high rooting frequency. High survival rate (86%) of these plants in soil indicates that the plants of de novo origin are equally strong and healthy like the plants of meristem origin.

Fig. 5.5: (A) Shoots rooted in rooting media after four weeks of culture.
(B) Shoot cultured in liquid rooting medium on filter paper bridge.
(C) Shoots in sand soil mixture for hardening.

Histological studies confirmed the *de novo* origin of the caulogenic buds in the cotyledon explants cultured in medium with TDZ. At lower concentration (4.54 μM) within 10 days of withdrawal of TDZ, divisions in the cells leading to formation of shoot buds from the adaxial surface of the explant was noted (Fig. 5.6A).

The inhibitory influence of TDZ on differentiation of the meristematic cells is possibly less in lower concentrations. On the contrary at higher concentration (13.12 μM) due to rapid multiplication of cells and suppression of differentiation process dome shaped masses appeared on the surface of the explants (Fig.5.6B). On withdrawal of TDZ, level of TDZ starts reducing from the outer layers of cells resulting in initiation of differentiation (Fig. 5.6C). On examination of the sections taken from the explants after withdrawal of TDZ for four weeks (Fig. 5.6D) it was noted that the whole tissue was meristematic and number of adventitious buds and shoot primordia were visible on the surface of the explants. Since there were no preexisting meristems in the cotyledon presence of meristematic cells, caulogenic buds and shoot primordia indicates regeneration of shoots via *de novo* organogenesis. Mehta et al. (2005) reported development of abundant meristematic zones in the epidermal and sub epidermal layers in tamarind. Similar observations were reported in peanut seedlings (Victor et al. 1999), *Cercis Canadensis* (Distabanjong and Geneve, 1997), and *Phaseolus vulgaris* (Malik and Saxena, 1992b).

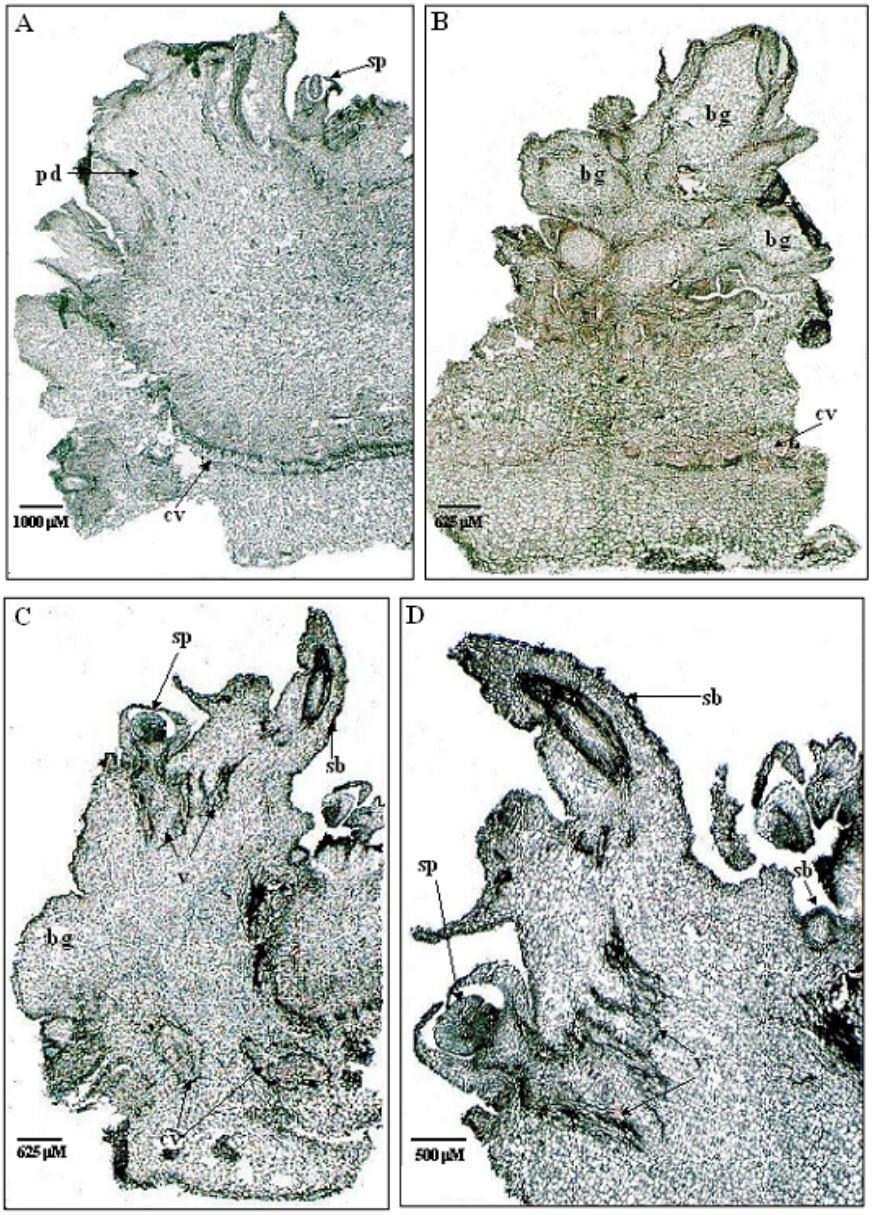

Fig. 5.6: (A) Section of explant exposed to lower concentration of TDZ (4.54µM) followed by 10 days in MS media. Periclinal cell division (pd) and differentiation of shoot primordia (sp) may be noted on the surface of explant. Meristematic expansion through periclinal activity was seen above vasculature (cv)

(B) A section of explant exposed to higher concentration of TDZ (13.12µM) followed by 10 days in MS media. Rapid proliferation of meristmatic activity was triggered by high TDZ, resulting in the formation of meristematic buldges (bg).

(C) A section of explant exposed to optimal (11.35µM) of TDZ followed by 30 days in MS media. Both proliferation and differentiation of mertistematic cells lead to formation of buldges (bg), shoot buds (sb) and shoot primordia (sp). *De novo* origin of buds is confirmed by distinctive vasculature (v).

(D) A magnified view of above explant (11.35µM) showing *de novo* origin of shoot buds (sb), shoot primordia (sp) and vasculature (v).

(b) Embryoaxis Culture:

After 30 days of culture in MS media, some bud like protuberances appeared on the surface of the TDZ pretreated embryoaxis especially near to the wounded site where the cotyledons were attached. On repeated transfers to MS media these protuberances differentiated into shoot primordia. The frequency of response and the number of adventitious buds were scored after four passages in GR free MS media.

In TDZ free media, the embryoaxis explants germinated normally with the frequency of 70 % and there was no adventitious bud formation (Fig. 5.7A). Whereas, bud like protrusions were induced in all concentrations of TDZ in both 10 days and 20 days exposed cultures (Table 5.5). The frequency of response ranged from 60% in 22.71μM to 82 % in 0.45μM TDZ in 10 days exposed explants. In 20 days TDZ preconditioned explants, the frequency of *de novo* caulogenic response ranged from 62% in 22.71μM to 85% in 0.45μM TDZ. In both 10 and 20 days exposures, the adventitious bud like formation increased with increase in concentration of TDZ. The average number of protrusions ranged from 1.7±0.6 to 19± .8 in 10 days exposed explants. While in 20 days explants it ranged from 3.0±1.3 to 22.1±0.8. In all the concentrations of TDZ, bud like protrusions were more in 20 days exposed cultures, implying longer exposure in TDZ elicit better *de novo* response. Adventitious bud formation was more at the region of the axis where the cotyledon was attached (Fig. 5.7B & C). The entire epicotyl portion became morphogenic (Fig. 5.7D; 5.7E) with increase in either concentration or exposure of TDZ.

Table 5.5: Denovo response in Embryoaxis using TDZ

Conc. of TDZ in μM	Frequency of response (%) mean ± sd.		Average number of *de novo* bud like protrusions* mean ± sd.	
	10 days	20 days	10 days	20 days
Control	0 ±0 [44]	0 ± 0 [46]	0.0 ± 0.0 (31)	0.0 ±0.0 (35)
0.45	82 ±16 [38]	82 ± 20[38]	1.7 ± 0.6 (30)	3.0 ± 1.3(30)
2.27	70 ±10 [47]	76 ± 10 [39]	2.0 ± 1.3 (33)	4.4 ± 1.7(34)
4.54	78 ±19 [36]	85 ± 24 [36]	3.7 ± 1.7 (30)	6.4 ± 2.6 (30)
6.71	80 ±19 [38]	81 ± 19 [44]	4.2 ± 2.0 (33)	7.9 ± 2.2 (35)
9.08	70 ±12 [46]	70 ± 14 [48]	6.6 ± 2.9 (32)	9.9 ± 2.6 (34)
11.35	76 ±09 [36]	80 ± 08 [32]	10.3 ± 2.3 (27)	12.8 ±2.8 (27)
13.12	68 ±16 [41]	68 ± 16 [43]	12.3 ± 2.5 (28)	16.4 ± 2.8 (27)
22.71	60 ±07 [40]	62 ± 07 [39]	19.0 ± 3.8 (24)	22.1 ± 3.8 (24)
Anova	NS	NS	S 1%	S 1%

[Numbers in the square parentheses represent the number of sterile explants; Numbers in the circular parentheses represent the number of explants responded].

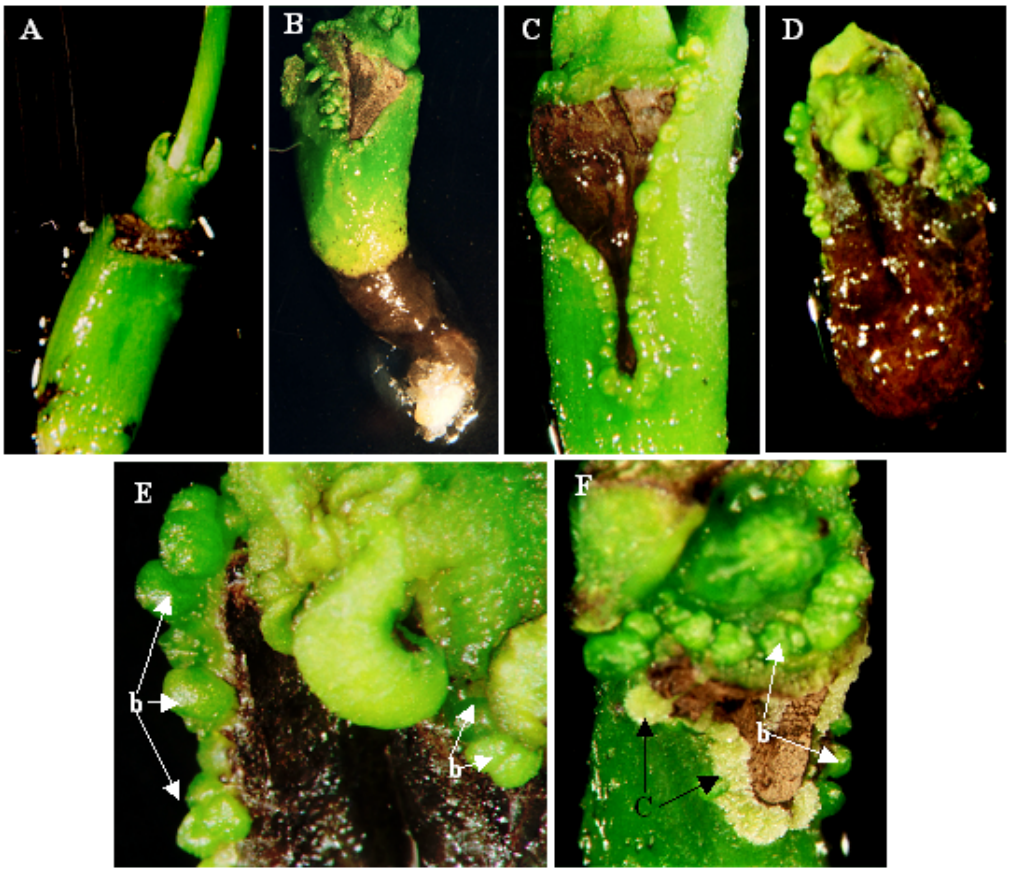

Fig.5.7: (A) Germination of embryoaxis cultured in control medium.
(B) Induction of protrusions in explants pretreated with 6.71µM TDZ, at the cut margin of wounded cotyledon attachment site.
(C) Induction of protrusions in explants pretreated with 11.35µM TDZ, all over cut margin of wounded cotyledon attachment site.
(D) Induction of protrusions in explants pretreated with 13.12µM TDZ. Differentiation of shoots from plumule region and root in hypocotyls region is restricted.
(E) A magnified view of a portion of embryoaxis (Fig. 5.7D) *de novo* origin of protrusions (b) and entire plumular region became morphogenic due to high dose of TDZ exposure.
(F) Protrusions (b) were dedifferentiated into callus(c) in explants pretreated with 22.7µM TDZ, after six passages in GR free MS media.

The explants were transferred repeatedly to GR-free MS media for differentiation of shoots from the meristematic protrusions. Even after eight to ten passages of transfer, the protrusions did not differentiate in to shoot. Some of these protrusions dedifferentiated into callus especially in explants (Fig. 5.7F), exposed in TDZ concentration above 11.35 µM. Repeated shifting of the TDZ pretreated *Pongamia* embryo axis to GR free failed to induce differentiation of shoot from the de *novo* buds. Hence, the explants were tested in different culture conditions to trigger bud differentiation.

(i) The radicle part of the axis was eliminated and the epicotyl region with the meristematic bud was cultured in MS medium for two passages.

(ii) Embryoaxis explants with protrusions were cultured in lower concentration of TDZ (0.45μM) supplemented MS medium for four weeks followed by the transfer to GR free MS media. This medium was effective in induction and elongation of shoot cultures.

(iii) Based on the seedling experiments in *Pongamia*, embryoaxis (Chapter 3) TDZ pre treated embryoaxis explants were cultured in MS media containing BA (4.45μM) for four weeks. With seedling explants, the caulogenic buds produced from pre existing meristem differentiated and elongated in BA containing medium.

(iv) Gibberlic acid is known for its effect in promoting shoot elongation (Chaturvedi and Jain, 2004). Embryoaxis explants with TDZ induced meristematic protrusions were cultured in MS medium supplemented with 0.45μM Gibberlic acid for four weeks.

