STUDIES ON *SEMECARPUS ANACARDIUM* L. FOR *IN VITRO* REGENERATION AND IDENTIFICATION OF BIOLOGICALLY ACTIVE COMPOUNDS

A THESIS
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BY

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Dedicated to my Parents,
Uncle and Aunty

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Bhuban Mohan Panda

CERTIFICATE OF THE GUIDE

Certified that the work incorporated in the thesis entitled **"Studies on *Semecarpus anacardium* L. for *in vitro* regeneration and identification of biologically active compounds"** submitted to the **University of Pune** for the award of **Doctor of Philosophy in Biotechnology** by **Mr. Bhuban Mohan Panda** was carried out by him 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.

Date:
Place: NCL, Pune

Dr. Sulekha Hazra
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DECLARATION

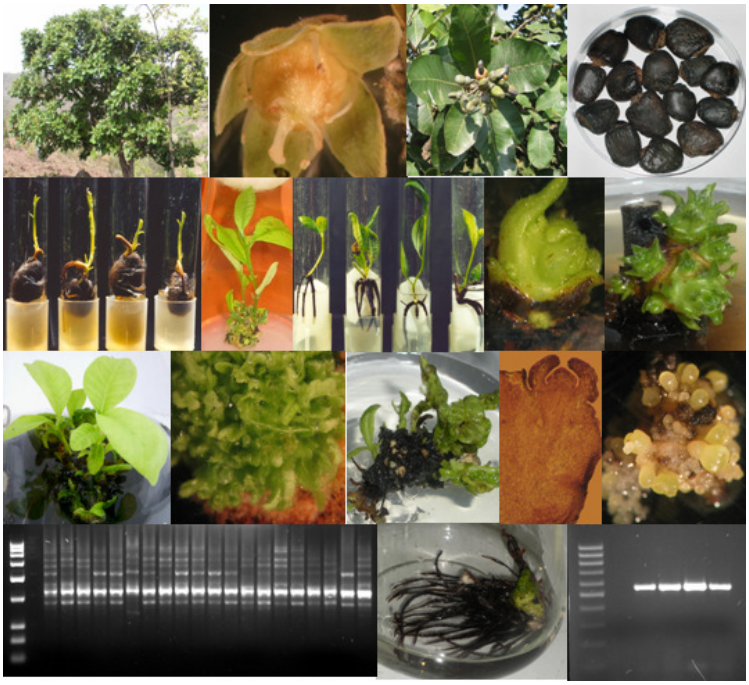
I hereby declare that the thesis entitled **"Studies on *Semecarpus anacardium* L. for *in vitro* regeneration and identification of biologically active compounds"** has been carried out by me in the Plant Tissue Culture Division, National Chemical Laboratory, Pune under the guidance of Dr. Sulekha Hazra. The work is original and has not been submitted in part or full by me for any degree or diploma to any other University. I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

Date: **Bhuban Mohan Panda**
Place: Pune

Key to abbreviations

ANOVA	**Analysis of variance**
BAP	**6-Benzyl amino purine**
°C	**Degree Celsius**
2,4-D	**2,4-Dichlorophenoxyacetic acid**
cm	**Centimeter**
DNA	**Deoxyribose Nucleic Acid**
MS	**Murashige and Skoog medium (1962)**
WPM	**Woody Plant Medium (Llyod and McCown, 1981)**
B5	**Gamborg's medium (1968)**
SH	**Schenk and Hildebrandt (1972)**
GA_3	**Gibberellic acid**
GR	**Growth Regulators**
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)**
ISSR	**Inter simple sequence repeats**
Taxim	**Cefotaxime (antibiotic)**
IBA	**Indole butyric acid**
bp	**Base pairs**
UV	**Ultra violet (light)**
TDZ	**Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)**
IAA	**Indole Acetic acid**
wks	**Weeks**
PVP	**Polyvinyl pyrollidone**
CTAB	**Cetyl Trimethyl Ammonium Bromide**
NAA	**Naphthalene acetic acid**
rpm	**revolution per minute**
sd	**Standard Deviation**
ppm	**Parts per million**
PPM	**Plant Preservative Mixture**
PGR	**Plant Growth Regulator**
µM	**Micro molar**
KN	**Kinetin (6-furfurylaminopurine)**
NS	**Non Significant**
mM	**Milli Molar**
HPLC	**High Performance Liquid Chromatography**
µm	**Micro meter**
YMB	**Yeast maltose Broth**
Picloram (PIC)	**4-amino-3,5,6-trichloro picolinic acid**
H_2SO_4	**Sulfuric acid**
NaOCl	**Sodium Hypochlorite**
MTDDT	**Mature Tree Derived Defoliated Twigs**
hr	**Hours**
min	**Minutes**

ABSTRACT



Abstract

Semecarpus anacardium L. commonly known as marking nut or bhilawa, is a deciduous forest tree native to India. This plant is described in Ayurveda as a potent source of drug against variety of ailments. The fruit nut of *Semecarpus contains* number of alkaloids, flavonoids, biflavonoids, and other compounds. Various formulations of the nut are used against many diseases like atherosclerosis, adjuvant arthritis, and hepato cellular carcinoma. The nut also possesses antitumour activity. Other proven uses of S*emecarpus anacardium* nut are, its cytotoxic activity in cancer anticholesterol activity, antihelminthic action, syphilis and a cure for neurological disorders. Exudes of stem bark valuable for scrofulous, veneral and leprous infection. The resinous liquid from nut can be used in lacquers, varnishes, enamels, tanning materials and ion exchange resins. The Kernel of nut contains 20-25% sweet semidrying non-edible oil. Oil used as wood preservative and as lubricant. The suitability of this oil for production of biofuel is not explored. Vegetable oils can be effectively used as biofuel on transesterification. The available literature on this species is restricted to chemical and medicinal aspects only. Biotechnological approaches offer the potential to alter qualitative and quantitative improvement in the products obtained from trees. There is no literature related to *in vitro* regeneration in this plant. There is a lot of scope for genetic modification of *Semecarpus anacardium* for which there is need to develop a reproducible *in vitro* regeneration system. Till date, no molecular studies have been done on this plant. There was a need for suitable *in vitro* regeneration system and molecular level studies for *in vitro* regeneration, diversity analysis and to understand the metabolic pathways for secondary metabolite production in this plant.

The present research topic "Studies on *Semecarpus anacardium* L. for *in vitro* regeneration and identification of biologically active compounds" was taken to address some of the above limitations. Objectives of the study,

1. Standardization of protocol for clonal propagation from seedling explants.
2. Optimization of conditions for micropropagation of mature trees.
    a. Genetic evaluation of mature trees in natural stands.
    b. Micropropagation of trees and genetic fidelity of the propagules.
3. Standardization of reproducible protocols for *de novo* morphogenesis.
4. Protocols for genetic transformation using *Agrobacterium rhizogenes* to establish hairy root culture.
5. Studies on isolation, purification and quantification of some of the chemical compounds.

The data generated from this research program is compiled in seven chapters of this thesis. The literature used in course of the research is listed in the section "References". A summary of the outcome of this research and the list of reports generated from the data are appended at the end of the thesis.

Chapter 1: Introduction

This chapter includes literature survey on *in vitro* morphogenesis on tree species, latest trend in molecular studied on *in vitro* morphogenesis of plants particularly trees. Molecular marker studies on plants for diversity/fidelity analysis. Genetic transformation of trees with different genes to get transgenic plants has been described. Establishment of hairy root culture for isolation of bioactive compounds from different plant was enlisted in this chapter. Tissue culture approach for secondary metabolite productions also included in this chapter. Detail literature survey on *Semecarpus anacardium* and the significance and the objectives of the study are included in this chapter.

Chapter 2: Materials and Methods

In this chapter, general methodology followed throughout the course of study has been explained. The source of various chemicals, glassware, plasticware and other materials utilized during the course of work, glassware cleaning and preparation, basic media compositions and preparation, DNA isolation, PCR amplification, culture conditions etc. have been discussed. Materials and methodology related to specific experiments and studies have been included separately in respective chapters.

Chapter 3: Aseptic germination of seedlings and micropropagation from seedling explants.

Mature nuts of *Semecarpus anacardium* were collected from the trees growing in the natural forests. Germinating the seeds under aseptic condition was the major constraint and the seeds isolated from the pseudo fruits needed elaborate surface sterilization treatments to eliminate the microbes prior to culture for germination. Concentrated H_2SO_4 treatment for 20 minutes followed by bavistin, savlon and sodium hypochlorite were essential to obtain around 30% of sterile cultures. On testing several media formulations germination was achieved in 60% of the seeds in half strength WPM medium without sucrose. Incorporation of growth regulators remained ineffective in increasing the germination frequency.

Growth regulators (GR) tested for establishment of shoot cultures from seedling derived nodal explants included, 6-benzyl amino purine (BAP), Kinetin (KN) and Thidiazuron (TDZ). Thidiazuron and BAP supported proliferation of multiple buds. Among the concentrations tested, BAP 4.44µM produced increased number of multiples. A combination of BAP 4.44µM and KN 4.64µM was optimum considering the overall response in the nodal buds. Nodal explants were cultured for four weeks in growth regulator containing medium followed by transfer to growth regulator free WPM medium every four weeks till the shoot buds become 1-3 cm in length. Single exposure of the explants in 2.27µM TDZ followed by repeated transfers in GR free, charcoal containing WPM medium induced optimum number of multiples per culture. *In vitro* raised shoots developed brittle roots in charcoal containing agar/phytagel gelled WPM medium

limiting the survival of the plants in soil. This limitation was overcome by using liquid medium for rooting. From the three auxins tested, IBA was more effective for induction of roots than IAA and NAA. Rooting was 100% in half strength liquid WPM medium with 2.46µM of IBA. Rooted plants survived on transfer to sand soil 1:1 mixture were hardened and acclimatized in green house. The data generated from these experiments were compiled and communicated for 3 publications.

Chapter 4: Clonal propagation from mature tree derived nodal explants.

(a) Mature trees of *Semecarpus anacardium* were identified from 5 locations. DNA was isolated from the tender leaves of these plants and PCR amplification of the DNA was performed using 100 ISSR primers. Fifteen primers show good reproducible amplification and polymorphism. Analysis of the ISSR-PCR data demonstrated high degree of variation within the trees. This observation advocates the need for reliable regeneration protocol for rapid propagation of elite genotypes.

(b) Mature tree derived nodal buds were collected during the month of April, May and June. Like in seeds, the major constraint encountered in mature tree derived tissues was to obtain microbe free explants for culture. After extensive trials, a procedure involving fungicide, antibiotic and sodium hypochlorite treatments was standardized to obtain 50-70% microbe free explants.

Bud-break in mature plant derived nodal cuttings was observed after 7-15 days of culture in WPM medium and with growth regulators GA_3, BAP, KN, TDZ singly and in combinations of BAP and KN. Media containing TDZ were more effective. Explants pre-cultured in TDZ, sprouted producing multiples buds which differentiated and elongated in growth regulator free WPM medium containing antibiotics for 4 passages of 4wks each. Similar to the seedling culture derived shoots (Chapter 3), rooting was induced in half strength liquid WPM medium with IBA. Rooting was 100% in medium with 7.38µM IBA. Rooted shoots survived in sand:soil mixture and the hardened plants were transferred to green house. Fifteen clonally propagated plants from a single mature tree were analyzed for clonal fidelity using the selected ISSR primers as described in section (a). All the clones show similar banding pattern. The data generated is being compiled for publication.

Chapter 5: *De novo* morphogenesis in cotyledon explants of *Semecarpus anacardium*

Surface sterilized nuts were cut and the cotyledons were separated. These were cultured in WPM basal medium supplemented with varying concentrations of BAP, KN, 2,4-D, PIC, IAA, IBA, NAA, GA_3 and TDZ. Caulogenic buds and embryogenic mass like structures appeared on the surface of the cotyledons in TDZ containing media. The caulogenic buds differentiated to form shoot primordia and shoot in GR free WPM with charcoal (0.2%). Differentiation of the buds to shoot was slow and needed 4-5 passages of four weeks each to achieve the height of 3-4cm. The shoots rooted in half strength liquid WPM with IBA. Plantlets were hardened and transferred to

green house. Histological studies confirmed the *de novo* origin of the caulogenic buds.

In TDZ containing medium, caulogenic response in the cotyledon explants was associated with formation of well defined globular cell masses and calli. Bipolar and heart shaped embryo like structures appeared from these globular masses on culturing in WPM medium with charcoal for several passages. Callus formed on the cotyledon explant was compact and gave rise to small embryo like structures after several passages in WPM medium with charcoal. Efforts made to convert these embryo-like structures to plantlets remained futile. These structures either dedifferentiated into callus or gave rise to more embryo like structures in each passage in growth regulator free medium.

Chapter 6: Identification of biologically active compounds in *in vitro* cultured tissues.

Amentoflavone, a biflavonoids having potential anticancerus, neuroprotective, antiviral and anti-inflammatory activity, was detected in the leaves of the plants developed and maintained *in vitro*. Cotyledon-derived callus tissue also produces Amentoflavone. The biflavnoid was not detected in roots and stems of *in vitro* raised plants. Increased Amentoflavone was detected in the shoot cultures maintained in 4% sucrose containing medium. Among the carbon sources tested, maltose at 2% produced more amentoflavone.

Chapter 7: Genetic transformation of *Semecarpus anacardium* using *Agrobacterium rhizogenes.*

Three *Agrobacterium rhizogenes* strains (ATCC15834, LBA9402, A4) were tested for transformation of *Semecarpus anacardium*. Experiments were conducted using leaves, internodal segments and *in vitro* raised shoot explants. Surface of the explants was pricked repeatedly with a sterile needle, keeping the explant in bacterial suspension. Rhizogenesis was observed in explants co-cultured with strain ATCC15834 for 30 minutes for infection followed by co-cultivation for 4 days. Root induction was noted after 20-25 days of culture in antibiotic containing medium. Roots developed in clusters from the site of infection. From the three explants tested shoots was more responsive. Among the bacterial strains ATCC15834 was more virulent than A4 and LBA9402. DNA was isolated from the roots. Integration of *rol* genes (A, B and C) was confirmed by PCR. The growth of the hairy roots was slow.

Summary:

The important observation of the research proposal **"Studies on *Semecarpus anacardium* L. for *in vitro* regeneration and identification of biologically active compounds"** has been presented in this section.

CHAPTER 1

INTRODUCTION

Since ancient time human depends on plant world to fulfill most of the requirements. The most basic needs, food, shelter and clothing are still provided by plants. Apart from these basic needs man's dependence on plants are many fold in terms of fire wood, furniture, aromatic compounds, medicine, fodder for domestic animal and other minor products. Plants are one of the most important sources of medicine (Tripathi and Tripathi 2003). 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. Vegetable oils of some tree species are in the process of utilization for the commercial production of biofuels. Tree borne edible oil seeds are also used in cosmetic and Pharmaceutical industries. Moreover plant polysaccharides are also used for the production of fuel-grade ethanol. In addition plants produce a diverse assortment of organic compounds that do not participate directly in its growth and development. These substances, traditionally called secondary metabolites, are often useful for various purposes. Economically important plants serves as source of industrial oils, resins, tannins, saponins, natural rubber, gums waxes, dyes, pharmaceutical and many specialty products. The essential and useful raw products derived from trees have potential to drive demand for greater tree productivity.

Most medicinal plants especially trees are not cultivated and are collected from wild. Thus the increased commercial demand leads to indiscriminate collection of plant parts. This leads to serious threat to the existence of plants when reproductive parts like inflorescences and seeds are harvested. Thus, there is a need for cultivation and conservation of medicinally important trees. There has been relatively little effort towards crop improvement either through conventional breeding or application of biotechnological approach, considering the potential usefulness demonstrated by woody trees. 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, vegetables and ornamental plants, the tree species have been left far behind (Tzfira *et al*. 1998).

LIMITATIONS OF CONVENTIONAL TREE BREEDING

In contrast to agronomic and horticultural species, the long regeneration cycle 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 period between seed germination and flowering. Most of the useful tree traits can be assessed only when the tree reaches sexual

maturity. Limited knowledge on the genetic maps of most trees is yet another limitation. There is lack of knowledge on genes for hybridization and expression of new traits, which are responsible 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 (Tzfira *et al.* 1998). For majority of trees, traditional breeding approaches are simply not a realistic means for achieving genetic improvement (Dean *et al*.1997).

POTENTIALS OF PLANT BIOTECHNOLOGY

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):

1. *In vitro* aseptic germination either to get microbe free seedling or to improve the germination frequency of the seeds.
2. Clonal propagation of superior genotypes using tissue-culture techniques.
3. Somatic-cell techniques such as somatic hybridization, exploitation of somaclonal variations and to induce mutation.
4. Molecular breeding to complement classical breeding (use of marker-assisted selection).
5. Direct introduction of genes for specific traits via genetic engineering.
6. Production of important high value secondary metabolites of medicinal importance through hairy root culture or in bioreactor.

Development of **tissue culture techniques** and molecular genetics allowed the exploration of new pathways for the improvement of trees that could compensate 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 limited time frame. *In vitro* regeneration of organs, tissues, cells, protoplasts is not seen as an alternative to stem cuttings for commercial propagation, 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 isolated virtually from any living being, from viruses to higher plant to animals and inserted into desired tree, thus broadening the range of genes available outside the current boundaries of the genome. (b) Individual 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 (Peña and Séguin 2001).

Advanced biotechnological methods for **genome analysis** and identification of novel **molecular markers** have been successfully applied to the model tree species, Poplar (Taylor 2002; Jansson and Douglas 2007). 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 polymorphism 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 can be identified (Dean *et al*. 1997; Jones *et al*. 2009). 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, ETS and Microsatellites have been widely used to produce dense genetic maps of different *Populus* species (Confalnieri *et al.* 2003). 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 needed 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 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. It also provides an alternative for 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). The term *in vitro* culture covers a wide range of techniques under sterile conditions including seed germination, micropropagation, meristem culture and callus culture. Germination of seeds in some species can be greatly increased by the use of *in vitro* methods, where low or no germination is achieved using conventional techniques, due to dormancy or specific germination requirements. This phenomenon is attributed to three mechanisms including hard seed coverings, chemical inhibitors including growth regulators, phenolics, etc. and morphological aspects such as undeveloped embryos. This character of the seeds limits the success of conventional propagation. *In vitro* techniques are useful tools to supplement propagation of desirable trees for planting out and for research (Sansberror *et al.* 2003).

Seed dormancy and the capacity of seeds to germinate are often associated with the seed coat. This factor is checked first if seeds do not germinate (Budy *et al*. 1986; Velempini *et al*. 2003). A

hard seed coat causes exogenous dormancy in seed and influences germination negatively by establishing a barrier that interferes with the water uptake required for imbibition, and gaseous exchange for respiration (Yang *et al*. 2007). To improve the germination frequency various pre-treatments for seeds are described (**Table 1.1**). When fewer seeds are available, it is often necessary to use the proliferation stage to provide the required number of plants. The resulting proliferating cultures can then be treated as standard micropropagation cultures (Fay 1994).

Table 1.1 Various pre-treatments used for optimum *in vitro* germination of seeds of plants.

Plant species	Pre treatment	Time Duration	Reference
Terminalia sericea	Soaking in water	24 hrs	Likoswe *et al.* 2008.
	Fire and soaking	24 hrs	
Areca triandra	H_2SO_4	98%, 15 min	Yang *et al.* 2007.
	H_2O_2	10% 20 min	
Sesbania sesban	H_2SO_4	-----	Dan and Brix 2007.
Astragalus cyclophyllon	H_2SO_4	98%, 10 min	Keshtkar *et al.* 2008.
	Hot water	80ºC, 5 min	
Piliostigma thonningii	H_2SO_4	98%, 15 min	Ayisire *et al*. 2009
	Physical scarification	------	
Gmelina arborea, Bauhinea variegata, Dalbergia latifolia and Hardwikia bipinnata	Hot Water	50ºC	Mahendra *et al.* 2005.
Acacia koa	Mechanical scarification and Soaking	24 hrs	Tanabe and Honda 1999.
Annona cherimola	Soaking	24 and 48 hrs	Padilla and Encina 2003.
Pistachia khinjuk	H_2SO_4	20 min	Baninasab and Rahemi 2008
Helianthemum sp.	H_2SO_4	5 min	Perez-Garcia and Gonzalez-Benito 2006.
	Dry heat (100ºC)	10 min	
	Hot water (80ºC)	5 min	
Tithonia diversifolia	Hot water (80ºC)	10 min	Akinola *et al.*2000.
	Hot water (100ºC)	10 min	
Koelreuteria paniculata	GA3 (100 PPM)	Germinated in the medium	Rehman and Park 2000.
	GA3(100 PPM)+ chilling at 4ºC	30 days	
Tamarindus indica	49% H_2SO_4	60 min	Muhammad and Amusa 2003.
	Hot water (100ºC)	60 min	
Pongamia pinnata	Hot water	30 min	Kumar *et al.* 2007.
Cassia occidentale Indegofera astragalina Sesbania pachycarpa	H_2SO_4	15, 30, 60 min	Sy *et al.* 2001.
Psidium guajava	5% H_2SO_4	12 hrs	Ali *et al*. 2007.

The primary objective of *in vitro* propagation or micropropagation is to capture the total genetic superiority of the parent material. 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 modified trees/selected elite variety that arises as a single plant (for field-testing and commercialization).
- Vegetative propagation of difficult-to-propagate species (Abortion of fertilized embryo, less seed viability, poor germination etc.).
- Year round production of clones.
- Large number of plants can be produced in a small space.
- Physical, chemical and environmental factors can be greatly controlled.
- Production of genetically uniform plants large-scale planting in seed orchards to facilitate production of superior quality seeds.
- Elimination of pathogens from the infected stock using meristem culture.
- Possibilities of rejuvenation and invigoration of mature tissues.

Micropropagation systems fall into three broad categories: Axillary bud proliferation, *De novo* organogenesis and Somatic embryogenesis (SEs) (Dean *et al.* 1997). 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:

In this method, development and multiplication of the growing meristem is achieved. The principal advantage of axillary shoot methods for tree multiplication is that all propagules are derived from preformed buds, thereby enhancing the likelihood that propagules will be true to type. The economical and ecological importance of forest trees necessitates the application of this technique for their clonal propagation (Tomar and Gupta 1988b). With concerted efforts, micropropagation protocol using pre-existing meristem from mature tissues of many of the woody tree species has been achieved (**Table 1.2).**

Table 1.2 Reports on clonal propagation in woody tree species

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Acacia albida	Root	Shoot regeneration	Plant in soil	Ahee and Duhoux 1994
Acacia catechu Willd.	Mature nodal segment	Shoot proliferation	Plant in soil	Kaur *et al*. 1998
	Shoot tip	Shoot proliferation	Plant in soil	Kaur and 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 and 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
A. mearnsii	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
Aegle marmelos	Shoot nodes	Shoot regeneration	Plantlet	Ajithkumar and Seeni 1998
Albizzia julibrissin	Roots	Shoot regeneration	Plantlet	Sankhla *et al*. 1996
	Root segment	Shoot regeneration	Plant in soil	Hosseini-Nasr and Rashid 2002
Annona squamosa	Mature and Seedling	Shoot proliferation	Plant in soil	Lemons and Blake 1996
Anacardium occidentalae	Shoot nodes	Shoot proliferation	Plant in soil	Boggetti *et al*. 1999
	Seedling explants	Shoot proliferation	Plant in soil	Das *et al.*1996

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Artocarpus heterophyllus	Shoot tip	Shoot proliferation	Plant in soil	Amin and Jaiswal 1993
Artocarpus altilis	Mature nodal buds	Shoot proliferation	Plant in soil	Murch *et al*. 2008
Azadirachta indica	Seedling explants	Shoot proliferation	Plant in soil	Thakur *et al*. 1998
Balanites aegyptica L.	Seedling explants	Shoot proliferation	Plant in soil	Siddique and Anis 2009
Bauhinia purpurea L.	Mature Explants 15-18 year old tree	Callus regeneration	Plant in soil	Kumar 1992
Bauhinia. variegata L.	Mature nodal explants 6-8 year old tree	Axillary shoot proliferation	Plant in soil	Mathur and Kumar 1992
Catalpa ovata	Nodes	Axillary shoot proliferation	Plant in soil	Lisowska and Wysokinska 2000
Caelastrus paniculatus Wild.	Mature nodal buds 12 year old tree	Shoot proliferation	Plant in soil	Martin *et al*.2006
Caesalpinia pulcherrima Sw.	Mature (20 yrs.) nodal bud	Shoot proliferation	Plant in soil	Rahman *et al*. 1993
Cedrela fissilis Vellozo	Seedling explants	Shoot regeneration	Plant in soil	Da costa Nunes *et al*. 2003
Cercis yunnanensis	Seedling explants	Shoot regeneration	Plantlet	Cheong and Pooler 2003
Ceropegia candelaebrum	---	Shoot regeneration	Plantlet	Beena *et al*. 2003
Ceratonia siliqua	Mature buds from12 year old female	Shoot regeneration	Plant in soil	Romano *et al*. 2002
Cinnamomum camphora	Mature shot top and nodal explants of 12 year old tree	Shoot regeneration	Plant in soil	Babu *et al*. 2003
Crataeva nurvala	Mature explants	Shoot regeneration	Plant in soil	Walia *et al*.2007
Dalbergia sissoo Roxb.	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
	Seedling,shoot tip, nodes of *in vitro* shoots	Shoot proliferation	Plant in soil	Ravishankar and Chandra 1989
	Mature Nodal explant of 60-80 yr old tree	Shoot proliferation	Plant in soil	Raghava Swamy *et al*.1992

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Eucalyptus tereticornis	4 year old trees	Shoot proliferation	Plant in soil	Sharma and Ramamurthy 2000
Ficus carica	Shoot tip from 10 year old tree	Shoot proliferation	Plant in soil	Pasqual and Ferreira 2007
Gmelina arborea Roxb.	Nodal buds from seedling	Shoot proliferation	Plant in soil	Sukartiningsih *et al*.1994
Juglans regia L	Nodal buds from seedling	Shoot proliferation	Plant in soil	Saadat and Hennerty 2002
Lichi chinensis	Nodal buds from seedling	Shoot proliferation	Plant in soil	Das *et al.* 1999
Litsea cubeba	Stem cutting of 6 year old tree	Shoot proliferation	Plant in soil	Mao *et al.* 2000
Mimosa tenuiflora (Willd.) Poiret	Axillary node	Shoot Proliferation	Plant in soil	Villarreal and Rojas 1996
Pistacia vera L.	Mature nodal bud	Shoot proliferation	Plant in soil	Onay 2003
Murraya koenigii	Mature nodal buds	Shoot proliferation	Plant in soil	Babu *et al*.2000
Myrica esculenta	Mature nodal buds	Shoot proliferation	Plant in soil	Bhatt and Dhar 2004
Ocotea bullata	Mature nodal buds	Shoot proliferation	Plant lets	Kowalski and van Staden 2001
Oroxylum indicum	Cotyledonary nodes	Shoot proliferation	Plant in soil	Dalal and Rai 2004
Peltophorum pterocarpum (DC.) Backer ex K. Heyne	Shoot tip, nodal segment, cotyledonary node	Shoot proliferation	Plant in soil	Uddin *et al*. 2005
Petrocarpus Santalinus L.	Mature explants	Shoot proliferation	Plant in soil	Prakash *et al.*2006
Pinus pinea	Mature axillary buds	Shoot proliferation	Plant in soil	Cortizo et al.2009
Pinus pinaster	Mature buds	Shoot proliferation	Plant in soil	Diego *et al.* 2008
Pinus sylvestris	Mature Explants	Shoot proliferation	Plant lets.	Andersone and Ievinsh 2002
Pittosporum napaulensis (DC)	Mature Explants	Shoot proliferation	Plant in soil	Dhar *et al*. 2000
Populus sp.	Explants from Green house raised plants	Shoot proliferation, organogenesis	Plant lets	Noel *et al*.2002
Populus trichocarpa	Shoot tips	Shoot proliferation	Plant in soil	Kang *et al*. 2009
Poinciana regia (Boj.)	Seedling explants	Callus	Plant in soil	Bajaj 1989

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Prosopis alba Griseb	Seedling shoot tip, nodal stem segment	Shoot proliferation	Plantlet	Jordan 1987
P. tumarugo F. Phil.	Seedling shoot tip or nodal stem segment	Shoot proliferation	Plantlet	Jordan 1987
P. chilensis	Young and mature nodal buds	Shoot proliferation	Plant in soil	Caro *et al.* 2002
Prunus fruticosa	Bud from 10 year old tree	Shoot proliferation	Plant in soil	Pruski *et al*. 2005
Prunus armeniaca L.	Meristem from mature buds	Shoot proliferation	Plant in soil	Perez-Tornero and Burgos 2007
Prunus tomentosa	Bud from 10 year old tree	Shoot proliferation	Plant in soil	Pruski *et al.* 2005
Prunus avium	Mature explants	Shoot proliferation	Plant in soil	Durkovic 2006
Psidium guajava	Shoot Tip	Shoot proliferation	Plant in soil	Amin and Jaiswal 1986
Psoralea corylifolia L.	Cotyledonary node	Shoot proliferation	Plantlet	Jayakumar and Jayabalan 2002
	Ex vitro seedling	Shoot proliferation	Plant in soil	Faisal *et al*. 2006
Pterocarpus marsupium	Cotyledonary node	Shoot proliferation	Plant in soil	Chand and 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
Pongamia pinnata	Mature explant	Shoot proliferation	Plant in soil	Sujatha and Hazra 2007
Robinia pseudoacacia	Hypocotyl	Shoot proliferation	Plantlet	Hosseini-Nasr 2003
Robinia pseudoacacia	Mature nodal buds	Shoot proliferation	Plantlets in soil	Gou *et al*.2006
	Hypocotyl	Shoot proliferation		Hosseini-Nasr 2003
Robinia ambigua	Mature Explants	Shoot proliferation	Plant in soil	Zhang *et al*. 2007

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Salix pseudolasiogyne	Nodes from 20 year old tree	Shoot proliferation	Plant in soil	Park *et al.* 2008
Sesbania drummondii	Nodal explant from seedling	Shoot proliferation	Plant in soil	Cheepala *et al*. 2004
Sesbania rostrata	Seedlings stem, leaves, roots of micropropaga-ted plant	Callus Caulogenesis	Plantlet	Pellengrineschi and Tepfer 1993
Simmondsia chinensis	Mature explants	Shoot proliferation	Plant in soil	Singh *et al*. 2008
Sophora toromiro	Seedling explants	Shoot proliferation	Plantlet	Jordan *et al*. 2001
Stereospermum personatum D.C	Seedling explants	Shoot proliferation	Plant in soil	Shukla *et al*. 2009
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 and Schaffiner 1995
Syzygium cumini	Shoot tip	Shoot proliferation	Plant in soil	Yadav *et al*. 1990
Tamarindus indica L.	Seedling Shoot tip	Shoot proliferation	Plant in soil	Kopp and Nataraja 1990
Taxus mairei	Mature nodal buds	Shoot proliferation	Plant in soil	Chang *et al*. 2007
Tamarindus indica L**.**	Seedling Shoot tips, nodes of shoot regenerated from shoot tip	Shoot proliferation	Plant in soil	Jaiwal and Gulati 1992
	Nodal explants from Seedling	Shoot proliferation	Plant in soil	Kopp and Nataraja 1992
Ulmus minora	Mature nodal explants	Shoot proliferation	Plant in soil	Conde *et al*. 2008
Ulmus parvifolia	Nodal buds from 20 year old plant	Shoot proliferation	Plant in soil	Thakur and Karnosky 2007
Warburgia salutaris	Mature nodal buds	Shoot proliferation	Plantlets	Kowalski and van Staden 2001

With the research progress in plant genetics and molecular biology, our understanding of *in vivo* plant shoot meristem development, plant cell cycle, and cytokinin signal transduction has advanced significantly. These research advances have provided useful molecular tools and resources for the recent studies on the genetic and molecular aspects of *in vitro* shoot

organogenesis. A few key molecular markers, genes, and probable pathways have been identified from these studies that are shown to be critically involved in *in vitro* shoot organogenesis. Furthermore, these studies have also indicated that *in vitro* shoot organogenesis, just as in *in vivo* shoot development, is a complex, well-coordinated developmental process, and induction of a single molecular event may not be sufficient to induce the occurrence of the entire process. Further study is needed to identify the early molecular event(s) that triggers dedifferentiation of somatic cells and serves as the developmental switch for *de novo* shoot development (Zhang and Lemaux 2004). In shoot meristem development, several regulatory genes have been identified that include maize KNOTTED1 (KN1) (Vollbrecht *et al.* 1991), *Arabidopsis* SHOOT MERISTEMLESS (STM) (Long *et al.*1996), WUSCHEL (WUS) (Laux *et al.* 1996; Mayer *et al.* 1998), and CLAVATA 1-3 (CLV1-3) (Leyser and Furner 1992; Clark *et al.* 1996, 1997; Kayes and Clark 1998; Fletcher *et al.* 1999). Evidence has also been presented that interactions exist at the molecular level among these three processes: cytokinin reception, cell cycle, and shoot meristem development (Riou-Khamlichi *et al.* 1999; Rupp *et al.* 1999). With the identification of these critical genes from plant genetic and molecular studies can be done by using these as useful molecular markers to understand *in vitro* shoot organogenesis at the molecular level. Genetic and molecular approaches have also been directly used in defining the molecular elements more directly involved in shoot organogenesis *in vitro* (Zhang and Lemaux 2004).