(v) Light plays major influence on the growth, development and morphogenesis of plants (Ellis and Webb, 1993). Shoot elongation could not be achieved in the embryo axis cultures incubated in light, Therefore the cultures in MS medium were incubated in dark for 8 weeks.

Unlike the adventitious buds in cotyledons, efforts taken to differentiate the *de novo* buds in embryoaxis remained futile. This irreversible suppression of differentiation in the TDZ pre treated embryo axes cannot be explained with the present state of knowledge. Similar observation was reported in chestnut (San Jose 2001). In chestnut, only few embryoaxes explants regenerated buds after culture on TDZ medium. The authors suggested that possibly the cytokinin activity of TDZ was excessive causing excessive disorganization of the meristematic zone.

Histological studies: On microscopic examination of the longitudinal sections of the embryo axes cultured in 4.54 μM TDZ (Fig. 5.8A) proliferation of morphogenic cells in the epidermal and subepidermal layers was noticed near the cotyledon attachment site. Vasculature of embryoaxis was intact and differentiation of existing meristem was initiated at plumular site as the lower concentration has not affected the differentiation of meristems. Longitudinal sections taken from the tissues precultured in 11.35μM TDZ (Fig. 5.8B), showed more morphogenic activity in the cotyledon attachment site and in the epicotyl portion. Small meristematic protrusions all over the epicotyl and cotyledon attachment surface indicate high morphogenic activity. Absence of morphogenic activity in the hypocotyl and vasculature indicate that TDZ does not influence the cells of this part of the axis. In the tissues exposed to high TDZ concentration (13.12 μM), intense morphogenic activity and mass formation (Fig. 5.8C & D) was noticed near the cotyledon attachment site and on the surface of the epicotyl.

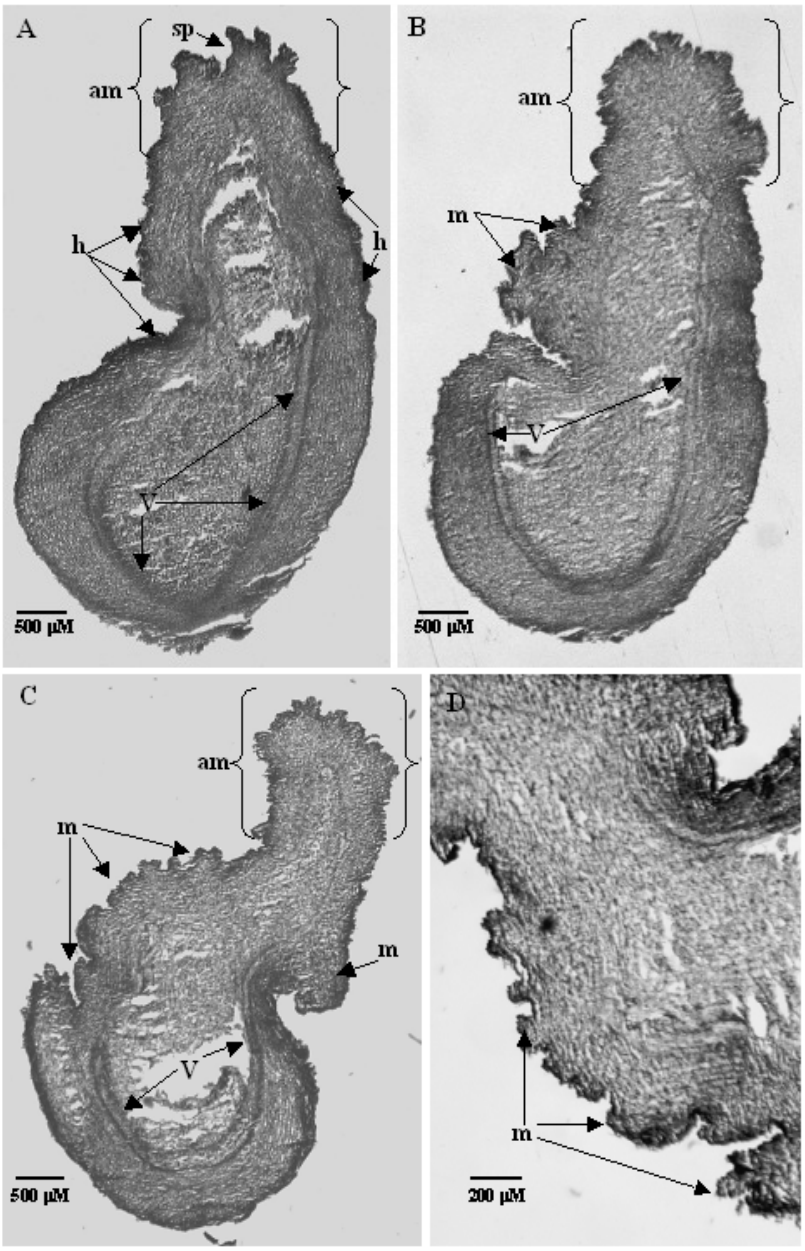

Fig. 5.8: (A) Section of explant exposed to lower concentration of TDZ (4.54µM) followed by four weeks in MS. Differentiation of shoot primordia (sp) at plumular end of existing apical meristem (am). Hump formation (h) was noted on the surface of the explant by meristematic expansion was seen above the vasculature (V).

(B) A section of explant exposed to higher concentration of TDZ (11.35µM) followed by four weeks in MS. Rapid proliferation of meristematic activity was triggered by TDZ, resulting in formation of meristematic masses(m). Proliferation of apical meristem was also observed.

(C) A section of explant exposed to higher concentration of TDZ (13.12µM) followed by 4 weeks in MS. Rapid proliferation of meristmatic activity was triggered by TDZ resulting in formation of meristematic masses(m). Due to intense proliferation of cells, shape and structure of explant got modified.The epicotyl portion of the vasculature not visible distinctly unlike the hypocotyl portion due to the proliferation of tissues.

(D) Magnified view of a portion of the explant C showing masses (m).

B. Somatic Embryogenesis

All MS media combinations used for induction of somatic embryos were supplemented with 6% sucrose. For induction of embryogenesis, sucrose has been identified as one of the major factor (Thorpe, 1988). It plays a dual role in plant tissue cultures. It influences the cultures as a carbon source and as osmoticum (Mhaske et al., 1998).

(i) Cotyledon:

When the cotyledon explants were transferred to hormone free media after culturing in various auxin containing media, rooting was observed in control as well as in the lowest concentration of all auxins (0.4µM) from the cotyledon nodal region. In NAA concentration above 13µM, adventitious rooting was observed from all over the surface of the cotyledon. Callusing was predominant in the explants irrespective of the auxin used. Sporadically some embryogenic mass like structures appeared in some of the explants (Fig. 5.9 A, B & C). However these structures did not differentiate further and eventually degenerated.

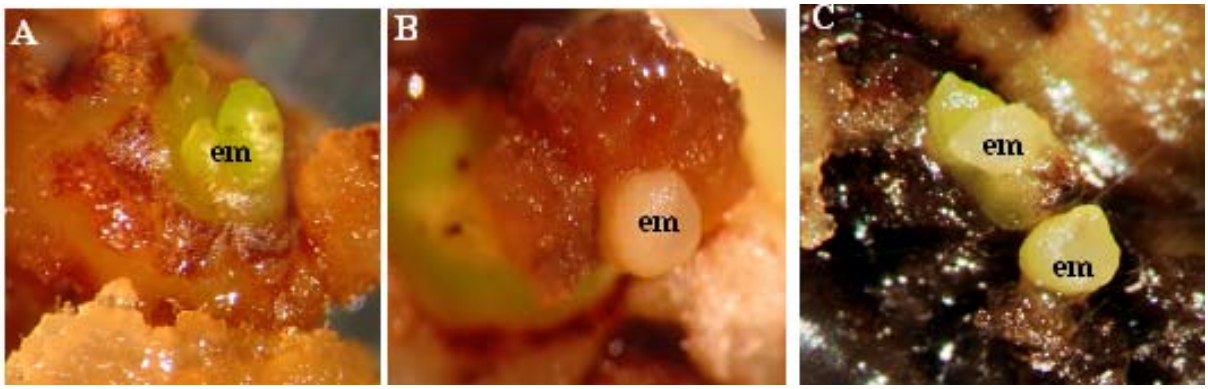

Fig. 5.9: (A) Embryogenic mass like structure (em) appeared on the surface of cotyledon cultured initially in 22.6µM DEM for 4 weeks followed by 13.6µM DEM for 4weeks.
(B) Globular embryo like structure appeared upon callus tissue from cotyledon explant cultured initially in 22.6µM 2,4-D for 4 weeks and followed by 13.6µM 2,4-D.
(C) Embryo likestructures appeared upon callus tissue of explant precultured in 12.4µM Picloram for 4 weeks and followed by transfer to 0.4µM Picloram for 4 weeks.

(ii) Embryoaxis:

Most of the reports on somatic embryogenesis of tree legumes used embryoaxis as explant. In our study white, translucent embryo like structures were formed on the plumular region of the embryoaxis (Fig. 5.10A&B) explants derived from semi mature green pods and cultured in control media. This observation was reproduced on repeated experimentation.

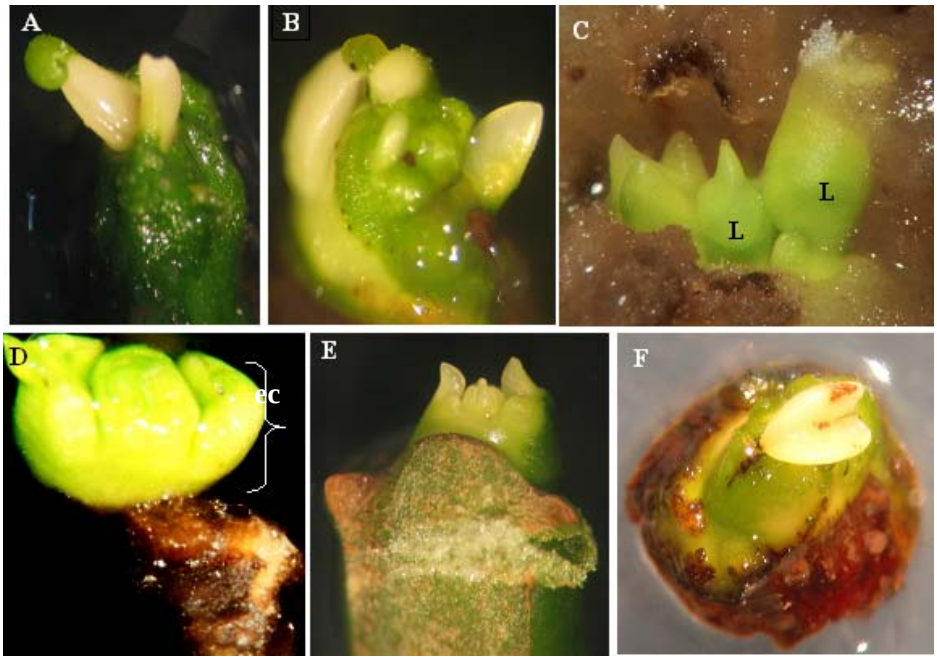

Fig. 5.10: (A & B) Peculiar structures in GR free MS medium with 6% sucrose on plumular region of the embryo axis explants.
(C) Embryo derived leaflets (L) base become swollen on culturing in 4.14µM Picloram for six weeks
(D) Entire epicotyl portion(ec) of embryonal axis became swollen on culturing in 12.4µM Picloram after six weeks of culture.
(E) In the explants pretreated with 11.7µM 2,4,5-T, the embryo derived leaflets become swoolen and transluent
(F) Colourless opaque horseshoe shaped structures were formed at the plumular end of zygotic embryo axis. in MS medium with 13.6 µM 2,4-D.

The apical meristem of an embryoaxis is usually covered with several layers of leaf primordia or leaflets. Among the auxins tested, in the explants cultured in 0.4, 2.0 & 4µM concentration of Picloram, swelling was noticed at the base of these leaflets in the plumule of the axes (Fig. 5.10C) after four weeks of culture. Above 4 µM concentration of Picloram, the plumular region of embryoaxis becomes swollen (Fig. 5.10D) green and morphogenic. In other auxins, embryoaxis explants were callused and sporadically showed some translucent structures (Fig.5.10 E&F). Although morphogenic structures were obtained, no further growth towards embryogenesis was achieved.

(iii) Immature Seed:

The immature seeds were classified (1 to 6) according to their approximate size (Fig. 5.11 A & B). From the different sizes of immature seeds used, embryos of size no.1 always degenerated. The results of the experiments conducted to identify the suitable auxin for SE in *Pongamia* is described

(Table 5.6). We considered the mass like swelling as an initial response for the induction of somatic embryogenesis (Fig. 5.11C). In control medium, no mass like structures were observed whereas these structures were seen in several other media tested (Table 5.6). The frequency of the morphogenic mass formation ranged from 2.5 to 48.3%. Seed sizes from 2 to 6 were responsive in culture, but the results were inconclusive. Conversion of smatic embryos to plantlets could not be achieved. Different media combinations, variation in incubation conditions were tested to differentiate these proembryogenic mass like structures. In lower concentration of auxin, the mass like structures callused or degenerated.