De novo Morphogenesis:

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

Organogenesis: Organogenesis is the formation of wide range 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 induction of localized meristematic activity by treatment with growth regulators. This leads to formation of primordium and eventually the formation of organs. Like axillary bud proliferation, juvenile tissues have been reported as capable of regeneration via organogenesis in number of woody species. In these studies, explants have been prepared from tissues as diverse as foliar disks, cotyledons, hypocotyl sections, and nodal stem sections etc. Organogenesis has been used to produce transgenic trees of poplar (Jonsson and Douglas 2007), Eucalyptus sp. (Poke *et al.* 2005) Birch, Liquidambar sp., black locust (Igasaki *et al.* 2000), and *Ulmus spp.* (Gartland *et al.* 2000).

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 provides an attractive model system for studying zygotic embryogenesis, particularly because zygotic embryos are encased by maternal tissues and are difficult to access using biochemical and

molecular tools. In contrast to zygotic embryogenesis, somatic embryogenesis is a nonsexual propagation process where somatic cells differentiate into somatic embryos. Thus somatic embryogenesis has been viewed as a tool for massive propagation of commercial crops and as a potential model system for the study of the regulation of gene expression required for the earliest developmental events in the life of higher plants, such as the developmental mechanism of embryogenesis (Zhu and Perry 2005). Somatic embryogenesis has been cited by many authors as the *in vitro* regeneration system of choice for economial production of clonal populations of forest trees (Gupta *et al*. 1991; Nehra *et al*. 2005). A major advantage is the potential for very high frequency regeneration, as well as their liquid cultures allow 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.

Somatic embryogenic cultures of woody species often initiated from seed or seedling tissues (Giri *et al*. 2004). Studies employing immature embryo or seed explants established that developmental stage of the embryo explant was critical in determining embryogenic response (Trigiano 1992; Dunstan *et al*. 1995; Paiva neto *et al*. 2002; Arulselvi and Krishnaveni 2009; Prakash and Gurumurthi 2009). 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 plants to achieve high probability of success, high conversion frequency, high reproducibility and production of normal somatic embryos. *De novo* morphogenic regeneration systems via organogenesis and embryogenesis (both direct and indirect) have been developed for a number of tree species (**Table 1.3)**.

Table 1.3 Reports on morphogenetic studies in trees.

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Acacia arabica	Immature zygotic embryos	Indirect embryogenesis	Plant in soil	Nanda and Rout 2003
Acacia catechu Willd.	Immature cotyledon	Indirect embryogenesis	Plant in soil	Rout *et al.* 1995
A. magnium	Cotyledon	Direct organogenesis	Plant in soil	Douglas and Mcnamara 2000
A. mangium Willd.	Immature zygotic embryo explants, seedling	Indirect organogenesis	Plant in soil	Xie and Hong 2001
A. nilotica (Linn.) Willd.	Immature endosperm	Indirect embryogenesis	Plantlet	Garg *et al*. 1996
A. sinuata	Seedling hypocotyl	Indirect organogenesis	Plant in soil	Vengadesan *et al.* 2000
	Cotyledon	Indirect organogenesis	----	Vengadesan 2003
Aegle marmelos	Zygotic embryo	Direct embryogenesis	Plant in soil	Islam *et al*. 1996
Albizzia lebbeck L.	Seedling root, hypocotyl	Indirect organogenesis	Plantlet	Upadhyaya and Chandra 1983
	Seedling hypocotyl, cotyledon, root, leaf rachis	Indirect Organogenesis	Plant in soil	Varghese and Kaur 1988
	Mature Petiole, stem	Indirect organogenesis	Plant in soil	Gharyal and Maheswari 1990
A. procera	Seedling leaflet	Direct organogenesis	Plant in soil	Kumar *et al.* 1998
	Seedling hypocotyl, cotyledon, root,	Indirect Organogenesis	Plant in soil	Varghese and Kaur 1988
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.richardiana King.	Seedling hypocotyl	Organogenesis Indirect embryogenesis	-- Plantlet	Tomar and Gupta 1988a
Artocarpus heterophyllus	cotyledons and epicotyls	Indirect embryogenesis	Plantlet	Roy and Debnath 2005

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Alhagi camelorum Fitch.	Seedling cotyledon, leaf, root, stem	Direct and Indirect organogenesis	--	Bharal and Rashid 1981
Azadirachta indica	Callus	Indirect organogenesis	Plant in soil	Gautam *et al*. 1993
	Cotyledon	Indirect organogenesis	Plant in soil	Su *et al.*1997
Bauhinia purpurea L.	Mature stem segments 15-18 yr. old tree	Callus regeneration	Plant in soil	Kumar 1992
Betula platyphyllla	Protoplast	Callus regeneration	---------	Watika *et al*. 1996
Calliandra tweedii	Internodal, petiolar explants	Direct embryogenesis	Plant in soil	Shashi kumar *et al*. 2002
Castanea dentana	Ovules	Indirect embryogenesis	------	Xiang *et al*. 1999
Dalbergia. latifolia Roxb.	Shoot callus of 5-yr old tree	Indirect organogenesis	Plant in soil	Lakshmi Sita *et al.*1986
	Seedling Hypocotyl	Indirect organogenesis	Plantlet	Sudhadevi, and Nataraja, 1987a; 1987b
	Mature Shoot callus	Callus regeneration	Plant in soil	Ravishankar and Chandra, 1988
Dalbergia. latifolia Roxb.	Seedling hypocotyl segment	Indirect regeneration from cell suspension derived callus	Plant in soil	Pradhan *et al*. 1998
Dalbergia sissoo Roxb.	Seedling Hypocotyl	Indirect organogenesis	Plantlet	Sharma and Chandra 1988
	Cells of cambium 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
Eucalyptus camaldulensis	Mature zygotic embryos and cotyledons	Indirect Embryogenesis	Plant in soil	Prakash and Gurumurthi 2009
Eucalyptus tereticornsis	Cotyledons	Indirect Embryogenesis	Plant in soil	Prakash and Gurumurthi 2004
Eucalyptus globulus Labill.	Cotyledons	Indirect Embryogenesis	Plantlets	Pinto *et al*. 2002
Eucalyptus globulus	Mature zygotic embryos	Direct embryogenesis	Plantlets	Pinto *et al*. 2008

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Elaeagnus angustifolia L.	Immature-cotyledon	Indirect organogenesis	Plant in soil	Karami *et al*. 2009
Hardwickia binata	Zygotic embryos	Direct embryogenesis	Plantlet	Chand and Singh 2001
Helicteres isora L.	Nodal explants	Indirect organogenesis	Plant in soil	Shriram *et al.* 2008
Indigofera enneaphylla L.	Cotyledon	Indirect organogenesis	Plantlet	Bharal and Rashid 1979 1981
Litchi chinensis Sonn.	Young leaflets vegetative flushes mature tree	Indirect Embryogenesis	Plant in soil	Raharjo and Litz 2007
Leucaena leucocephala	Cotyledon	Shoot proliferation	Plantlet	Saffi and Borthakur 2002
M. tenuiflora (Willd.) Poiret	Root segment	Direct organogenesis	Plantlet	Kackar *et al.* 1992
	Hypocotyl segments, cotyledons	Somatic embryogenesis and shoot buds		
Myrciaria aureana	Cotyledon	Direct embryogenesis	---	Motoike *et al*. 2007
Parkia timoriana	Cotyledon	Indirect somatic embryogenesis	--	Thangjam and Singh 2006
Pistacia vera L.	Mature leaves	Direct organogenesis	Plant in soil	Tilkat *et al*. 2009a Tilkat and Onay 2009b
Prunus avium L.	Leaves and internodes	Direct organogenesis	Plant in soil	Matt and Jehle 2005
Populus ciliata	Leaves	Shoot regeneration	Plant in soil	Thakur and Srivastava 2005
Populus deltoides	Leaves, roots, internodes	Direct organogenesis	Plant in soil	Yadav *et al*. 2009
Robinia pseudoacacia	Zygotic embryo	Embryogenesis	Plantlet	Merkle and Wiecko 1989
Robinia pseudoacacia	Immature seeds	Indirect embryogenesis	Plant in soil	Arrillaga *et al.* 1994
Sesbania aculeata (Pers)	Seedling hypocotyl	Adventitious Organogenesis	Plant in soil	Bansal and Pandey 1993
Sesbania bispinosa (Jacq.) W.F. Wight	Seedling hypocotyl, cotyledon	Direct organogenesis	Plant in soil	Kapoor and Gupta 1986
Sesbania grandiflora	Seedling hypocotyl, cotyledon	Indirect organogenesis	Plantlet	Shankar and Ram 1990

Plant species	Explant	Pathway of morphogenesis	Status	Reference
Sesbania grandiflora	Cotyledon pieces	Direct organogenesis	Plant in soil	Detrez *et al.* 1994
Swainsona salsula.	Cotyledon	Direct Organo genesis	Plant in soil	Yang *et al*. 2001
Tamarindus indica L.	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

The patterning mechanisms responsible for generating any plant organ are complex and involve many gene hierarchies, and co-ordinated spatially and temporally regulated programmes of cell divisions, gene expression, and hormone function. Historically, it has been observed that there are genetic and also physiological factors that trigger *in vitro* embryogenesis in various types of plant somatic cells. Number of genes and proteins involved in SEs had been discovered so far (Raghavan 2006; Karami *et al.* 2009). However, the molecular bases of those triggering factors and the genetic and biochemical mechanisms leading to *in vitro* embryogenesis are still unknown (Vergne and Dumas 2000; Nakano *et al*. 2006; Karami *et al.* 2009). Understanding the key factors promoting vegetative-to-embryogenic transition and identification of genes involved in the induction of competence for embryogenesis and subsequent embryo development presents a challenge for modern molecular biology. Currently, the trend is towards recognition of embryo development that is comprised of variety of different stages involving specific gene expression (Goldberg *et al*. 1989; Hughes and Galau 1989; Kermode 1990; Feher 2008).

During the past two decades, considerable efforts have been made to identify genes with altered expression patterns during SEs. Various systems have been exploited to understand the mechanisms of gene regulation during SEs and carrot has served as the model system (Chugh and Khurana, 2002; Feher *et al.* 2003; Ikeda *et al.* 2003, 2004; Raghavan 2006; Zhu and Perry 2005; Quint and Gary 2006). Analysis of the proteome and transcriptome has led to the identification and characterization of certain genes involved in SEs (Zdravkovic-Korac *et al.* 1999; Chugh and Khurana 2002; Thibaud-Nissen *et al.* 2003; Ben *et al*. 2005; Imin *et al.* 2005; Rensing *et al.* 2005; Che *et al.* 2006 a, b; Srinivasan *et al.* 2007). Most of these genes, however, are up-regulated only in the late developmental stages, suggesting that they do not play a direct role in the vegetative to-embryogenic transition (Karami *et al.* 2009). The general aspects about the specific genes involved in SEs are reviewed (Phillips *et al*. 2004; Feher 2008). The discovered genes are listed in **Table 1.4**

Table 1.4 Genes involved in organogenesis and embryogenesis

Gene	Putative function	References
CYCD3	Involved in acquisition of competence for organogenesis	Sugiyama 1999; Fletcher 2002
SRD3 SRD1, SRD2	Competence for dedifferentiation of shoots	Sugiyama 1999,2000
ESR1	Enhances shoot regeneration, vegetative-to-organogenic transition	Zuo *et al*. 2002
CRE1 CK11	Cytokinin receptor	Zuo *et al*. 2002 Fletcher 2002
CLV WUS	Preserve stem cell identity in shoot apical meristems	Fletcher 2002; Groβ-Hardt and Laux 2003
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
HBK2 HBK3	Proliferation of the apical pole during Somatic embryogenesis	Hjorrtswang *et al*. 2002
PgAGO	Control of Apical meristem	Tahir *et al.* 2006
ZLL	Maintaining the meristematic cells in pluripotent state	Moussian *et al*. 1998
LEC2	Initiates ectopic somatic embryogenesis	Zuo *et al*. 2002; Stone *et al*. 2008
WUS (PGA6), SERK,	Involved in the vegetative-to-embryogenic 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; Groβ-Hardt and Laux 2003
LEC1, FUS3, ABI3	Regulates embryo maturation	Suzuki *et al*.2007; Von Arnold *et al.* 2002
Pickle	Suppress embryogenic program	Li *et al*. 2005; Handerson *et al.* 2004.
BABY BOOM	Alters hormone levels or hormone signalling pathways to induce somatic embryogenesis and organogenesis	Srinivasan *et al.* 2008.
SERK1	Appearance of competent cells	Schmidt *et al.* 1997 Nalon *et al*. 2009

POTENTIAL OF MOLECULAR TECHNIQUES

Development of molecular techniques for genetic analysis has led to a great increase in our knowledge of tree genetics and our understanding of structure and behaviour of genomes which is useful for improvement of tree species. These molecular techniques, in particular use of DNA

markers have been used in monitoring DNA sequence variation in and among the species thereby increasing their chances of finding a marker that segregates with the specific phenotype of interest. 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 germplasm of existing species 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 provide accessibility of novel alleles. RAPD markers have been use (Karp *et al.* 1996) in investigation of distribution of variability in natural populations of various species and provided the foundation for effective breeding and gene conservation strategies. 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). 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 tree species like populus, eucalyptus etc. for studying the variation and clonal fidelity during micropropagation.

DNA markers are generally classified as hybridization based markers i.e. Restriction Fragment Length Polymorphism (RFLPs), and polymerase chain reaction (PCR) based markers like 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; Agarwal *et al.* 2008; Jones *et al*. 2009).

Table 1.5 Comparision 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 polymorphic 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

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 4A . 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; Wolfe 2005).

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 microsatellites 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 wheat (Nagaoka and Ogihara 1997), *Camellia sinensis* (Devarumath *et al*. 2002), strawberry (Anita 2004), *Swertia chirayita* (Joshi and Dhawa 2007) etc. 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.

PRODUCTION OF SECONDARY METABOLITES FROM PLANTS AND TISSUE CULTURE

Economically important plants serve as sources of industrial oils, resins, tannins, saponins, natural rubber, gums, waxes, dyes, pharmaceuticals and many specialty products. For the sake of convenience, plant chemicals are often classified as either primary or secondary metabolite (proteins and nucleic acids are generally excluded from this classification). Primary metabolites are substances widely distributed in nature, occurring in one form or another in virtually all organisms. Secondary metabolites are compounds biosynthetically derived from primary metabolites but more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group (species, genus, family, or closely related group of families). Plants produce and accumulate a vast number of different natural products, also called secondary metabolites. Although tens of thousands of secondary metabolites have been chemically identified

(Gershenzon *et al.* 2007), the biological roles of most of these compounds remain obscure. Many natural compounds are of commercial and industrial importance, imparting colors and scents to flowers, fruits and vegetables, and are also key ingredients in medicinal and nutraceuticals. Many studies have indicated that natural products accumulated in plants have clear ecological roles such as protection against predation, protection against fungal and bacterial diseases or against adverse climatic conditions (Langenheim 1994). Additionally, many natural products serve as signal molecules to attract pollinators and seed-dispersers, or mediate pathogenic, parasitic, or symbiotic interactions. Still, the biological roles of most specialized compounds in the plants producing them are unknown (Schwab *et al*. 2008). Secondary metabolites are frequently accumulated by plants in smaller quantities than are primary metabolites. In addition, secondary metabolites, in contrast to primary metabolites, tend to be synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification difficult. As a result, secondary metabolites that are used commercially as biologically active compounds (pharmaceuticals, flavors, fragrances, and pesticides) are generally higher value-lower volume products than the primary metabolites. Thus, compared to primary metabolites (bulk chemicals), many secondary metabolites can be considered as specialty materials or fine chemicals. Examples of commercially useful plant secondary metabolites are nicotine, the pyrethrins, and rotenone, which are used in limited quantities as pesticides, and certain steroids and alkaloids, which are used in drug manufacturing by the pharmaceutical industry. The steroids and alkaloids include steroidal sapogenins, Digitalis glycosides, the anticancer Catharanthus (formerly Vinca) alkaloids, belladonna alkaloids (for example, atropine, hyoscyamine, and scopolamine), cocaine, colchicine, opium alkaloids (codeine, morphine, and papaverine), physostigmine, pilocarpine, quinine, quinidine, reserpine, and d-tubocurarine. Other secondary plant metabolites are used in limited quantities as pharmacological tools to study various biochemical processes. Compared to the relatively low cost of primary or bulk metabolites, secondary plant metabolites are often valued at several dollars to several thousand dollars per pound (Balandrin *et al*.1985). In recent years there has been renewed interest in screening higher plants for novel biologically active compounds. This has resulted in development of therapeutics used in treatment for cancer, AIDS, hypertension, malaria and others (Khan and Khanum 1998). When compared with the intact plant, cultured plant cells often produce different quantities with different profiles of secondary metabolites and these quantitative and qualitative features may change with time (Yabata 2006).

The advantages of plant tissue cultures for production of bioactive compounds over the conventional production are as follows:

1. It is independent of geographical and seasonal variations and environmental factors – the synthesis of bioactive secondary metabolites runs in controlled environments and the negative biological influences that affect secondary metabolites production in nature are eliminated (microorganisms and insects).

2. It offers a defined production system, which ensures the continuous supply of products, uniform quality, and yield.

3. Selection of cell/ hairy root lines with ability to produce high quantity of secondary metabolites.

4. Novel compounds that are not normally found in parent plant can be produced in cell or hairy root culture.

5. It allows the efficient downstream production of secondary metabolite directly from the culture medium of cell masses.

6. Plant cell can perform stereo- and regio-specific biotransformations for the production of novel compounds from cheap precursors.

7. With automatization of cell growth control and regulation of metabolic processes, cost price can decrease and productivity increases.

There are a number of successfully established and commercialized plant cell cultures producing a high amount of different secondary metabolites (**Table 1.6**). However, this technology is still being developed and despite the advantages, there is a variety of problems to be overcome before it can be adopted for the production of useful plant secondary metabolites.

Table 1.6 Groups of natural products that were so far isolated from tissue and suspension cultures of higher plants

Phenylpropanoids	**Alkaloids**	**Terpenoids**	**Quinones**	**Steroids**
Anthocyanins	Betalaines	Carotenes	Anthroquinones	Cardiac glycosides
Coumarins	Quinolizidines	Monoterpenes	Benzoquinones	Pregnenolone
Flavonoids	Furonoqui nones	Sesquiterpenes	Naphthoquinones derivatives	
Hydroxycinnamoyl	Triterpenes	Diterpenes derivative		
Isoflavonoids	Lignans	Isoflavonoids		
Harringtonines	Phenolenones			
Indoles	Proanthocyanidins			
Purines	Stilbenes			
Tropane	Tanins alkaloids			
	Acridines			

Rao and Ravisankar, 2002.

POTENTIAL OF GENETIC TRANSFORMATION

Advancement of molecular genetics in forest trees, 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 hard wood tree species by different methods but *Agrobacterium tumefaciens* mediated transformation was most widely used (Merkel and Nairan 2005). Choice of explants

having competence for transformation and regeneration is a crucial factor. Regeneration through organogenesis, somatic embryogenesis definitely offers the advantage of both multicellular and single cell regeneration and currently appears to be the most promising approach to introdue new genes into woody species. Transformation by *Agrobacterium rhizogenes* was mostly confined for establishment of hairy root culture, secondary metabolite production and is disscussed in the next section of this chapter.

Targets for Tree Transformations:

Gene engineering focuses on the traits that are a subject of standard breeding. Trunk is the major harvested organ in trees and breeding programs are usually aimed directely at improving trunk performance and wood quality. Among these the reduction of the proportion of juvenile wood, increase of wood density, reduction of lignin content, modifying the type of lignin and increasing cellulose content can be mentioned (Balocchi and Valenzuela 2004). Additional breeding targets include improving root-system and tree-canopy performance, pest resistane and tolerance to abiotic stresses. The development of genetically modified trees has increased in the recent years, having more than 200 field trials involving at least 15 forest species (Valenzuela *et al*. 2006). The main characteristics used have been herbicide tolerance and marker genes. Most of the species represented on the field trials are hardwoods (77%), with Populus being the most used, accounting for 55% of the field trials. A number of economically important genes (for example, herbicide resistance, insect and disease resistance, reduced lignin, and growth traits) have been transferred to produce transgenic plants in several forest tree species (**Table 1.7**).

Table 1.7 Genetic transformation of some of the tree species by different genes.

Gene	Species	Reference
Herbicide resistance genes aroA,BAR,CP4	Populus sp	Fillatti *et al*. 1987; Donahue *et al.* 1994; Meilan *et al.* 2002; Li *et al.* 2008a, b
	Eucalyptus	Harcourt *et al.* 2000
	Pinus radiata	Bishop-Hurley *et al*. 2001
	Picea abies	Charity *et al*. 2005
Insect resistance gene (Bt)	*Populus* sp	Leple *et al*. 1995; Wang *et al*. 1996; Meilan *et al*. 2000; Hu *et al*.1999;Yang *et al*. 2003
	Pinus radiata	Grace *et al.* 2005
	Pinus taeda	Tang and Tian 2003
	Picea glauca	Lachance *et al*. 2007
Stress tolerance (CaPF1)	*Pinus strobus*	Tang *et al.* 2007
Lignin modification genes (CAD, 4Cl, COMT, CAld5H)	Populus	Hu *et al*. 1999 Pilate *et al*. 2002 Baucher *et al*. 2003 Li *et al.* 2003 Halpin *et al*. 2007 Hancock *et al*.2007
Pt COMT promoter	*Betula pendula*	Tiimonen *et al.* 2007
Bacterial and fungal resistant D4E1,ChitIV, STS, ESF39A, ech42	*Populus tremula* 9 P. alba *Betula* *Populus* *Ulmus Americana* *Picea mariana, Populus nigra* 9 P. maximowiczii	Mentag *et al.* 2003 Pasonen *et al*. 2004 Seppa¨nen *et al.* 2004 Newhouse *et al*. 2007 Noe¨l *et al.* 2005

PLANT TRANSFORMATION BY *AGROBACTERIUM RHIZOGENES*

Biotechnological advances offer an impressive option to supplement the ongoing efforts on developing genetically modified germplasm for achieving sustainable production of food, fodders, and medicinal compounds (Rao and Ravisankar 2002). Several different techniques (*Agrobacterium*-mediated, electroporation of intact tissues, microprojectile bombardment etc.) have been tested for gene delivery to plant cells. Two major methods, namely *Agrobacterium*-mediated and particle bombardment have been extensively employed for genetic transformation of plants (Chandra *et al*. 2003). Each method has its limitations regarding the competent target tissues and genotype. *Agrobacterium* infection depends on biochemical factors within plant, and its infection is more genotype dependent. Likewise, electroporation requires the DNA to be in contact with the plasma membrane of competent cells and is most effective with protoplasts, plant cells that have had their cell walls removed (Li *et al.* 1995). Molecular analysis of plants

obtained by biolistic transformation generally reveals a complex pattern of transgene integration and resulted in high frequency of multiple transgene insertions (Fu *et al.* 2000; Ozias-Akins and Gill 2001). This led to infertility, transgene silencing, and was found to be one of the major drawbacks of the biolistic technique. Transgene silencing is often observed when multiple copies of a transgene are integrated, or when the sequence of the inserted genes is homologous to that of an endogenous gene and triggered transgene methylation (Muller *et al.* 1996; Chandra and Pental 2003). In addition, delivery of longer DNA fragments is challenging because the sequence can break while delivering. Although the fate of introduced DNA is not clear, ligation of the transgenic DNA fragments before integration is proposed to account for the observation of arrays of transgenic DNA integrated at the same site into the plant genome. This can result in reduction of transgene expression by co-suppression (Pawlowski 1998). *Agrobacterium* mediated transformation remains to be the most opted method as it is devoid of most of the above limitations inherent in the direct gene delivery method. In addition, the integration of the transgene is better defined in *Agrobacterium*-mediated transformation (Chandra and Pental 2003) and this is the most attractive method because of the ease of the protocol coupled with minimal equipment costs. This technique efficiently introduces variability in transgenic plants due to stable integration and appropriate expression of the limited copy numbers of the transgenes. These advantages make the system adaptable to many different crops (Finnegan and Elroy 1994; Gould 1997; Kumpatla *et al.* 1998; Hansen and Wright 1999).

The *Agrobacterium* mediated transformation method itself includes several steps. The co-cultivation step is a crucial period when the two different biological elements (1) plant explant, and (2) *Agrobacterium* share the same space and conditions, and many parameters should be tested to satisfy both partners and guarantee a successful outcome (Hansen and Wright 1999). Most commonly used species of *Agrobacterium* are *A.rhizogenes* and *A. tumefeciens.*

Biology and life cycle of A. rhizogenes:

Agrobacterium rhizogenes is known as a soil-borne gram-negative bacterium that induces hairy roots in infected plant tissues by transferring *T-DNA* of the Ri-plasmid into the plant genome. *A.rhizogenes* (formerly *Phytomonas rhizogenes*) was first identified more than 70 years ago (Riker *et al.* 1930; Hildebrand 1934; White 1972) as the causative agent of the plant disease known as hairy-root syndrome or root-mat disease. *A.rhizogenes* is a close relative of the better-known *A. tumefaciens*, which is the causative agent for crown gall disease and the best-characterized species among the genus *Agrobacterium.*

To date, the basic understanding of the molecular mechanisms of genetic transformation of plants by members of the *Agrobacterium* genus relies on extensive studies using *A. tumefaciens* (Tzfira *et al.* 2004). The overall process of infection is considered similar in both species. *Agrobacterium*

infects wounded plant cells because of the production of phenolics compounds like acetosyringone that attract *A. rhizogenes*. Bacteria travel to the wound site by chemotaxis. Subsequently causing infection of plant cells by *Agrobacterium* at wound site, followed by transfer and integration of *Agrobacterium* derived genetic material (T-DNA) into the plant genome results in development of hairy root disease. Hairy-root disease is characterized by plagiotropic root growth, a high degree of lateral branching, profusion of root hairs, and enhanced growth rates similar to that of undifferentiated callus, although the tissue maintains a highly differentiated and functional root organ (Christey 2005; Veena and Taylor 2007). The most common *A. rhizogenes* strains identified to date include agropine-type strains (Ri plasmids pRiA4, pRi1855, pRiHRI, pRi15834, and pRiLBA9402), mannopine-type strains (Ri plasmid, pRi8196), cucumopine-type strains (Ri plasmid, pRi2659), and mikimopine-type (Ri plasmid pRi1724) (Christey 2005).

Hairy-root expression

The mechanism of hairy-root formation is not completely understood (Tzfira *et al*. 2005). Hairy-root formation can be broadly broken down into four steps: activation, processing, movement of T- DNA from the bacteria into the plant cell, and subsequent induction of root formation and growth. Of the four processes, the first three steps are quite well understood given the homologies in Ti and Ri plasmids and existing models of pathogenesis provided by extensive studies of *A. tumefaciens* strain C58. How genetic transformation by *A. rhizogenes* leads to the phenotype of hairy-root formation and proliferation still remains unclear (Veena and Taylor 2007).

The Ri plasmid of *A. rhizogenes* and the Ti plasmid of *A. tumefaciens* are broadly similar in terms of composition as well as structure. Comparative studies of the complete sequence of several Ri and Ti plasmids indicate that many of the mechanisms of activation, processing, and movement of the T-DNA from the bacterial to the plant cell are conserved between the two types of plasmids (Huffman *et al*. 1984). In Ri plasmid, T-DNA is flanked by 24-bp direct repeats, known as border sequences. The wild-type T-DNA carries a set of oncogenes and opines catabolism genes, the expression of which in transgenic plant cells lead to neoplastic growth of tissues. The production of opines, amino acid derivatives almost exclusively used by the bacteria as a carbon and nitrogen source. Ri plasmid also contains genes responsible for processing T-DNA, for its attachment and transfer to plant cell, and for anabolism of opines in transformed plant tissues. The observation of considerable homologies between Ri and Ti plasmids in the virulence (vir) region and similarities in the mechanisms of T-DNA processing, transfer, and integration (Moriguchi *et al*. 2001) strongly suggests the existence of a common mechanism of gene transfer. Ri plasmids possess 8-bp repeated sequences, named T-DNA transfer stimulator

sequences (TSS), that enhance efficiency of T-DNA transfer to the plant genome (Moriguchi *et al*. 2001).

The least understood aspect of hairy-root disease is the mechanism of prolific root induction and growth. *A.rhizogenes* strains contain a T-DNA region located on the Ri plasmid that carries genes involved in root initiation and development (*rol*-genes) genes concerned with opine biosynthesis, and genes of unknown function (Veena and Taylor 2007). A second T-DNA may be present that contains genes involved in auxin biosynthesis (aux1 or iaaM and aux2 or iaaH) along with further genes of unknown function. Ri plasmids with two T-DNA, left and right (T_L and T_R, respectively), are termed "split" T-DNA. The Ri plasmid of cucumopine and mannopine-type strains consists of only one T-DNA region, whereas that of agropine-type strains are a split T-DNA, consisting of two T-DNA regions, T_L and T_R, each ranging in size from ~15–20 kb. Whereas both T_L-DNA and T_R-DNA are known to be transferred and integrated independently into the host plant genome, the transfer of T_L-DNA is essential for induction of the hairy-root syndrome (Veena and Taylor 2007).

Structure of Ri plasmids of A. rhizogenes

Several loci on the T_L-DNA of Ri plasmids have been shown to be essential for hairy root induction (*rol* genes for root oncogenic loci). The T_L-DNA of the agropine-type Ri plasmid consists of at least four loci, *rolA*, *B*, *C*, and *D* (Veena and Taylor 2007). In the early 1990s, it was demonstrated that a combination of *rolA*, *rolB*, and *rolC* loci was sufficient for producing the hairy-root phenotype, depending upon plant species and tissue type (Christey 2001). The capacity of *rolA*, *B*, and *C* genes to induce neoplastic roots with faster growth rates than normal tissues is equivalent to that of the whole T_L-DNA of the Ri plasmid (Spano *et al*.1988). The T_R-DNA found only in agropine-type Ri plasmids of *A. rhizogenes* consists of genes (*aux1*, *aux2*, *rolB TR*, *mas1*, *mas2*, and *ags*) that control opines and auxin biosynthesis (Christey 2001). The *aux* genes are considered to play an ancillary role in hairy-root induction and are not essential for production of hairy roots (Veena and Taylor 2007).

Development of transgenic using A. rhizogenes

The natural host range of *A. rhizogenes* appears to be limited to a small number of plant species: apple, cucumber, tomato, or melon etc. (Weller *et al*. 2004). However, under laboratory conditions, more than 450 different species of plants are found to be susceptible to infection by *A. rhizogenes* (Veena and Taylor 2007), including a diverse range of dicotyledonous and monocotyledonous plant families and some gymnosperms. Most plant tissues and organs, including the hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, and tuber, susceptible to be infected and genetically transformed *by A. rhizogenes*, with the

resulting production of hairy roots (Giri *et al*. 2000). However, the response varies depending upon the *A. rhizogenes* strain and its interaction with the plant species and tissue type.

An important feature of *A. rhizogenes*-induced roots is their unique ability to grow *in vitro* in the absence of exogenous plant growth regulators (Rao and Ravishankar 2002). The use of *A. rhizogenes*-mediated transformation enables the development of transgenic plants via marker-free selection through use of hairy root morphology as the primary indicator of transformation. Due to high rates of co-transformation the genes of interest on the binary vector are likely to also be present. GUS and GFP reporter genes were used by (Puddephat *et al*. 2000) to select transgenic root cultures, eliminating the need to use antibiotic resistance or herbicide resistance as marker genes. Alternatively, having the genes of interest incorporated in the Ri TDNA also allows for use of hairy root morphology as the primary indicator of transformation eliminating the need for additional marker genes (Christey 2001). *A.rhizogenes* is confined to specific uses with potential applications for plant breeding and plant improvement including: root system alteration, use of *A. rhizogenes* and *rol* genes for altered phenotype, and the introduction of desirable foreign genes (Christey 2001).

In some species, the difficulty in regenerating transgenic plants has been circumvented by development of rapid and efficient transformation protocols using *A. rhizogenes* to produce hairy roots on "composite" plants (an untransformed plantlet with hairy roots). These composite plants have been used in studies focused on root characteristics such as nodulation in *Lotus.japonicus* (Stiller *et al*. 1997; Martirani *et al*. 1999), soybean (Narayanan *et al*. 1999) and barrel medic (Boisson-Dernier *et al*. 2001). Rather than production of composite plants, binary and co-integration vectors introduced into *A. rhizogenes* strains have been used to transform several plant species, including *Eustoma grandiflorum* (Handa 1996), melon (Toyoda *et al*. 1991), petunia (Kiyokawa *et al*. 1992b), *Tylophora indica* (Chaudhuri *et al*. 2005). The high biosynthetic potential of transformed root cultures was largely neglected for years, and the investigations that were performed on them mainly focused on the mechanisms underlying hairy root syndrome. However, during the mid-1980s and early 1990s, there were several investigations on their production of biologically active substances, especially alkaloids (Kamada *et al*. 1986; Flores *et al*.1987), which revealed the biosynthetic capacity of the transformed root systems. Nowadays, hairy root cultures receive more attention as biological matrices for producing valuable metabolites, as they have several attractive features, including high genetic stability (compared to undifferentiated cultures) and relatively fast growth rates (compared to normal roots). Furthermore, growth regulators are not required for their cultivation, which is an important consideration, as some hormones are toxic (2,4-dichlorophenoxyaceticacid, for example), so their presence in many end products is unacceptable. In addition, use of hairy root systems is advantageous for the production of a number of secondary metabolites that are

synthesized in plant roots and then accumulated in aerial parts of the plant (for example, the naphthoquinone derivative lawsone), as such metabolites are accumulated at very low amounts, or not at all, in undifferentiated or shoot cultures (Shanks and Morgan 1999). **Table 1.8** lists some of the phyto-chemicals produced by hairy root cultures.

Table 1.8 Report of hairy root induction in some medicinal plants and isolation of bioactive compounds.

Plant Species	Explant used	*Agrobacterium rhizogenes* Strain	Name of secondary metabolite	References
Artemisia annua	Shoot tips	*A. rhizogenes* LBA9402	Artemisinin	Giri *et al.* 2001
Tylophora indica	Shoots	*A. rhizogenes* A4	Tlyophorine	Chaudhuri *et al.* 2005
Arachis hypogaea	Leaves	*A. rhizogenes* R1601	Resveratrol	Kim *et al*. 2008
Taxus brevifolia	Shoot culture	*A. rhizogenes* ATCC 31798	Taxol	Huang *et al*. 1997
Solanum khasianum	Leaves	*A. rhizogenes* LBA 9402	Solasodine	Jacob and Malpathak 2005
Ruta graveolens L.	Seedlings	*A.rhizogenes* 15834	Acridone-type compounds	Kuzovkina *et al.* 2004
Salvia miltiorrhiza	Plantlets	*A. rhizogenes* ATCC15834	Diterpenoid tanshinones.	Yan *et al*. 2005
Saussurea involucrata	Seedlings	*A. rhizogenes* R1601	Syringin, Rutin and Hispidulin	Fu *et al*. 2006
Ammi majus	Plantlets	*A. rhizogenes* LBA 9402	Coumarins and furanocoumarins	Kro´licka *et al*. 2001
Artemisia annua L.	Leaf blade and petiole	*A.rhizogenes* 1601	Artemisinin and Stigmasterol	Xie *et al*. 2001
Lithospermum erythrorhizon	Hypocotyl	*A. rhizogenes* 15834	Shikonin	Yazaki *et al*. 1998
Atropa belladonna	Stems of shoot cultures	*A. rhizogenes* (MAFF 03-01724)	Littorine	Nakanishi *et al.*1998;Kamada *et al.*1986
Catharanthus roseus	Seedling	*A.rhizogenes* 1855	Ajmalicine	Moreno-Valenzuela *et al*. 1998

Plant Species	***Explant used***	***Agrobacterium rhizogenes* Strain**	**Name of secondary metabolite**	**References**
Centella asiatica (L.)	Leaves and petioles	*A. rhizogenes* R1000	Asiaticoside	Kim *et al.* 2007
Hedysarum theinum	Geminated shoots	*A. rhizogenes* A4	Ononin	Vdovitchenko *et al*. 2007
Stizolobium hassjoo	Leaves	*A. rhizogenes* A4	L-*DOPA*	Huang and Chau 2006
Catharanthus roseus	Seedling	*A. rhizogenes* 15834	Tryptamine Tabersonine	Hong *et al.* 2006
Datura stramonium L.	Hypocotyls	*A. rhizogenes* A4	Hyoscyamine	Amdoun *et al.* 2009
Harpagophytum procumbens	Leaves	*A. rhizogenes* ATCC 15834	Coumaric acid, ferulic acid,	Ludwig-Mu¨ller *et al.* 2008
Rhamnus fallax Boiss	Stem cuttings 3 - 4 cm	*A.rhizogenes* A4	Anthraquinones	Rosić *et al.* 2006
Fagopyrum esculentum	Leaves	*A. rhizogenes* R1000	Rutin	Lee *et al.* 2007
Atropa belladonna	Leaf	*Agrobacterium rhizogenes* 15834	Scopolamine	Bonhomme *et al.* 2000
Datura talula L.	Leaf	*A. rhizogenes* A4	Gastrodin	Peng *et al.* 2008
Gmelina arborea	Cotyledon	*A. rhizogenes* 15834	Verbascoside	Dhakulkar *et al*. 2005
Echiniacea Sp	Hypocotyls	*A. rhizogenes* A4	Alkmide	Romero *et al.* 2009
Papaver bracteatum	Seedlings	*A. rhizogenes* 15834	Benzylisoquinoline	Rostampour *et al*. 2009
Rubia tinctorum L.	Cotyledon	*A. rhizogenes* 15834	Anthraquinones	Ercan and Taskin 1999
Silybum marianum (L.)	Hypocotyls	*A. rhizogenes* 15834	Silymarin	Rahnama *et al.* 2008
Azadirachta indica	Seedlings	*A. rhizogenes* LBA 9402,	Azadirachtin	Stadive *et al.* 2007

Semecarpus anacardium

Ayurveda, the literature on traditional Indian medicines describe *Semecarpus anacardium* as a potent source of drugs against variety of alignments. The plant is also known as "ardha vaidya" (Nadkarni 1976).

Systematic classification (Bentham and Hooker 1862-83)

Kingdom-Planta. Division-Dicotyledons. Group-Polypetalae. Series-Disciflorae. Order-Sapindales. Family-Anacardiaceae. Genus-*Semecarpus*. Species-*anacardium*.

General characteristics:

Distribution

This is a common forest tree widely distributed in tropical region of south and Southeast Asia. Most common throughout hotter parts of India (Wealth of India 1999).

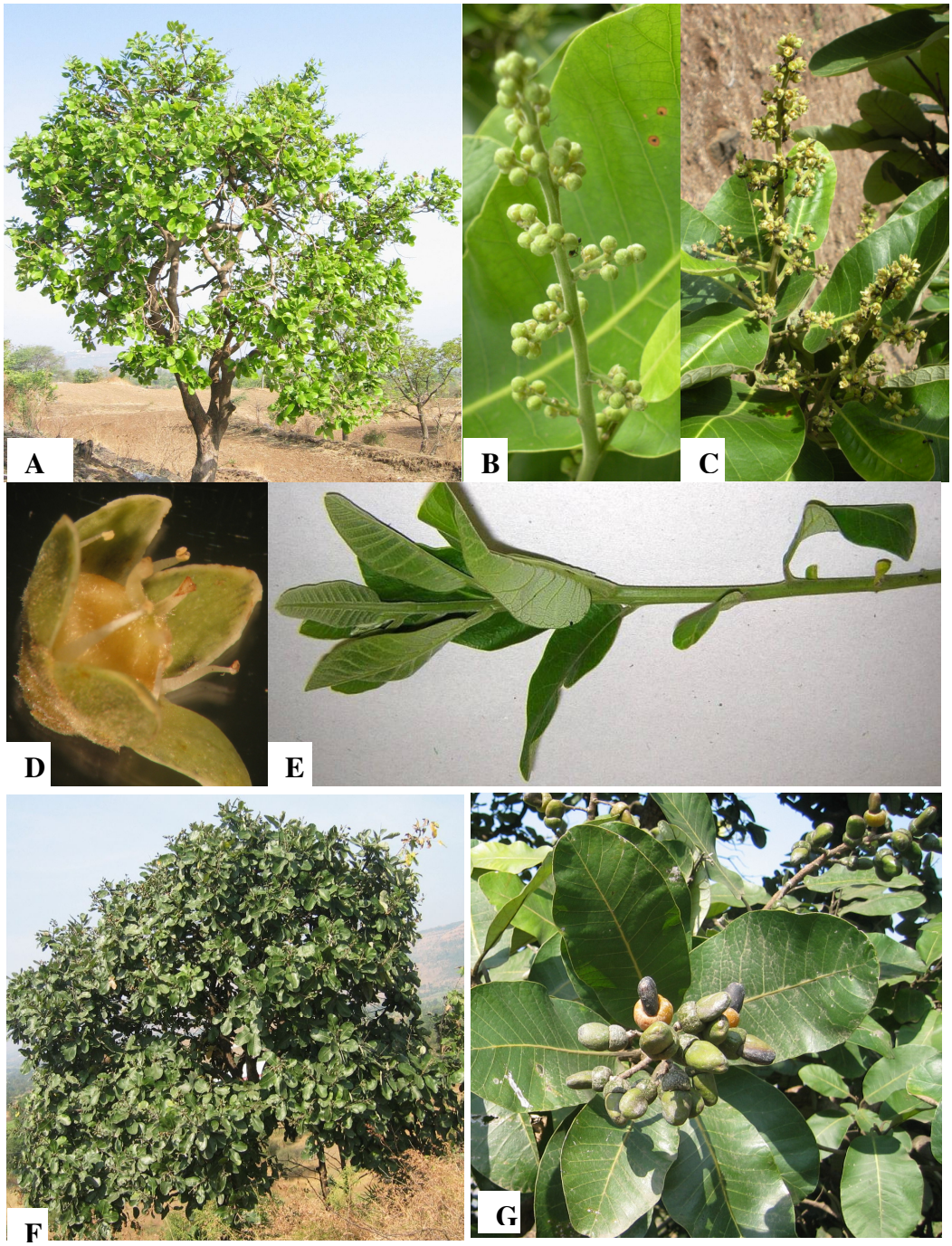
Plant description

A moderate sized deciduous tree, 12-15 m in height. Bark dark brown, rough, leaves simple large (17.5-60 cm) x 10-30 cm, obovate, oblong with upper smooth shiny and lower hairy surface. Flowers are small dull greenish yellow, dioecious in terminal panicles. Fruit- A drupe 2.5cm long, laterally flattened, kidney bent, obliquely ovoid, smooth and shining. Fruit turns black when ripe, situated on a fleshy orange colored receptacle. The pericarp is differentiated in to epicarp, mesocarp and endocarp. Mesocarp contains an acrid irritant yellowish oily secretion. Seeds have poor viability (Anonymous, Wealth of India 1999). Reports on chemical compounds from different parts are listed in **Table 1.9.**

Table 1.9: **Chemical compounds from various parts of the plant:**

Plant Parts	**Chemical compounds**	**References**
Stem	Tetrahydroamentoflavone 3,4,2',4'tetrahydroxy chalcone and 7,3',4'-trihydroxy flavone	Selvam *et al.* 2005 Selvam and Jachak 2004
Leaf	Saponins and amentoflavone	Ishratullah *et al*. 1977
Nuts	3-(n-pentadec-8-enyl) catecol, 1,2-dihydroxy-3-pentadecaenyl-8'-benzene and 1,2 -dihydroxy-3-(pentadecadienyl-8',11') benzene, biflavanoids like 3',8-binaringenin and 3'.8-biliquiritigenis, Anacardic acid, Semecarpol	Rao *et al*.1973
	Semecarpuflavanone, Jeediflavanone, Galluflavonone, Anacardioflavanone, Semecarpetin	Murthy 1983 a, b, 1985, 1988, 1992
	Tetrahydroamentoflavone	Iqblahmad *et al*. 1981
	Anacardic acid, Semecarpol	Rastogi and Mehrotra 1991
	3-(8-(Z),11-(Z)-pentadecadienyl) catechol	Nair *et al*. 2009
Kernel	Anacardioside	Gil *et al*. 1994

Different parts of the plant are seen in **Fig. A-I**.



A. Mature tree of *Semecarpus anacardium* with freshly sprouted twigs in the month of May.
B. Unopened floral buds
C. Inflorescence with opened and unopened floral buds
D. Flower of *Semecarpus anacardium*.
E. Freshly sprouted twig of *Semecarpus anacardium*.
F. Mature tree of *Semecarpus anacardium* bearing fruits.
G. A bunch of fruit of *Semecarpus anacardium* with mature and pre-mature nuts. The pre-mature nuts were used for *de novo* organogenesis.

H I

H. Mature nuts with orange colored ripened pseudo-fruit.
I. Mature dried nuts of *Semecarpus anacardium* after removing the pseudo-fruits used for germination studies.

Kernel of nut has following composition:

Moisture-3.8%, Proteins-26.4%, Fat-36.4%, Fibers-1.4%, Carbohydrates-28.4% Minerals-3.6% (Ca-295mg/100g, P-886mg/100g, Fe-6.1mg/100g), Vitamins (Thiamin-0.38mg/100g, Riboflavin-0.15mg/100g, Nicotinic acid-2.74mg/100g). Essential amino acids like arginine, lysine, histidine, leucine, isoleusine, methionine, threonine, phenylalanine, valine; tryptophan has been reported from kernel (Anonymous, Wealth of India 1999). Kernel contains 20-25% reddish brown semidrying oil with a pleasant taste. Fatty acid analysis of the oil has the following composition:

Myristic acid 0.28%
Palmitic acid 12.44%
Stearic acid 7.96%
Oleic acid 64.28%
Linoleic acid 15.04% (Agarwal and Bholanath 1961)

Common use of the plant

The nutshell liquid is used in varnish, paints, plastics, and allied industries (Anonymous, Wealth of India 1999). The chlorinated derivative of the phenolic constituents of nutshell liquid is used in production of insecticide, antiseptics, herbicides and termite repellents. The kernel tastes like almond and is eaten. Oil of kernel is used as preservative for wood. Wood of the tree is used in making light furniture and for packing material.

Medicinal use of the plant:

The fruit oil is claimed to be useful in treating leprosy, neuritis, rheumatoid arthritis, piles, asthma, cough, sexually transmitted diseases like syphilis and gonorrhea, veneral disorder, nematicidal etc. (Kirthikar and Basu 1933; Nadkarni 1976). Several Ayurvedic preparation containing *S. anacardium* such as bhallataka rasayana, amritha bhallataki, kalpaamurathaa and brihat bhallataka lehya are marketed in India. These are suppose to posses rejuvenating properties, which increases longevity, brings glow to the face sweetness in tone, improvement in vision (Sreenivasacharyulu 1931).

"Siddha" preparation of the *S. anacardium* nut i.e. nut boiled in milk and mixed in "ghee" has been shown to be useful for many diseases. Anticancerous activity of the siddha preparation has been proven (Premalatha *et al*. 1997, 1999 and 2000) against aflatoxin B_1 induced hepatocellular carcinoma induced in female albino Sprague-Dawley rats of Wistar stain. Two of the compounds from the nut namely 1,2 dihydroxy-3-pentadeca-7', 10'-dienyl benzene and 1,2dihydroxy-3-pentadeca-8'-enylbenzene demonstrated cytotoxic activity against human cancer cell lines (Young *et al*. 1999). The chloroform extract of the fruits was reported to possess anticancer properties and was effective in Yoshida ascites sarcoma in rats (Gothoskar and Ranadive 1971) and P388 lymphocytic leukaemia in mice (Gothoskar *et al*. 1971). The acetylated oil of the fruits was also effective against and the P388 lymphocytic leukaemia (Pathak *et al.* 1983). Water, alcohol and oil extract of *S.anacardium* are antimutagenic against benzopyrene-induced mutagenecity (Kotharia *et al*. 1997). Ethanolic extract of *S. anacardium* on male albino rat shows reduction in sperm motility and density (Sharma *et al*. 2003).

Increase in phagocytic activity of neutrophils, significant decrease in level of reactive oxygen species, lysomal enzymes and increased accumulation of neutrophils around joints of adult male wistar rats suffering from adjuvant arthritis has been observed on administration of siddha preparation of *S. anacardium* (Vijayalakhmi *et al*. 1996, 1997; Mary *et al.* 2003; Ramaprasatha *et al*. 2005, 2006; Mythilypriya *et al.* 2007, 2008, 2009; Tripathi *et al.* 2008). Significant increase in the level of Lipid peroxides (LPO), ROS and decreased levels of antioxidant enzymes in arthritic rats are restored back to normal level when treated with siddha preparation of *S.anacardium* nut (Ramprasatha *et al.* 2005). The effect of ethanolic extract of dried nuts of *S.anacardium* on blood glucose level was investigated in both normal and alloxan induced diabetic rats. The extract significantly lowered blood glucose level after 3 hours of administration, which is comparable to tolbutamide (Kothai *et al.* 2005). The same siddha formulation has been active against prostate cancer, lung cancer cell lines (Ranga *et al.* 2004), breast cancer cell lines (Sowmyalakshmi *et al*. 2005), breast cancer induced in Sprague-Dawley rats (Veena *et al.* 2006; Mathivadhani *et al.* 2006, 2007,2008).

There are reports of cholesterol lowering effects of *S. anacardium.* Administration of nutshell extract to cholesterol fed rats resulted in significant reduction in serum cholesterol by 73.3% and serum LDL cholesterol 80%. The extract feeding also prevented the accumulation of cholesterol/triglycerides in liver, heart, muscles and aorta (Tripathy and Pandey 2004), Similar cholestrol lowering activity has been reported (Veena *et al* 2006). Ethyl acetate extract of *S.anacardium* nuts contain Tetrahydroamentoflavone (THA). This biflavonoid (THA) act as a cyclooxygenase inhibitor. The *in vitro* cyclooxygenase (COX-1) catalyzed prostaglandin biosynthesis assay of THA gave an IC_{50} value of 29.5 μM (COX-1) and 40.5% inhibition at 100 μg/mL (COX-2). The *in vivo* carrageenan induced paw edema assay resulted in dose dependent anti-inflammatory effect of THA and the activity was comparable to that of ibuprofen, one of the well-known NSAIDs (Selvam and Jachak 2004). The methanolic extract of stem bark has been shown to have acetylcholinesterase (AChE) inhibitory activity (*in vitro*) (Vinutha *et al*. 2007).

The juice of pericarp has antibacterial properties. Sulfonate and arsenic derivative of nutshell liquid shows marked bactericidal activity against *Bacillus pyogenes, E.coli, Staphylococus* and *Streptococcus pneumaticus* (Godbole and Pender 1960). Boiled methanol extract of *S.anacardium* leaf was active against *Pseudomonas aerginosa and P. fluorescens* (Bonjar and Nik 2004). Alcoholic extract of dry nuts of *S.anacardium* showed dose dependent antifungal activity *in vitro* against *Aspergillus fumigatus* and *Candida albicans*. At 400 mg/ml concentration, growth of both the fungi was inhibited and considerable reduction in size of cells and hyphae was observed, sporulation was also decreased (Sharma *et al.* 2002).

SCOPE OF RESEARCH ON *SEMECARPUS ANACARDIUM*

With the present state of knowledge, it is apparent that there is no literature on tissue culture studies in *S.anacardium*. Thus, there is need to develop reliable tissue culture protocols for *in vitro* regeneration of. this multipurpose tree species. 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 yielding trees in terms of secondary metabolites and seed oil content will contribute to industrial applications. Protocols developed for *de novo* organogenesis and embryogenesis will be useful for understanding the basic processes of morphogenetic pathways and causes of recalcitrance in tree species in general and Anacardiaceae in particular. *In vitro* culture provides the only route for generation of genetically engineered genotypes of trees.

S .anacardium which represent a substantial variability in phenotype as well as chemo type, and need to be studied 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. 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.

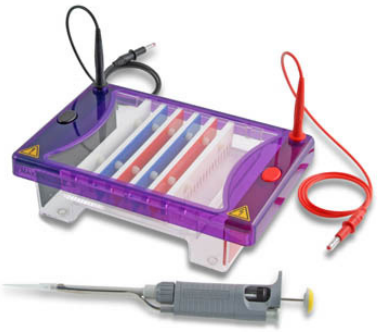
OBJECTIVES OF THE PRESENT STUDY

The present research program entitled **"Studies on *Semecarpus anacardium* L. for *in vitro* regeneration and identification of biologically active compounds"** was taken up with the following objectives:

1. Standardization of protocol for clonal propagation from seedling explants.
2. Optimization of conditions for micropropagation of mature trees.
    (a) Genetic evaluation of mature trees in natural stands.
    (b) Micropropagation of trees and genetic fidelity of the propagules.
3. Standardization of reproducible protocols for *de novo* morphogenesis.
4. Protocols for genetic transformation using *Agrobacterium rhizogenes* to establish hairy root culture.
5. Studies on isolation, purification and quantification of some of the chemical compounds using axenic culture derived plant organs.

CHAPTER 2

MATERIALS AND METHODS



INTRODUCTION

This chapter describes the general techniques and materials routinely practiced in plant tissue culture, transformation, isolation of DNA, PCR amplification and isolation of bioactive compounds. Materials and methods, specific to particular experiment, are dealt in details in respective chapters.

2.1 PLANT TISSUE CULTURE.

2.1.1 MATERIALS

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

Glasswares:

Test tubes (25x150mm), conical flasks (25, 50, 100, 250, 500, 1000, 2000 and 5000ml capacity), pipettes (0.1, 0.2, 1, 2, 5, 10ml capacity), Beakers (100, 250, 500, 1000, 2000 and 5000ml), separating funnel (250, 500 and 1000ml), petridishes (150x20 and 200x20mm), round bottom flask (250, 500, 1000ml) and measuring cylinders (10, 25, 100, 500 and 1000ml capacity) of Borosil, India, were used for culturing the tissues, for preparation of media and for reagents etc. Jam bottles used for culture are procured locally. Autoclavable, screw cap bottles (100, 250 and 500ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (Borosil, India), slides (Blue Star, India) and cover slips (Micro-Aid, India).

Plasticwares:

Plasticwares including sterile disposable plastic petriplates of size 60x15 mm and 90x15 mm diameter were procured from Tarson or Laxbro, Pune, India. Klin wrap, used for sealing the petriplates and bottles were bought locally. Micropipette of different precision measurements (0.2, 2, 10, 20, 100, 200 and 1000µl) was procured from Gilson, USA. Micro tips (10, 200 and 1000µl), eppendorff tubes (0.5, 1, 1.5 and 2ml), coupling jars, syringe filter, centrifuge tubes, floating rack, -20°C mini cooler, cryo boxes slide boxes, PCR tube racks and micro tip boxes were procured from Tarson, Pune, India. Sterile disposable needles (1ml) were from Hindustan syringes and Medical Devices Ltd., Faridabad, India.

Chemicals:

Chemicals used for surface sterilization procedures were Bavistin (50% Carbendazim BASF, India), Savlon containing chlorhexidine gluconate1.5% v/v and strong cetrimide solution 3% w/v (Johnson and Johnson, India) and sodium hypochlorite (Merck, India) with 4% available

chlorine. Antibiotic, Cefotaxime (Alkem, India) was often used for controlling the growth of the contaminating bacteria in plant tissue culture.

All major, minor, inorganic salts and vitamins used for preparation of culture media and for other experiments were of analytical grade (BDH, Merck, Hi-Media and Qualigens Fine Chemicals, India). Sucrose was procured from Hi-Media (India). Agar agar (bacteriological grade) and phytagel used as gelling agent in the semisolid culture medium were procured from Hi-media and Sigma respectively. Activated charcoal was procured from Sarabhai M Chemicals, India. Growth regulators including 2,4-dichlorophenoxy acetic acid (2,4-D), 4-amino-3,5,6-trichloropicolinic acid (Picloram), Kinetin (KN), 6-benzyl adeninepurine (BAP), Zeatin (ZE), N-phenyl-N'-1, 2,3-thidiazol-5-ylurea (Thidiazuron, TDZ), Indole acetic acid (IAA), Indole butyric acid (IBA), Gibberllic acid (GA3) and Naphthalene acetic acid (NAA) were obtained from Sigma (U.S.A.). Plant preservative mixture (PPM) was procures from Sameer Science Lab, Jabalpur, India. 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 and Eosin stain was from S.D. fine chemicals, India; paraffin wax (m.p. 58-60°C) from Merck, India Ltd. Haematoxylin from Hi-Media Laboratories Pvt. Ltd., Bombay, and DPX-4 [189-(2-chloro-N- (4-methoxy-1, 3, 5-triazin-2-yl amino carbonyl) benzene sulphanamide)] mountant was from BDH, India.

***Equipments**:*

pH meter:

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/ Citizen) was used for quick weighing and for analytical purposes. This is a single pan balance with sensitivity 0.1mg operating on 230 V 50 H_2 AC mains. Precision of ± 0.005g, weighing range 0-120g, digital read out was used for making stock solutions of growth regulators and for other fine weighing.

Autoclave:

The autoclave (Natt 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. The decontamination procedure 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 60 minutes. The culture media were autoclaved for 20min.

Laminar airflow ultra clean unit:

All aseptic manipulations were carried out in this unit. In laminar (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.

Other equipments used in the study includes:

Milli-RO water system (Millipore, USA) for deionized water, Water bath (Julabo, Germany),. 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/Canon), membrane filter sterilizing unit (Laxbro, Pune) and Micropipettes (Gilson / Tarson) were used at different stages of the study. With the exception of pipetman, microtome, microscopes and camera, different companies in India fabricate all other small equipments used in course of this study.

Source of Explants:

Mature seeds of *Semecarpus anacardium* were obtained from mature fruits extracted from the trees growing locally in the month of December. These seeds were used for developing seedlings *in vitro*. Sprouted buds from the mature plants were collected in the months of April, May and June. Semi mature seeds prior to ripening were collected in the month of November and used in the *de novo* morphogenesis experiments. Cotyledon explants were excised from these nearly mature seeds and cultured. The above explants were often used immediately for culture or occasionally stored for 1-2 days in cold room maintained at 4ºC before culturing.

2.1.2 METHODS

Preparation of glassware and instruments:

Glassware sterilization:

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

Tubes and flasks were plugged with absorbent cotton (Safe Surgical Industries, Beawar, India). Forceps, scalpels, millipore filter units etc. were wrapped in aluminum foils and then with brown paper. These were packed in autoclavable bag and autoclaved. 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 sterile papers under aseptic conditions and disposed after use. Microtips used for aseptic addition by micropipettes 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 60minutes 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 MS (Murashige and Skoog, 1962), WPM (Loyd and Mc Cown, 1980), SH (Schenk and Hilderbrandt, 1972) and B5 (Gamborg et al, 1968), etc. are now available. Concentrations of the macro and microelements, salts and organic constituents of the basal media are listed in **Table 2.1**.

Table 2.1 Compositions of basal media mg/lit

Ingredient	Basal Medium			
	MS	**SH**	**B5**	**WPM**
Macronutrients				
KNO_3	1900	2500	2528	400
NH_4NO_3	1650	-	-	-
$CaCl_2.2H_2O$	440	200	150	96
$MgSO_4.7H_2O$	370	400	246	370
KH_2PO_4	170	300	-	170
K_2SO_4	-	-	-	990
$(NH_4)_2 SO_4$	-	-	134	-
$Ca (NO_3)_2.4H_2O$	-	-	-	556
Micronutrients				
$MnSO_4.4H_2O$	22.3	10.0	10	22.3
$ZnSO_4.7H_2O$	8.6	1.0	2.0	8.6
H_3BO_3	6.2	5.0	3.0	6.2
KI	0.83	1.0	0.75	-
$CuSO_4.5H_2O$	0.025	0.1	0.025	0.025
$Na_2MoO_4.2H_2O$	0.25	0.2	0.25	-
$CoCl_2.6H_2O$	0.025	0.1	0.025	-
$FeSO_4.7H_2O$	27.8	15	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	20	37.3	37.3
Vitamins				
Myo-inositol	100	1000	100	100
Thiamine-HCl	0.1	5.0	10.0	0.1
Nicotinic acid	0.5	5.0	1.0	0.5
Pyridoxine-HCl	0.5	0.5	0.1	0.5
Glycine	2.0	-	-	2.0

Stock solutions of the media ingredients were prepared by dissolving weighed amounts of these salts in double distilled water. Appropriate aliquots of these solutions were mixed to prepare the media. Stock solutions of growth regulators (GR) were prepared by adding few drops of solvent

(EtOH, 1N NaOH, Ethanol, DMSO etc.) in the weighed amount of growth regulator to dissolve. After dissolution, the volume was made by addition of sterile double distilled water. The solutions were stored in refrigerator in sterilized bottles. **Table 2.2** describes the list of solvent, diluents and stock concentration of growth regulators used in the study.

Table 2.2 Preparation and Concentration of Growth Regulators

Growth regulators	Molecular weight	Solvent	Concentration of stock	Storage	Addition Before (B)/After (A) autoclaving
BAP	225.26	1 N NaOH	10mg/100ml	4ºC	B
KN	215.22	1 N NaOH	10mg/100ml	4ºC	B
TDZ	220.20	1N NaOH	10mg/100ml	4ºC	B
ZEA	219.25	1N NaOH	10mg/100ml	4ºC	A
GA3	346.4	Ethanol	10mg/100ml	4ºC	A
IAA	175.20	1 N NaOH	10mg/100ml	4ºC	A
NAA	175.20	1 N NaOH	10mg/100ml	4ºC	A
IBA	203.24	1 N NaOH	10mg/100ml	4ºC	A
PIC	241.46	1N NaOH	10mg/100ml	4ºC	B
2,4 D	221.04	Ethanol	10mg/100ml	4ºC	B

For media preparation calculated amounts of aliquots were added from stock solutions. Sucrose was weighed and added in required quantity (1-8%) and allowed to dissolve. The pH of the media was adjusted to 5.8 using 1N NaOH/1N HCl after mixing all the constituents except the gelling agent. The volume was made up with double distilled water. Gelling agents (Agar Agar / phytagel) were added and autoclaved for 20min. at 121°C and 15 lbs/(inch)^2. Autoclaved media were poured aseptically in sterile petridishes, as and when required for culturing tissues. For 60mm Petridishes 10ml medium was poured and 20ml medium was poured in 90mm dishes. For culturing in tubes and bottles, agar was added in the media and heated on water bath for the agar to melt. Molten medium was dispersed into sterile culture tubes (20ml of media) or bottles (50ml of media) after thorough mixing and was autoclaved. Media additive, sterilants like antibiotic Taxim (Cefotaxime, Alkem, India) and PPM (Plant preservative mixture) in required concentration were added aseptically to the autoclaved semisolid medium before distributing in culture vessels.

Sterilization:

Except the heat labile materials sterilization of all required materials was carried out by autoclaving at 121°C, 15 lb psi for 20 minutes. The heat labile materials were sterilized

aseptically using the 0.22µm pore size millipore filtration membranes procured from Advanced microdevices (P) Ltd., Ambala, India.

Culture conditions:

Cultures were incubated in 16hr photoperiod at 50µmol m^{-2} s^{-1} light intensity at 25±2°C. During hardening procedures, the cultures were incubated in continuous light (24h).

Transfer of plantlets to soil and Hardening:

In vitro raised plantlets were carefully taken out of the test tubes. The roots were washed gently under tap water to remove the adhering medium. Washed plantlets were transferred to autoclaved soil: sand (1:1) mixture in plastic cups and were covered with transparent polypropylene bags to maintain high humidity and were incubated in continuous light (24hr) for two weeks. The plants were watered once a week. The top corners of polypropylene bags were cut after two weeks to gradually expose the plants to the outside environment. After four weeks the cups were shifted in green house and maintained. After 3-4 weeks the plants were shifted in earthen pots containing a mixture of soil: sand (1:1).

Histological Preparations:

Tissues were prepared for histological studies following the methods described by Ruzin 1999. Tissues were cut into small pieces (approx 3x4mm) and were fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48hr at room temperature. These were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (mp 58-60°C). Serial sections of 10µm 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 study under microscope.

Microscopy and Photography:

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

Statistical Procedures:

Statistical methods were used for comparison of treatment means during optimization of the parameters for somatic embryogenesis using different explants. Completely Randomized Designs were used. The data was analyzed using Microsoft excel package. 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 ($P<0.01$/$P<0.05$), wherever applied. Graphs were plotted using Origin 6.1 software.

2.2 MOLECULAR CHARACTERIZATION

2.2.1 MATERIALS

Source of plant material:

Tender leaves from freshly sprouted buds of mature trees were collected. The leaves are cleaned and stored at -20°C. The preserved leaves, leaves from hardened plants, hairy roots produced from *Agrobacterium rhizogenes* transformation and non-transformed roots were used for DNA isolation.

Equipments:

Milli-RO water system (Millipore, USA) for deionized water, Horizontal electrophoresis unit (Tarson/ Bioera, India) for identifying DNA and PCR products., Spectrophotometer, Perkin & Elmer, USA , UV Transilluminator, (Syngene, UK) for gel visualization under ultraviolet radiation, Water bath (Julabo), Spinwin (Tarson), Spectrophotometer (Perkin & Elmer, USA), PCR (Veriti 96 well thermal cycler, Applied Biosystems, USA) were used for experiment.

Reagents and Chemicals for ISSR:

1. Tris-HCl pH 8.0 (1M); EDTA pH 8.0 (0.5M); NaCl (5M); CTAB (Sigma Chemicals) (20%); Chloroform:Iso-amylalcohol (24:1 v/v); Polyvinyl pyrrolidone; β-mercaptoethanol; cold isopropanol and ethanol (70%)
2. Extraction buffer: 100mM Tris-HCl (pH 8.0), 25mM EDTA, 1.5M NaCl, 2.5%
3. CTAB, 0.2% β-mercaptoethanol (Sigma Chemicals) (v/v) (added immediately before) and 1% PVP (Sigma Chemicals) (w/v) (added immediately before use).
4. High salt TE buffer: 1M NaCl, 10mM Tris-Cl (pH 8.0) and 1mM EDTA.
5. Agarose (Sigma Chemicals, India)
6. Electrophoresis buffer: Tris-acetate-EDTA (0.5X)
7. Loading buffer: Bromophenol blue (Sigma Chemicals) (0.25%) and glycerol (30%)
8. Fluorescent dye: Ethidium bromide (Sigma Chemicals) (10mg/ml).
9. Marker: 100 base pair DNA ruler (Bangalore Genei, India).
10. Enzymes: RNAase A (10mg/mL) and Taq DNA Polymerase (Bangalore Genei, India).
11. Buffers: *Taq* DNA Polymerase buffer with $MgCl_2$ (Bangalore Genei, India).
12. Nucleotides: dNTPs (G, A, T, C) (Sigma Chemicals).
13. The chemicals used for ISSR-PCR were obtained from "Sigma Chemical Co.," USA or Bangalore genei, India. Bioresource Pvt Ltd, Pune, synthesized ISSR primers locally. Primers specific to *Rol* gene were synthesized from MWG biotech Bangalore, India.