Table 5.6: Induction of embryogenic mass like structures in various growth regulators

No.	Media Conc. (μM)	Frequency of response (%) mean ± s d.	Responded Embryo size (1……6)
1.	MS control	0.0 ± 0.0	-
2.	0.45-2,4 D	18.3 ± 27.7	3,5
3.	2.26-2,4 D	40.8 ± 24.7	3,4,5
4.	4.52-2,4 D	25.0 ± 25.0	3,5
5.	13.6-2,4 D	12.5 ± 13.8	2,4
6.	22.6-2,4 D	9.2 ± 14.6	4,5
7.	45.2-2,4 D	27.5 ± 9.1	2,3,4,5,6
8.	0.54- NAA	27.0 ± 28.3	2,4,6
9.	2.69- NAA	42.3 ± 36.7	2,3,4,5
10.	5.37- NAA	22.5 ± 15.8	2,4
11.	16.1- NAA	26.7 ± 26.7	2,4,5
12.	26.8- NAA	12.0 ± 13.7	2,4,5
13.	53.7- NAA	24.0 ± 27.4	2,3,4,5
14.	0.41-PIC	25.8 ± 42.1	3,4
15.	2.07- PIC	48.3 ± 29.1	2,4,5
16.	4.14- PIC	27.3 ± 22.0	3,4
17.	12.4- PIC	13.3 ± 29.8	1,2,3
18.	20.7- PIC	30.2 ± 30.6	2,3,4
19.	41.4- PIC	18.5 ± 12.5	2,4,5
20.	0.45- DEM	21.7 ± 21.7	3,4,5,6
21.	2.26- DEM	35.4 ± 38.7	2,3,4,6
22.	4.52- DEM	22.5 ± 28.2	2,3,4,5
23.	13.6- DEM	18.9 ± 13.2	2,3,4
24.	22.6- DEM	25.8 ± 30.7	2,3,4,5,6
25.	45.2- DEM	2.5 ± 5.6	5
26.	0.39- 2,4 T	13.2 ± 14.1	3,5,6
27.	1.96- 2,4 T	15.0 ± 22.4	4,5,6
28.	3.91- 2,4 T	17.3 ± 16.7	2,3,5
29.	11.7- 2,4 T	27.3 ± 24.3	2,3,4,5
30.	19.6- 2,4 T	18.2 ± 12.7	2,5,6
31.	39.1- 2,4 T	6.7 ± 14.9	2
	Anova	**NS**	

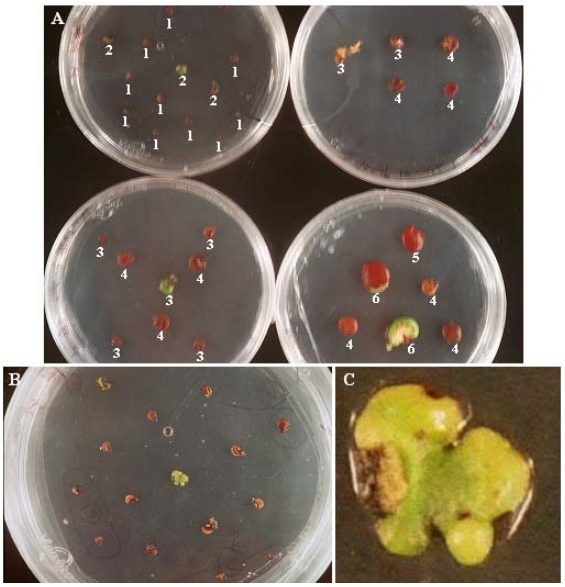

Fig. 5.11: (A) Classified sizes of immature seeds (1 to 6) cultured for inducing somatic embryos.
(B) Immature seeds of size 1& 2 cultured in 45.2μM 2,4-D containing MS medium showing mass like structures.
(C) A close view of mass like structure obtained with immature seed (Size 2) cultured in 2,4-D containing MS.

5.3 CONCLUSION

TDZ triggers meristematic activity in the *P. pinnata* cotyledon cells. The proximal segment of the cotyledon have highest morphogenic potential and the best condition for optimum caulogenic response is exposure to TDZ 11.35 μM for 20 days with abaxial side in contact of medium. Elimination of TDZ from media allowed bud differentiation and shoot elongation. The shoots rooted in rooting medium and survived on transfer to soil. *De novo* origin of the organogenic buds was confirmed using histological techniques. This is the first report on *in vitro* regeneration of *P. pinnata* via adventitious organogenesis. This protocol may find application in studies in genetic transformation, isolation of somaclonal variants and in induction of mutants. It may also find application in study of the opposite roles of TDZ in induction and differentiation of buds. Adventitious buds were induced in the embryoaxes cultured in TDZ but these did not differentiate into shoots by the methods tested. It may need further experimentation to obtain differentiation in these buds. Studies undertaken to induce somatic embryos in *Pongamia* using different explants like immature seeds, cotyledon and embryoaxis from green pods remained unsuccessful.

A part of this work has been communicated for publication

***In Vitro* Regeneration of *Pongamia pinnata* via adventitious organogenesis**

CHAPTER 6: MOLECULAR CHARACTERIZATION

6.1 INTRODUCTION

One of critical challenges facing the world today is the conservation of biological diversity and use of its components for the benefit of humanity. Apart from consideration of vast numbers of plant species on this earth which is often referred to as taxonomic diversity, the genetic diversity exists in individuals within a species or a population must also be considered for conservation (Hawkes et al., 2000). Such genetically significant variation might include plant height, tolerance to water-logging, fecundity and resistance to disease etc. Expression of these genetic traits in each individual results from an interaction between the individuals's genotype and the local environment. There can be various distinct forms of the same gene, referred to as alleles, in different individuals of a species, and thus at the genetic level variation may also be encountered. In *Trifolium pratense* where self incompatibility is controlled by a single multi allelic gene expressed in the pollen, it has been estimated that approximately 200 alleles exist for this single gene (Lawrence, 1996).

Genetic diversity between individuals enables evolution and adaptation of species to take place within a changing environment and is thus essential for the long term survival of a species (Newton et al., 1999). It is this variation between individuals of the same species that ensures that the species as a whole can adapt and change in response to natural (eg. changing environment) and artificial (plant breeders selection criteria) selection pressures. Assessment of genetic variation is therefore, of key importance to the development of effective conservation strategies.

The diversity within the species has been increasingly recognised as a tangible, economic resource directly equivalent to a country's mineral wealth, thus they have been referred to as Plant genetic resources. People conserve diversity because they wish to exploit it (Maxted et al., 1997). The exploitation of genetic diversity for crop improvement has been the major driving force for the exploration and *ex situ/ in situ* conservation of plant genetic resources.

Characterization and evaluation of germplasm:

For genetic resources to be put to use, it is necessary to know what valuable variation they contain. Characterization and evaluation of germplasm have been achieved largely on *ex situ* materials and must obviously take place before the choice can be made of what material will be worth using for any particular purpose. Identification of an elite with high seed oil content or high seed producing ability or with ability to produce seed oil of desired composition in *Pongamia*, has biodiesel utility.

Characterization and evaluation as applied specifically to plant genetic resources refers to the description of the material in a collection (Phenotypic characterization). Characterization usually involves taxonomic confirmation of the accessions and biochemical or molecular discrimination between germplasm accessions using highly heritable and easily visible traits.

Classical methods of estimating genetic diversity or relatedness among the plants rely upon morphological characters. Several studies based on morphological characters were carried out to understand the degree of variation within the species. In *Gaultheria fragrantissima*, Paulsamy et al., (2007) estmated the degree of variability present between the four ecological variants and reported the leaf thickness and oil content were highly correlated with the population density. Kaura et al., (1998) studied the seed morphology (seed length and seed weight) and oil content in Neem from five provenances and reported that seed oil content from most of the provenances was not consistantly and significantly correlated with the morphological parameters of the seed. Molecular and biochemical markers avoid many of the complications of environmental factors by looking directly at the variation controlled by genes or DNA fragments.

Isozymes have been the markers of choice for studies of ecological genetics in natural populations of plants. From isoenzyme analysis Hamrick et al., (1992) concluded that tree species maintain more intraspecific variation than other life forms but generally display less variation between populations, particulary in the case of widespread species with out-crossing breeding systems and wind or animal dispersed seeds.

Development of wide range of molecular markers represent a powerful and potentially rapid method for evaluating genetic diversity for conservation, as well as assisting in the evalution process for agronomically important traits (Newsbury and Ford-Lloyd,1997). Discovery of PCR (Mullis, 1986) facilitated the development of marker based gene tags, map based cloning of agronomically important genes, synteny mapping, marker assisted selection of desirable genotypes etc., thus giving a new dimension to concerted efforts of breeding (Gupta PK, 1996). DNA markers offer several advantages over traditional phenotypic markers, as they provide data that can be analysed objectively.

Introduction to DNA markers:

Genetic polymorphism is classically defined as the simultaneous occurrence of two or more discontinuous variants or genotypes of a trait in the same population (Joshi, 2001). Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is very expensive and

laborious. A wide variety of techniques have, therefore been developed in the past few years for visualization of DNA sequence polymorphism.

The term DNA fingerprinting was introduced for the first time by Sir Alec Jeffery in 1985, to describe bar code-like DNA fragment patterns generated by hybridization with multilocus probes after eletrophoretic separation of genomic DNA fragments. The emerging pattern make up a unique feature of the analysed individual and are currently considered to be the ultimate tool for biological individualization. DNA fingerprinting / DNA marker system is used in describing the combined use of several single loci and is one of those techniques used in detection of polymorphism.

Properties of DNA markers:

DNA markers are *ubiquitous, innumerable, discrete, nondeleterious, inherited by Mendelian laws, unaffected by the environment, and are free of epistatic and pleiotropic interactions* (Beckman and Soller 1986; Tanksley et al., 1989). These markers offer several advantages over morphological and isozyme markers (Joshi et al., 1999; Gupta PK, 1996):

1. They are unlimited in number because the RFLP and PCR techniques are able to detect differences at the DNA level in both translatable and non translatable DNA regions.
2. They are phenotypically and environmentally neutral because sequence polymorphisms are detected directly at the DNA level and not by assaying gene products which may be influenced by alterations in gene coding sequence or by changes in the level of gene expression.
3. DNA markers mediated selection in breeding programs can be carried out in a non destructive process to the original plant because only a small amount of tissue is required.
4. They enable selection for specific recessive genotypes in a heterozygous form where the recessive genotype cannot express because a dominant allele masks a recessive allele. This advantage can be achieved with markers which are co-dominant in nature.
5. They do not show either dominant recessive interaction or epistasis and are devoid of pleotropic effect.
6. DNA markers in the genome can be considered as entry point to the genes. Thus, they facilitate the chromosome walking techniques for gene cloning.
7. DNA markers are not tissue specific and can be used at any stage of the plant growth.

   An ideal DNA marker should have the following properties (Weising et al., 1995):

   - ❖ High polymorphism
   - ❖ Co-dominant inheritance

- ❖ Frequent occurence in genome
- ❖ Selective neutral behaviour
- ❖ Easy access
- ❖ Easy and fast assay
- ❖ High reproducibility
- ❖ Easy exchange of data between laboratories

It is not easy to find a molecular marker which would meet all the above criteria. Depending upon the type of study to be undertaken, a marker system can be identified that would fulfil at least a few of the above characteristics (Weising et al., 1995).

Types of DNA markers:

Various types of molecular markers are utilized in evaluation of DNA polymorphism and are generally classified as hybridization based markers and polymerase chain reaction based markers. In hybridization based markers, the DNA profiles are visualized by hybridizing the restriction enzyme digested DNA, to a labelled probe, which is a DNA fragment of known origin or sequence. PCR based markers involve *in vitro* amplification of particular DNA sequences or loci with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography. Information about few representative marker systems is as given below (Joshi et al., 1999).

Single or Low copy probes

Restriction Fragment Length Polymorphism (**RFLP**) are simply inherited Mendelian characters and codominant in nature. They have their origin in the DNA rearrangements that occur due to the evolutionary processes, point mutations within the restriction enzyme recognition site sequences, insertions or deletions within the fragments and unequal crossing over (Shlotterer and Tautz, 1992). Botstein et al., (1980) used RFLPs for the first time to construct a genetic map. In RFLP analysis, restriction enzyme digested genomic DNA is resolved by gel electrophoresis and then blotted on to a nitrocellulose membrane. Specific banding patterns are then visualized by hybridization with radioactive labelled probe. The utility of RFLPs has been hampered due to the requirement of large amount of DNA and specific radioactive labelled probe. Moreover, the assay is time consuming and labor intensive. RFLP markers converted to PCR based markers are Sequence Tagged Sites (**STS**),

Allele Specific Associated Primers (**ASAP**), Expressed Sequence Tag markers (**EST**) and Single Strand Conformation Polymorphism (**SSCP**) (Orita et al., 1989).

Arbitrary sequence markers:

Random Amplified Polymorphic DNA (**RAPD**) marker assay in which nucleotide sequence polymorphism is detected by using a single primer of arbitrary nucleotide sequence. Some variations from the RAPD technique includes DNA amplification fingerprinting (**DAF**), Arbitrary primed PCR (**AP-PCR**), Sequence characterized amplified regions for amplification of specific band (**SCAR**), Cleaved amplified polymorphic sequences (**CAP**s), Randomly amplified microsatellite polymorphisms (**RAMPO**) and Amplified Fragment Length Polymorphism (**AFLP**).

Multilocus probes:

About 30-90% of the genomes of virtually all species is constituted of repetitive DNA, which are highly polymorphic in nature. These regions contain genetic loci comprising several alleles differing from each other with respect to length, sequence or both. The repetitive DNA play an important role in absorbing mutations in a genome and inherited mutations forces together from the basis for a number of marker systems that are useful for several applications in plant genome analysis. The major forms of repetitive DNA are **Microsatellites** and **Minisatellites.** They are multilocus probes creating a complex banding patterns usually nonspecies specific and occur ubiquitously. The patterns generated by these probes are known as **Oligonucleotide prints**. Minisatellites are tandem repeats with a monomer repeat length of about 11-60 bp, while microsatellites or short tandem repeats/simple sequence repeats (**STR**s / **SSR**s) consist of 1-6 bp long monomer sequence that is repeated several times. These loci contain tandem repeats that vary in the number of repeat units between genotypes and are referred as variable number of tandem repeats (**VNTR**s). Microsatellites and minisatellites form an ideal marker system creating complex banding patterns by detecting multiple DNA loci simultaneously. They are dominant fingerprinting markers and codominant Sequence Tagged Microsatellite Sites (**STMS)** markers. They exist as many alleles in a population, their level of heterozygosity is high and they follow Mendelian inheritance. Minisatellite and Microsatellite sequences converted in to PCR based markers are Sequence Tagged Microsatellite Sites (**STMS**), Directed amplification of Minisatellite region DNA (**DAMD**), Inter Simple Sequence Repeats (**ISSR**). STMS method includes DNA polymorphism using specific primers designed from a sequence data of a specific locus. Where ISSRs can be used in the species with unknown sequence data. The potential of ISSR markers have been exploited in the present study.