List of ISSR Primers Screened for variation analysis.

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

2.2.2 METHODS

DNA isolation protocol:

The DNA was isolated using Khanuja's protocol (Khanuja *et al.* 1999) with minor modification. The protocol is as follows:

1. About 150mg of leaf tissue was ground into fine powder with mortar and pestle in liquid nitrogen.
2. The powder was transferred to 2ml eppendorf tube and added 1ml of freshly prepared extraction buffer. Mixed by inversion to slurry.

3. The mixture in eppendorf incubated at 65 ºC in a water bath for 40-50 mins with occasional mixing.
4. Added 1ml of chloroform: isoamyl alcohol (24:1) and mixed gently by inversion for 15 min.
5. Spinned at 10000 rpm for 10 min at 25-30ºC.
6. Carefully transferred the upper clear aqueous layer to another 2ml microfuge tube.
7. Added 200µl of 5M NaCl and mixed gently (do not vortex).
8. Added 500µl of cold Isopropanol and by careful mixing produced fibrous nucleic acid that can be scooped and transferred to 2ml eppendorf tube and centrifuged.
5. Discard the supernatant and wash the pellet with 70% ethanol.
6. Dried the pellet in vacuum for 15 min and dissolved it in 300µl high salt TE buffer.
7. Added 5 µl of RNAase A and incubated at 37 ºC.
8. Added 300µl of chloroform: isoamyl alcohol (24:1) and mixed by gentle inversion.
9. Spinned at 10,000 rpm for 10 min at 25-30ºC.
10. Transferred the aqueous layer to a fresh 2ml tube eppendorf and added equal volume of isopropanol.
11. Spinned at 10,000 rpm for 10 min at 25-30ºC.
12. Washed the pellet with 70% ethanol. Dried the pellet in vacuum and dissolved the dried pellet in 200µl of sterile milli Q water. Stored at 4ºC until required.

The purity of the DNA was assessed with the ratios of absorptions at 260 nm and 280 nm. DNA concentrations were determined by running aliquots of DNA samples on a 0.8% agarose gel electrophoresis with concentration marker.

PCR Protocol for ISSR Markers:

The PCR protocol described by Raina *et al.* (1995) was followed and carried out in a

1. total volume of 25µl containing following components: 3µl of genomic DNA (30ng); 2.5µl of 10X *Taq* buffer with 1.5mM $MgCl_2$, 2.5µl of dNTPs (1mM/µl); 0.16µl of *Taq* Polymerase (3u/µl); 1.5µl of Primer (1.5pm/µl); 1µl of Spermidine (20mM) and 15.34µl of sterile milliQ water.
2. PCR amplifications were performed on a Veriti thermal cycler (Applied Biosystems) under the following program: Initial denaturation at 94^{o}C for 7min, followed by 40 cycles of denaturation at 94^{o}C for 30s, annealing at 52^{o}C for 45s, extension at 72^{o}C for 2 min with a final extension at 72^{o}C for 7min **(Fig. 2.1)**.
3. Negative controls, containing all PCR components except DNA were also set up and run with each set of reactions.
4. Low range DNA ladder (100bp-3000bp) was used for band sizing.
5. The ISSR products were loaded on 1.5% agarose gel stained with ethidium bromide for electrophoresis in 0.5X TAE at a constant current 50mA, for 2hr.

6. Visualization and documentation of gel was undertaken in Gel Documentation system (Syngene, UK).

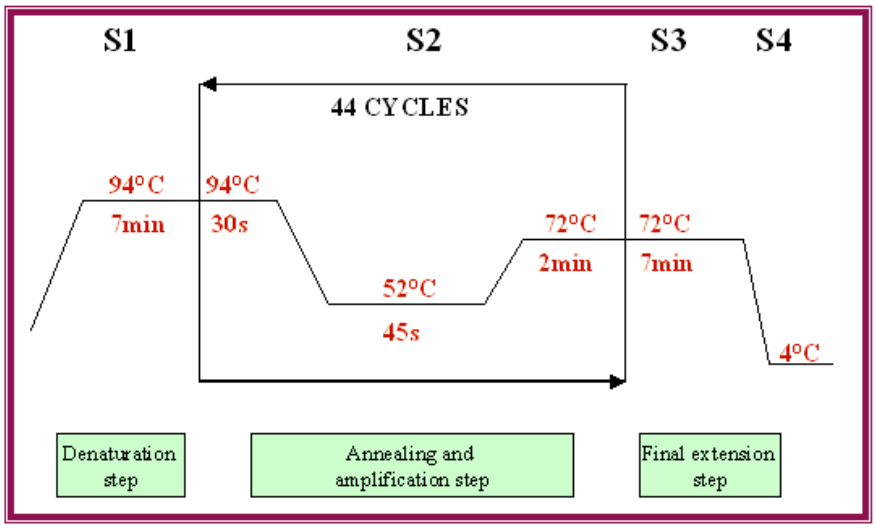


Fig. 2.1. Schematic diagram of PCR process for ISSR primers.

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.

PCR protocol for rol genes:

Genomic DNA isolation from the putatively transformed and untransformed roots, was carried out as described above.

Stock solutions:

Solution A: 50mM Glucose, 10mM EDTA, 0.1% Triton X-100, 25mM Tris-HCl pH 8.0, containing 2 mg/ml lysozyme freshly added.

Solution B (lysis buffer): 0.2M NaOH, 1% SDS (w/v). Solution C (5M Potassium acetate pH 4.8): Prepared by adding glacial acetic acid to 5M Potassium acetate until pH 4.8.Sodium acetate 3 M, pH 5.2: Prepared by dissolving 40.8gm Sodium acetate in 100ml water and the pH was adjusted with 3 M Acetic Acid.

RNAse A: stock 10.0mg/ml.

Plasmid DNA isolation:

Plasmid DNA from the *Agrobacterium* strains was isolated using the alkaline lysis method (Sambrook *et al.* 1989) with minor modifications.

1. About 1.5ml of overnight grown culture (16 hr) was taken in 1.5ml eppendorf tube and pelleted out at 10,000 rpm for 1min.

2. Supernatant was discarded and the pellet was re-suspended in 100µl of solution A by vortexing for 5s.
3. The mixture was incubated in ice for 10min.
4. To the mixture, 200µl of freshly prepared solution B was added and the content were mixed by gentle inversion. Again the mixture was incubated on ice for 5min.
5. To this, 150µl of 5M Potassium acetate pH 4.8 was added and the contents were mixed by vortexing.
6. The mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to another eppendorf.
7. To the above mixture 3µl of RNAase A (10mg/ml) was added and the mixture was incubated at 37ºC for 15 min.
8. To this, 500µl of Phenol:Chloroform:Isoamylalcohol (25:24:1) was added, the contents were mixed by inversion and centrifuged at 10,000 rpm for 10min.
9. The supernatant was collected in another eppendorf and 40µl of 3M Sodium acetate pH 5.2 and 1.0ml of cold (-20ºC) absolute ethanol were added. The contents were mixed and incubated at -20ºC for 1hr.
10. The mixture was centrifuged at 12000rpm for 15min and the pellet was washed with 70% cold (-20ºC) ethanol and air dried at room temp.
11. The pellet was re-suspended in 40µl of sterile deionized water.

PCR condition:

The PCR reactions were carried out in a total 50µl volume and consisted of 200ng of DNA, 10 pm/ µl primer, 200mM dNTP, 1U of Taq DNA polymerase, 1X PCR buffer and 1.5 mM $MgCl_2$. DNA amplifications were performed in a thermal cycler (Veriti thermal cycler, Applied Biosystems) using the programme: initial denaturation at 94ºC for 5min, followed by 35 cycles of 94ºC for 1min, 52.5ºC (for *rolB* gene)/62ºC (for *rolA* and *C* gene) for 1.5min and 72ºC for 2 min and a final extension at 72ºC for 10min (Fig.2.2). The amplification products were visualized on 25% w/v agarose gel stained with ethidium bromide (0.5µg/ml)

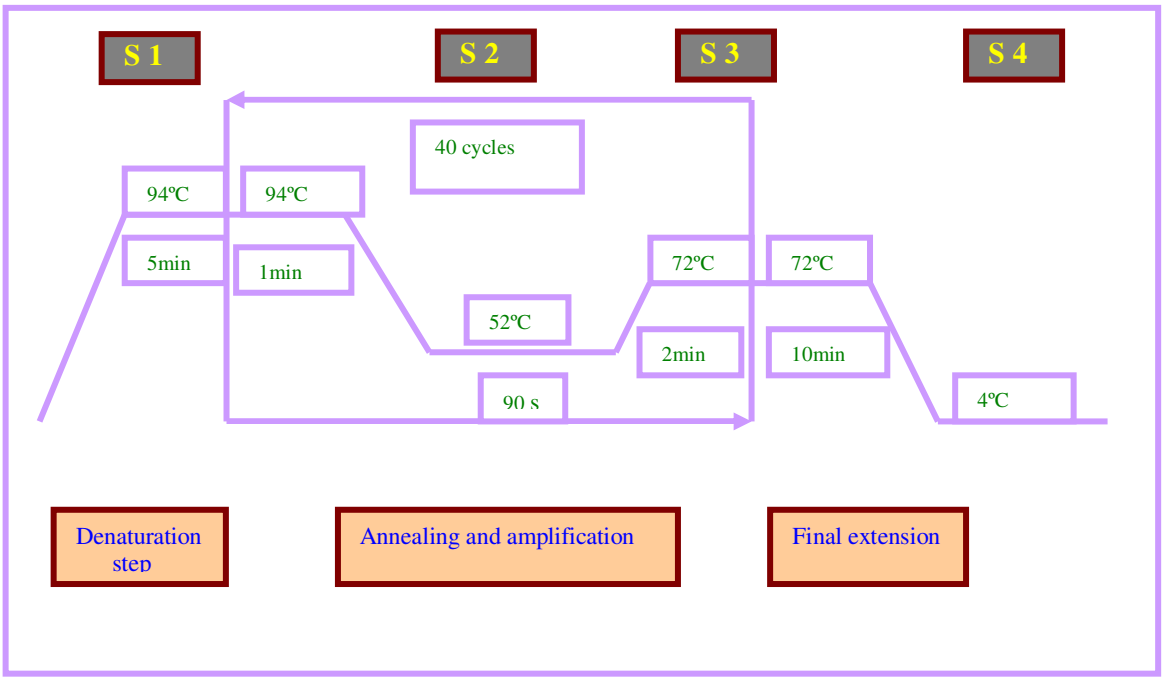


Fig. 2.2 Schematic diagram of PCR process for *rol* primers.

CHAPTER 3

ASEPTIC GERMINATION OF SEEDLINGS AND MICROPROPAGATION FROM SEEDLING EXPLANTS

3.1 INTRODUCTION

Conventionally *S. anacardium* is propagated through seeds. The seed viability of this species is limited (30-35%) and it decreases with time of storage (Panda and Hazra 2009). This characteristic of the seeds reduces the rate of natural propagation. Moreover, the seeds are utilized in Ayurvedic preparations. Thus, there is need for alternate propagation methods for germplasm preservation, rapid propagation and for production of biologically active compounds. This chapter of the thesis describes the experiments conducted to standardize protocols for aseptic germination and micropropagation of this species using seedling derived explants.

The process of micropropagation can broadly be divided into four steps:

Step I - Establishment of culture:

This stage begins with the excision of meristem tissue from an identified stock plant. The explant is treated with surface sterilants to remove contaminating organisms. Using aseptic techniques the explants are cultured onto appropriate nutrient medium. A rapidly proliferating culture is established under optimum culture conditions.

The major constraints in establishing sterile cultures of woody tissues are microbial contamination and interference of phenolics exudates (Jain and Ishii 2003). Use of fungicides and antibiotics limits the microbial infection to some extent. Use of antioxidants like ascorbic acid, PVP and charcoal helps in reducing the interfering effects of phenolics. For most micropropagation work the explant of choice is an apical or axillary bud. In general, younger tissues such as terminal or axillary shoot buds regenerate better than older and mature tissues of the same plant (Giri *et al*. 2004).

Step II - Proliferation of shoots in culture:

In this stage, shoots are proliferated in culture, which then serves as the source of explants for subsequent propagation for plant production and to maintain the stock culture. Multiplication of shoots is achieved by sub-culturing the shoots at regular intervals in appropriate medium.

Step III – Rooting:

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 *ex vitro*. For rooting *in vitro*, shoots are cultured either in growth regulator free medium or subjected to treatment of auxin prior to transferring to an auxin free medium for root induction.

For *ex vitro* rooting, shoots are isolated and treated as small cuttings. The cut end of these shoots are treated with auxin/s and planted in soil. These are then placed in a high humidity chamber for rooting. Rooting *ex vitro* has several advantages (Maene and Debergh 1983; Debergh and Read 1991; Kris *et al*. 2005):

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

Step IV – Hardening of tissue culture raised plants:

This stage involves transfer of plantlets from aseptic condition to green house and ultimately to the environmental conditions. Plantlets develop within culture vessel under aseptic condition, on a medium containing sugar and nutrients, in an atmosphere with high relative humidity and low levels of light. All these factors 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 establish in soil and adapted to ambient conditions under which plants are normally grown. High relative humidity is 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. Younger the tissues better the *in vitro* response. The age of the stock plant, physiological status of the explant and its developmental stage as well as its size determine the success of *in vitro* protocol (Franclet 1987). Mature plant derived explants are reported to be highly recalcitrant *in vitro*. Moreover, high degree of contamination in mature tissues poses difficulty 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 1993). Juvenile explants have been extensively employed for clonal propagation of woody plants and forest trees (Giri *et al*. 2004). 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 requirements of the plant for its growth and maturity.

Reports of successful micropropagation of various tree species are reviewed extensively (Trigiano *et al*. 1992; Giri *et al*. 2004). In forest tree species, the success has been largely restricted to seedling materials (Rodriguez and Vendrame 2003). *In vitro* protocols, using seedling explants, have been successfully employed for rapid propagation and manipulation of woody plants such as *Madhuca longifolia* (Rout *et al*. 1993); *Swartzia madagascariensis* (Berger and Schaffner 1995); Cashewnut (Das *et al*. 1996); *Dalbergia sissoo* (Pradhan *et al*. 1998); *Gymnema sylvestre* (Komalavalli *et al*. 2000); *Bauhinia vahlii* (Bhatt and Dhar 2000); (Saffi and Borthakur 2002); *Schinopsis balansae* (Sansberro *et al*. 2003); *Cercis yunnanensis* (Cheong 2003); *Ceropegia candelaebrum* (Beena *et al*. 2003); *Leucaena leucocephala*, Mulberry (Thomas *et al*. 2003); *Pterocarpus marsupium* (Chand and Singh 2004; Anis *et al*. 2005); *Sesbania drummondii* (Cheepala *et al*. 2004); *Peltophorum pterocarpum* (Uddin *et al*. 2005); *Pongamia pinnata* (Sujatha and Hazra 2006); *Maytenus canariensis* (Gutiérrez-nicolás *et al*. 2008) and *Stereospermum personatum* D.C (Shukla *et al*. 2008) etc.

In the present investigation, juvenile tissues from *in vitro* germinated seedling derived explants of *Semecarpus anacardium* L. were studied for the establishment of *in vitro* regeneration protocol. 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 mature explants. This strategy is often followed in tree species for establishment of micropropagation protocol (Giri *et al*. 2004). Seeds are germinated *in vitro.* After the seedling attains an appropriate size, the nodal explants are excised from seedlings and are used for establishment of culture. Factors that influence establishment of cultures include surface sterilants, microbial interference, growth regulators, organic supplements, sucrose concentration, type of culture vessel, incubation conditions etc.

Production of multiple shoots from axillary and apical meristems is the most widely followed method of *in vitro* propagation of plants (George 1993). Using suitable nutrient media and growth regulators, multiple shoots are regenerated from shoot tip or axillary bud. These shoots can be rooted to produce plantlets, and also used for further multiplication cycles. Thus single explant can give rise to a large number of plantlets in a short period. Such a method has been successfully employed for propagation of several fruit trees (Jaiswal *et al*. 2004).

Semecarpus anacardium trees bear seeds in winter. Seasonal bearing of seeds and poor viability (Anonymous 1999) limits the success of conventional propagation. Poor germination frequency restricts propagation in various species. To improve the germination frequency various pre-

treatments are described (**Table 1.1**). *Semecarpus* nuts were highly recalcitrant and major constraints encountered in establishment of seedling cultures included poor germination frequency, acute microbial contamination and heavy leaching of phenolics. Standardization of a convenient and effective method for *in vitro* germination of seeds was necessary for establishment of seedling culture.

The present study was undertaken with the following objectives:

1. Standardize of an effective surface sterilization procedure and *in vitro* germination of *Semecarpus anacardium* seeds.
2. Development of a protocol for micropropagation of shoots using the meristems of *in vitro* grown seedling derived nodal explants.
3. Refinement of the micropropagation protocol using Thidiazuron (TDZ) to increase the number of buds induced from single meristem

The results of the above experiments are described in the following three Sections

SECTION A:

In vitro germination of *Semecarpus anacardium* L. seeds.

Semecarpus anacardium nuts were highly recalcitrant and major constraints encountered in establishment of seedling cultures included poor germination frequency, acute microbial contamination and heavy leaching of phenolics. The present section describes the experiments conducted to overcome these limitations using various pre-treatments and standardization of a protocol to achieve rapid, uniform and high frequency germination of *S. anacardium* seeds.

3A.1 MATERIALS AND METHODS

Pre-treatments for optimum germination:

Mature seeds of *Semecarpus anacardium* (**Fig. 3Aa**) were collected during December from trees growing naturally in different locations around Pune, Maharashtra, India. The seeds were sun dried for 3-4 days. These were washed thoroughly under running tap water and subjected to various treatments.

Following pretreatments were applied before surface sterilization:

- **i.** Mechanical scarification (Removing manually the pericarp and mesocarp),
- **ii.** Treatment with 50% sulfuric acid (Qualigens) for 20 min ,
- **iii.** Treatment with concentrated sulfuric acid (98.08%) for 20 min,
- **iv.** Cold treatment at 4˚C for 7 days,

v. Treatment with 2% Bavistin (50% Carbendazim, BASF, India) for 24 hrs,

vi. Treatment with 10N sodium hydroxide (Qualigens) for 20 minute,

vii. Hot water treatment at 80˚C for 20 minutes.

Following pretreatments were applied under aseptic condition after surface sterilization with Sodium hypochlorite.

viii. Treatment with plant preservative mixture (Sameer Science Lab, Jabalpur, M.P.) for one-hour,

ix. Quick dip in absolute alcohol and flaming of the seeds,

x. Overnight treatment with 2%(w/v) 8-Hydroxyquinoline (Qualigens) solution,

xi. Overnight treatment with 3% copper sulphate (Qualigens),

xii. Treatment with 3% Sodium chloride (Qualigens) for 12 hrs.

Surface sterilization:

Seeds were treated with 2% Bavistin with a few drops of liquid detergent Exalin (Merck) and placed on Shaker at 90 rpm for one hour. These were washed several times in sterile distilled water and treated with 6% solution of Savlon for 15 min. This was followed by washing with sterile distilled water to eliminate Savlon. Finally the seeds were treated with sodium hypochlorite (Merck) with 4% available chlorine for 45 min. The seeds were then rinsed 5-6 times in sterile distilled water. All operations were carried out under aseptic conditions. Surface sterilized seeds were cultured in half strength basal woody plant medium (WPM) (Lloyd and Mc Cown, 1980).

Germination of seeds:

The seeds were treated with concentrated sulfuric acid for 10, 20, 30 and 40 min under non-sterile condition. Acid treated seeds were surface sterilized and were cultured in two basal media; WPM and MS (Murashige and Skoog, 1962). Full strength basal media with and without charcoal and/or sucrose; similarly half strength basal media with and without charcoal and/or sucrose were used. WPM medium with GRs including 6-benzylaminopurine (BAP), kinetin (KN), thidiazuron (TDZ), and gibberellic acid (GA_3) (Sigma chemicals) were tested to achieve optimum germination frequency. Gibberellic acid (GA_3) was added aseptically to sterile medium whereas other growth regulators were added before sterilization of medium. Seeds with emerged radical were scored for germination.

Effect of storage on in vitro seed germination:

Seeds were cultured every month for six months from the date of collection in December. Acid treated and surface sterilized seeds were cultured in half strength WPM medium. Seeds with emerged radical were scored for germination after 4wks of culture.

The pH of all the media were adjusted to 5.8 before sterilization. Media were sterilized by autoclaving at 121°C for 20 minutes at 1.06 kg cm^{-2}. Phytagel (Sigma) (0.2%) was used for gelling the medium. All cultures were incubated in darkness at 25±2°C for 4wks.

All experiments were repeated four times with 10 replicates each. Number of seeds germinated and number of non-contaminated seedlings were counted. All the data were subjected to analysis of variance (ANOVA). The difference among treatment means was tested using a Duncan multiple range test (DMRT) at probability level ($P<0.01$). Student's t-test was conducted between *ex vitro* germination percentage of five month old seeds that were treated with Sulfuric acid.

Morphological studies of seeds before and after concentrated sulfuric acid treatment:
Sulfuric acid treated and un-treated seeds were cut open horizontally and vertically through the micropylar end with the help of secator. The changes in the seed surface and in the cut seeds were studied microscopically using a stereomicroscope.

3A.2 RESULTS AND DISCUSSION

Seeds of *S. anacardium* were collected in December. Germination frequency of these seeds in soil ranges from 25-30%. Whereas, it was 33% when germinated *in vitro* on WPM medium supplemented with 2% sucrose. *In vitro* germination was noted within 10-15 days of inoculation in comparison to 25-30 days in *ex vitro* condition.

In the preliminary experiments, all seeds cultured *in vitro* were lost due to fungal and bacterial contamination. Several seeds leached phenolics causing blackening of the medium. To optimize an effective method of surface sterilization and for establishment of seedling cultures of *Semecarpus anacardium*, various pre-treatments were tested (**Table 3A.1**).

Table 3A.1 Effect of different pretreatment on *Semecarpus anacardium* seed germination

Treatments	**Condition of treatment**	**Aseptic seeds (mean ± sd) %**	**Germination (mean ± sd) %**
Control	Surface-sterilized seed	0 ± 0 ^{e}	33 ± 9.57 ^{gh}
Mechanical treatment	Seed coat removed mechanically before surface sterilization	100 ± 00 ^{a}	00 ± 00 ^{j}
50% H_2SO_4	20min	00 ± 00 ^{e}	40 ± 8.16 ^{cd}
Conc. H_2SO_4	10min	20 ± 8.16 ^{cd}	35 ± 12.91 ^{efg}
Conc. H_2SO_4	20min	**73 ± 9.57 ^{b}**	**63 ± 5.00 ^{a}**
Conc. H_2SO_4	30min	100 ± 00 ^{a}	27 ± 9.57 ^{h}
Conc. H_2SO_4	40min	100 ± 00 ^{a}	00 ± 00 ^{j}
Cold treatment	Seeds pretreated at 4°C for a week	00 ± 00 ^{e}	38 ± 5.00 ^{cdef}
24 hr bavistin	On shaker 90 rpm	00 ± 00 ^{e}	39 ± 16.07 ^{cde}
NaOH 10N	20 min	25 ± 7.5 ^{c}	29 ±13.14 ^{h}
Hot water (80°C)	Hot water for 20min	00 ± 00 ^{e}	35 ± 5.77 ^{efg}
8-HQLS 2%	Over night soaking (12hr)	00 ± 00 ^{e}	35 ±12.90 ^{efg}
Flame	Surface sterilized seeds dipped in alcohol and flamed	00 ± 00 ^{e}	23 ± 5.00 ^{i}
PPM 2%	1hr after surface sterilization	00 ± 00 ^{e}	38 ± 26.29 ^{cdef}
$CuSO_4$ 3%	Overnight soaking (12hr)	00 ± 00 ^{e}	43 ±23.12 ^{c}
NaCl 3%	Overnight soaking (12hr)	00 ± 00 ^{e}	56 ± 20.46 ^{b}
ANOVA		S1%	S 1%

Mean followed by the same letters within a column do not differ significantly at $P \leq 0.01$ according to DMRT.

Manual removal of the seed coat with forceps and scalpel helped in controlling microbial contamination but the seeds lost viability. This was possibly due to increased production of phenolics from the injured parts of the seeds or due to injury to the embryo. The medium turned dark brown within a day of culturing. Surface sterilization was ineffective in all other treatments except when seeds were pre-treated with concentrated H_2SO_4 and 10N NaOH. These two treatments were effective in eliminating the microbial contamination in 73 and 25% of the seeds, respectively. However, germination frequency of seeds treated with concentrated H_2SO_4 was 63% whereas it was only 29% on treatment with 10N NaOH. The liquid obtained after H_2SO_4 treatment was thick and dark brown due to phenolics and other chemicals (unidentified). Rinsate from several rinses after acid treatment was brown indicating additional leaching of the compounds. This was obvious from the difference in color of the seeds before (**Fig. 3Aa**) and after (**Fig. 3Ab**) acid treatment.

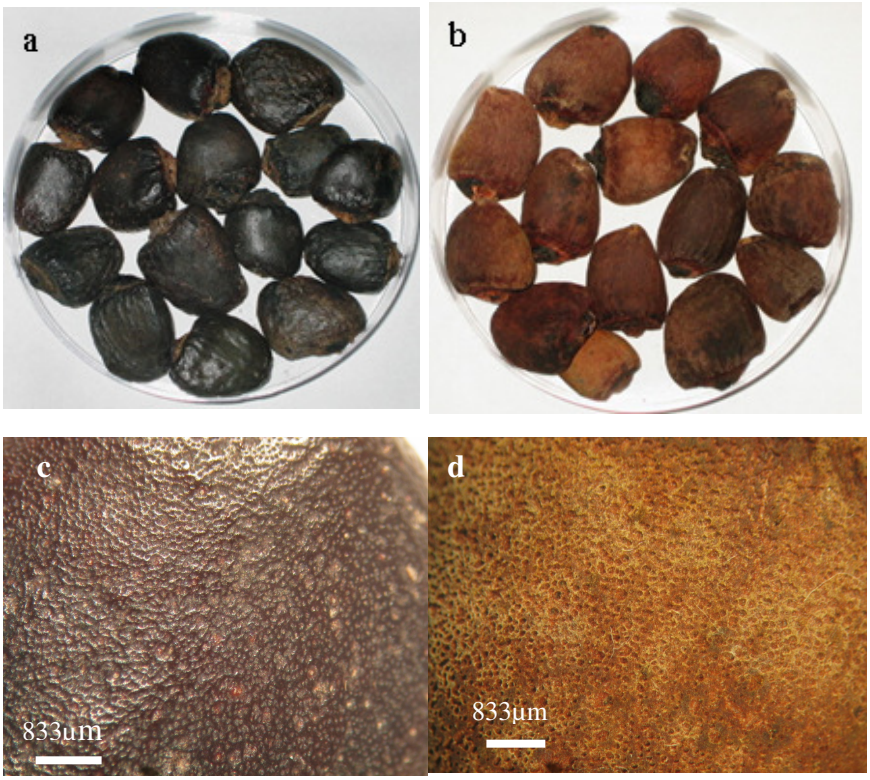


Fig. 3A a. Seeds of *S. anacardium*; **b.** Change in surface color of *S. anacardium* seeds due to elimination of the dark brown pigments on concentrated sulfuric acid treatment; **c.** Surface of the seed with granular undulation before sulfuric acid treatment.; **d.** Absence of granular undulation and presence of innumerable pores on the seed surface after concentrated sulfuric acid treatment for 20 minutes.

The surfaces of the seeds before and after H_2SO_4 treatment were examined microscopically. Dark brown undulations, noted on the surface of the untreated seeds (**Fig. 3Ac**) were absent after 20 min acid treatment (**Fig. 3Ad**). The acid treated seeds were yellowish-to-light brown in color suggests removal of the brown deposits. On the surface of the acid-treated seeds (**Fig. 3Ad**) innumerable pores were apparent. Presumably microbes are harbored in the undulations of the seed surface and the granular dark brown deposits restrict the sterilants to access these. Thus, surface sterilization without acid treatment was ineffective. Treatment with concentrated H_2SO_4 dissolved the granular deposits and allowed the sterilants to reach the contaminants. Fungal contamination was predominant in seeds of *S. anacardium* and appeared within a few hours of culture. Bavistin, a systemic fungicide, used successfully in establishing *Pongamia* seedling culture to control fungal contamination (Sujatha and Hazra 2006) failed to restrict fungal growth in *S. anacardium*. Surface sterilization with NaOCl after 10N NaOH treatments achieved 25% microbe-free cultures but the germination frequency was 29% (**Table 3A.1**). Soaking seeds in hot water is an inexpensive and easy method to soften the seed coat and stimulate water uptake

by seeds in tropical species (Smith and Bent 1993). Hot water treatment enhanced germination of *Tamarindus indica* L. (Muhammad and Amusa 2003) and *Tithonia diversifolia* (Akinola *et al*. 2000). Hot water, or 8-hydroxyquinoline sulfate treatment were ineffective in surface sterilization of *S. anacardium* seeds. Flaming of the seeds reduced germination frequency to 23%. Soaking the surface-sterilized seeds in PPM at 2% for 1hr was ineffective against the contaminants. Overnight soaking of seeds in 3% $CuSO_4$ or 3% NaCl failed to control contamination although germination frequency increased to 42 and 56%, respectively.

The presence of anthocyanins and phenolics and hardness of the seed coat inhibited germination (Bhattarai *et al.* 2008). H_2SO_4 has been used effectively for treatment of hard-coated seeds. The optimum time for H_2SO_4 treatment depends on the hardness of the coat. It varied from 3 min in *Parkia biglobosa* (Aliero 2004) to 90min in *Sesbania rummondii* (Elastin 1984). Soaking in 50% H_2SO_4 for 20min (**Table 3A.1**) failed to reduce contamination in *S. anacardium* seeds, but the germination frequency was higher (40%) than the untreated seeds (33%). Optimum germination frequency (63%) with reduced contamination was achieved in seeds treated with concentrated H_2SO_4 for 20 min. Contamination was eliminated in *S. anacardium* seeds by increasing the period of H_2SO_4 treatment to 30 and 40min but the germination percentage decreased to 27% and 0% respectively. Surfaces of the seeds treated with H_2SO_4 for various time periods were examined microscopically (**Fig. 3A e-h**). Presence of residual granular brown deposits on the surface of the seeds treated for 10min (**Fig. 3Ae**) indicated incomplete removal of the deposits. Seeds treated for 20min did not show any deposit and distribution of the pores was obvious (**Fig. 3Af**). Longer treatment (30 and 40min) in acid created larger pores (**Fig. 3A g-h**). Reduction in the germination frequency in seeds treated for longer period in acid could be due to injury to the embryos (Sy *et al*. 2001).

Microscopic examination of the dissected seeds of *S. anacardium* revealed three layers around the seed (**Fig. 3Ai**). The outer layer is a compact epicarp with a smooth surface. The middle layer was spongy with channels, which were filled with phenolics. The innermost one was a hard, stony endocarp. Outer surface of the epicarp was absent in the seeds treated with concentrated H_2SO_4 (**Fig. 3Aj**). In mature seeds of *S. anacardium* the micropylar end is devoid of endocarp (**Fig. 3Ak**). Acid treatment caused erosion of the epicarp (**Fig. 3Aj**) which possibly allowed excretion of inhibitory phenolics and entry of water into the seed to facilitate germination of the embryo.

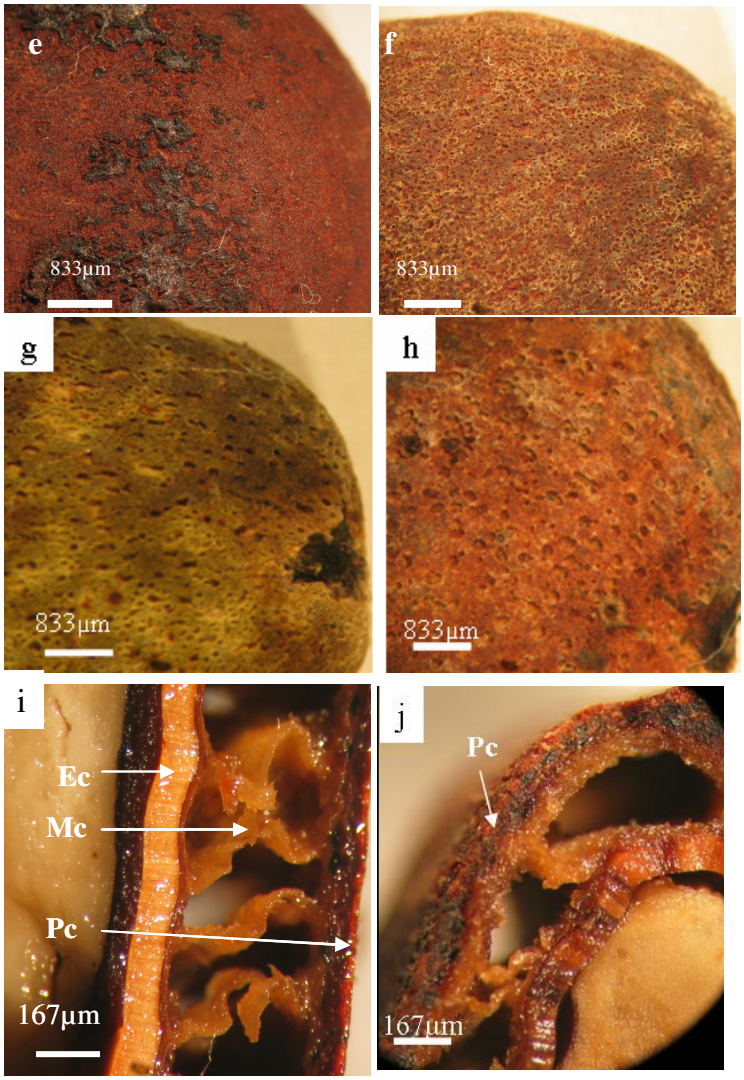


Fig. 3A **e.** Surface of seed treated with sulfuric acid for 10 minutes. Presence of residual pigment indicates incomplete removal; **f.** Surface of seed after 20 minutes acid treatment. Absence of pigment, and presence of numerous pores suggest complete removal of pigment layer; **g** and **h.** Seed surface after 30 and 40 minute acid treatment. Appearance of larger pores indicates erosion of seed coat. **i.** Horizontally cut seed showing epicarp (Pc), mesocarp (Mc), endocarp (Ec); **j.** Horizontally cut acid treated (20 minute) seed with, eroded epicarp (Pc) while Mesocarp (Mc) and endocarp (Ec) are unaffected.

In *Rhus* species (Li *et al*. 1999) anatomical changes were noted in the endocarp after 1hr of H_2SO_4 treatment. A clearly outlined micropylar region (**Fig. 3Ak, 3Al**) was seen in the untreated seed. After treatment for 20min with concentrated H_2SO_4 the erosion of the epicarp was obvious (**Fig. 3Am**). When acid treatment was increased to 40min, cracks were noted in the micropylar region (**Fig. 3An**) and in surroundings areas. Failure in germination of these seeds could be due to injury of the embryos caused by entry of acid through these cracks. The use of concentrated H_2SO_4 for longer duration had lethal effects on the embryos of the seeds in *Piliostigma thonningii* (Ayisire *et al*. 2009). Failure of a seed to germinate in favorable environmental conditions is defined as seed dormancy (Bentsink and Koornneef 2008). This phenomenon is

attributed to three mechanisms including hard seed coverings, chemical inhibitors including growth regulators, phenolics, etc. and morphological aspects such as undeveloped embryos. Exogenous dormancy was associated with (i) impermeability to water (ii) hardness of the seed coat which does not allow the embryo to expand during germination and (iii) presence of inhibitors in the outer coverings.

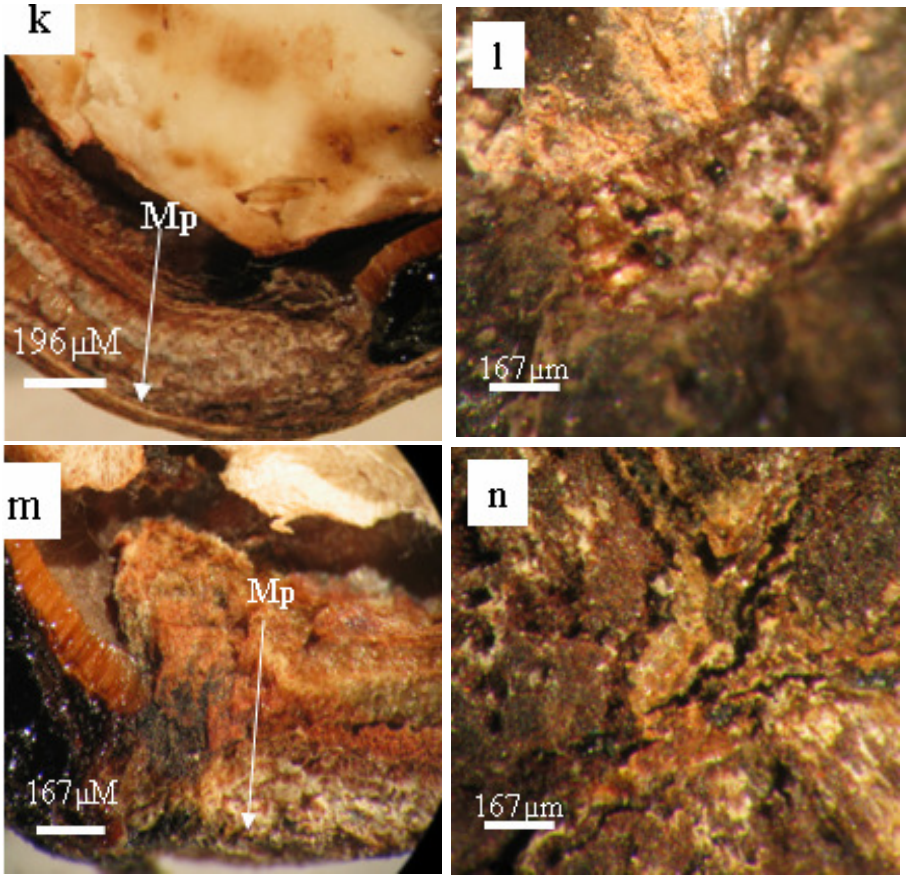


Fig. 3A k. Vertically cut untreated seed section. Intact micropylar region before acid treatment is apparent; **l.** Surface appearance of micropylar region of untreated seed; **m.** Vertical section of acid treated (20min) seed cut through micropylar (Mp) region. The micropylar (Mp) region appears distorted but no cracks were seen; **n.** Micropylar region of seed after acid treatment for 40 minutes. Appearance of cracks suggests damage in this region due to over exposure in sulfuric acid.

A hard seed coat and presence of phenolics in *S. anacardium* influenced germination negatively possibly by establishing a barrier that interfered with the water uptake required for imbibition, and gaseous exchange for respiration. The seed coat leached enormous amount of phenolics in culture. Scarification of seeds with concentrated H_2SO_4 not only made the seeds imbibe water but also eliminated some of the inhibitory phenolics that might reduce the dormancy, resulting in enhanced germination frequency. The seedlings obtained by germinating the seeds were contamination free and healthy (**Fig. 3Ao).** These seedlings were used as source of explants for culture establishment.

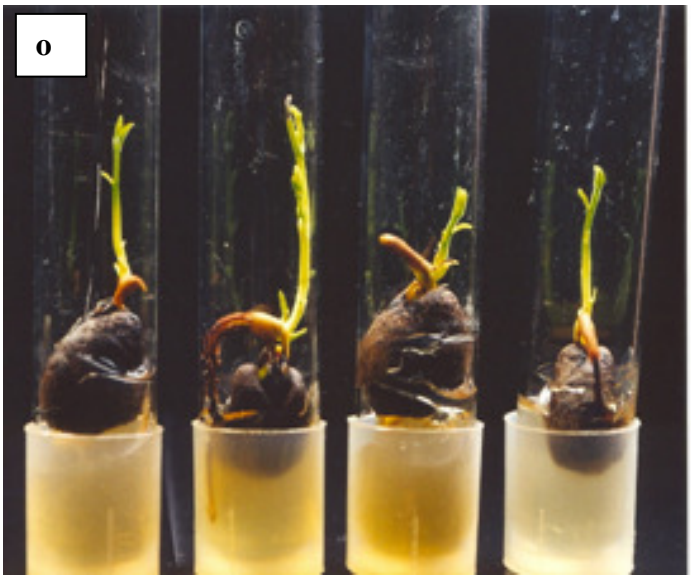


Fig. 3A o. *In vitro* raised seedlings of *S.anacardium* obtained from seeds pretreated with concentrated sulfuric acid for 20 minutes.

Seed dormancy and the capacity of seeds to germinate were associated with a hard seed coat in *Corchorus olitorius* (Velempini *et al*. 2003). In *Anacardiaceae* the cause of dormancy was physical due to water impermeability of the pericarp (Baskin and Baskin 1998). In *S. anacardium*, germination increased from 33 to 63% in seeds treated with concentrated H_2SO_4 for 20min. Treatment with acid partially digested the outer surface of the pericarp and rendered it permeable to water. Erosion of the seed surface due to acid treatment was obvious (**Fig. 3Aj**). Thus, a passage for the release of phenolics and water imbibition was created. Production of a thick, dark brown liquid after acid treatment indicated rapid release of phenolics, which was eliminated by repeated washing with water. These phenolics could be growth inhibitory. Increase in germination frequency to 63% (**Table 3A.1**) in acid-treated seeds supports this observation. On opening of the pores on the seed surface, the inhibitory compounds were released and water entered to initiate the process of germination. Acid treatment was also effective in controlling contamination in 73% of the cultures. This could be due to destruction of the harboring microbes, which came in contact with the concentrated H_2SO_4.

Several media combinations with or without GRs (**Table 3A.2**) were tested to enhance the germination frequency. Half-strength WPM without sucrose was more suitable for germination of *S. anacardium* seeds.

Table 3.A.2 Effect of media composition and growth regulators on germination of *Semecarpus anacardium* seeds.

Media	**Germination (mean ± sd) %**
MS	17 ± 15.3 ^{cd}
MS charcoal	23 ± 05.8 ^{bcd}
1/2 MS	37 ± 15.3 ^{abcd}
1/2 MS with out sucrose	57 ± 15.3 ^{ab}
WPM	34 ± 11.5 ^{abcd}
WPM charcoal	50 ± 26.5 ^{abc}
1/2 WPM	50 ± 10.0 ^{abc}
1/2 WPM with out sucrose	**63 ± 11.5** ^{a}
WPM BAP 2.22 µM	13 ± 05.8 ^{d}
WPM BAP 4.44 µM	20 ± 17.2 ^{cd}
WPM BAP 8.88 µM	17 ± 11.5 ^{cd}
WPM KN 2.32 µM	30 ± 17.3 ^{bcd}
WPM KN 4.64 µM	17 ± 15.3 ^{cd}
WPM KN 9.28 µM	27 ± 05.8 ^{bcd}
WPM BAP 2.22 µM + 2.32 KN µM	10 ± 17.3 ^{d}
WPM BAP 4.44 µM + 4.64 KN µM	13 ± 05.8 ^{d}
WPM TDZ 0.227 µM	20 ± 10.0 ^{cd}
WPM TDZ 2.27 µM	7 ± 05.8 ^{d}
WPM TDZ 4.54 µM	3 ± 05.8 ^{d}
WPM TDZ 9.08 µM	3 ± 05.8 d
WPM GA_3 0.29 µM	30 ± 17.3 ^{bcd}
WPM GA_3 1.44 µM	30 ± 26.5 ^{bcd}
WPM GA_3 2.88 µM	27 ± 23.1 ^{bcd}
WPM GA_3 5.76 µM	23 ± 32.1 ^{cbd}
ANOVA	**S1%**

Mean followed by the same letters within a column do not differ significantly at $P \leq 0.01$ according to DMRT.

Germination was 63% in this medium compared to 56% in half-strength MS medium without sucrose. Between the two basal media, WPM has more sulphate and calcium. Moreover, NaOCl, which was used as surface sterilant following the pre-treatments, could have increased the germination frequency as reported in *Podophyllum hexandrum* Royle (Nadeem *et al*. 2000).

Uniform and consistent germination of seeds has been reported with the use of PGRs (Nadeem *et al*. 2000; Nikolic *et al*. 2006; Sujatha and Hazra 2006). None of the media formulations with PGRs (**Table 3A.2**) was effective in enhancing the germination frequency of *S. anacardium* seeds *in vitro*. On the contrary, the frequency was reduced on incorporation of PGRs in half-

strength WPM medium without sucrose in which 63% germination was achieved. This suggests that failure of the *S. anacardium* seeds to germinate was due to dormancy associated with the hard seed coat and the phenolics and is not due to lack of any of the PGRs tested (**Table 3A.2**).

Results of the experiment conducted to assess viability of *S. anacardium* seeds over the period of storage demonstrated that germination frequencies of the seeds *in vitro* and in soil were similar in freshly collected seeds and after one month of storage (**Table 3A.3**). Seeds exhibited a gradual drop in viability after two (55%), three (32%) and four months (22%) of storage. The viability of the seeds was lost completely after 6 months.

Table 3A.3 Reduction in seed viability on storage.

Time of culture	**Germination (mean± sd)*%**
December	64 ± 0.58 [a]
January	63 ± 2.52 [ab]
February	55 ± 7.64 [abc]
March	32 ± 7.65 [d]
April	22 ± 7.46 [d]
ANOVA	**S 1%**

*H_2SO_4-treated seeds,
Mean followed by the same letters within a column do not differ significantly at $P \leq 0.01$ according to DMRT.

The germination frequency of the seeds (with or without H_2SO_4 treatment) was 4 and 21%, respectively (**Table 3A.4**) when planted in soil five months after collection (April). This result corroborates with the data (22%) generated by culturing H_2SO_4-treated seeds *in vitro* in April (**Table 3A.4**). Storage period influences seed germination in plants like *Annona cherimola* Mill (Padilla and Encina 2003) and *Avicennia marina* (Farrant *et al*. 1986).

Table 3.A.4 Seed germination in soil after storage for 4 months*

Treatment	**Percentage of germination**
Control	4
Control	21
t-test	S 5%

*Seeds collected in December and tested in soil in April.

3A. 3 CONCLUSION

The protocol standardized for seed germination may find application in obtaining an increased number of seedlings from freshly collected seeds to supplement conventional propagation of this species. The experiment demonstrated that concentrated H_2SO_4 not only helped in increasing the permeability of the hard seed coat and excretion of phenolics but also acted as a surface sterilant to a limited extent. Improved germination in the absence of sucrose supplement is also an

important observation in this study. Since establishment of seedling culture is a major constraint in propagation of several tropical tree species this protocol may find application in increasing germination frequency *in vitro*, to provide microbe-free explants for further studies on micropropagation and genetic transformation.

SECTION B:

Optimization of culture conditions for micropropagation of S*emecarpus anacardium* L. from axenic shoot culture derived nodal explants.

In this section the experiments conducted to standardize a protocol for micropropagation of *Semecarpus anacardium* L using axenic seedling derived nodal buds are described. The information generated in this study will be useful for optimization of a protocol for rapid propagation of mature *S.anacardium* plant using *in vitro* techniques.

3B.1 MATERIALS AND METHODS

Establishment of sterile cultures:

Seedlings of *S.anacardium* were obtained following the method described in Section A (Panda and Hazra 2009). From each seedling, 4-5 nodal explants (1-2cm) were excised. Keeping in view the efficacy of BAP and KN in large number of micropropagation protocols, several media formulations (list not included) containing these GRs either singly, or in combinations with three basal media viz., MS (Murashighe and Skoog 1962), WPM (Lloyd and Mc Cown 1980) and B5 (Gamborg et al. 1968), were tested for regeneration, by culturing varied number of nodal explants obtained from the aseptic seedlings. The number of explants and the number of replicates in each media formulation varied. These explants were then cultured for 4 weeks in growth regulator (GR) free WPM medium containing 2% sucrose and 0.2% charcoal. Explants showing axillary bud sprouting were maintained for four passages of 4 weeks each in GR free medium to eliminate the carry over effect. The shoots elongated sufficiently to obtain 3-4 numbers of nodal explants from each. Subsequently these cultures were maintained in GR free medium for several passages. These shoot cultures served as source of explants for the experiments conducted to optimize a protocol for rapid propagation of *S. anacardium*.

Culture conditions

The pH of all media was adjusted to 5.8 before sterilization. Media were autoclaved at 121°C for 20 minutes at 1.06 $kg^{-}cm^{-2}$. The experiments were carried out in culture tubes with cotton plugs. All the media were supplemented with 2% sucrose. Phytagel (0.2%) was incorporated in the medium for gelling. Cultures were incubated for 4wks in 16hr photoperiod at 25±2°C with

irradiance of 50µmolm-^{2}s-^{1} provided by cool white fluorescent lamps (Phillips). All experiments were repeated four to five times with ten replicates in each repeat.

Parallel experiments were conducted to determine the requirement of various factors for optimum sprouting, elongation and growth of *S. anacardium* shoots in culture.

Effects of basal salt formulation:

Media formulations such as MS (Murashige and Skoog 1962), WPM (Loyd and Mc Cown 1980), SH (Schenk and Hilderbrandt 1972) and B5 (Gamborg *et al*. 1968) were used to evaluate the response of axillary buds. Number of explants responding, number of buds per explant, length of the shoot, number of leaves per shoot and percentage of explant with phenolics were noted after 4wks to find out the suitable media formulation. The experiments were conducted in GR free medium with 2% sucrose, and 0.2% phytagel.

Cytokinins:

Woody plant medium (WPM) supplemented with various concentrations and combinations of BAP and or KN were tested to optimize the requirement of these GRs for optimal sprouting of buds from the axenic culture derived nodal explants, proliferation and elongation of shoots. The sprouted buds from these media were subsequently maintained in GR free semisolid WPM medium with 2% charcoal and 0.2% phytagel for three passages of 4wks each (12wks) for multiplication and elongation of the shoots. At the end of every passage the number of shoots per explant and the length of the primary shoot in each culture were noted before transferring the explants to fresh medium of similar composition. The primary shoot (length of the sprouted nodal explants cultured in the medium) and its multiples which elongated to 1-3cm in 12wks were isolated from the primary explant and the number of shoots obtained per explant were scored. After excising the elongated shoots the explant with remaining shoot primordia were transferred to fresh medium and incubated for 4wks to obtain more number of elongated shoots. This process of isolation of shoots and re-culturing in medium of same composition was repeated for 4 passages (altogether 28wks). The number of elongated shoot isolated from the explant in each passage was scored. These shoots were treated for induction of roots.

Effects of carbon source and sucrose concentration:

Carbon source like sucrose, glucose, fructose and maltose at 2% was tested for optimum growth of the nodal explants. Different concentrations (1-5%) of sucrose were also tested. Number of explants responding, number of buds per explant, length of the shoot, number of leaves per shoot and percentage of explant with phenolics were noted after 4wks. The experiments were conducted in GR free WPM medium with 0.2% phytagel.

Gelling agents:

Two gelling agents including 0.7% agar (Hi-media, India) and 0.2% Phytagel (Sigma, USA) were tested for multiplication and elongation of *S. anacardium* shoots in culture. The gelling agents were incorporated in WPM basal medium with 2% sucrose. The pH of the agar and phytagel containing media was checked before and after autoclaving. The axenic shoot culture derived nodal explants were used for this experiment and the explants were cultured for 2 passages of 4wks each in these media. Number of explants sprouted, number of buds per explant and the lengths of the primary shoots were noted after 4wks.

Antioxidants:

Four antioxidants including ascorbic acid 0.02%, citric acid 0.02%, poly vinyl pyrollidone 0.8% and charcoal 0.2% were tested for enhancement of bud sprouting, shoot multiplication and growth. Number of explants responding, number of buds developed per explant, length of the shoots and number of leaves per culture were noted in the antioxidant supplemented media, after 4wks. The experiments were conducted in GR free WPM medium with 2% sucrose, and 0.2% phytagel.

Culture vessels:

Influence of three types of culture vessels including cotton plugged culture tubes (25x150 mm), cotton plugged Erlenmeyer flasks (250 ml) and jam bottles (300ml) with polypropylene caps were tested. The experiment was followed upto 2 passages. Number of explants responding, number of buds per explant and lengths of the shoot were noted after 4wks. The experiments were conducted in GR free WPM medium with 2% sucrose and 0.2% phytagel.

Rooting:

Elongated shoots (1-3cm) obtained from the cluster of buds induced by cytokinins and maintained in GR free medium for four or more passages were tested for rooting. Auxins including IAA, IBA, NAA (Sigma, USA) at concentrations of 2.85µM, 2.46µM and 2.69µM respectively were incorporated aseptically in half strength WPM liquid medium in tubes. Elongated shoots (1-3cm) were cultured in these media on filter paper support. After 4wks of culture in light, percentage of rooting, number of roots per explant and root lengths were scored.

Hardening and Acclimatization:

The rooted plantlets were transplanted in sterilized soil: sand (1:1) mixture in plastic cups. These were covered with transparent polythene bags to maintain high humidity during hardening for 4wks in 24hr light. Thereafter, the plantlets were acclimatized for 4wks by exposing gradually to *ex vitro* conditions. The hardened plants were transferred to earthen pots in green house.

Ex vitro rooting:

In vitro raised shoots (2-3cm) were tested for *ex vitro* rooting. Cut ends of the shoots were coated with Seradix B (May and Baker, India Ltd), a commercial preparation often used for rooting of cuttings. These shoots were planted in plastic cups containing autoclaved soil-sand mixture in 1:1 ratio. The cups with the explants were covered with polyethylene bags to prevent loss of moisture. After 8wks in 24hr light, number of shoots stabilized in soil was scored and the plants were transferred to green house.

Statistical analysis:

All the data generated, were subjected to Analysis of Variance (ANOVA) and Duncan's multiple range tests (DMRT).

3B.2 RESULTS AND DISCUSSION

Poor germination frequency, acute microbial contamination, slow growth and heavy leaching of phenolics were some of the limitations encountered in tree tissue culture restricting the progress of work. Obtaining seeds of *S. anacardium* free of microbes was a serious constraint for establishment of seedling cultures (Panda and Hazra 2009). Optimization of a protocol for micropropagation of a forest tree demands extensive trials involving large number of nodal explants. In *S. anacardium* obtaining microbe free nodal explants from seedlings or from naturally growing trees (unpublished data) was a challenge. Keeping this in view microbe free shoot culture was established as stock cultures. These shoots maintained in GR free medium served as source of nodal explants for optimization experiments. A similar approach has been followed for explant establishment in *Stereospermum personatum* D.C, (Shukla *et al*.2008), a tree species. Interestingly, the shoot cultures of *S. anacardium* initiated in cytokinin containing medium could be maintained for several passages in medium devoid of GR. However, the incubation period taken by these shoot cultures to attain optimum growth got extended in later passages.

This strategy of using axenic cultures as source of experimental material for optimization of a micropropagation protocol has several advantages. (i) Time lost in obtaining microbe free seedlings as source of explant is avoided; (ii) Large number of explants could be obtained at a time from these shoots to carry out parallel experiments with varying culture conditions. (iii) The shoot cultures were not maintained as clones of the seedlings. Thereby, a condition of random distribution of different clones was established. Thus it is speculated that the protocol standardized will be effective for all genotypes of *S.anacardium*. Being a cross pollinated species there is genetic variation among the population of plants (Chapter 4A).

Selection of basal salt formulation for nodal explants:

From the four basal media tested for sprouting of buds and elongation of shoots from nodal explants of *S.anacardium*, cultures in WPM gives better response. The number of responding explants in WPM medium was 88% with mean shoot length of 0.39cm **(Table 3B.1; Fig. 3Ba)**. Number of buds developed in each explant was similar in all the basal media tested. Although the number of leaves (1.9) developed per explant after 4 weeks of culture medium was not significantly higher in WPM but was optimum. Percentage of explants which leached phenolics into the medium was reduced in WPM basal medium compared to other salt formulations tested. Concentrations of the salts in WPM medium was lower than in MS, SH or B5. Weaker salt formulations are reported to promotes axillary bud differentiation in forest trees (McCown and Sellmer 1987)

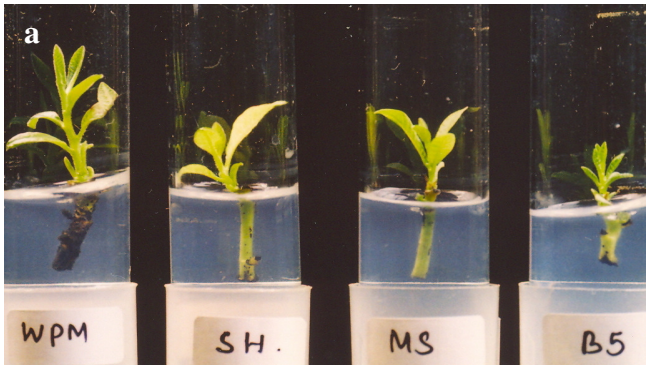


Fig. 3B a. Effects of Basal media on growth of nodal explants from axenic cultures of *Semecarpus anacardium.*

Table 3B.1 Effect of basal salt formulation on growth of axenic nodal explants from shoot culture after 4wks.

Basal media	Percentage of response	Shoot length after 4wks (mean ± sd) in cm	Number of buds after 4 wks (mean ± sd)	Number of leaves after 4 wks (mean ± sd)	Phenolics after 4wks (mean ± sd)%
WPM	88 ± 3.3^{a}	0.39 ± 0.12^{a}	0.2±0.06	1.93±0.85	50 ± 8^{b}
MS	76 ± 10.6^{b}	0.28 ± 0.01^{b}	0.17±0.06	1.02±0.03	70 ± 10^{a}
SH	73 ± 8.5^{bc}	0.26 ± 0.02^{bc}	0.25±0.05	1.3±0.26	60 ± 18^{ab}
B5	68 ± 7.5^{d}	0.18 ± 0.04^{d}	0.22±0.08	0.92±0.51	70 ± 8^{a}
ANOVA	S1%	S1%	NS	NS	S1%

40 explants in 4 repeats.

Effects of cytokinins:

Axenic shoot derived axillary buds of *S.anacardium* sprouted at high frequency (78-100%) in the media tested (**Table 3B.2a**). However, it was optimum (100%) in WPM basal medium supplemented with BAP 4.44µM, KN 4.64µM, phytagel 0.2% and sucrose 2%.

Table 3B.2a: Effect of BAP and KN on axillary bud sprouting and multiplication of axenic nodal explants of *Semecarpus anacardium*

Media	*Sprouting in WPM + Cytokinin		*Number of buds formed in the axils of the shoot in medium without GR		
Concentration	**Frequency After 4wk (%)**	**Number of buds after 4wk (mean ± sd)**	**4wk (mean ± sd)**	**8 wk (mean ± sd)**	**12 wk (mean ± sd)**
WPM	82.5	0.05 ± 0.1^{h}	0.05 ± 0.1^{h}	0.11 ± 0.12^{i}	0.22 ± 0.17^{h}
BAP2.22µM	87.5	1.03 ± 0.12^{cd}	1.95 ± 0.7^{cde}	3.02 ± 1.48^{abcde}	4.15 ± 2.31^{cde}
BAP4.44µM	97.5	1.92 ± 0.66^{a}	3.87 ± 1.4^{a}	5.97 ± 2.23^{a}	8.1 ± 3.0^{a}
BAP8.88µM	77.5	1.78 ± 0.9^{ab}	3.65 ± 1.6^{ab}	5.47 ± 2.63^{ab}	7.3 ± 3.61^{ab}
KN2.32µM	92.5	0.84 ± 0.4^{defg}	1.75 ± 0.8^{defg}	2.5 ± 1.16^{bcdefg}	3.65 ± 1.91^{cdefg}
KN4.64µM	95	0.9 ± 0.34^{def}	1.75 ± 0.5^{defg}	2.35 ± 0.5^{bcdergh}	3.3 ± 0.81^{cdefgh}
KN8.28µM	82.5	0.95 ± 0.54^{de}	1.85 ± 1.08^{def}	2.97 ± 1.41^{abcdef}	4.0 ± 1.9^{cdef}
BAP2.22µM+ KN2.32µM	92.5	0.95 ± 0.31^{de}	2.25 ± 0.51^{bcd}	3.37 ± 1.22^{abcd}	4.5 ± 1.9^{bcd}
BAP4.44µM+ KN4.64µM	100	1.65 ± 0.27^{abc}	3.17 ± 0.66^{abc}	4.32 ± 0.60^{abc}	5.5 ± 1.1^{abc}
ANOVA	NS	S1%	S1%	S1%	S1%

* 40 explants in four repeats. $^{a-i}$ Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at $P\leq0.05$.

The multiple buds (7.3/explant**, Table 3B.2a**) which developed in 8.88µM of BAP, failed to grow and turned necrotic in GR free medium. Number of buds noted in the cultures initiated in BAP 4.44µM was optimum (8.1±3.0) but the elongation was only 0.66±0.15 cm (**Table 3B.2b**) even after three passages in medium without GR. On the contrary buds sprouted in KN 4.64µM, attained the optimum height of 2.10cm after the 3 passages in GR free medium, but the multiples were only 3.3/explant (**Table.3B.2a**).

Incorporation of BAP singly produced more number of multiples (**Fig. 3Bb**) whereas incorporation of KN singly supported elongation (**Fig. 3Bc**). Optimum bud proliferation (1.92) with 98% sprouting of the buds (**Table 3B.2a**) after 4wks of initiation of culture, was achieved in medium with BAP 4.44µM, whereas optimum elongation (0.3cm) was achieved in medium with KN 4.64µM (**Table 3B.2b**). Similar finding has been reported in number of tree species like *Cornus florida* (Kaveriappa et al. 1997), *Melia azedarach* (Thakur *et al*. 1998), *Madhuca longifolia* (Rout *et al*. 1993) and *Gymnema sylvestre* (Komalavalli *et al*. 2000) where BAP incorporated medium produced more number of bud than that in KN. In order to obtain multiplication and elongation of shoots in single step combinations of BAP and KN were tested. Elongation of *S.anacardium* shoots in BAP 4.44µM+ KN 4.64µM, and BAP 4.44µM media was slow and after 4 weeks of initiation, the shoots were short (**Table 3B.2b**) and were not suitable for induction of rooting. However, differentiation of the buds and elongation of the shoots was

achieved on repeated transfer of these cultures for 3 passages in medium devoid of GR.

Table 3B.2b: Effect of BAP and KN on elongation of shoot developed from axenic shoot derived nodal explants of *Semecarpus anacardium*

Concentration	***Shoot length 4 wk in cm (mean ± sd)**	***Mean shoot length in WPM GR free medium(mean ± sd)**		
		4 wk	**8 wk**	**12 wk**
WPM	0.21±0.03	0.36 ± 0.06^{bcdef}	0.48 ± 0.15^{def}	0.72 ± 0.23^{cdef}
BAP2.22µM	0.16±0.05	0.31 ± 0.05^{cdefg}	0.43 ± 0.10^{defg}	0.64 ± 0.18^{cdefgh}
BAP4.44µM	0.11±0.04	0.30 ± 0.10^{defgh}	0.39 ± 0.08^{efgh}	0.66 ± 0.15^{cdefg}
BAP8.88µM	0.04±0.04	0.06 ± 0.04^{i}	0.14 ± 0.07^{i}	0.2 ± 0.069^{fgh}
KN2.32µM	0.19±0.15	0.52 ± 0.25^{bc}	0.94 ± 0.24^{ab}	1.90 ± 0.76^{ab}
KN4.64µM	0.3±0.2	0.69 ± 0.18^{a}	1.04 ± 0.36^{a}	2.10 ± 1.29^{a}
KN9.28µM	0.2±0.1	0.53 ± 0.17^{ab}	0.72 ± 0.35^{c}	1.31 ± 0.65^{abc}
BAP2.22µM+ KN2.32µM	0.16±0.09	0.38 ± 0.17^{bcde}	0.54 ± 0.24^{cde}	1.22 ± 0.70^{abcd}
BAP4.44µM+ KN4.64µM	0.17±0.04	0.43 ± 0.05^{bcd}	0.60 ± 0.16^{cd}	1.21 ± 0.49^{abcde}
ANOVA	NS	S1%	S1%	S1%

40 explants in four repeats. $^{a–h}$ Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at $P\leq0.05$.

It was noted that KN containing media supported differentiation of the shoot primordia and elongation of shoots. In medium with BAP 4.44µM and KN 4.64µM, in which sprouting was 100%, both multiplication and elongation were moderate (1.65 and 0.17cm, respectively) after 4wk of initiation of culture. Repeated sub-culturing of the explants in GR free medium supported further elongation of the shoots irrespective of the primary medium in which it sprouted. Considering the frequency of sprouting, number of multiples developed from the axillary meristem, elongation of the shoot, opening of the leaves and overall appearance of the cultures the WPM basal medium supplemented with both BAP 4.44µM and KN 4.64µM was identified for optimal sprouting and proliferation of the axillary meristem (**Fig. 3Bd**) in single step. In this medium, the frequency of response was 100%, the number of multiples produced in each explant in 16wk was 5.5±1.1, and the mean height of the shoots was 1.21±0.5cm.

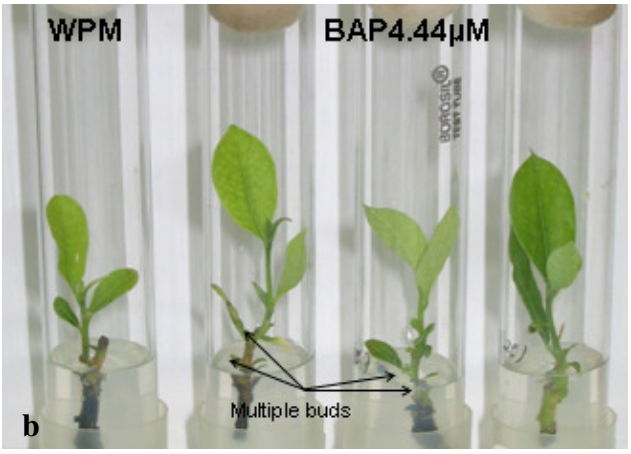


Fig. 3B b. Differentiation of seedling derived axillary meristem to form shoot after 20wk in GR free WPM medium. Explants were pre-cultured in BAP 4.44µM containing WPM medium for 4wk. The axils of the sprouted shoots show new shoot primordia.