Inter simple Sequence Repeats (ISSR) and its applications:

Inter simple sequence repeats are the regions which lie within the microsatellite repeats. Zietkiewicz et al., (1994) reported this technique for the first time. The technique involves use of a single primer composed by microsatellite sequences plus a short arbitrary sequence (anchor) which target to a subset of 'simple sequence repeats' (SSRs) or microsatellites and amplify the region between two closely spaced and oppositely oriented SSRs. The primers can be dinucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide and number of combinations could higher with increasing number of nucleotides eg. for dinucleotide (4)^2= 16, (4)ˆ3=64, (4)ˆ4=256 etc. The primers used can be either unanchored or more usually anchored at 3' or 5' end with 1 or 4 degenerate bases extended into the flanking sequences.

ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Gupta et al., 1994; Tsumura et al., 1996; Joshi et al., 1999; Wang et al., 1998). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wang et al., 1998; Reddy et al., 2002).

This technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility possibly due to the use of longer primers (16-25 mers) as compared to RAPD primers (10- mers) which permits the subsequent use of high annealing temperature (45-60º C) leading to higher stringency. This technique is found to be stable across wide range of PCR parameters (Bornet et al., 2001; Reddy et al., 2002) and the potential for integrating ISSR-PCR into programs of plant improvement is enormous (Table 6.1).

Table 6.1 : Applications of ISSR-PCR Technique

Application	Reference
Genomic fingerprinting	
Chrysanthemum cultivars	Wolff et al., 1995
Potato cultivars	Prevost & Wilkinson, 1999
Cocoa germplasm	Charters & Wilkinson, 2000
Cannabis sativa	Kojima et al., 2002
Juglans regia	Pollegioni et al., 2003
Clonal fidelity	
Potato	Matthew et al., 1999
Cauliflower	Leroy et al., 2000
Camellia sinensis	Devarumath et al., 2002
Mandrin	Scarano et al., 2002

Genetic diversity and phylogenetic analysis	
Barley (*Hordeum vulgare*)	Sanchez et al., 1996
Barley cultivars	Fernandez et al., 2002
Maize in bred line (Zea mays)	Kantety et al., 1995
Finger millet (*Elucine* sp)	Salimath et al., 1995
Sorghum (chinese)(*sorghum bicolor*)	Yang et al., 1996
White lupin germplasm (*Lupinus albus*)	Gilbert et al., 1999
Wheat (*Triticum* sp)	Nagoka & Ogihara 1997
Indian tetraploid wheat	Pujar et al., 2002
Rice cultivars	Virk et al., 2000
Indian elite rice cultivars	Davierwala et al., 2000
Oryza	Joshi etal., 2000
Oryza granulata	Qian et al., 2001; Wu et al., 2004
Gymnosperms, Douglas fir and sugi	Tsumura et al., 1996
Oilseed rape cultivars (*Brassica napus)*	Charters et al., 1996
Citrus cultivars (Citrus sp)	Moreno et al., 1997
Trifoliate orange germplasm (P.trifoliata)	Fang et al., 1997
Plantago major sub sp.	Wolff & Morgan-Richards,1998
Grapevine germplasm (*Vitis vinifera)*	Moreno et al., 1998
Sweet potato, wild relatives (*Ipomoea sp*)	Huang & Sun, 2000
Red current germplasm (*ribes sp*)	McGregor et al., 2000
Eucalyptus species	Van Der Nest et al., 2000
Psammochola villosa	Li et al., 2001
Mentha species	Khanuja et al., 2000
Apple cultivars	Goulao et al., 2001
Pear cultivars	Monte-Corvo et al., 2001
Olea europaea cultivars	Pasqualone et al., 2001
Symplocos laurina, *Gaultheria fragrantissima* and *Eurya nitida*	Deshpande et al., 2001
Juglans regia	Potter et al., 2002
Morus species	Vijayan et al., 2003
Date palm	Hamama et al., 2003
Eurya nitida	Bahulikar et al., 2004
Pea germplasm (*pisum sativum*	Lu et al., 1996
Soya bean (*Glyine Max*)	Wang et al., 1998
Vigna sp	Ajibade et al., 2000
Peanut	Raina et al., 2001
Cicer	Rajesh et al., 2003
Phaseolus vulgaris L.	Galvan et al., 2003
Trigonella foenum-graecum & T. caerulea	Dangi et al., 2004
Genome mapping	
Saturating RFLP linkage map in Barley	Becker & Heun, 1995
Construction of a genetic linkage map in Einkorn Wheat	Kojima et al., 1998
Soyabean	Wang et al., 1998
Genetic mapping of Japanese and european types of larch	Arcade et al., 2000
Saturating genetic linkage map in Citrus	Sankar & Moore, 2001
Saturating RFLP/ RAPD linkage maps in Citrus	Sankar et al., 2001

Determining SSR motif frequency	
Distribution pattern of micro satellites across eukaryotic genomes	Gupta et al., 1994
Analysis of microsatellite frequency in rice cultivars	Blair et al., 1999
Recovery of micro satellite sequences in the mustard genome	Varghese et al., 2000
Microsatellites markers for Salix reinii, Pinus densiflora, Robinia pseudoaacia	Lian et al., 2001
Gene tagging and use in marker assisted selection	
RF-1 gene for fertility restoration in rice	Akagi et al., 1996
Race specific markers in fungi	Hantula et al., 1996
Gene for resistance to *Fusarium* wilt race-4 in Chikpea	Ratnaparkhe et al., 1998
Temperature senstitive genetic male sterility in rice	Hussain et al., 2000
Fgr gene for modulating fructose to glucose ratio in tomato	Levin et al., 2000
Genome/species speifi markers in *Lolium* and *Festuca*	Pasakinskiene et al., 2000
Putative genome /species specific markers in *Oryza*	Joshi et al., 2000
Quantitative traits in Maize	Domenyuk et al., 2002
Seed size in wheat	Ammiraju et al., 2001
Protein ontent in wheat	Dholakia et al., 2001
Kernal hardness in wheat	Galande et al., 2001
Stem and leaf rust in wheat	Khan et al., 2005
Yellow berry tolerance in wheat	Ammiraju et al., 2002
Kernal size and shape in wheat	Dholakia et al., 2003
Evolutionary Biology	
Diploid hybrid speciation in *Penestemon*	Wolfe et al., 1998
Diplotaxis species	Martin & Sanchez-Yelamo, 2000

Pongamia Genomics: Use of ISSR markers:

Being an out breeding species variation in the tree characters like height, spread, volume and physico-chemical characters of fruit such as pod length, shape, weight, volume, number of seed/pod, oil content, color of oil, variability in bearing habit and yield etc. exists. However, there is no information available on the genetic variation within *Pongamia* individuals/ accessions.

Variation in the responses was noted in the experiments conducted to standardize protocols for clonal propagation of *Pongamia*, with seedling (Sujatha and Hazra, 2006) and mature explants (Sujatha and Hazra, 2007) from different sources of explants. In an experiment conducted with the nodal buds

collected from ten different trees, differential response on sprouting was noted (Chapter 4). However, there is no literature on the genetic diversity in *Pongamia.*

Maintenance of variation within the species is essential for their long-term survival and fitness. A good understanding of level and distribution of genetic variability within the species is vital for the development of strategies for their effective conservation and utility. Hence, there is a need to study the existing variation within the species. Furthermore, occurrence of genetic variation is a matter of great concern where commercial success in micropropagation depends solely on the maintenance of clonal uniformity (Bonga and Durzan, 1987). It is, therefore, extremely important to ascertain the suitability of a micropropagation protocol developed for a particular species, in terms of production of identical plants. This is all the more important for tree species and perennial crops where the life span is long and the performance of *in vitro* derived plants can be ascertained only after their long juvenile stage. Under this scenario, molecular markers play an important role in the assessment of genetic uniformity, variability and characterization of germplasm of *Pongamia*.

In the present study, the ISSRs were exploited to generate information on

A. The variation among the *Pongamia* trees (**Variability Studies**) and
B. The similarity / variability within the clones (**Fidelity Studies)**

Subsequently seed oil content and pod morphology were also studied in an attempt to establish correlation with the variations noted among the trees studied using ISSR markers.

6.2 EXPERIMENTAL PROTOCOL

A. Variability studies

A population of *Pongamia* trees growing locally was used as starting material for *in vitro* regeneration studies. From them, ten trees were identified for variability studies using ISSR markers, seed oil content, pod morphology and seed morphology. Six trees from the population at location A (Range hills) and four trees from the population at location B (Pashan) were chosen.

I. Morphological Characterization: Both Pods and seeds were collected from the selected ten trees and the morphological characters were studied. Shape and size of the pods and number of seeds per pod were scored. Color and shape of the seeds were noted.

II. Extraction and Quantification of Oil: A simplified method (Eckhardtjems,1997) was used for quantification of oil in the seeds collected from ten identified trees. Approximately 10g seeds of each tree were taken and were crushed using mortar and pestle. The crushed seed powder was transferred to 250 ml Erlenmeyer flask. To dissolve the fat substances from the tissue, 100ml of hexane was added and kept over night. After overnight incubation at room temperature, the hexane solution was separated from the tissue debris and transferred to a pre weighed Erlenmeyer flask for evaporation. Complete evaporation of hexane left the oil behind. The quantity of oil eluted was determined from the weight of the flask and oil from each sample. This experiment was repeated thrice. Oil content was expressed in grams per 10 g of seeds.

III. Genetic analysis: DNA was isolated from fresh leaf material collected from ten trees, using miniprep method reported by Khanuja et al., 1999. The details of DNA isolation, checking of purityand PCR protocols are described in chapter 2.

Various parameters including (a) Spermidine concentration (10,15 & 20mM/reaction), (b) Sample DNA concentration (20ng and 25ng/ reaction) and (c) *Taq* polymerase (0.16, 0.18 and 0.2µl/ reaction) were tested to optimize PCR conditions. These parameters were tested in three randomly chosen DNA samples using ISSR primer 808.

Hundred ISSR Primer of Set #9 (801…900) obtained from University of British Columbia (UBC), Canada, British Columbia were screened for amplification using two randomly chosen DNA samples of *Pongamia*. Five 'UBC set #9' ISSR primers, which gave reproducible and unambiguous amplification products were chosen (807, 808, 809, 881 & 890) for variability studies. DNA samples of ten trees were amplified using these five primers. PCR amplification was done individually for each primer and the experiment was repeated twice. A low range (3 Kb) DNA ladder was used for band sizing. Presence/absence of each scorable fragment was recorded in a binary data matrix and the data were analyzed using Win Dist and Win Boot software programs.

B. Fidelity studies on in vitro raised plantlets

Among the *in vitro* regenerated plantlets, 12, 13 and 12 propagules from three mother explants were tested for fidelity analysis using ISSR primers. , P1, P2 & P3 are the three mother samples tested. DNAs were isolated from fresh leaf of the mothers and their respective *in vitro* regenerated propagules using miniprep method of Khanuja et al., 1999. The details of DNA isolation, purity checking and PCR protocols are described in chapter 2.

Fidelity analysis: Three UBC set #9 ISSR primers (807, 808 & 809), which gave reproducible and precise amplification products were chosen for fidelity analysis of forty DNA samples as above. PCR amplification was done individually for each primer and the experiment was repeated twice. A low range DNA ladder (3 Kb) was used for band sizing. Presence/absence of each scorable fragment was recorded in a binary data matrix and the frequency of each band in samples was determined.

6.3 RESULTS AND DISCUSSION

Ten pongam trees were selected for ISSR analysis. Six trees (Figure 6.1) were from location A (Rangehills) and four trees (Figure 6.2) were from location B (Pashan). The locations are approximately 5 km. apart. The trees growing in the location A & B, from which nodal explants were taken for clonal propagation studies were selected.

DNA isolation:

Initially several protocols like Doyle and Doyle method (1987), Murray and Thompson's method (1980), Lodhi's CTAB method (1994), Khanuja's method (1999) were tried to extract DNA from *Pongamia* leaves. Difficulties were encountered from the stage of cell lysis to DNA separation in case of procedures described by Doyle and Doyle (1987), Murray and Thompson (1980) and Lodhi (1994). Low DNA yield and poor PCR amplification were also the problems encountered. Khanuja's DNA extraction protocol (1999) yielded better quality and quantity of DNA. Out of ten samples of DNA extracted, three DNA samples (P1, P2 & P3) were used for optimizing conditions of PCR.

Conditions optimized for PCR:

Microsatellite fingerprints obtained using various **concentrations of spermidine** are shown in Figure 6.1 (lane1 to.9). Varied band pattern among three samples ((P1, P2 & P3) of DNA was noticed. However, the pattern was consistent with spermidine concentration. Use of 20mM spermidine produced more intense bands. The present result shows no modification of pattern when spermidine concentrations are varied and hence 20mM spermidine was used for the study.

The experiment conducted to study the effect of **DNA concentration** to be used per reaction produced clear fingerprints in both the concentrations (20 and 25ng/reaction) tested (Fig. 6.1 lane 10 to 15). The concentration of 20ng DNA was therefore chosen for PCR. Different **concentrations of *Taq* polymerase** (0.48, 0.54 and 0.6units/ reaction) were tested for optimum amplification in three DNA samples. The amplified products are as shown in Fig. 6.1 (lane16 to 24). There was no change

in the banding pattern with enzyme concentration in any of these three samples tested, however, with increase in enzyme concentration, the sharpness in the bands was lost with all three DNA samples. Thus Taq polymerase concentration of 0.16 µl (0.48 unit) was optimum for PCR amplification of *Pongamia* DNA. Finally, Spermidine 20mM, genomic DNA 20ng and 0.16µl of *Taq* polymerase were optimum for PCR amplification of *Pongamia* DNA.

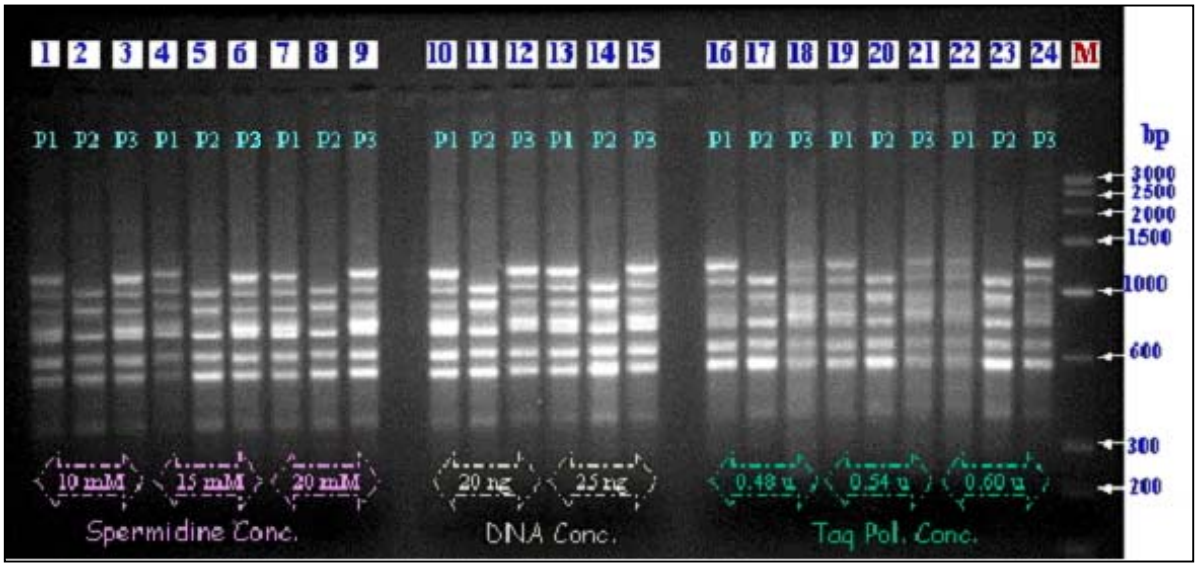

Fig. 6.1: Agarose gel electrophoresis pattern of PCR amplification products using ISSR primer 808.
Lane 1 to 9: Inter microsatellite fingerprints with various concentrations of spermidine used.
Lane 10 to 15:Inter microsatellite finger prints with different DNA concentrations tested.
Lane 16 to 24: Inter microsatellite finger prints with different concentrations of *Taq polymerase*.
P1, P2 & P3: DNA samples tested. M: Low range DNA ruler (3kb)

Selection of primers:

Initially hundred ISSR primers were screened for amplification using two DNA samples of *Pongamia* (Fig. 6.4 and 6.5). Out of 100 primers, 60 primers did not produce any amplification product, 19 primers gave weak fragments and 21 primers produced clear, intense bands (Table 6.2).

The results of the ISSR primer screening in *Pongamia* suggest that di nucleotide repeat motifs occur at high frequencies while tri, tetra and pentanucleotide appear less frequent along the *Pongamia* genome. Even in the crops like bean, rice, maize and soyabean, it was found that di and tri nucleotide SSR occur along the genomes at higher frequencies than tetra nucleotide repeats (Yu et al., 1999; Rangwen et al., 1995; Akagi et al., 1996b; Chin et al., 1996).

Table 6.2: Nucleotide sequence of the primers producing clear amplification products

Sr. N	Primer Name	Sequence 5'-3'
1	**UBC-807**	AGAGAGAGAGAGAGAGT
2	**UBC-808**	AGAGAGAGAGAGAGAGC
3	**UBC-809**	AGAGAGAGAGAGAGAGG
4	UBC-810	GAGAGAGAGAGAGAGAT
5	UBC-811	GAGAGAGAGAGAGAGAC
6	UBC-812	GAGAGAGAGAGAGAGAA
7	UBC-815	CTCTCTCTCTCTCTCTG
8	UBC-818	CACACACACACACACAG
9	UBC-834	AGAGAGAGAGAGAGAGYT
10	UBC-835	AGAGAGAGAGAGAGAGYC
11	UBC-836	AGAGAGAGAGAGAGAGYA
12	UBC-840	GAGAGAGAGAGAGAGAYT
13	UBC-841	GAGAGAGAGAGAGAGAYC
14	UBC-845	CTCTCTCTCTCTCTCTRG
15	UBC-848	CACACACACACACACARG
16	UBC-856	ACACACACACACACACYA
17	UBC-866	CTCCTCCTCCTCCTCCTC
18	UBC-867	GGCGGCGGCGGCGGCGGC
19	UBC-876	GATAGATAGACAGACA
20	**2UBC-881**	GGGTGGGGTGGGGTG
21	**UBC-890**	VHVGTGTGTGTGTGTGT

[Y= (C, T); V=(A, C, G); H= (A, C, T)]
The highlighted ones are selected primers

From the 21 primers, which helped in amplification of the DNA fragments of *Pongamia,* five primers (807, 808, 809, 881 & 890) were chosen for variability analysis based on the quantity and quality of their amplified fragments and their polymorphism. Out of five primers, four were dinucleotide repeats (807,808,809 & 890) and one was pentanucleotide repeat (881).

Analysis of polymorphism:

The amplification products using five primers, 807,808, 809, 890 & 881 were separated on agarose gel (Fig. 6.2, 6.3, 6.4, 6.5 & 6.6).

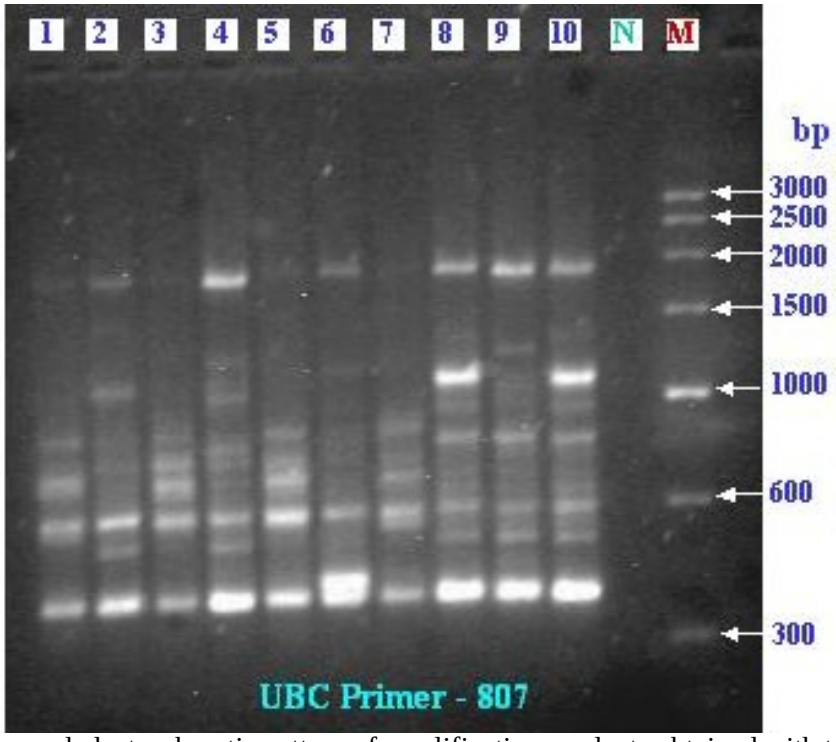

Fig. 6.2: Agarose gel electrophoretic pattern of amplification products obtained with ten trees using the primer 807. **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification. Lane 1-10: Trees in order.

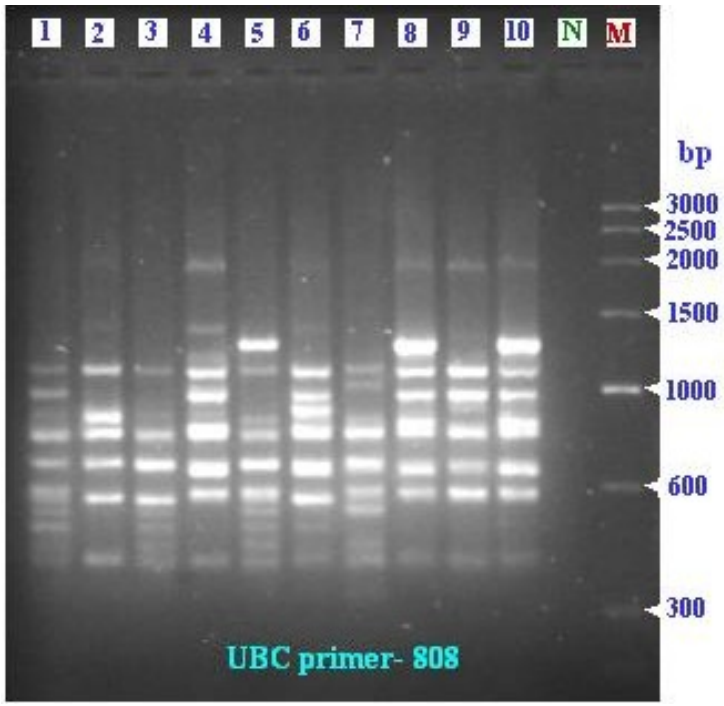

Fig. 6.3: Agarose gel electrophoretic pattern of amplification products obtained with ten trees using the primer 808. **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification. Lane 1-10: Trees in order.

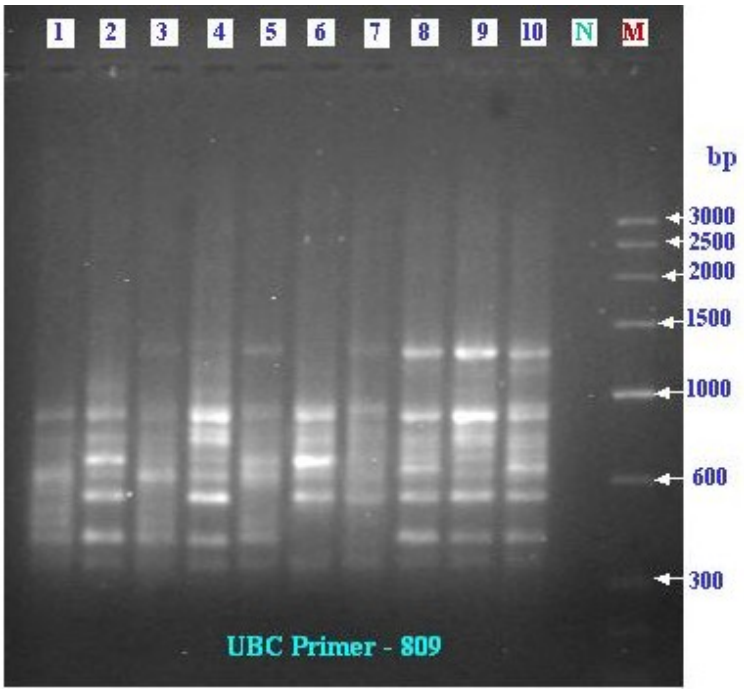

Fig. 6.4: Agarose gel electrophoretic pattern of amplification products obtained with ten trees using the primer 809. **M:** Low range DNA ruler (3 kb). **N:** **N**egative control of PCR amplification. Lane 1-10: Trees in order.

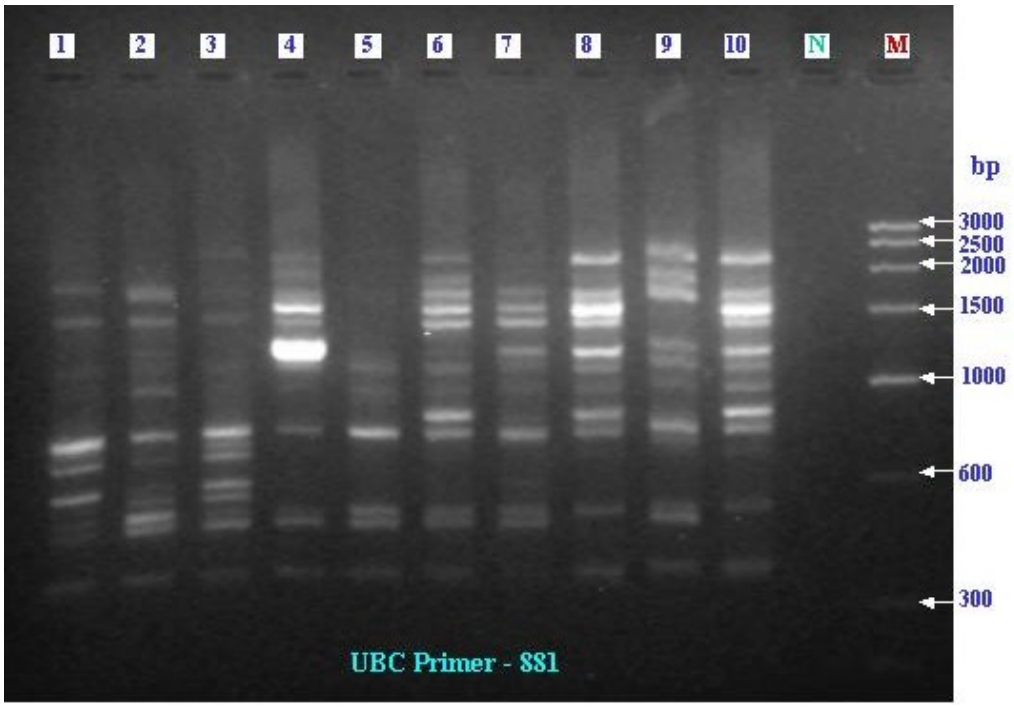

Fig. 6.5: Agarose gel electrophoretic pattern of ISSR amplification products obtained with ten trees using the primer 881. **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification. Lane 1-10: Trees in order.