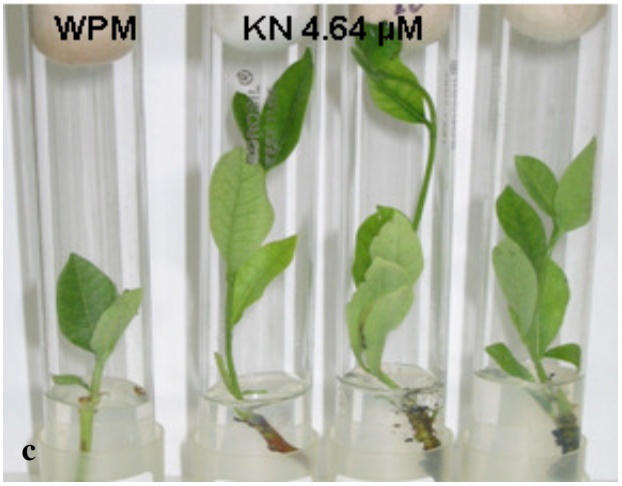


Fig. 3B c. Shoot development in the seedling derived axillary meristem in GR free WPM medium after pre-culture of the explant in KN 4.64µM containing WPM medium for 4wk. Long internodes due to influence of KN.

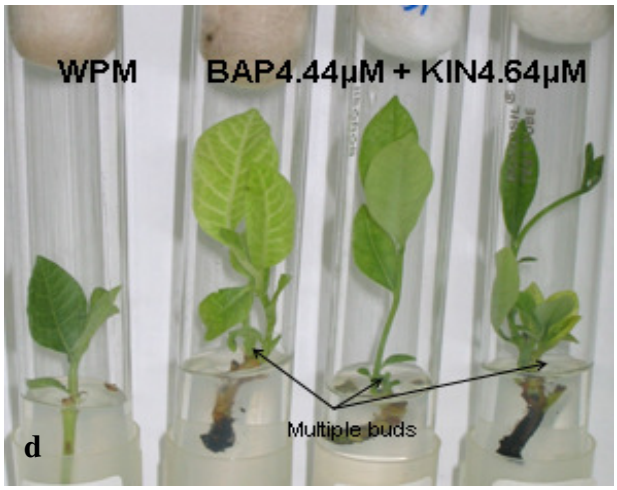


Fig. 3B d. Moderate elongation of shoot buds and new shoot primordia in the axils after 20wk in GR free WPM medium. Explants were pre-cultured for 4wk in BAP 4.44µM+KN4.64µM containing WPM medium.

On maintaining these shoots for 28wks in GR free medium it was observed that more number of shoots appeared in each passage (**Table 3B.3**). Number of shoots produced from each explant pre-cultured in combination of BAP 4.44µM and KN 4.64µM for 4wks produced 6.16 shoots after seven passages (28wks) in GR free medium.

Table 3B.3 Number of shoots produced from each explant every 4 wks.

Conc. of GRs	*Number of shoot/explants in GR free medium (mean ± sd)					
	12 wk	**16 wk**	**20 wk**	**24 wk**	**28 wk**	**After 7 passages**
WPM	0.17± 0.06^{cd}	0.37± 0.06^{de}	0.46± 0.06^{de}	0.83± 0.21^{cdef}	0.6± 0.17^{e}	2.43± 0.35^{h}
BAP 2.22µM	0.33±0.12^{cd}	0.5±0.2b^{cde}	0.83±0.15^{cd}	1.07±0.12^{cde}	0.83±0.15^{bcde}	3.56±0.16^{ef}
BAP 4.44µM	0.67±0.15^{a}	1.03±0.062^{a}	1.27±0.06^{a}	1.57±0.21^{a}	1.70±0.26^{a}	6.24±0.17^{a}
BAP 8.88 µM	0.1±0.17^{d}	0.23±0.23^{e}	0.3±0.1^{e}	0.63±0.21^{f}	0.57±0.21^{e}	1.83±0.29^{i}
KN 2.32 µM	0.33±0.06^{cd}	0.47±0.17^{cde}	0.57±0.06^{de}	1.07±0.25^{cde}	0.63±0.12^{e}	3.07±0.18^{g}
KN 4.64 µM	0.47±0.06^{bc}	0.7±0.2^{abcd}	0.97±0.25^{c}	1.15±0.46^{c}	1.13±0.15^{bc}	4.42±0.62^{c}
KN 9.28 µM	0.33±0.15^{cd}	0.53±0.25^{bcde}	0.63±0.29^{cde}	1.13±0.38^{cd}	1.03±0.15^{bcd}	3.65±0.24^{e}
BAP2.22µM + KN2.32 µM	0.33±0.06^{cd}	0.8±0.17^{abc}	0.97±0.15^{c}	1.07±0.12^{cde}	1.17±0.06^{b}	4.34±0.17^{cd}
BAP4.44µM +KN 4.64 µM	0.63±0.15^{ab}	0.9±0.1^{ab}	1.3±0.1^{ab}	1.57±0.06^{b}	1.76±0.1^{a}	6.16±0.27^{ab}
ANOVA	S1%	S1%	S1%	S1%	S1%	

* 40 explants in four repeats. $^{a–f}$ Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at P≤0.05.

Effects of carbon source and sucrose concentration:

Exogenous carbohydrates play an important role in plant tissue culture. They have essential function in plant metabolism since they serves as carbon and energy sources (Thorpe, 1974) and act as osmotic agents (Brown *et al.* 1979). Besides their role in metabolic pathways, there are strong evidences of the role of sugars as signaling molecules (Smeekens 2000). The preference of specific carbohydrates has been shown to be species- specifics or even line specific (Taber *et al*. 1998). Therefore, the carbohydrate requirement needs to be optimized for a specific species/genotype and explant (Gemas and Bessa 2006). In *S.anacardium* optimum sprouting of nodal buds (86%) was achieved in medium with sucrose 2% (**Table 3B.4**). In media containing other carbohydrates such as glucose, fructose and maltose the sprouting responses were 79, 72 and 81% respectively. Shoot length of sprouted bud in sucrose containing medium was 0.4cm which is higher than that with other carbon source tested (**Table 3B.4).**

Table 3B.4 Effects of carbon source concentration on growth of axenic seedling derived nodal explants after 4 wks.

Carbon source (2%)	Percentage of response (mean ± sd)	Shoot length in cm (mean ± sd)	Number of buds (mean ± sd)	Number of leaves (mean ± sd)
Sucrose	86±2	0.4±0.03	0.36±0.15^{a}	1.29±0.47^{a}
Glucose	79±6	0.3±0.01	0.23±0.11^{b}	0.95±0.32^{ab}
Fructose	73±5	0.29±0.02	0.22±0.08^{b}	1.06±0.32^{abc}
Maltose	81±7	0.33±0.01	0.29±0.13^{b}	0.44±0.14^{d}
ANOVA	NS	NS	S 5%	S1%

40 explants in four repeats

Fig. 3B e. Effects of different carbohydrates on growth of axenic culture derived nodal buds.

Number of buds and number of opened leaves per explant were also higher in sucrose containing medium (**Table 3B.4; Fig. 3Be**). As sucrose at 2% gave better response, we extend our study to determine the optimum sugar requirement by incorporating different concentrations of sucrose. The nodal buds gave similar response in terms of shoot elongation and number of bud formation in media containing 2% and 3% of sucrose (**Table 3B.5; Fig. 3Bf**). Hence for further studies on *in vitro* propagation and also in other studies 2% sucrose was incorporated in the media.

Table 3B.5 Effects of sucrose concentration on growth of axenic seedling derived nodal explants after 4wks

Sucrose conc. (%)	**Frequency (%)of response (mean ± sd)**	**Shoot length in cm (mean ± sd)**	**Number of buds (mean ± sd)**	**Number of leaves (mean ± sd)**
1	76±3.6	0.16 ± 0.06^{c}	0.26 ± 0.15^{b}	0.33 ± 0.23^{c}
2	83±4.5	0.36 ± 0.18^{a}	0.46 ± 0.2^{ab}	1.3 ± 0.17^{a}
3	85±2.8	0.33 ± 0.1^{ab}	0.5 ± 0.16^{a}	0.97 ± 0.16^{b}
4	80±2.3	0.25 ± 0.1^{abc}	0.31 ± 0.18^{ab}	0.72 ± 0.34^{abc}
5	78±1.5	0.22 ± 0.07^{bc}	0.25 ± 0.19^{b}	0.74 ± 0.23^{abc}
ANOVA	NS	S5%	S5%	S5%

40 explants in four repeats

Fig. 3B f. Effects of different sucrose concentration on growth of axenic culture derived nodal buds.

Influence of gelling agents:

Out of the two gelling agents tested, Phytagel 0.2% was comparatively more effective in bud sprouting and shoot growth (**Table 3B.6**) but the difference in frequency of response was not significant statistically. Phytagel promoted opening of leaves in addition to elongation of the

shoots (**Fig. 3Bg**). Number of buds noted in phytagel containing medium was significantly higher after 4 weeks of culture and the shoot elongation was more after 8 weeks. Similar growth enhancing effect of phytagel was reported in Persian walnut (Sharma and Ramamurthy 2000; Saadat and Hennerty 2002). It was demonstrated (Mackay *et al.* 1988) that phytagel having low gel strength, render water availability to the explants whereas agar decreases it. In the course of the present study, it was observed that the pH of the media (with either phytagel or agar), adjusted to 5.8 before autoclaving, reduced to 5.6± 0.02 in agar gelled medium and to 5.21±0.03 in phytagel gelled medium upon autoclaving. Change in pH on incorporation of agar and/or phytagel in culture medium has been reported (Wetzstein *et al*. 1994). It needs to be tested, whether the superior growth of the shoot cultures of *S.anacardium* shoots in Phytagel containing medium was due to high water availability of the cultures or due to the reduction in pH to 5.2 after autoclaving.

Table 3B.6 Effects of gelling agents on sprouting, multiplication and shoot elongation of axenic shoot derived nodal explants of *Semecarpus anacardium*

Gelling agent	pH after autoclaving	Sprouting Frequency (%) (mean ± sd)	Number of buds (mean ± sd)		Shoot elongation (mean ± sd) in cm		Explants with phenolics (%)
		4 wk	4 wk	8 wk	4 wk	8 wk	
Phytagel	5.21±0.03	90.8±6.29	0.34±0.14	0.35±0.14	0.35±0.1	0.49±0.07	55%
Agar	5.60±0.02	80.5±8.24	0.04±0.08	0.28±0.3	0.21±0.13	0.31±0.06	80%
ANOVA	S1%	NS	S 5%	NS	NS	S1%	S 5%

40 explants in four repeats.

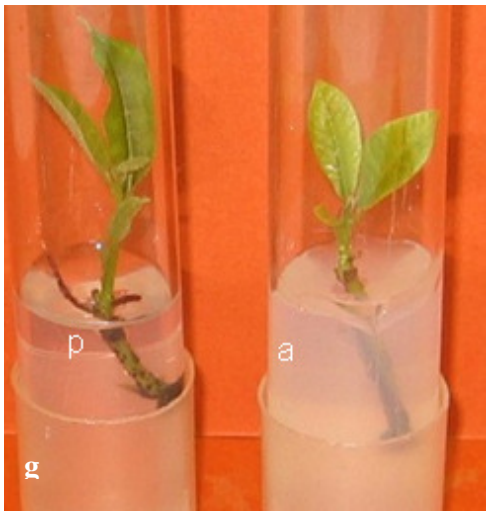


Fig. 3B g. Enhanced elongation of nodal bud derived shoot in Phytagel medium (p). Elongation and Leaf differentiation retarded in medium with agar (a).

Inhibitory influence of phenolics in tree tissue culture is well documented (Onay 2003). In the present study it was observed that 80% of the cultures in agar gelled medium released phenolics whereas in phytagel medium it was only 55%. Relatively reduced growth of the shoots in agar gelled medium could be due to inhibitory influence of the increased amount of exuded phenolics in this medium.

Influence of antioxidants:

As a member of *Anacardiaceae*, *S.ancardium* tissues posses large amount of polyphenols, flavonoids etc. (Rao *et al*. 1973) which leaches into the medium causing browning of the media and restriction of growth of the explants *in vitro.* Antioxidants were incorporated in the culture medium to eliminate/reduce the inhibitory phenolics released by the tissues in culture. *S.anacardium* explants although taken from *in vitro* grown shoots, released phenolics in culture causing browning of media and inhibition of shoot growth. From the four media additives tested to reduce the phenolics released in the medium, activated charcoal at 0.2% was significantly more favorable for the growth of *S.anacardium* shoots in culture (**Table 3B.7, Fig. 3Bh**). Frequency of responding explants after 4 weeks of culture in WPM medium with charcoal was 91% and the shoot length was 0.43±0.21 cm as against 67% and 0.2±0.05 in control medium.

Table 3B.7: Effect of antioxidants on sprouting of buds, elongation of shoot, number of leaves and explants releasing phenolics, of *Semecarpus anacardium* after 4wk in WPM.

Antioxidants	***Sprouting Frequency (%) (mean ± sd)**	**Number of Buds (mean ± sd)**	**Shoot length in cm (mean ± sd)**	**No of leaves (mean ± sd)**	**% of explants with Phenolics (mean ± sd)**
Control	67.2 ± 12.33^{b}	0.13±0.05	0.2 ± 0.05^{b}	0.51 ± 0.49^{b}	48 ± 1.9^{bc}
Charcoal 0.2%	90.83 ± 6.88^{a}	0.25±0.1	0.43 ± 0.21^{a}	1.2 ± 0.49^{a}	00 ± 00^{e}
Ascorbic acid 0.02%	66.3 ± 10.68^{bc}	0.18±0.05	0.17 ± 0.12^{bc}	0.35 ± 0.42^{bcd}	50 ± 29^{b}
Citric Acid 0.02%	63.96 ± 15.12^{bcd}	0.13±0.05	0.17 ± 0.12^{bc}	0.29 ± 0.43^{bcde}	43 ± 12^{bcd}
PVP 0.8%	58.54 ± 9.95^{bcd}	0.15±0.05	0.15 ± 0.09^{bc}	0.2 ± 0.24^{bcde}	82 ± 22^{a}
ANOVA	S 5%	NS	S 1%	S 5%	S1%

*5 repeat, 10 explants in each repeat. $^{a-e}$Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at P≤0.05.

Other antioxidants failed to control the release of phenolics. On the contrary in presence of PVP, the release of phenolics was increased resulting in reduction of sprouting frequency. Growth enhancing effect of charcoal has been reported in tree species of Anacardiaceae *in vitro* like *Schinopsis balansae* (Sansberro *et al*. 2003) and Cashewnut (Das *et al.* 1996). The phenolics released by the tissues during subculture often inhibit the growth and morphogenesis (Onay 2003). The growth-promoting role of activated charcoal may be due to adsorption of undesirable inhibitory substances (Pan and Staden 1998; Thomas 2008).

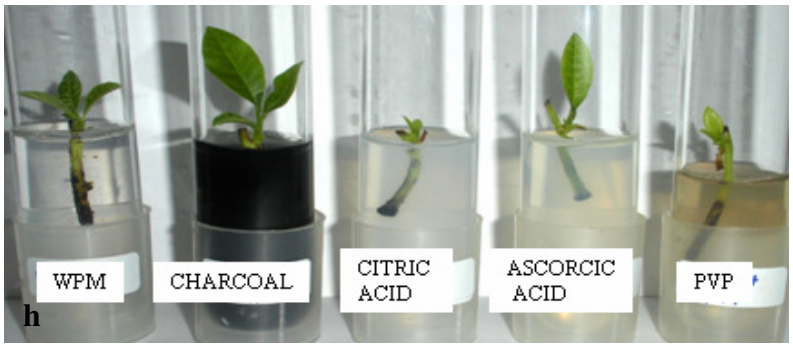


Fig. 3B h. Effect of antioxidants on shoot growth. In charcoal containing medium elongation is optimum and shoots demonstrate vigorous growth with opened leaves. Note the poor shoot growth in PVP containing medium, which turned brown due to leaching of phenolics from explant.

Influence of culture vessels:

In commercial laboratories the jam bottles are culture vessel of choice for mass propagation of plants. However this culture vessel was not suitable for the *S.anacardium* shoot cultures. Percentages of responding nodal explants after four weeks of culture was 88.2±12.19, 84.2±8.84, and 38.4±29.83, in flasks, test tubes and bottles respectively (**Table 3B.8**).

Table 3B.8: Effect of culture vessel on shoot elongation and multiplication of axenic nodal explants of *Semecarpus anacardium*

Culture vessel	**Sprouting Frequency (%) 4wk (mean± sd)**	**No. of buds**		**Shoot elongation**	
		4 wk (mean ± sd)	**8 wk (mean ± sd)**	**4 wk in cm (mean ± sd)**	**8 wk in cm (mean ± sd)**
Flasks	88.2±12.19^{a}	0.38±0.88^{a}	0.38±0.08^{ab}	0.16±0.07^{a}	0.75±0.15^{a}
Tubes	84.2±8.84^{ab}	0.38±0.88^{ab}	0.43±0.08^{a}	0.16±0.06^{ab}	0.68±0.07^{ab}
Bottles	38.4±29.83^{c}	0.08±0.04^{c}	0.23±0.05^{c}	0.06±0.06^{c}	0.39±0.14^{c}
Anova	S1%	S1%	S 1%	S 5%	S 1%

*4 repeat, 10 explants in each. $^{a–c}$ Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at P≤0.05.

Shoot growth of the *S.anacardium* explants was similar in cotton plugged Erlenmeyer's flasks and culture tubes. In these vessels the shoot growth was more vigorous with expanded leaves compared to the cultures in plastic capped glass bottles. Shoot lengths in flasks and tubes were 0.75±0.15cm and 0.68±0.07cm compared to 0.39±0.15cm in bottles. The leaves in the shoots cultured in tubes (**Fig. 3Bi**) and flasks (**Fig. 3Bj**) were fully open and dark green in appearance whereas shoots in bottles (**Fig. 3Bk**) were stunted with reduced internodal distances and bearing smaller yellowish green leaves. Cotton plugs of culture vessels permit escape of moisture and exchange of gases with outer environment while maintaining the aseptic condition. The bottles often require sealing with cling film to maintain the aseptic condition. This restricts the escape of

moisture and gases. Thus, the moisture, carbon dioxide, ethylene etc increase in the microenvironment of the tissues in culture. Some of these factors may have adverse effect on growth of *S.anacardium* shoots in bottles. Similar observation was noted in cultures of *Pongamia pinnata* (Sujatha and Hazra 2006).

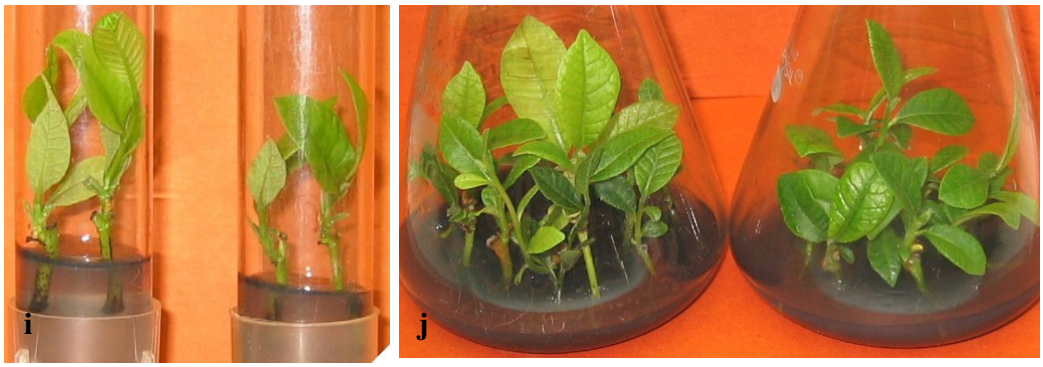


Fig. 3B Comparison in growth of nodal explants in cotton plugged tubes (**i**) Erlenmeyer's flasks **(j)** these explants shows elongation with opened leaves

Fig. 3B k. plastic capped bottle demonstrate limited elongation and reduced leaf size in shoots maintained in plastic capped bottles after 8 wk of culture under identical condition.

Influence of culture vessel on *in vitro* response of woody species has been studied extensively (McClelland and Smith 1990, Joshi *et al*. 2009). Results of *in vitro* growth and morphogenesis of *Dianthus caryophyllus* L., evs. ScaniaWhite Sim, Angeline, and Pink Calypso in four different culture vessels provided evidence (Fal *et al.* 2002) that the environmental differences detected inside the culture vessels affect micropropagation due to specific sensitivity of each plant to the gas exchange and medium desiccation determined by the vessel type.

Rooting and hardening:

S.anacardium shoots often rooted spontaneously in GR free semisolid medium in which it was maintained. However, these roots were brittle. This problem was evident while removing the rooted shoots from semisolid medium and also during transfer to soil. This limitation was overcome by rooting in half strength WPM liquid medium in tubes on filter paper support in presence of auxins. The shoots did not root in full strength WPM medium (**Table 3B.9**), whereas 43% rooting was achieved in the cultures on reduction of the salt strength to half WPM.

Table 3B.9 Effect of different auxins on rooting of shoot of *Semecarpus anacardium* explants after 4wk in culture.

Medium	* Frequency (%) of rooting(mean ± sd)	Number of roots/Explant(mean ± sd)	Root length(mean ± sd) in cm
WPM	0 ± 0^{c}	0 ± 0^{d}	0±0
1/2WPM	42.5 ± 3.54^{bcd}	0.58 ± 0.04^{cd}	1.55±0.07
1/2WPM 2.46 µM IBA	100 ± 38.89^{a}	4.5 ± 1.56^{a}	3.55±0.42
1/2WPM 2.85 µM IAA	62.5 ± 3.89^{abc}	1.85 ± 1.98^{abc}	1.53±1.87
1/2WPM 2.69 µM NAA	82.5 ± 3.54^{ab}	3.15 ± 0^{ab}	1.50±0
ANOVA	S 5%	S 5%	NS

*4 repeat, 10 explants in each repeat. $^{a-d}$ Duncan multiple range notation. Means followed by the same superscripts within a column do not differ significantly at P≤0.05.

The frequency of rooting increased with incorporation of auxin in liquid half strength WPM medium. Among the auxins tested, IBA at 2.46µM was most effective in inducing roots in all the explants. Root length and number of roots were also higher. In IAA 2.85µM and NAA 2.69µM roots were associated with callusing and frequency of rooting were 62.5 and 82.5% respectively (**Table 3B.9, Fig. 3Bl**).

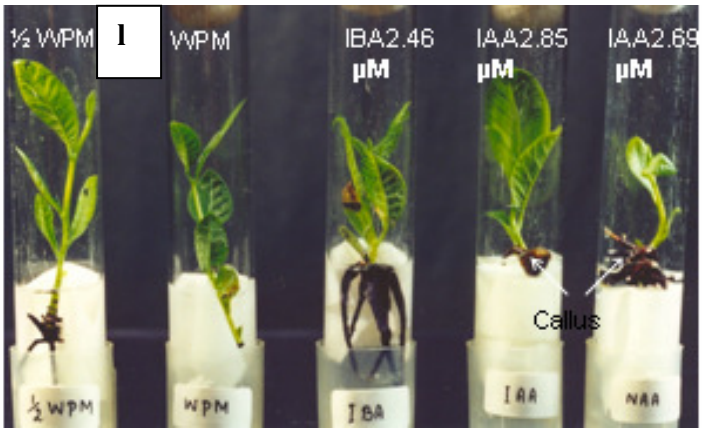


Fig. 3B l. Rooting of shoots in different auxin containing liquid medium after 4wk of culture. Good root growth in medium containing IBA. In full strength WPM there was no rooting and moderate root growth in half strength. Limited root growth was noted in media with IAA or NAA.

Of the 45 rooted plants 41 survived (**Fig. 3Bm**) on transfer to soil: sand mixture under non-sterile condition giving the survival rate of 91%. The positive influence of IBA in root induction in tree species of *Anacardiaceae* and other family members has been reported in Cashew (Mneney *et al.* 2001; Das *et al.* 1996; Boggetii *et al*. 1999; Ananthakrishn *et al*. 2002), Mulberry (Thomas *et al*. 2003) and *Pistachio* (Onay *et al*. 2003). After one month of hardening the *S.anacardium* plants were transferred to green house for further acclimatization (**Fig. 3Bn**).

Fig. 3B m. Hardened plants in hardening room after 4 wk of transfer in sand soil mixture 1:1.**n.** Hardened plants in green house before transferring to bigger pots. All plants survived on transfer from hardening room to green house.

All the *in vitro* raised plants survived in green house and grew well on transfer to earthen pots (**Fig. 3Bo**).

Fig. 3Bo Plants growing in pots after six month of transfer in green house.

Ex vitro rooting:

Experiment conducted to obtain rooting of shoots in soil: sand mixture under non-sterile condition was successful as 16 out of 20 shoots rooted *extra vitrum* and survived. Although rooting cum survival was 80% compared to 100% and 91% respectively in *in vitro* approach, the *ex vitro* rooting protocol was simpler, faster and economical as rooting and hardening are achieved in single step. All plants raised via *ex vitro* rooting survived in soil and grew well on transfer to green house (**Fig. 3Bp**). In spite of careful handling, at times the roots of *in vitro* raised shoots got damaged. This resulted in reduced (91%) survival of rooted plants on transfer to soil. This limitation was avoided by rooting the shoots directly in soil. *Ex-vitro* rooting responses of *in vitro* propagated shoots were reported in cherry species (Kris *et al*. 2005).

Fig. 3B p. *Ex vitro* rooted shoots after 8wks of transfer in sand soil mixture 1:1 and incubation in hardening room in 24hr light. Shoots rooted and hardened simultaneously to obtain hardened plant in one step.

3B.3 CONCLUSION

This is the first report describing a protocol for micropropagation of *S.anacardium.* Obtaining microbe free explants was a limiting step for standardization of the micropropagation protocol. Being a tree species the mature tree derived explants harbored several microorganisms. Moreover the explants were recalcitrant in culture. Some of these constraints were overcome by using seedling derived explants. The micropropagation protocol described in this report may find application in standardization of protocol for propagation of identified trees after refinement. The shoot cultures can also be used in study of secondary metabolite production. Shoot cultures have been used successfully for secondary metabolite production from plants (Review: Bourgoud *et al*. 2001).

SECTION C:

Effects of TDZ on morphogenesis of nodal explants from axenic shoot cultures

Media containing WPM basal salts with BAP and KN were formulated to establish shoot cultures of *S.anacardium* using seedling derived nodal explants (Section B). These media supported the growth of the shoots. After 32wks of culture, optimum number of 6 shoots /explant was obtained in the media containing BAP 4.44 and KN 4.64µM. To refine the protocol and to obtain increased number of buds from single meristem, we tested the effect of Thidiazuron (TDZ). Thidiazuron is identified as the most active cyokinin like substance for woody plant tissue culture (Lu 1993; Huettman and Preece 1993; Murthy and Saxena 1998). Before the discovery of TDZ as a GR, amino purine cytokinins have been used extensively for micropropagation of plants via organogenesis. Comparative studies of aminopurine cytokinins and TDZ remain complicated. The requirement of TDZ to stimulate axillary shoot proliferation is extremely low as against high requirement of amino purine cytokinins (Huettman and Preece 1993). The activity of this GR varies widely depending on its concentration, exposure time, the explant and species tested (Murthy *et al*.1998). TDZ is recognized for its targeted influence on meristematic tissue and proliferation of caulogenic buds in trees (Mehta *et al*. 2005; Sujatha and Hazra 2007; Siddique and Anis 2009), shrub (Sujatha *et al*. 2005) and herbaceous species (Mithila *et al*. 2003; Shan *et al*. 2005; Joshi *et al*. 2008). This report describes for the first time a protocol for clonal propagation of *S.anacardium* from the shoot culture derived meristems using TDZ.

3C.1 MATERIALS AND METHODS

Shoot cultures maintained in WPM basal medium supplemented with BAP 4.44µM and KN 4.64µM were cultured for four passages of 4wks each in WPM basal medium with charcoal (0.2%). These shoots were used as source of explant for refinement of the protocol for rapid propagation using TDZ. Nodal explants, 1-2cm in length, carrying 1-2 axillary meristems were isolated from these shoots and cultured in tubes (150x25mm) containing 20ml of semisolid medium. The media were composed of WPM basal salts supplemented with varying concentrations (0.0045, 0.045, 0.227, 0.45, 2.27, 4.54 and 9.08µM) of TDZ and 2% sucrose. After 4 wk of incubation, number of explants with swollen buds, or with shoot primordia or small shoots was scored (**Table 3C.1**) as responding explants. The number of shoot primordia that appeared after 4 weeks in TDZ containing medium was scored and their lengths were noted. Thereafter the cultures were transferred to semisolid WPM medium devoid of GRs and with 2% sucrose and 0.2% charcoal. Number of buds and shoot primordia, per explant (**Table 3C.1**) and the lengths of the shoots (**Table 3C.2**) were noted before transferring the cultures to fresh medium of similar composition after every 4wks. This process was repeated for 3 passages (12wks). The longer shoots in these cultures were excised and tested for rooting. The

original explants with additional shoot buds and shoot primordia were transferred to WPM medium devoid of GRs for elongation. The process of excising the elongated shoots and re-culturing the explant in GR free medium was followed for four more passages to recover additional shoots for rooting. Number of shoots excised in every passage was scored (**Table 3C.3**) and the total number of shoots produced by each explant was determined after 7 passages (28wks) in WPM medium. The isolated shoots were rooted on filter paper support in half strength liquid WPM medium with 2.46µM IBA. All cultures were incubated at 25±2°C in 16hr photoperiod with an irradiance of 50µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Phillips). Rooted plantlets were transplanted in plastic cups (11cm height x 8cm diameter) filled with autoclaved soil: sand mixture (1:1) and covered with transparent polythene bags to maintain high humidity. The plantlets maintained for four wks in 24hr light for hardening were transferred to green house for further acclimatization and transfer to soil in pots for maintenance.

The pH of all media was adjusted to 5.8 before sterilization by autoclaving at 121°C for 20 minutes at 1.06kg cm^{-2}. Phytagel (0.2%) was used as gelling agent. Indole Butyric Acid (IBA) was added aseptically to liquid rooting medium. All the experiments were repeated four times with ten replicates in each repeat.

Statistical Analysis

Mean and Standard deviation for frequency of response, shoot elongation, number of multiples, number of shoots extracted in each passage was determined. These data were subjected to Analysis of Variance (ANOVA) and Duncan's multiple range tests (DMRT)

3C.2 RESULTS AND DISCUSSION

Shoot cultures of *S.anacardium* established using seedling culture derived nodal explants following the protocol described in the Section B, could be maintained for several passages in GR free medium for proliferation of shoots. However, the number of shoots obtained from each explant was 1-6. The advantages of using axenic shoot cultures as source of nodal explants are discussed (Section B).

In WPM medium without GR, bud break, sprouting and differentiation of leaf primordia was noted in 10d **(Fig. 3Ca)**. In the explants cultured in medium with lower concentrations (0.0045, 0.045µM TDZ) there was no response after 10 days of culturing. In media with 0.227 or 0.45µM TDZ enlargement of the axillary buds were observed. In higher concentrations (2.27-9.08µM), the axillary buds enlarged to develop into round structures and the meristem lost its characteristic bright green color and form (**Fig. 3Cb**) after10 days.

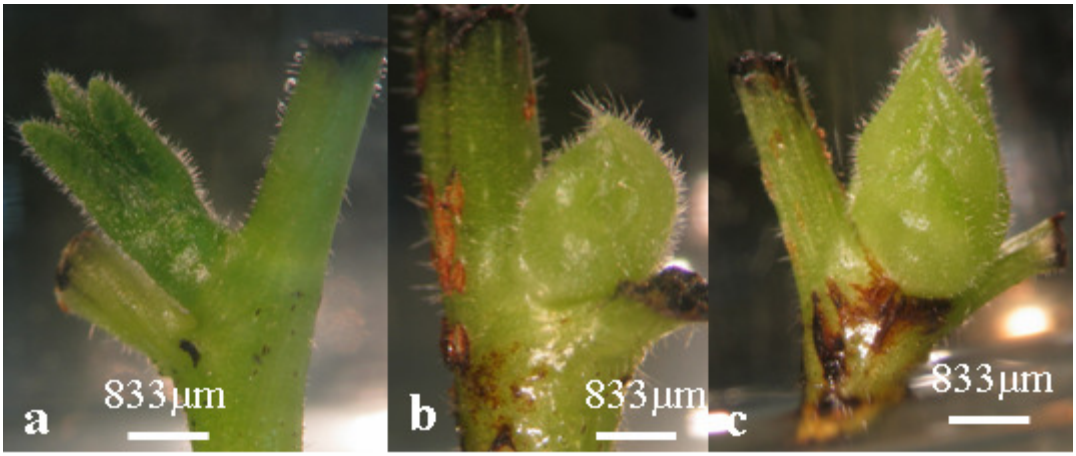


Fig. 3C a. Bud break from nodal explant of *S.anacardium* in WPM medium after 10 days of culture**; b.** Swelling of nodal bud in TDZ containing media after 10 days of culture**; c.** Swelling of the nodal bud after 20 days culture in medium containing TDZ. The size of the swelling was more in the media with higher concentration of TDZ.

After 20d in the same medium, the swellings extended and became more obvious (**Fig. 3Cc**). In the explants cultured for 4wks in lower concentrations (0.0045, 0.045µM) of TDZ, the bud break was noted in a few explants. However, the elongations of these shoot primordia was limited. The explants with the swollen axillary meristem were 98 - 100% (**Table 3C.1**) after 4wks of culture in media with higher concentrations of TDZ (2.27µM and 4.54µM). In explants pre-cultured in lower concentration (0.0045µM and 0.045µM), very few multiple buds were seen after 4wks of culturing. More buds appeared in these explants on further culturing in GR free medium for 4wks where elongation of shoots was found to be limited. Number of buds per explant, cultured in 0.0045µM TDZ was 0.56±0.10, in four wks. In GR free medium the number increased in each passage to 1.8±0.69, 2.93±1.31 and 4.0±1.38 after four, eight and twelve wks respectively (**Table 3C.1**). In the explants pre-cultured in 2.27µM, number of meristematic buds was more whereas in higher concentrations (4.45-9.08µM) sprouting of the axillary meristem was restricted.

Table 3C.1 Effect of TDZ on shoot multiplication of i*n vitro* culture derived nodal explants of *Semecarpus anacardium* after 4wks of culture.

Conc. of TDZ in µM	Frequency (%) of response (mean ± sd)	Multiple shoot buds in 4wks (mean ± sd)	Shoot length in cm after 4wk (mean ± sd)	Number of buds in WPM (mean ± sd) After every passage of 4 wks		
				4wk	8wk	12 wk
0	83±5	0.05±0.01	0.21±0.03	0.05 ±0.01^{g}	0.12 ±0.15^{f}	0.37 ±0.29^{f}
0.0045	75±5.77	0.56±0.10	0.23±0.12	1.8 ±0.69^{cdefg}	2.93 ±1.31^{ef}	4.0 ±1.38d^{ef}
0.045	78±15	0.05±0.01	0.10±0.11	2.23 ±0.37^{bcdef}	3.81 ±0.88^{cdef}	6.01 ±0.71^{cdef}
0.227	88±9.6	0±0	0.06±0.08	3.41 ±1.19^{abcd}	7.49 ±2.67^{abc}	12.26 ±4.09^{abc}
0.45	93±9.6	0±0	0±0	3.51 ±1.0^{abc}	7.18 ±2.2^{abcd}	11.01 ±2.98^{abcd}
2.27	98±5	0±0	0±0	4.67 ±1.65^{a}	10.25 ±3.8^{a}	18.51 ±8.65a
4.54	100±00	0±0	0±0	3.75 ±0.77^{ab}	8.32 ±1.9^{ab}	16.45 ±6.9^{ab}
9.08	100±00	0±0	0±0	2.8 ±0.41^{bcd}	6.1 ±0.96^{bcde}	10.5 ±1.24^{abcde}
ANOVA	S1%	S1%	NS	S1%	S1%	S1%

40 Explants, 4 repeats, 10 in each repeat

[a-g] Duncan multiple range notation. Mean followed by the same superscripts within a column do not differ significantly at $P \leq 0.01$.

Development of multiple buds was noted from the axillary meristems of the explants on eliminating TDZ from medium. Explants cultured in lower concentrations responded faster compared to the ones cultured in higher concentrations. More buds appeared on repeated transfers in GR free media. Multiplication of buds was scored up to 16wks (**Table 3C.1**). Out of these 16wks the explants were in TDZ containing medium for 4wks followed by 12wks in TDZ free medium. Optimum number of bud formation 18.51±8.65 was observed in TDZ (2.27µM) pre-treated explants (**Table 3C.1**). With every passage in GR free medium, the buds that appeared earlier elongated and additional buds regenerated. Mean shoot length in 0.0045µM TDZ was 0.23±0cm after four wks (**Table 3C.1**). It increased to 0.54±0.27, 0.68±0.26, and 0.88±0cm after four, eight and twelve wks in GR free medium respectively (**Table 3C.2**). Explants pre-cultured in 2.27µM of TDZ recorded shoot length of 0.46±0.32, 0.7±0.38 and 0.91±0.49cm on 4th , 8th and 12th wk in GR free medium respectively (**Table 3C.2**). This observation confirms the earlier reports (Sujatha and Hazra 2007, Joshi *et al*. 2008) that suggest that TDZ stimulate proliferation of the meristematic cells of the axillary meristems and restricts the proliferation of the shoot buds to shoots. In *Pongamia pinnata* (Sujatha and Hazra 2007) the axillary bud in the mature nodal explants was distended to form a compact meristematic mass. In peanut (Joshi *et al*. 2008), tamarind (Mehta *et al*. 2004, 2005) and soybean (Shan *et al*. 2005) it is

demonstrated that TDZ selectively influence the meristematic cells and induce proliferation of these cells to form compact meristematic mass while inhibiting the differentiation of shoots.

Table 3C.2 Length of shoot primordia/buds nodal explants of *S. anacardium* in WPM medium after every passage of 4wks.

Conc. of TDZ in µM	**Shoot length in WPM (mean ± sd) in cm**		
	4 wk	**8 wk**	**12 wk**
0	0.34±0.10	0.44±0.06	0.69±0.21
0.0045	0.54±0.27	0.68±0.26	0.88±0.34
0.045	0.29±0.20	0.45±0.22	0.59±0.16
0.227	0.31±0.21	0.54±0.20	0.72±0.12
0.45	0.27±0.19	0.47±0.29	0.86±0.53
2.27	0.46±0.32	0.7±0.38	0.91±0.49
4.54	0.31±0.25	0.49±0.35	0.60±0.51
9.08	0.20±0.11	0.32±0.12	0.41±0.16
ANOVA	NS	NS	NS

40 Explants, 4 repeats, 10 in each repeat.

In concurrence with the earlier reports (Mehta *et al*. 2004; Sujatha and Hazra 2007; Ahmed and Anis 2007), differentiation of shoot buds in *S.anacardium* was triggered on withdrawal of the GR and more number of buds developed and sprouted with every passage in GR free medium. In *S.anacardium* on repeated transfers in medium without GR more buds appeared in all the TDZ treated explants. Callusing was noted in the basal cut end of almost all explants pre-cultured in higher concentrations of TDZ (2.27 -9.08µM). Callusing was restricted to the portion of explant in contact with the medium (**Fig. 3Cd**).

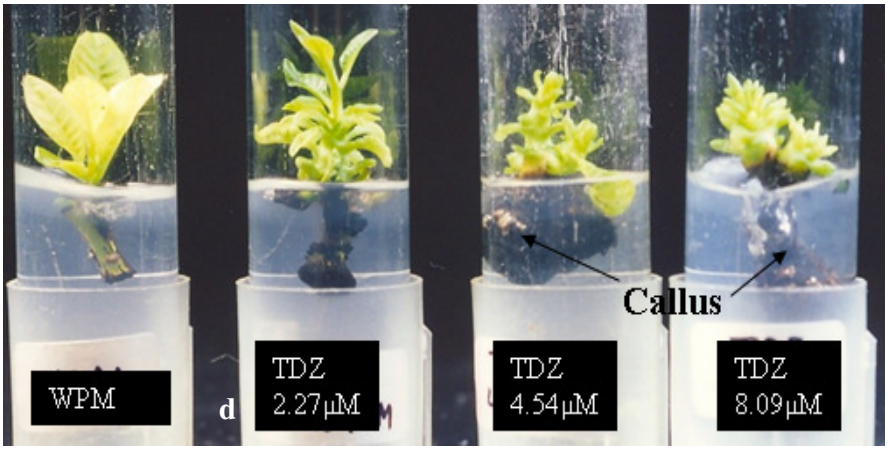


Fig. 3C d. Sprouted bud of *Semecarpus anacardium* after 8wks in WPM charcoal medium, Numbers of buds were more in the explants pre-cultured in higher concentrations of TDZ. However, the differentiation of these buds to shoots was reduced.

Similar callusing response has been observed in cut end of explants of *Abelmoschus moschatus* Medik. L. (Sharma and Shahzad 2006), cacti species (Giusti *et al*. 2002) and cocoa (Traore *et al*. 2003) that were in contact with the medium containing TDZ. The cluster of buds induced in the nodal explants of *S.anacardium* in presence of TDZ, however, did not elongate simultaneously in WPM GR free medium. Dominance of the elongated shoots on the remaining buds was noted in all explants treated with TDZ resulting in asynchronous differentiation of shoots **(Fig. 3C e-g)**. In each passage of GR free medium, only few (1-2) buds proliferated into shoot and rest of the buds remained stunted. On removal of the elongated shoots from the cluster of multiple buds, the remaining ones resumed elongation.

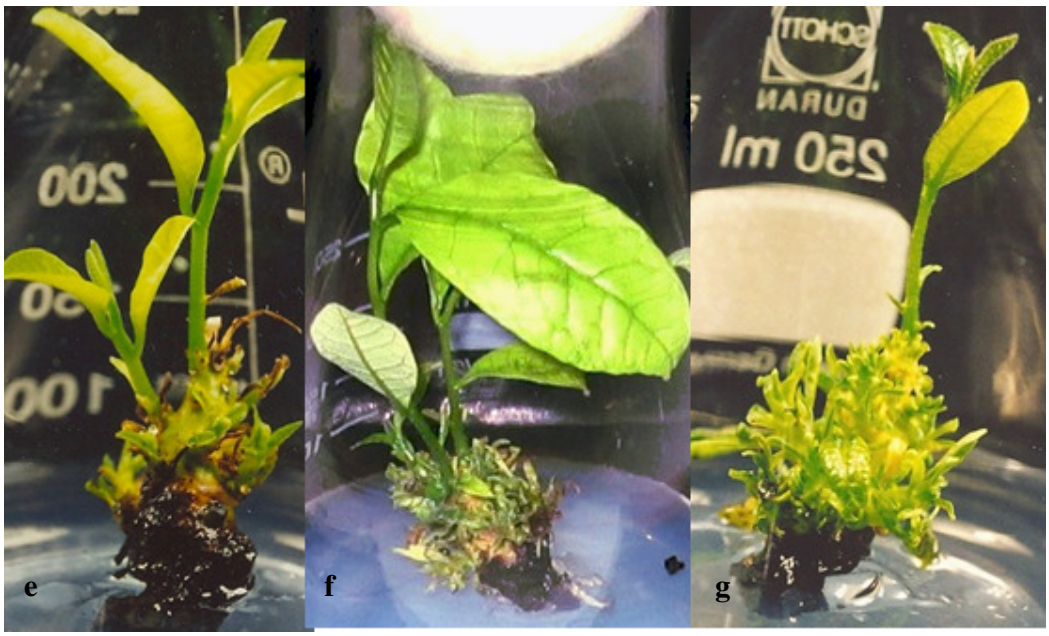


Fig. 3C e. Both multiplication and elongation (3-5cm) of shoot after 20wks in WPM medium. These explants were pre-cultured in medium with TDZ 0.45µM**; f.** Elongation of the buds after 20wks in GR free basal medium, in explants pre- cultured in WPM with 2.27µM of TDZ**; g.** Innumerable buds developed from the meristem of the explants cultured for 20wks in WPM medium after 4wks in TDZ 4.54µM. Stunted shoot buds indicate retarded differentiation and suppression of elongation due to influence of TDZ.

Apical dominance in tissue culture of tree species has been observed in cocoa, where removal of apical shoot promotes elongation of basal axillary buds (Traore *et al*. 2003). Number of shoots developed per explant was scored every 4wks, starting from 12wks upto 28wks of culturing in WPM GR free medium **(Table 3C.3)**. It is apparent from this table that 2.27µM TDZ produced optimum number of shoots. On evaluating the response of the explants, TDZ (2.27µM) was found to be the optimum concentration.

Table 3C.3 Effect of TDZ on shoot production from nodal explants every four wks in GR free medium.

Conc. of TDZ in µM	Number of shoot /explants in WPM (mean ± sd)					
	12 wk	**16 wk**	**20wk**	**24 wk**	**28 wk**	**After 7 passages**
0	0.17 (7) ± 0.06^{b}	0.37(15) ±0.06^{d}	0.43(17) ±0.06^{e}	0.83(33) ± 0.21^{fg}	0.6 (24) ±0.17^{g}	2.4 ±0.2^{g}
0.0045	0.47(19) ±0.06^{b}	0.47(18) ±0.15^{cd}	0.67(27) ±0.15^{de}	1.07(43) ±0.21^{f}	1.2(48) ±0.2^{f}	3.88 ±0.02^{f}
0.045	0.23(9) ±0.06^{b}	0.63(25) ±0.21^{cd}	0.9(35) ± 0.2^{de}	1.77(71) ±0.12^{e}	1.97(79) ±0.15^{de}	5.5 ±0.05^{e}
0.227	0.33(13) ±0.11^{b}	0.77(30) ±0.21^{bc}	1.87(74) ±0.15^{bc}	2.7(109) ±0.2^{c}	3.1(123) ±0.0.2^{c}	8.77 ±0.08^{c}
0.45	0.47(18) ±0.15^{b}	1.03(41) ±0.25^{b}	2.03(82) ±0.47^{b}	3.27(131) ±0.15^{b}	3.87(155) ±0.38^{b}	10.67 ±0.09^{b}
2.27	1.17(47) ±0.06^{a}	1.53(61) ±0.06^{a}	3.8(151) ±0.5^{a}	4.67(187) ±0.21^{a}	5.9(273) ±0.3^{a}	**17.07** ±0.12^{a}
4.54	0.37(15) ±0.32^{b}	0.67(26) ±0.15^{bcd}	1.33(53) ±0.57^{bcd}	2.27(91) ±0.15^{d}	2.57(103) ±0.31^{d}	7.21 ±0.11^{d}
9.08	0.17(6) ±0.15^{b}	0.33(13) ±0.12^{d}	0.35(14) ±0.31^{e}	0.43(18) ±0.15^{g}	0.41(17) ±0.17^{g}	1.69 ±0.07^{h}
ANOVA	S 1%	S 1%	S 1%	S 1%	S 1%	S1%

40 Explants, 4 repeats, 10 in each repeat .Figures in parenthesis indicate number of shoots.
$^{a-h}$ Duncan multiple range notation .Mean followed by the same superscripts within a column do not differ significantly at $P \leq 0.01$.

In TDZ (9.08µM) pre-treated cultures innumerable buds were formed, but majority of them failed to developed into shoots and often resulted in leafy structures (**Fig. 3Ch)** or turned brown and became necrotic later **(Fig. 3Ci**).

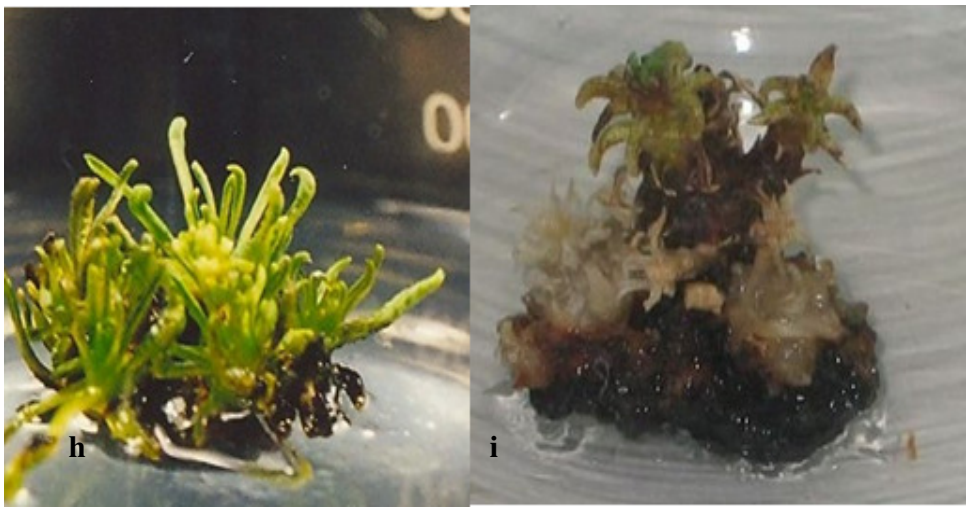
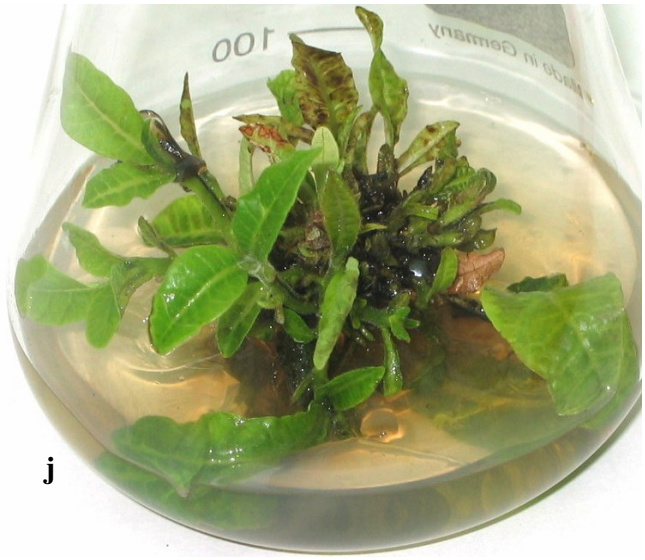


Fig. 3C h. Cluster of elongated leaf primordia developed in GR free medium from multiple buds induced in TDZ 9.08µM; **i.** Cluster of leaf primordia induced in TDZ 9.08µM and developed in GR free medium turned brown and necrotic on further transfers to growth regulator free medium.

Failure of elongation and fasciations in multiple shoot buds of *Oroxytlum indicum* by the influence of TDZ has been reported (Dalal and Rai 2004). However, in *S.anacardium* fasciation was not observed. Inhibition of shoot elongation may have been due to the high cytokinin activity of a phenyl group in TDZ (Huettmam and Preece 1993). A number of physiological effects of natural and synthetic cytokinins are well documented in literature, but the mode of action by which these plant GRs in particular the phenyl urea cytokinins control the process of growth and development are still not well understood (Toteva *et al*. 2000; Jones *et al*. 2007).

It is noted that TDZ molecule remains largely intact within the tissue in several forms (Susan *et al*. 2001). This provides a possible explanation for the long-term effect of TDZ in bud multiplication and differentiation of the shoots in *S.anacardium* after several passages in WPM medium without GR (**Fig. 3Cj**).



j

Fig. 3C j. Multiplication and elongation of shoot buds in GR free medium, initially cultured for 4wks in TDZ 2.27µM.

All the shoots developed from TDZ pre-treated explants rooted in semisolid WPM medium containing 0.2% charcoal, but the roots were brittle and often broke during transfer to sand-soil mixture resulting in poor survival of the plants. Reducing the salt concentration to half strength and eliminating the gelling agent from the medium overcame this limitation. All shoots (100%) rooted in liquid, half strength WPM medium containing 2.46µM of IBA (**Fig. 3Ck**). Filter paper bridge supported the shoots in liquid medium. The positive influence of IBA on root induction in tree species has been reported in Cashew (Mneney *et al*. 2001; Ananthakrishnan *et al*. 2002), Pistachio (Tilkat *et al*. 2008a), *Ulmus minora* (Conde *et al*. 2008) and *Balanites aegyptica* L. (Del.) (Siddique and Anis 2009).

Fig. 3C k. Rooting of *S. anacardium* shoots in liquid medium with IBA 2.46µM after 4wks of culture.

Fig. 3C l. Hardened plant in sand: soil mixture 1:1 after 4wks of transfer.

Rooted plantlets of *S.anacardium,* survived (90%) on transfer to sand: soil (1:1) mixture and hardened in 4wks (**Fig. 3Cl**). All plants survived on transfer to green house for acclimatization (**Fig. 3Cm**).

Fig. 3C m. Acclimatized plants in green house after 4wks of transfer

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Fig. 3C n. Plants growing in larger pots in green house.

This report describes micropropagation of *S.anacardium* from nodal buds of axenic shoot culture using TDZ. The present protocol is efficient than that was described in chapter 3B using BAP and KN. Nodal buds pre-cultured in 2.27µM of TDZ provided 17 shoots/explants after 28wks of culture compared to 6 shoot/explants in buds pre-cultured in 4.44µM BAP and 4.64µM KN. Though more number of shoot can be obtained by using TDZ as GR the time taken for differentiation of shoots was very long. This is the characteristic of tree tissues in culture. The slowness of the protocol can also be attributed to the inhibitory influence of the carry over TDZ on differentiation of the shoot buds. Therefore, the protocol may need further refinement to remove TDZ rapidly from the explant to shorten the time period taken in elongation of shoots from the meristematic dome. The characteristic influence of TDZ to produce a swollen mass of meristematic cells in the axillary meristems of nodal explants in culture (Sujatha and Hazra 2007) is thus further confirmed in *S.anacardium*.

3C.3 CONCLUSION

This micropropagation protocol optimized using the axenic shoot derived nodal explants of *S.anacardium* will find application in micropropagation of this plant from elite tree derived nodal buds. The advantages of using axenic shoot culture derived explants for standardization of protocol are discussed in the previous section (Section B). The responses noted in the explants under identical conditions were nearly uniform and there was no loss of culture due to contamination. The characteristic influence of TDZ to induce proliferation of meristematic cells of the axillary nodes into large number of totipotent meristematic cells may find application in developing transgenic and in induction of somaclonal variation.

CHAPTER 4

CLONAL PROPAGATION FROM MATURE TREE DERIVED NODAL EXPLANTS

4A.DIVERSITY STUDIES IN *SEMECARPUS ANACARDIUM* L. USING ISSR MARKERS.

4B.MICROPROPAGATION OF *SEMECARPUS ANACARDIUM* L. FROM MATURE TREE DERIVED NODAL EXPLANTS

4A.DIVERSITY STUDIES IN *SEMECARPUS ANACARDIUM* L. USING ISSR MARKERS

4A.1 INTRODUCTION

The PCR based random amplified polymorphic DNAs (RAPD) and inter simple sequence repeat polymorphisms (ISSR) have been widely applied to different species to survey a population's genetic structure, because their applications need no prior information concerning the target sequence on the genome (Williams *et al*. 1990; Zietkiewicz *et al.* 1994; Ilbi, 2003; Belaı¨d *et al*. 2006; Svetleva *et al.* 2006).

Inter-simple sequence repeat (ISSR) markers are generated from single primer polymerase chain reaction (PCR) amplifications in which the primers are based on dinucleotide or trinucleotide repeat motifs. ISSR markers were introduced in 1994 (Gupta *et al*. 1994; Zietkiewicz *et al.*1994) for studies of cultivated plants but have also been applied to characterized hybridization and hybrid speciation (Wolfe *et al*. 1998a, b; Archibald *et al*. 2004), population and conservation genetics (Esselman *et al.* 1999; Culley and Wolfe 2001), as well as systematic investigations in natural populations (Crawford *et al.* 2001; Wolfe and Randle 2001; Mort *et al.* 2003). The hypervariable nature of ISSRs combined with minimal equipment requirements and ease of use has made them extremely useful and cost-effective molecular markers for many ecological and taxonomical investigations (Yang *et al*. 1996; Wolfe *et al*. 1998b). The amplification and data-scoring protocols used for ISSR markers are similar to those used for random amplified polymorphic DNA (RAPD) enabling comparison of macromolecular markers. The only exception is that the annealing temperature for ISSR amplification is generally higher, resulting in a higher degree of stringency for amplified fragments (Wolfe and Liston, 1998).

The advantages of ISSR includes requirement of low quantities of template DNA, no constraint regarding sequence data availability for primer construction, random distribution throughout the genome, feasibility in generation of many informative bands per reaction, reliability and reproducibility (Ziekiewica *et al.* 1994; Nagaoka and Ogihara 1997; Ge 2001). Consequently, the ISSR has been widely used in marker assisted selection, genetic diversity analysis, DNA fingerprinting, evolutionary and molecular ecology (Reddy *et al*. 2002; Vijayan *et al.* 2006; Zhao *et al.* 2007a, b; Yao *et al* 2008; Naik *et al*. 2009). The effectiveness of ISSR primers lies in the ease of rapid production of reproducible amplicons when compared to RAPD (Wu *et al*. 2004). The number of amplification fragments (bands) generated per reaction varies with the primer–template combinations which are easily scored using agarose gel electerophoresis.

Genetic diversity between individuals enables evolution and adaptation of species within a changing environment and is thus essential for the long-term survival of a species (Bauert *et al.*

1998). 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 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.

S.anacardium is a highly out breeding species, in which cross-pollination takes place primarily by insects and wind. This leads to wide variations in characters like maturation time, shape of nut, oil content etc. Keeping in view the phenotypic variation among the *S. anacardium* plants, genetic variation was studied using ISSR markers.

4A.2 MATERIALS AND METHODS

Morphological characterization:

Seeds with pseudo-fruits were collected in late December from different naturally growing populations. The locations are Mulshi, Katrajghat, Sinhagad and Narayanpur around Pune, Maharashtra and Gillagada of Andhra Pradesh. Difference in morphological characters was noted. Shape and size of the nuts and pseudo-fruits were studied. Seeds with orange coloured pseudo-fruit and black colored nuts were considered ripe and mature. Difference in ripening time of pseudo fruits and nuts were observed. Nuts of *Semecarpus anacardium* L. from the above locations were collected, cleaned and morphological character of the nuts was studied closely. Nuts from all the trees can not be collected due to various constrains like long height of trees, in accessibility and very early maturity etc.

Oil extraction:

Extreme care was taken while isolating the kernel as the fluid spilled from the seeds is allergic and causes blisters on skin. Estimation of oil from the nuts was restricted to limited number of trees as sufficient numbers of nuts were not obtained from all the trees. Oil extraction was carried out following the procedure of Kadi and Fellag 2001. Approximately 10gms of the kernel was taken and crushed with mortar and pestle to fine powder. The crushed kernel was soaked in 50ml of hexane for 12hr. The hexane was decanted and collected in 250ml Erlenmeyer flasks. The process was repeated three times. All the hexane washes were pooled and evaporated at room temperature. The oil left behind after evaporation of hexane was collected. Weight of oil was determined gravimetrically in pre-weighed vials. Percentage of oil was calculated in w/w basis. ANOVA was performed on the percentage of oil contents.

DNA isolation and PCR:

Leaves of freshly sprouted buds of *Semecarpus anacardium* L. was collected from 4 different regions around Pune (Maharashtra) and from Gillagada (Andhra Pradesh). These were washed thoroughly with deionised water and the excess moisture from the surface was removed by drying over filter paper. These were stored at –20ºC until further use. DNA from the leaves was isolated following protocol described in Chapter 2. PCR protocol for ISSR markers (Raina *et al*. 1995) as described Chapter 2 was followed. PCR amplifications were performed on a Veriti thermal cycler (Applied Biosystems) under the following program: Initial denaturation at 94^{o}C for 7min, followed by 40 cycles of denaturation at 94^{o}C for 30s, annealing at 52^{o}C for 45s, extension at 72^{o}C for 2min with a final extension at 72^{o}C for 7min. Annealing temperature was varied depending on the primers used **(Table 4A.1)**. Hundred UBC primers were screened for the study. Out of the 100 primers screened only 27 primers gave amplification. In these 27 primers 15 primers gave reproducible and clear banding pattern.

Table 4A.1 List of Primers used for diversity study in *Semecarpus anacardium* L.

UBC Primer	Primer sequence	Annealing temperature
808	AGAGAGAGAGAGAGAGC	44ºC
811	GAGAGAGAGAGAGAGAC	44ºC
812	GAGAGAGAGAGAGAGAA	50ºC
816	CACACACACACACACAT	56ºC
821	GTGTGTGTGTGTGTGTT	56ºC
825	ACACACACACACACACT	52ºC
827	ACACACACACACACACG	47ºC
834	AGAGAGAGAGAGAGAGYT	53ºC
835	AGAGAGAGAGAGAGAGYC	56ºC
841	GAGAGAGAGAGAGAGAAYC	53ºC
842	GAGAGAGAGAGAGAGAAYG	53ºC
847	CAC ACA CAC ACA CAC ARC	60ºC
861	ACCACCACCACCACCACC	56ºC
862	AGCAGCAGCAGCAGCAGC	56ºC
867	GGCGGCGGCGGCGGCGGC	53ºC

A low range DNA ladder (100bp-3000bp) was used as molecular standard for band quantification. The PCR-ISSR products were loaded on 2% agarose gel stained with ethidium bromide for electrophoresis in 0.5X TAE at a constant current 50mA, for 2hr. Visualization and documentation of gel was undertaken in Gel Documentation system (Syngene, UK).

Data analysis of PCR products in Gel electrophoresis:

Size of different fragments in gel was analyzed by comparing with the ladder and using Gene tool software provided with the gel documentation system. Consistent, well-resolved fragments, in the size range of 100bp to 3000kb were manually scored. Reproducible and well defined bands obtained after PCR amplification using each primer were scored as 1 (present) or 0 (absent) in a binary matrix in Excel (MS Office) worksheet. The number of polymorphic loci (P) and percent polymorphism was calculated manually. Measurement of genetic diversity within and between populations was estimated using Multi Variant Statistical Package (MVSP) software free online version. The genetic associations were evaluated by calculating the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of un-weighted pair group method with arithmetic averages (UPGMA) and dendrogram was plotted using MVSP software. All the experiments including oil extraction, PCR and gel electrophoresis was repeated.

4A.3 RESULTS AND DISCUSSION

Morphological variation:

The study of seed morphological characters of the natural populations is often considered to be useful step in the study of the genetic variability. Morphological variation between the plants of *Semecarpus anacardium* L was noticed. Shape of the nut and maturation period varied in plants from same locality. Difference in shape of nuts and pseudo fruits from different location was obvious (**Fig. 4Aa - e**) even though the collection time was nearly same for all the locations. The ripening time of the seeds also varied as can be observed from the figure. Some of the pseudo fruits were already ripened whereas some others were still green and immature.

a

Fig. 4A a. Nuts from different trees of Mulshi Dam (MH) location with difference in shape of pseudo- fruits and the seeds.

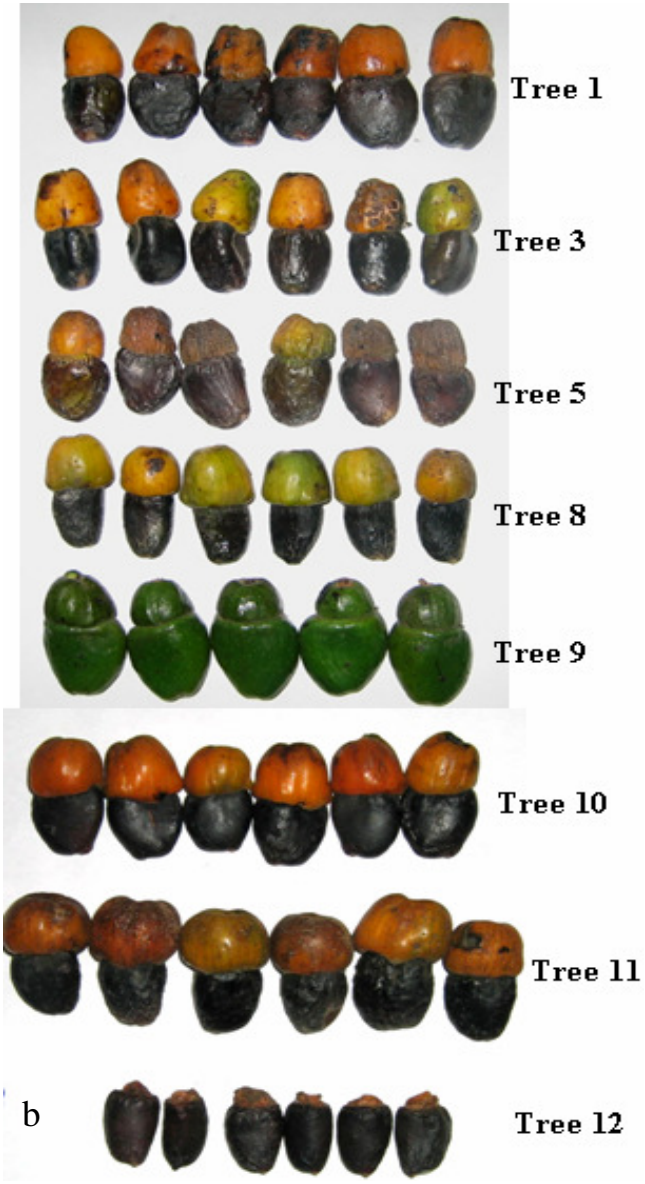


Fig. 4A b. Nuts from different trees of Katrajghat (MH) location with difference in shape of pseudo-fruits and the seeds**.**

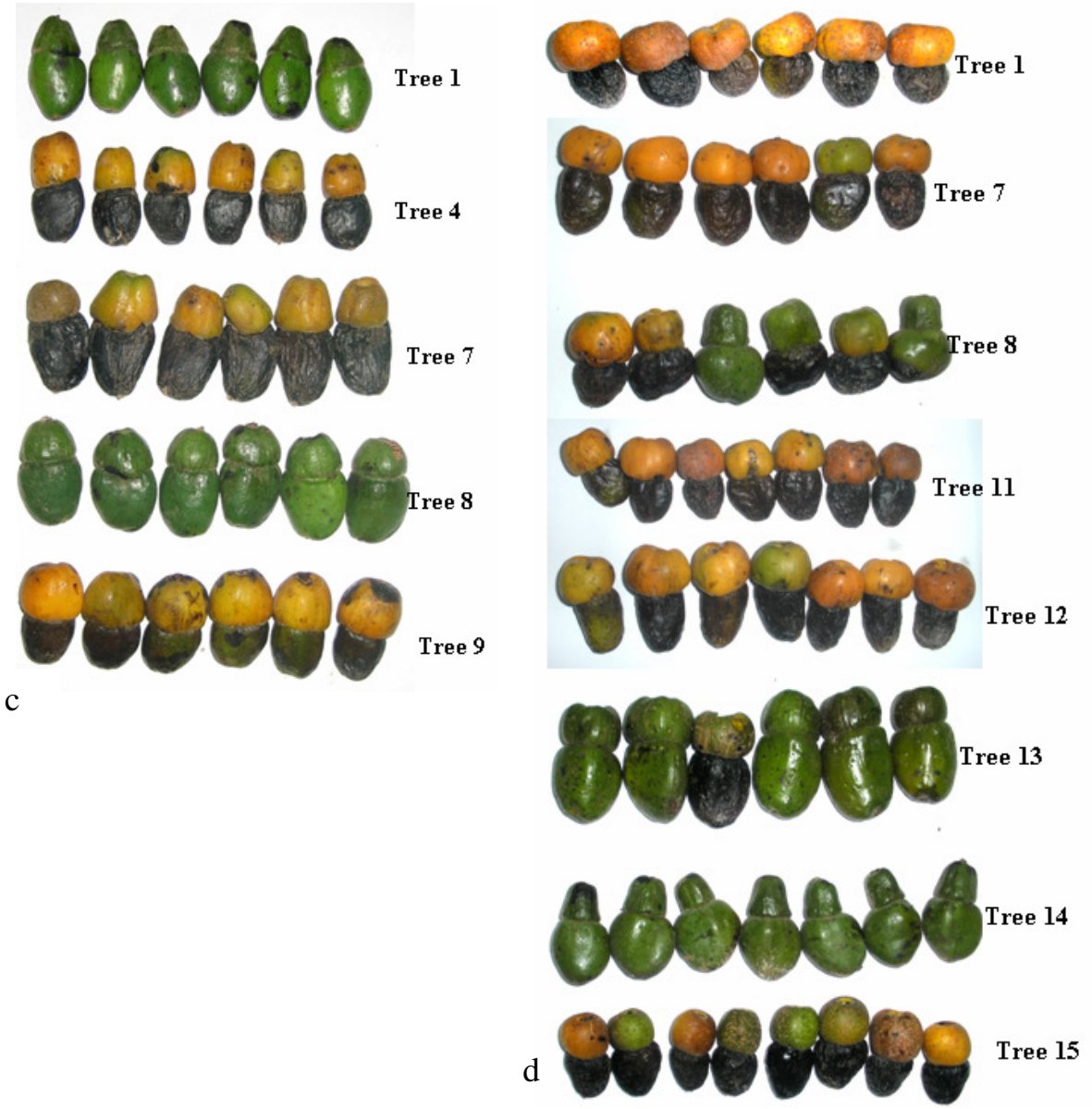


Fig. 4A c. Nuts from different trees of Sinhagad (MH) location with difference in shape of pseudo-fruits and the seeds**; d.** Nuts from different trees of Narayanpur (MH) location with difference in shape of pseudo-fruits and the seeds.