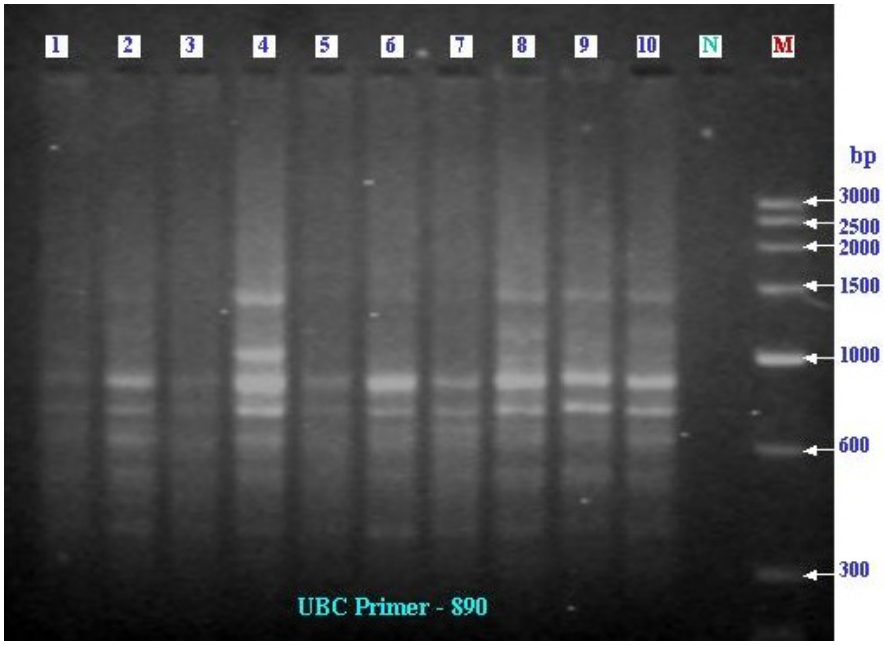

Fig. 6.6: Agarose gel electrophoretic pattern of amplification products obtained with ten trees using the primer 890. **M:** Low range DNA ruler (3 kb). **N:** **N**egative control of PCR amplification. Lane 1-10: Trees in order.

The data generated (Table 6.3) using these five primers were analyzed for variability studies. Five ISSR primers gave rise to 111 products with an average of 22.2 products. Out of which 75 bands were scored for polymorphic analysis in total with an average of 15 bands per primer. The minimum being 10 bands for the primer UBC-809 and the maximum 25 bands for primer UBC-881. An average level of polymorphism of 76% (range 63.6% for UBC-890 and 85.7% for UBC-807) was observed. The approximate size of the largest fragment was 2.5kb (UBC-890), whereas the smallest recognizable fragment was about 0.36 kb in size (UBC-809).

Table 6.3: Selected primers with the number of bands and their relative size

Primer ISSR	Total No. of bands	No. of bands scored	Percentage of polymorphic bands	Range (bp) of amplified bands
UBC-807	22	14	85.7	366-2000
UBC-808	23	15	73.3	433-1850
UBC-809	18	10	80	360-1410
UBC-881	32	25	76	480-2250
UBC-890	16	11	63.6	475-2500
Total	111	75	76% (average)	

ISSR markers used here proved to be able to assess the similarity / variability of *Pongamia* within a population. The source of polymorphisms may include deletion of a priming site and insertion or deletion causing changes to the DNA fragment size or renders priming sites too distant to support amplification. The simple sequence repeats change by amplification and transposition more frequently than single copy sequences (Pellegioni et al., 2003). They have a high mutation rate from generation to generation due to DNA slippage and chromosomal structural rearrangements (Pellegioni et al., 2003). The ISSR polymorphism level in *Pongamia* (76%) is comparable with the polymorphism reported in other species; 62% in Sweet potato (Huang et al., 2000); 61% pea (Lu et al., 1996); 60% in *Phaseolus vulgaris* (Metais et al., 2000) and 65.2 % in *Juglans* (Pollegioni et al., 2003).

The objective of the study was to assess the genetic similarity/distance within ten trees tested. The estimate of pair wise genetic distance between the ten selected trees is given in Table 6.4.

Table 6.4: Similarity matrix of selected *Pongamia* trees based on Jaccard's similarity index

	1	2	3	4	5	6	7	8	9	10
1	**1.00**									
2	**0.50**	**1.00**								
3	**0.71**	**0.44**	**1.00**							
4	**0.48**	**0.69**	**0.43**	**1.00**						
5	**0.55**	**0.39**	**0.75**	**0.38**	**1.00**					
6	**0.53**	**0.69**	**0.43**	**0.66**	**0.42**	**1.00**				
7	**0.63**	**0.56**	**0.64**	**0.64**	**0.55**	**0.59**	**1.00**			
8	**0.45**	**0.59**	**0.41**	**0.66**	**0.48**	**0.61**	**0.50**	**1.00**		
9	**0.52**	**0.62**	**0.46**	**0.70**	**0.45**	**0.53**	**0.52**	**0.78**	**1.00**	
10	**0.45**	**0.59**	**0.41**	**0.67**	**0.48**	**0.61**	**0.50**	**1.00**	**0.78**	**1.00**

The similarity matrix was calculated based on Jaccard's Similarity Coefficient. The genetic similarity indices ranged from 0.38 to 1.00. Among the six trees selected from the population of location A, tree-5 is closely related to tree-3, whereas low index between tree-5 and tree-4 reveals that they have least genetic similarity. In location B, an index of 1.0 substantiates absolute similarity between tree-10 and tree-8. This reveals that tree-10 and tree-8 are genetically similar genotypes. Tree-10 is also closely related to tree-9 with an index of 0.78. Overall, the plants selected from the population of

location B are very closely related than the plants selected from location A. The similarity coefficients were used to generate a tree for cluster analysis using Win Boot software, wherein UPGMA algorithm was used for clustering. It showed overall genetic relatedness among the selected trees (Fig. 6.7).

In this analysis, two distinct clusters could be identified. The first cluster comprised, Tree 1,3,5, & 7. The second cluster is formed by tree 2,6,4,8,10 & 9. Tree-3 & 5 are genetically closer to each other in the first cluster (50%), while tree-8 &10 are genetically similar genotypes (100%) in the second cluster. Tree 2 and 6 in the second cluster are also genetically closer. This is evident with all the primers tested (Fig. 6.6 to 6.10) supporting the observation. UPGMA cluster analysis reveals the tree-7; a tree from location B is closely related with the trees (1,3 & 5) in the location A (90%). In addition, Tree- 2, 4 & 6 closely related to the trees chosen from the population of location B (8, 9 &10) by 65%. Being an uncultivated species, the species classification of the genus *Pongamia* is not available. However, our study is not a diversity/ phylogenetic analysis, but the results of the cluster analysis allow visualising the high evolutionary potential of *Pongamia* as shown by their rich genetic variability.

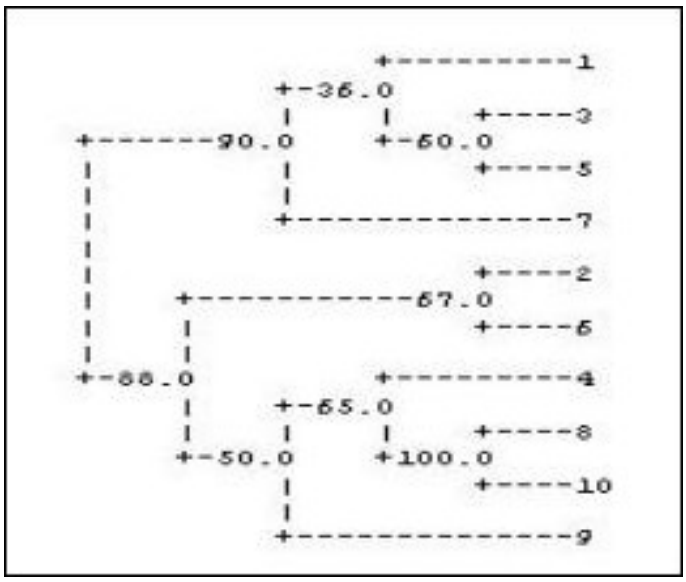

Fig. 6.7: Dendrogram showing genetic relationship among the ten trees of *Pongamia* generated by ISSR data using UPGAMA method. Numbers at the forks indicate the confidence limits for the grouping of those species, which are to the right of the fork.

In summary, ISSR markers successfully identified genetic variation among the selected trees of *Pongamia*. Genetic similarity between the trees of both locations suggests that these populations are in equilibrium in the ecosystem. High polymorphism (76%) was observed among the ten trees. Thus

efforts were made to correlate banding pattern with the morphological characters. As our ultimate objective is to improve *Pongamia* for its biodiesel utilities, characters related to oil content and seed morphology were mainly considered for the variation studies.

Quantification of oil:

Oil extracted from the seeds was quantified and percentage of oil was determined (Table 6.6). Amount of oil recovered from 10g of seeds ranged from 1.25g to 2.8 g. Maximum (2.8 ± 0.06 g/10g) amount of oil was recovered from the seeds of tree No.9 and tree No. 4 gave low yield (12.5%). The seeds of the trees 1,2,3,4,5 & 6 from the population taken from location A gave an average of 1.96 g of oil per 10 g seeds and the trees 7, 8, 9 & 10 from the population of location B gave an average amount of 2.57 g oil per 10g seed. Weight wise the seeds of tree No. 9 yielded more oil (28%), but volume wise it did not seem to be the highest (Fig. 6.8). This could not be explained with present state of knowledge. However, ossibility of higher specific gravity of this oil cannot be ruled out. From the literature, it is known that pongam trees yield approximately 24 - 27% of oil (Wealth of India, 1993). Consistency of our data was confirmed by low deviation (S1%) statistics (Table 6.6).

Table 6.6: Estimated Oil content from Pongam Seeds

Location	Trees	Oil content (g / 10g) mean ± sd	Percentage of oil obtained (w/w)
A	1	2.40 ± 0.08	24.0
	2	2.20 ± 0.16	22.0
	3	2.11 ± 0.06	21.1
	4	1.25 ± 0.03	12.5
	5	1.84 ± 0.10	18.4
	6	2.00 ± 0.17	20.0
B	7	2.68 ± 0.11	26.8
	8	2.33 ± 0.02	23.3
	9	2.80 ± 0.06	28.0
	10	2.48 ± 0.33	24.8
ANOVA		S1%	

Fig. 6.8: Oil extracted from approximately 30g of Pongam seeds excised from ten trees using solvent extraction (Hexane) method. Note. Low quantity of oil extracted from the seeds of tree-4.

The banding pattern obtained with pentanucleotide primer 881 (Fig 6.10) was chosen for correlation of oil content, as it produced more scorable (25) bands and clear banding pattern. There was noticeable doublet fragment of ~ 1250bp in the tree-4. Seeds of this tree demonstrated lowest oil content of 12.5%. However, other trees (7, 8, 9 &10) showed the fragment of this size, but the oil contents were 26.8, 23.3, 28.0, and 24.8% respectively. Possibility of this double fragment (tree 4) related to the character like oil yield cannot be ruled out with the present status of knowledge. Or else, correlates to other morphological characters, which are not taken into consideration in this study. No correlation could be made with the DNA banding pattern and the oil content. High oil content was observed in the tree-9, but there was no distinguishable banding pattern was observed.

Oil yield is the most important trait in oil producing plants like brassica, rapeseed, sunflower, soyabean etc. Oil content in *B. napus* is generally regarded as a character with high heritability (Becker 1999). Quantitative trait loci (QTL) analysis associated with oil content, especially with altered fatty acid, composition has been reported in many oil producing species, eg. Protein and oil yield in rape seed (Zhao et al., 2006); palmitate content in soybean (Cardinal et al., 2007); oil content and fatty acid composition in *B. napus* (Burns et al., 2003); linolenic content in *B. rapa* ssp. Oleifera (Tanhuanpàà & Schulman, 2002) and oleic acid content in *B. juncea* (Sharma et al., 2001).

Morphological characters:

Selected trees growing in close proximity in both locations appeared visually different. Pods of tree no.5 were elongated with narrow width (Fig. 6.9). However, pod shapes were closely similar in rest of the trees, but were smaller in tree no.2. Pods of tree no.8 and 10 had prominent veins on surface.

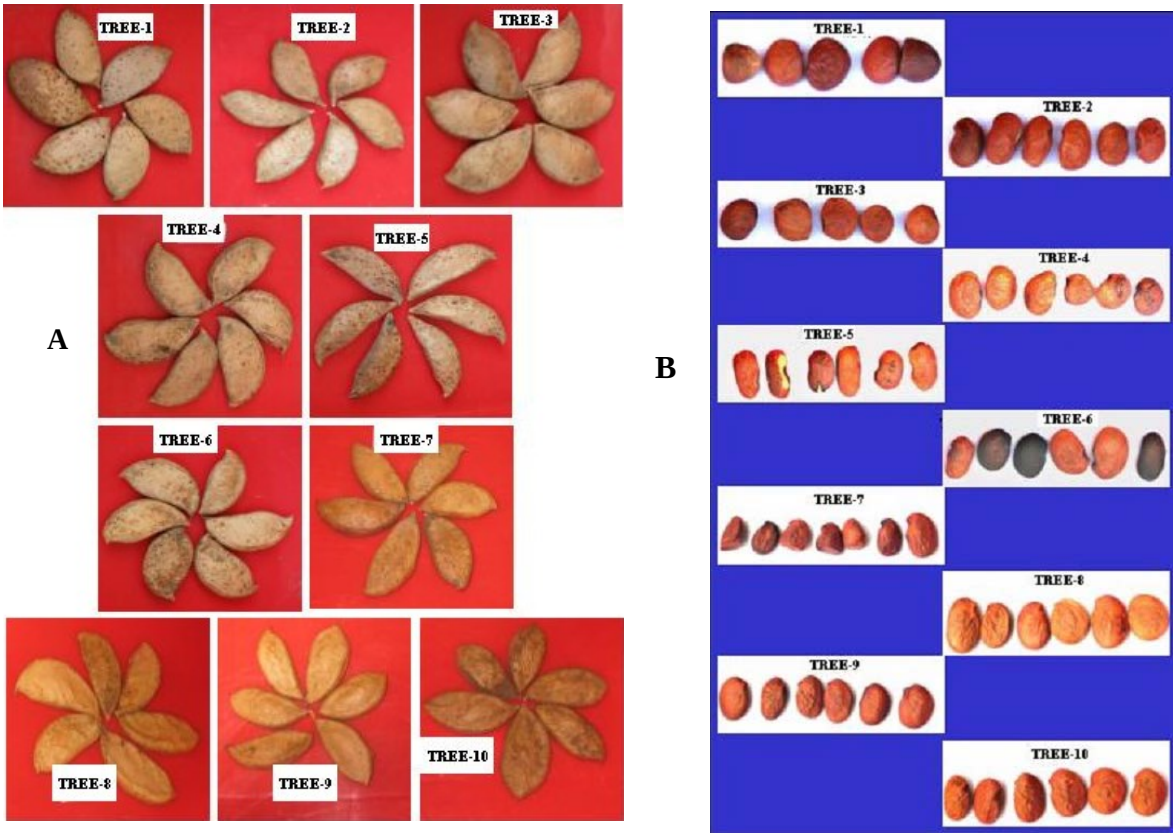

Fig. 6.9: (A) Pods collected from ten trees. Note. Narrow and elongated pods of tree 5; Prominent veins on the trees 8 and 10; Smaller size pod of tree 2.
(B) Seeds excised from the pods of ten pongam trees to extract oil.

The seeds collected from the trees exhibited variation in color (Fig. 6.10). There is a gradation of seed color from light reddish brown to dark brown. Several pods in Tree No. 7 were two seeded and smaller in size. Seeds were almost round in tree No. 3. While, elongated seeds were noted in tree No.5. While correlating the banding pattern (Fig. 6.2 – 6.6) with the characters studied, the fragment above 1500bp was absent in Tree-5 with all the primers used. Both pods and seeds are elongated in tree No.5. No definite correlation was observed with the banding pattern produced by the primers used and the pod size and shape, seed size and shape, and oil content. However, the present study reveals that both phenotypic and genotypic variations exist in *Pongamia*.

B. Fidelity studies on in vitro raised plantlets

Realizing the importance of genetic uniformity for commercial success in micropropagation (Bonga and Durzan, 1987). the genetic fidelity of the *Pongamia* plants regenerated *in vitro* were analyzed using ISSR markers. Conditions standardized for testing the naturally growing *Pongamia* trees were

extended to the plants regenerated using our protocol for clonal propagation of mature *Pongamia* trees (Chapter 4).

Varying numbers of plantlets were regenerated *in vitro* from each of the mother tree. Thirty seven (12 +13 +12) *in vitro* regenerated plantlets obtained from three mother plants P1, P2 & P3 were randomly selected for fidelity analysis. In the present study, three dinucleotide primers (807, 808 & 809) were chosen from the list of 21 primers, which gave good amplification (Table 6.2). The products of PCR reaction using these primers were size separated by gel electrophoresis in 1.5% Agarose gel.

Fidelity Analysis:

All the three primers produced amplification products that were monomorphic across all the micropropagated plants with respect to their mother plants (Fig. 6.11 - 807; Fig. 6.12 - 808; Fig. 6.13- 809).

In the present study three ISSR primers gave rise to 107 products (Table 6.7). Out of which 82 bands, were clear and reproducible. Primer 808 produced more bands on a gel in all three different clones tested. The average number of fragments obtained by the three selected primers was 27.3 bands. The size of the monomorphic bands produced by the primers 807, 808 and 809 was ranging from 1372-492bp, 1250-375bp and 1375-430 bp respectively. There were no polymorphic DNA fragments among the micropropagated plants. Where as polymorphism presents among the three mother plants tested . This result is in correlation with the results of variation studies.

Table 6.7: Selected primers with the number of bands and their relative size

Primer ISSR	Total No. of bands	No. of bands scored	Range (bp) of amplified products
UBC-807	28 (11+08+09)*	22 (09+05+08)#	1372-492
UBC-808	42 (18+14+13)	31(11+11+09)	1250-375
UBC-809	37 (14+13+10)	29 (10+10+09)	1375-430
Total	107	82 (27.3%)	

(* Numbers in the parentheses represent the total number of bands produced by different clones respectively; # Numbers in the parentheses represent the number of scored bands produced by different clones, respectively)

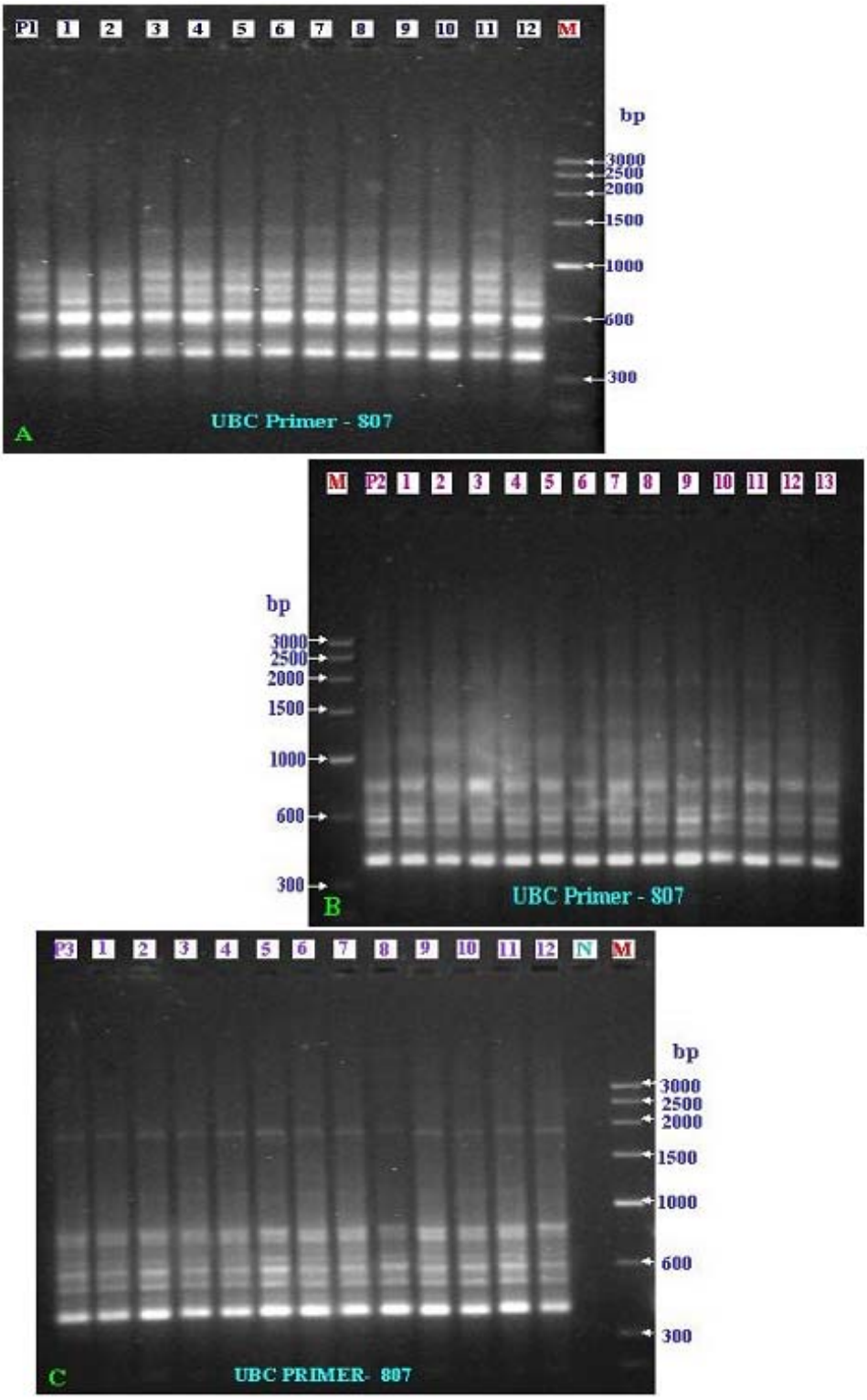

Fig 6.11: PCR amplification products obtained using the primer 807. A, B & C are products from three mother plants (**P1 +12**, **P2+13** & **P3+12**) with their *in vitro* raised plants respectively. **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification.

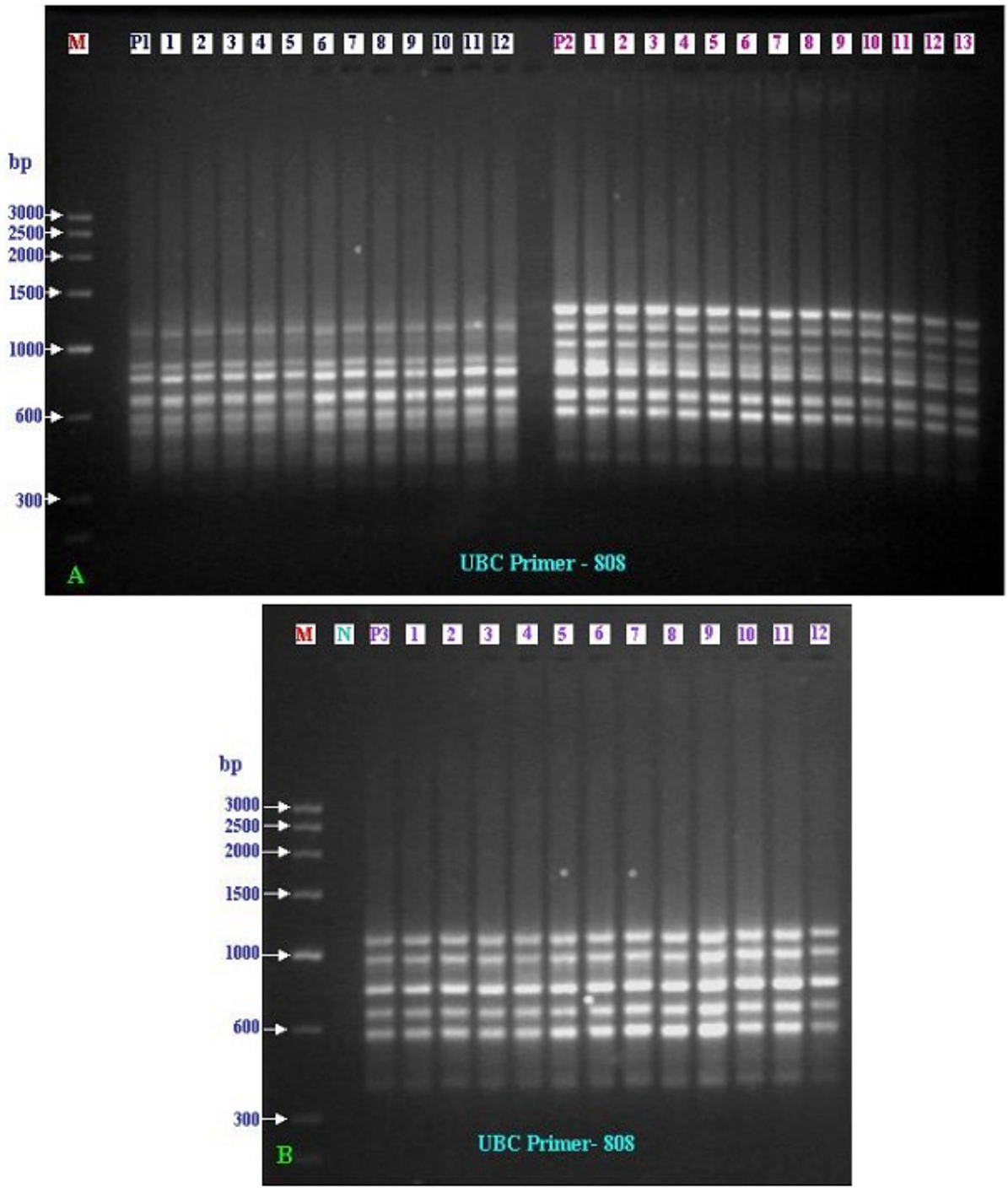

Fig 6.12: PCR amplified ISSR products obtained using the primer 808. Sub figure A shows the products of two mother plants (**P1+12 & P2+13**) with their respective micropropagated plants. Sub figure B represents third mother plant (**P3+12**) with its regenerated plants. **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification.

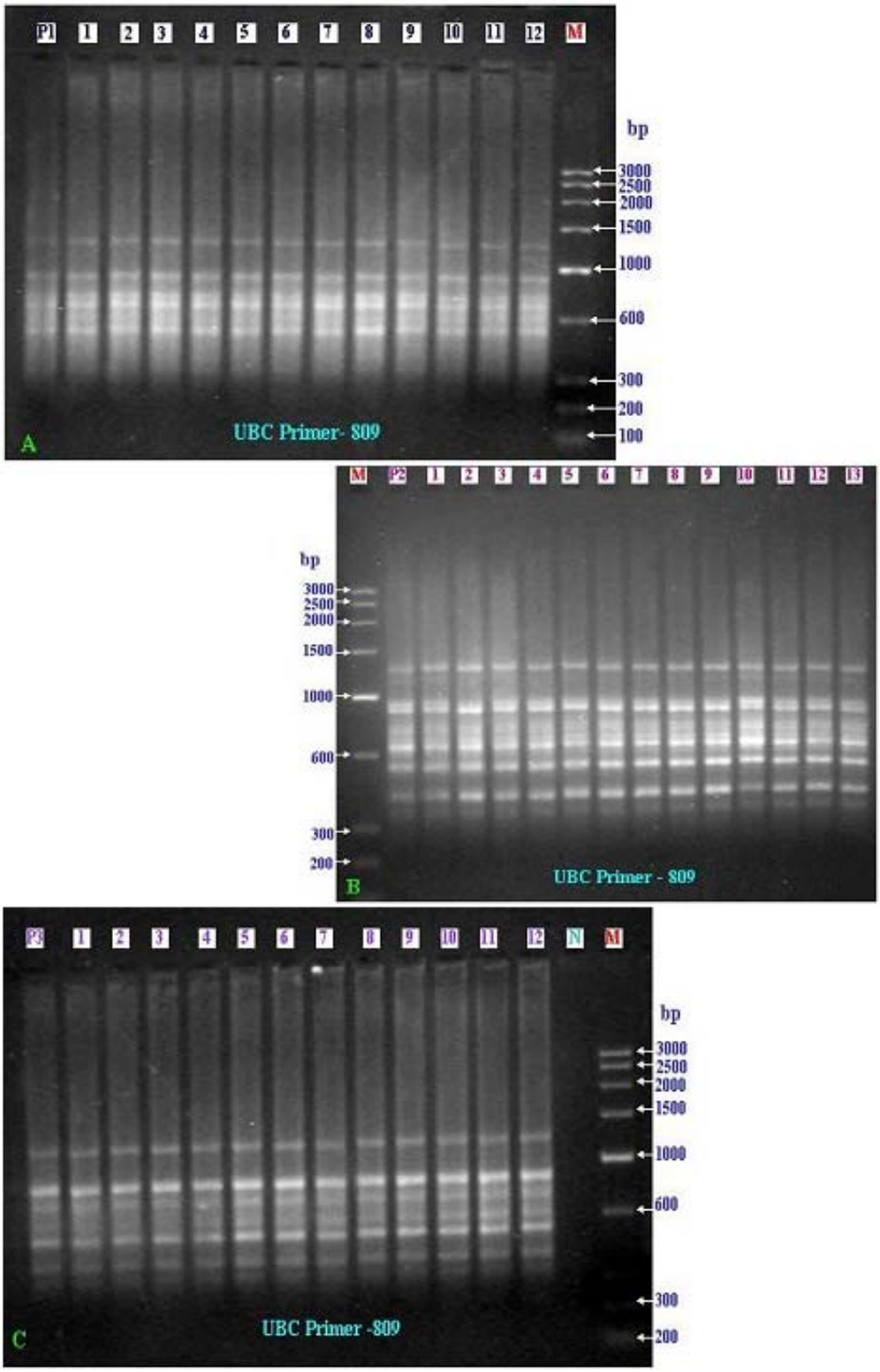

Fig. 6.13: Agarose gel electrophoretic pattern of ISSR amplification products obtained with the primer 809. A,B & C shows the products from three mother plants (**P1+12**, **P2+13** & **P3+12**) with their regenerated plantlets respectively. **M:** Low range DNA ruler (3 kb). **N:** Negative control of PCR amplification

The pattern of monomorphic bands in the *in vitro* raised plants was reported earlier in other plants using different molecular markers. In potato ISSR was used effectively for screening of somatic hybrids between S*olanum tuberosum* cv "Brodick" and *Solanum sanctae-rosae* (Matthews et al., 1999). The molecular markers were used for characterization of the new allotetraploid somatic hybrids of Mandarin (Scarano et al., 2002). Leroy and Leon (2000) used this marker for the detection of genomic instability in callus cultures of cauliflower. Devarumath et al, (2002) evaluated the genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* and *Camellia assamica*. These authors demonstrated that ISSR fingerprinting detected more polymorphic loci than RAPD finger printing.

6.4 CONCLUSION

Khanuja's DNA extraction protocol (1999) yielded good quality and quantity of DNA from *Pongamia* leaves. Spermidine 20mM with 20ng of genomic DNA and 0.16µl(0.48 unit) of Taq *Polymerase* was optimum for amplification of *Pongamia* DNA. ISSRs successfully identified the variation (76% polymorphism) among selected ten trees tested, and demonstrated uniformity in the propagaules produced *in vitro* using the protocol standardized for clonal propagation of *Pongamia* using mature tree derived explants. Variation in the morphological characters like pod shape and size; seed shape, size, and color was noticed among the selected ten trees. Oil content of the seed showed variation ranging from 12 to 28%. By determining the oil content in seeds, high oil producing *Pongamia* trees (elites) can be identified.

Fidelity studies undertaken on *in vitro* raised *Pongamia* plantlets produced no polymorphic band pattern with the primers used. On the contrary, polymorphism was noted among the three mother plants from which the explants were collected for micropropagation. The clones showed uniformity among them. Monomorphic pattern of *in vitro* raised clones of *Pongamia* validates the protocol developed for clonal propagation of *Pongamia.* High degree of variability among the selected trees in variation analysis suggests applicability of the tissue culture techniques for multiplication of high oil yielding *Pongamia* trees.

This part of work has been compiled for communication

Application of ISSR-PCR techniques in the germplasm characterization of wild and tissue cultured derived *Pongamia* species.

SUMMARY

SUMMARY

Pongamia pinnata (L) Pierre (Syn: Pongamia glabra. Vent), an oil producing tree legume, popularly known as Karanja, is the most widely available non-edible oil tree in India. This multipurpose tree species is well adapted for various agro climatic conditions and is one of the most extensively chemically investigated plants. Potential of the seed derived oil of *Pongamia* as a substitute for diesel is recognized and often identified as **"Bio-diesel"** (Vivek & Gupta AK, 2004). A large number of bioactive compounds have been isolated and characterized from various parts of this tree (flavonoids, furanoflavonoids), which has many industrial and medicinal uses (Wealth of India, V.3, 1998; Parmar, 1976).

Pongamia can serve as a source of high quality fuel and raw material for industries. Raising plantations of clonally propagated, high seed yielding trees will contribute to increased seed production. These plantations will also serve the purpose of seed orchards for production of superior quality seeds. *Pongamia* tree can be exploited as an alternate source of edible oil by silencing the related genes, which cause production of undesirable substances. For genetic modification, the first pre-requisite is a reproducible *in vitro* regeneration system. There is no literature on *in vitro* regeneration or tissue culture studies in this species

Pongamia species, which represent a substantial variability in phenotype as well as chemo type properties for the oil and its components, do need to be documented well for establishing phylogenetic relationships and unique marker profiles at DNA level. To date, there is no literature available regarding the molecular scaffold of this tree.

The present study was designed with the following objectives:

1. Optimization of conditions for propagation using juvenile tissues.
2. Optimization of conditions for clonal propagation using mature nodal buds.
3. Optimization of protocolfor *de novo* morphogenesis (organogenesis and somatic embryogenesis).
4. Assessment of variability/similarity among the trees and fidelity of the *in vitro* raised clones using ISSR markers (Molecular characterization).

The major constraint encountered in the study to establish a regeneration protocol for clonal propagation from seedling explants was to obtain seedlings under aseptic condition. Several factors were tested to obtain microbe free seedlings. Removal of seed coat prior to germination, controlled fungal growth partially but enhanced bacterial growth. Antibiotic Cefotaxime was ineffective in controlling bacterial contamination. Increased germination frequency of dry mature seeds was obtained in media with 8.88μM BA in MS medium. Cotton plugged culture vessels favored germination.

A protocol for *in vitro* propagation of this plant was standardized using seedling explants. This is the first report on *in vitro* regeneration of *Pongamia pinnata* **(Sujatha and Hazra, Journal of Plant Biotechnology 33(4): 263-270, 2006)**. Seedling derived nodal explants and cotyledon nodes with attached cotyledons were used as explants. Optimum shoot proliferation from nodal explants and cotyledon nodes was achieved in MS medium supplemented with 8.88μM BA and 3% sucrose. Reculturing of cotyledon node explants after removal of shoots produced more shoots from same site. This process was repeated for 8 cycles and 4-8 shoots were obtained in each cycle. This process offers an alternative to produce clones and to avoid the step of repeated seed germination *in vitro*. The shoots elongated and rooted (75%) in half strength MS basal medium supplemented with 0.22% activated charcoal. Plantlets survived on transfer to soil. The *in vitro* raised shoots rooted *extra vitrum* (67.5%) and hardened successfully.

To eliminate the seed borne contamination, cotyledon node explants were isolated from semi mature seeds obtained by dissecting the surface sterilized green pods. These were cultured in varying concentrations of TDZ for induction of shoots from cotyledon node meristem. After 20 days pretreatment in TDZ, cotyledon node explants with intact cotyledon and embryo axis explants responded in culture. On transfer to MS media for 2-4 passages, the buds induced in the cotyledonary meristem differentiated. Optimum TDZ concentration was 11.4 μM. Buds induced in high TDZ (13.6 & 22.7μM) took longer time to differentiate, delaying the rooting step. The shoots were rooted (80%) in half strength MS media supplemented with charcoal. The rooted shoots were hardened (75%) and transferred to green house successfully.

A protocol for clonal propagation of *Pongamia* using mature nodal buds was optimized **(Sujatha and Hazra, In Vitro Cellular and Developmental Biology, 2007 – In press)**. Four basal media formulations including MS, SH, B5 and WPM were tested. MS basal media was superior for mature

bud culture of *Pongamia*. Effects of various growth regulators including BA, KN, Z and TDZ were studied on sprouting of nodal buds. No multiples were produced in MS supplemented with BA and KN singly. Supplementing Adenine sulphate (AdS) with BA promoted production of 2-3 multiples from the nodal bud. Media with kinetin and AdS was ineffective. In TDZ, sprouting was completely suppressed and the meristems were swollen. Caulogenic buds appeared from the swelling on withdrawal of TDZ. Number of buds was more in the explants pretreated in increased concentration of TDZ. Clusters of buds elongated on transfer to GR free MS medium. In TDZ 0.45μM the response was optimum as the buds proliferated in this medium and the induced bud differentiated faster. Shoot cultures were maintained by subculturing in 0.45μM TDZ. Among the parameters tested for optimization, sucrose at 4% and pH 5.8 were optimum for shoot proliferation and growth. Reculturing of primary explants after cropping the shoots produced more shoots. This process was followed for 6 cycles to obtain additional shoots in each cycle. Shoots elongated and rooted (70%) in GR free MS medium. Rooted shoots survived in green house (65%). Repeated proliferation of caulogenic buds from same origin may find application in the rescue of endangered germplasm and in development of transgenics. This study not only describes a simple protocol for clonal propagation of an important tree species "*Pongamia pinnata*" for the first time, but also a system to study some of the processes of TDZ induced morphogenetic activities.

Deembryonated cotyledon from green pod of *Pongamia* were precultured for 10 days and 20 days in varying concentrations of TDZ in MS basal media and transferred to GR free MS basal media for induction of *de novo* organogenesis. Preculture of 20 days in 11.35μM TDZ yielded more number of buds than 10 days culture. Proximal segment of the cotyledon demonstrated highest morphogenic potential. The best condition for optimum caulogenic response is, exposure to TDZ 11.35μM for 20 days with abaxial side in contact of medium followed by elimination of TDZ from media to promote differentiation of these buds to elongated shoots. Plants were obtained by rooting of these shoots. *De novo* origin of the organogenic buds was confirmed using histological techniques. This is the first report on *in vitro* protocol of *Pongamia pinnata* via adventitious organogenesis (**Sujatha et al.,** communicated in **Trees-structure and Function)**. The protocol may find application in studies on genetic transformation, isolation of somaclonal variants and in induction of mutants. It may also find application in study of the opposite roles of TDZ in induction and differentiation of buds. Embryo axis was tested as an alternative explant for induction of *de novo* organogenesis. TDZ induced adventitious buds did not differentiate into shoots on transfer to GR free MS media. Efforts taken to differentiate these buds are described.

Efforts made towards regeneration of *Pongamia* via somatic embryogenesis using different explants like cotyledon, embryo axis and immature seeds from green pods produced embryo like structures which did not differentiate further to generate plantlets.

The trees (ten numbers) used as source of explants were studied to assess the variation/uniformity. Variations were noted in morphological characters like pod shape and size, seed shape and size, and color. Oil content of the seeds showed variation ranging from 12 to 28%. For molecular analysis DNA was extracted from the tender leaves of these trees using Khanuja's DNA extraction protocol which yielded good quality and quantity of DNA. Band patterns obtained from ISSRs were analysed using Jaccard's coefficient to study the extent of similarity or variability among the selected trees. Highly polymorphic band pattern were obtained within the selected trees. ISSRs identified the 76% variation (polymorphism) among the ten trees tested.

Fidelity studies undertaken on the *in vitro* raised *Pongamia* plantlets produced no polymorphic band pattern with the primers used. Monomorphic pattern of *in vitro* raised clones of *Pongamia* validates the protocol developed for clonal propagation of *Pongamia.* The variation existing in this species emphasizes the importance of micropropagation techniques for cloning the superior genotypes. In this study ISSR-PCR techniques are applied in the germplasm characterization of wild and tissue cultured derived *Pongamia* plants **(K.Sujatha et al.,** Manuscript under preparation).

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AUTHOR'S
PUBLICATION

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RESEARCH WORK COMMUNICATED/PUBLISHED

1. **K. Sujatha** and Sulekha Hazra. (2007) Micropropagation of mature *Pongamia pinnata*. Pierre. In Vitro Cell. Dev. Biol. Plant. (in press)

2. **K. Sujatha** and Sulekha Hazra (2006) *In Vitro* Regeneration of *Pongamia pinnata*. Pierre. J. Plant Biotechnol. 33 (4): 263-270.

3. **K. sujatha,** B.M. Panda and Sulekha Hazra (2006) *In Vitro* Regeneration of *Pongamia pinnata* via de novo organogenesis. J. Med. Arom. Plant Sci. (JMAPS) CIMAP, Lucknow (in press).

4. **K. Sujatha,** B.M. Panda and Sulekha Hazra. *In Vitro* Regeneration of P*ongamia pinnata* via adventitious organogenesis". Trees. (communicated).

5. **K. Sujatha,** Ashwini R, Gupta V.S. and Sulekha Hazra. Application of ISSR-PCR techniques in the germplasm characterization of wild and tissue cultured derived *Pongamia* species (manuscript under preparation).

6. Joshi M, **K. Sujatha** and Sulekha Hazra. Effect of TDZ on early embryogenesis and high frequency in vitro regeneration in Peanut. In Vitro Cell. Dev. Biol. Plant. (manuscript under preparation)

PRESENTATIONS AT NATIONAL SEMINARS / SYMPOSIA

1. **K. Sujatha** and Sulekha Hazra (2003) "*In Vitro* Regeneration of *Pongamia*, An oil producing tree Legume". Poster presented on National Science Day, NCL, Pune.

2. **K. Sujatha** and Sulekha Hazra (2004) "Tissue Culture in *Pongamia*". Poster presented on National Science Day, NCL, Pune.

3. **K. Sujatha** and Sulekha Hazra (2005) "Karanj (*Pongamia pinnata.* Pierre) A Renewable Energy Source". Poster presented on National Science Day at NCL, Pune.

4. **K. Sujatha,** B.M. Panda and Sulekha Hazra (2005) "In Vitro Regeneration of *Pongamia Pinnata* via de novo organogenesis". Poster presented and Abstract published. 'National Symposium on Plant Biotechnology: New Frontiers' (November 18-20, 2005) in the 27th Annual Meeting of Plant Tissue Culture Association (India) held at CIMAP, Lucknow.

5. **K. Sujatha**, B.M. Panda and Sulekha Hazra **(2006) "***In Vitro* Regeneration of *Pongamia pinnata* via *de novo* organogenesis". Poster presented on National Science Day at NCL, Pune.

WORKSHOP ATTENDED

CSIR sponsored **CIMAP's Winter School** on "***Recent Techniques in Gene Cloning, DNA Analysis and Functional Genomics***" held at CIMAP, Lucknow during 1-10 December 2005.

Best Performance Prize.