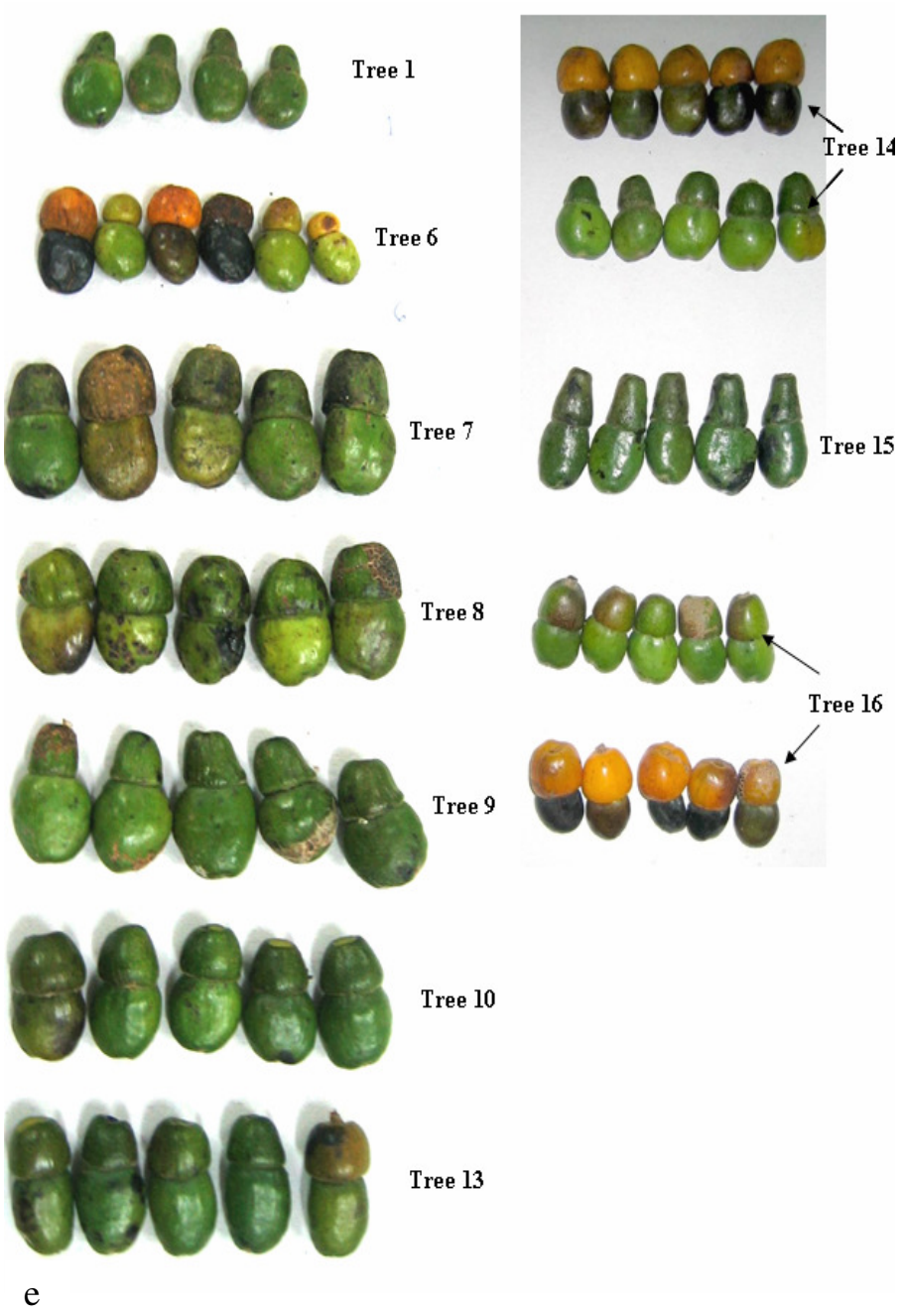


Fig. 4A e. Nuts from different trees of Gillagada (AP) location with difference in shape of pseudo-fruits and the seeds.

Variation in oil contents:

Significant variation was noticed in oil contents in the seeds of different trees (**Table 4A.2**). A maximum oil quantity of 46% was in measured in the tree no. 4 of Narayanpur (MH) location. Lowest amount of oil (19.5%) was noted in tree no. 5 of Katrajghat (MH) location. Tree no. 10 from the Katrajghat (MH) location yielded 40% of extractable oil. Plant from other trees of different location yielded varying amount of oil. There is considerable variation among the trees in terms of oil quantity (**Fig. 4Af - j**). The variation in oil content may be due to the possibility

that it is a polygenic and complex trait responsive to environmental effects that occur during plant development as has been observed in *Glycine max* (Miranda *et al.* 1998).

Table 4A.2 Percentage of oil content in Kernal of *Semecarpus anacardium* L from trees of different location.

Trees from different locality	**Oil quantity (mean±sd) %**
Mulshi 1	30.8 ± 0.7
Mulshi 2	34.5 ±0.80
Mulshi 3	32.6 ±0.7
Mulshi 8	27.7 ± 0.7
Katraj 5	19.5±0.6
Katraj10	40.9±0.19
Katraj11	33.8±0.25
Katraj12	33.7±0.3
Sinhagad 1	21.7±0.42
Sinhagad 8	21.4±0.13
Sinhagad 9	25.4±0.4
Narayanpur1	28.8±2.16
Narayanpur4	46.2±1.15
Narayanpur9	29.8±6.88
Narayanpur12	36.8±2.4
Gillagada AP 7	24.5 ± 0.5
Gillagada AP9	35.6±0.5
Gillagada AP14	29.7± 1
Gillagada AP16	24.4±0.1
ANOVA	S1%

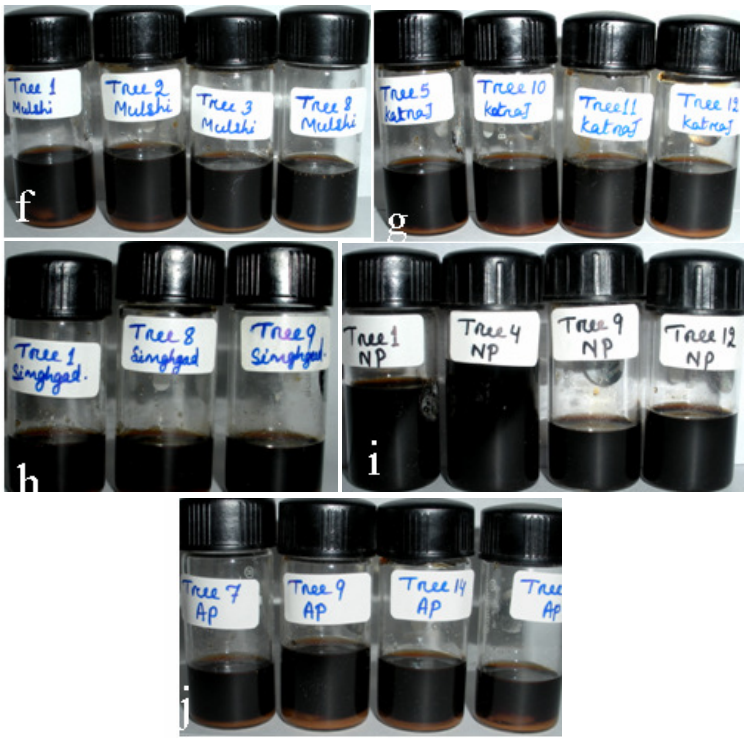


Fig. 4A f. Oil extracted from Trees from Mulshi dam (MH) location; **g.** Oil extracted from Trees from Katrajghat (MH) location**; h.;** Oil extracted from Trees from Sinhagad (MH) location **i.** Oil extracted from Trees from Narayangaon (MH) location.**; j.** Oil extracted from Trees from Gillagada (AP) location.

Genetic similarity and cluster analysis based on ISSR amplification data:

Different banding patterns in UBC primers were observed in trees from different locations. These representative amplification profiles show variation at the genotype level within the population. Variation in banding pattern was noticed with all the primers studied.

Trees from Mulshi dam location scored 194 bands out of which 160 were polymorphic. The band size ranged from 202bp in primer UBC 835 to 3000bp in UBC827. Highest number of bands (20) was noted with UBC 808. Lowest number of 08 bands was noted in UBC812 and UBC862. The representative PCR amplification profiles using UBC primer 812 **(Fig. 4Ak)** and 834 **(Fig. 4Al**) show variation within the population. Dendrogram analysis of the banding pattern from the trees of Mulshi dam location (**Fig. 4Am**) displayed two distinct clusters. Tree 19 and 20 that were clustered in one minor group showed a similarity index value of 0.659 between them. The major cluster consisted of rest of the trees. The similarity index between all the trees ranged from 0.496-0.795. The highest genetic proximity with a similarity index value of 0.795 was found between Tree 3 and 4.

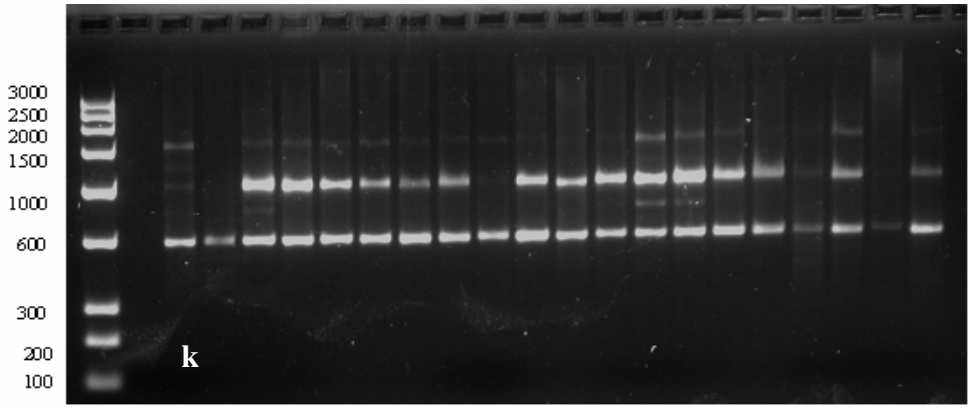


Fig. 4A k Agarose gel electrophoresis pattern of amplification products of DNA trees (1-20) of Mulshi dam location by using the primer 812 **L:** Low range DNA ruler (3kb). **N:** Negative control.

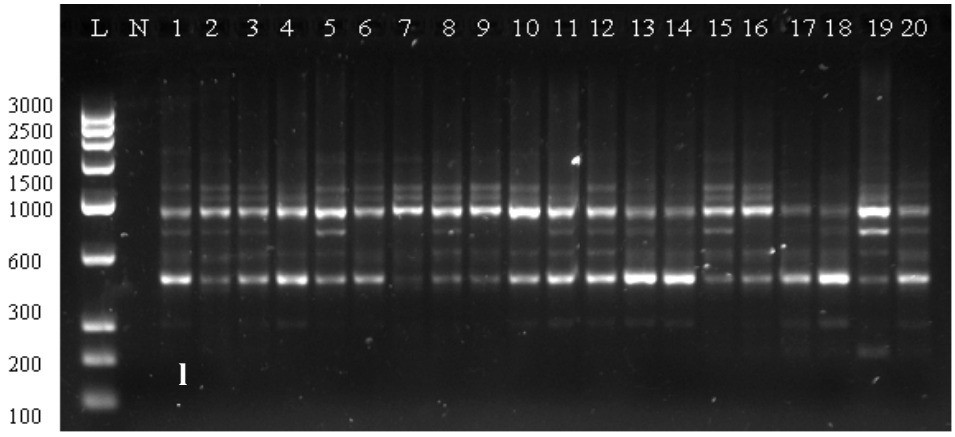


Fig. 4A l. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-20) of Mulshi dam location by using the primer 834 L: Low range DNA ruler (3kb). N: Negative control.

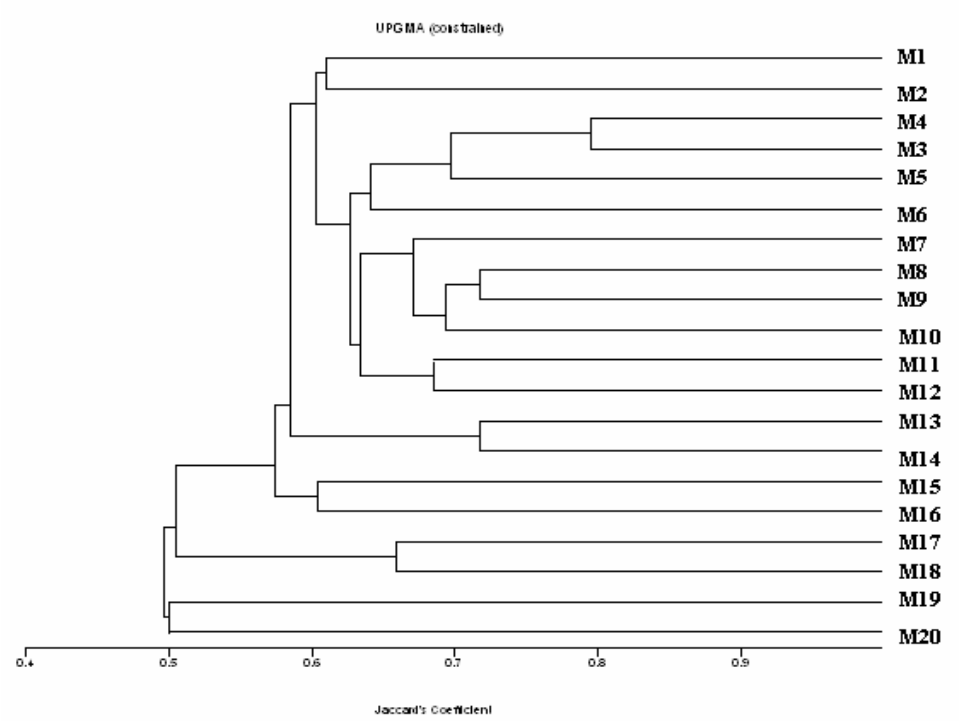


Fig. 4A m. UPGMA dendrogram analysis of plants of Mulshi dam (MH) location based on Jaccard's coefficient.

Thirteen trees of Katrajghat region were subjected to the analysis. DNA from 13 plants were subjected to ISSR analysis which scored 146 bands. Polymorphism was observed in 129 bands. UBC867 show 16 bands compared to 06 in UBC 825.Band size ranged from 200bp in UBC835 to 3313bp in UBC867. The representative PCR amplification profiles using UBC primer 812 **(Fig. 4An)** and 834 **(Fig. 4Ao**) showed variation within the population. The dendrogram generated (**Fig. 4Ap**) shows tree 1 forming a separate cluster and distinct from remaining trees. The similarity index of tree 1 with rest of the trees is 0.311. The trees from 2-13 that formed from one cluster was sub divided into two clusters one containing tree 2-9 and another comprising of trees 10-13. The over all genetic similarity index between the trees ranged from 0.311-0.844. Tree 3 and 4 are more closely related with similarity matrix value of 0.844 which is higher then that between any two plants in the location studied.

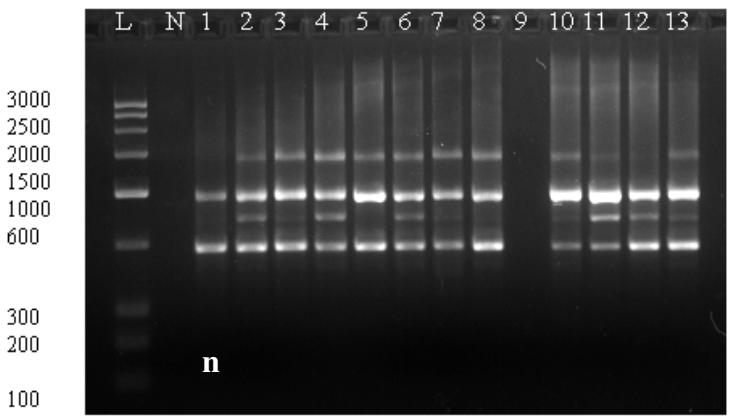


Fig. 4A n. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-13) of Katrajghat location by using the primer 812 **L:** Low range DNA ruler (3kb). **N:** Negative control

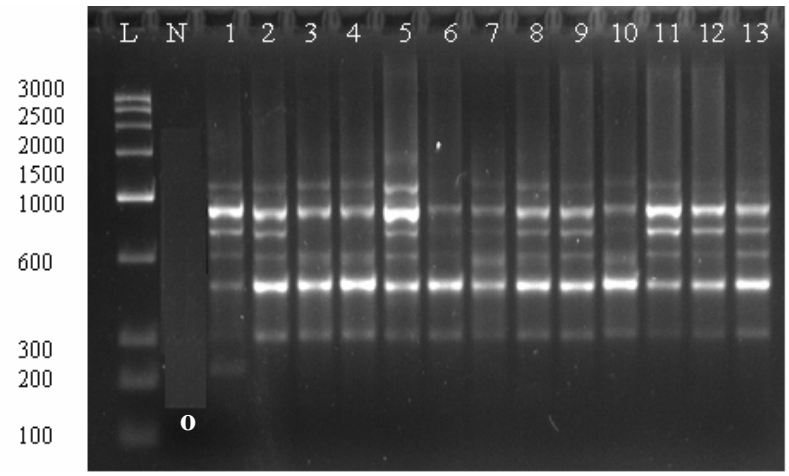


Fig. 4A o. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-13) of Katrajghat location by using the primer 834 **L:** Low range DNA ruler (3kb). **N:** Negative control.

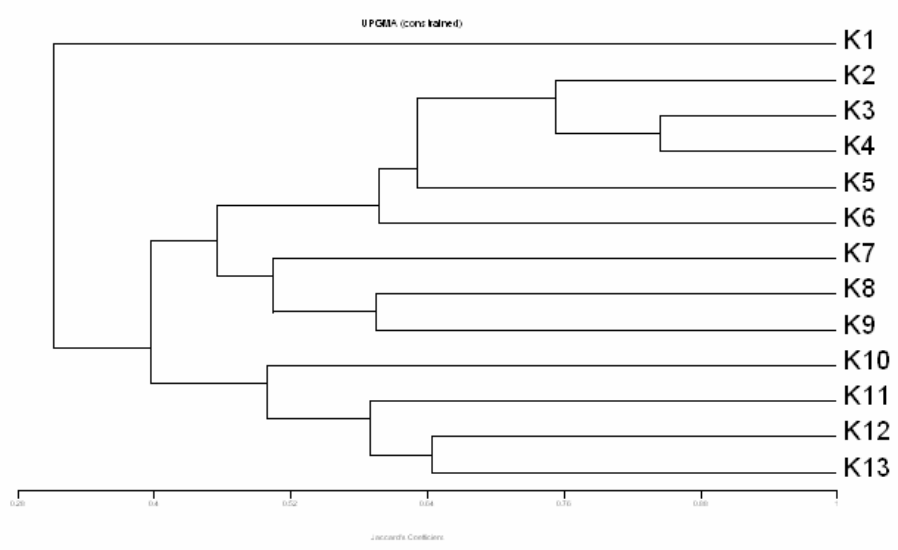


Fig. 4A p. UPGMA dendrogram analysis of plants of Katrajghat (MH) location based on Jaccard's coefficient.

The sample size of trees from Sinhagad location was 15. ISSR analysis generated 178 scorable bands of which 156 were polymorphic. Primer UBC827 shows a maximum of 21 scorable bands where as the lowest number of band of (05) was obtained with UBC835 and UBC821. Band size ranged from 193bp with UBC835 to 2223bp with UBC827. The representative PCR amplification profiles using UBC primer 812 **(Fig. 4Aq)** and 834 **(Fig. 4Ar**) showed variation within the population In trees of Sinhagad location, the dendrogram (**Fig. 4As**) produces two clusters, one minor cluster comprising of tree14 and 15 and another major cluster containing rest of the trees. The major cluster is again divided in two 2 clusters. One comprising of trees 1-9 while tree number 10-13 grouped in another. The genetic similarity ranged from 0.210-0.717. The genetic similarity index values between the trees of Sinhagad location reveal a wider genetic

diversity among the studied population. Tree 6 and 7 genetically close related with similarity index value of 0.717 where as Tree 14 and 15 are distantly related to rest of the tree.

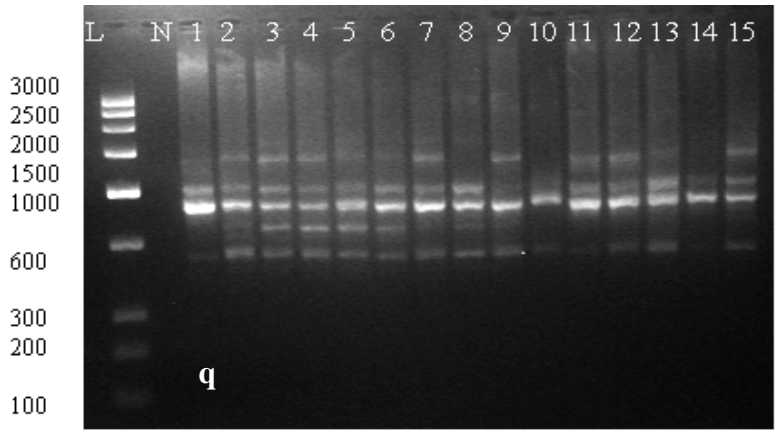


Fig. 4A q. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-15) of Sinhagad location by using the primer 812 **L:** Low range DNA ruler (3kb). N: Negative control

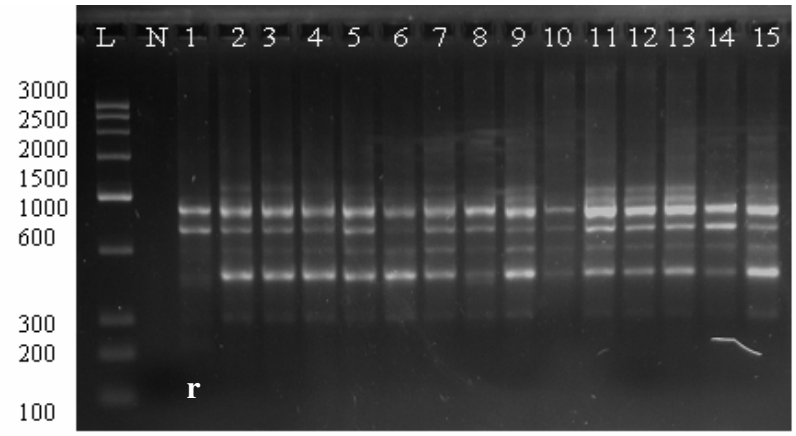


Fig. 4A r. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-15) of Sinhagad location by using the primer 834 **L:** Low range DNA ruler (3kb). **N:** Negative control.

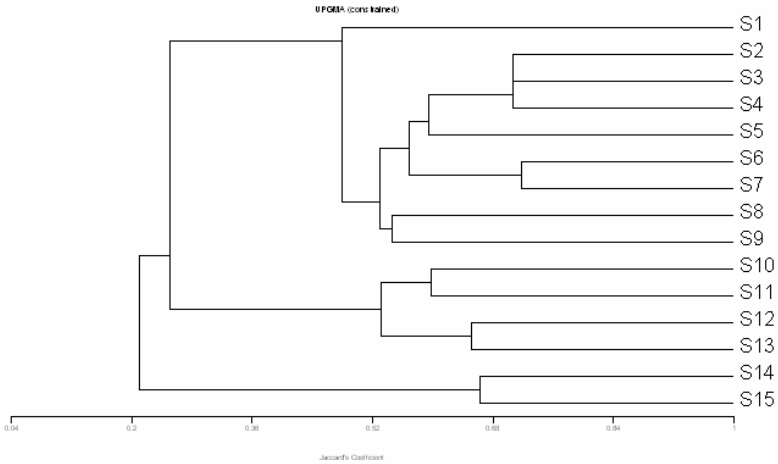


Fig. 4A s. UPGMA dendrogram analysis of plants of Sinhagad (MH) location based on Jaccard's coefficient.

From 12 plants of Narayanpur region ISSR markers yielded 147 bands out of which 115 were found to be polymorphic. UBC811 yielded highest number of 17 scorable bands. A lowest of 05 bands noticed with UBC816. Band size ranged from197bp with UBC835 to 3235bp with UBC825. The representative PCR amplification profiles using UBC primer 812 **(Fig. 4At)** and 834 **(Fig. 4Au**) show variation within the population. The dendrogram derived from trees of Narayanpur location (**Fig. 4Av**) was grouped in to two distinct clusters. One cluster contains trees from 1-6 and other contains trees 7-12.Genetic similarity between the plants ranged from 0.461-0.698.

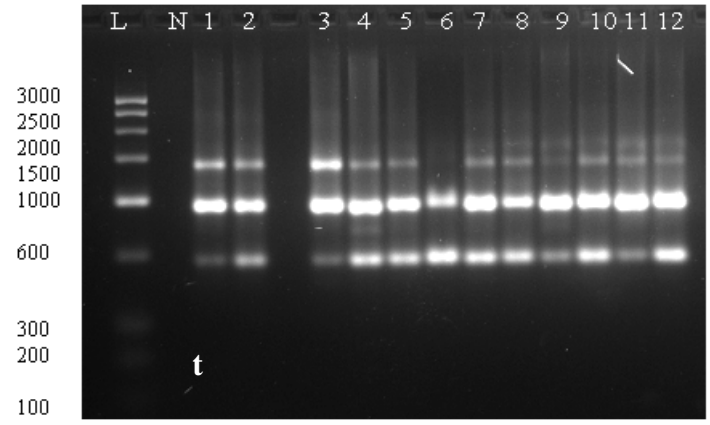


Fig. 4A t. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-12) of Narayanpur location by using the primer 812 **L:** Low range DNA ruler (3kb). **N:** Negative control.

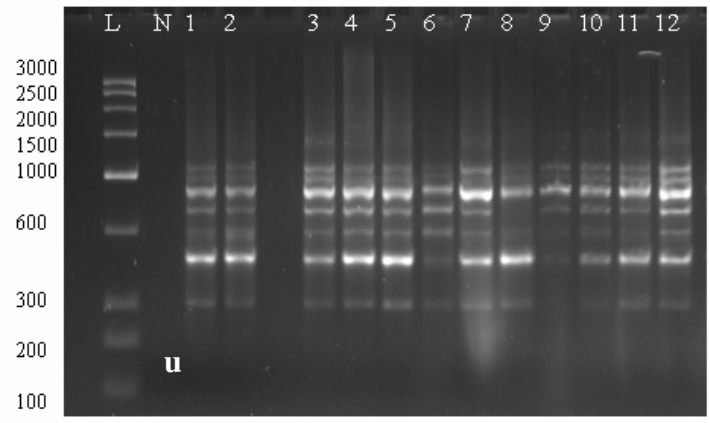


Fig. 4A u. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-12) of Narayanpur location by using the primer 834 **L:** Low range DNA ruler (3kb). **N:** Negative control.

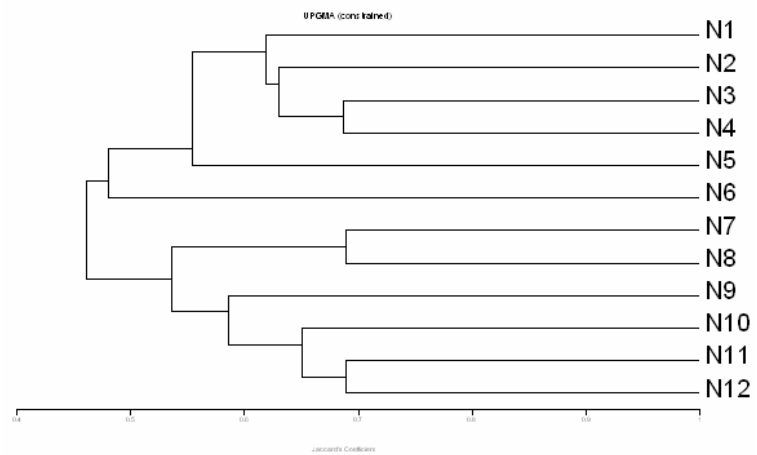


Fig. 4A v. UPGMA dendrogram analysis of plants of Narayanpur (MH) location based on Jaccard's coefficient.

DNA from 18 trees of Gillagada (AP) was subjected to ISSR analysis giving 191 scorable bands with 182 polymorphic. Smaller band size was 196bp with UBC835 and larger band size of

3243bp was obtained with UBC847. UBC811 generated a highest number of 20 bands, whereas lowest number of bands 06 was scored with UBC816. The representative PCR amplification profiles using UBC primer 812 **(Fig. 4Aw)** and 834 **(Fig. 4Ax**) showed genetic variability with in the population. The dendrogram (**Fig. 4Ay**) obtained from trees of Gillagada (AP) location revels close relationship between tree 4 and 5 with genetic similarity of 0.659. Tree18 formed a different group in the dendrogram having similarity of 0.421 with rest of the trees. The overall similarity index ranged from 0.421-0.659.

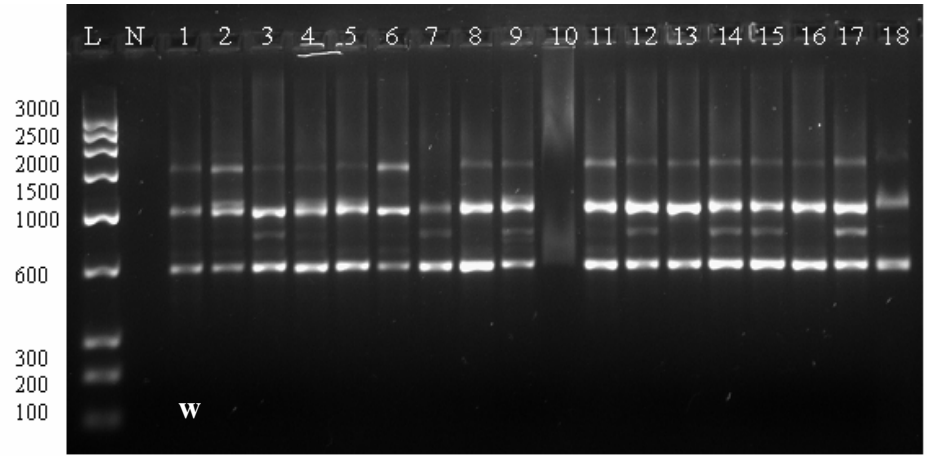


Fig. 4A w. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-18) of Gillagada (AP) location by using the primer 812 **L:** Low range DNA ruler (3kb). **N:** Negative control.

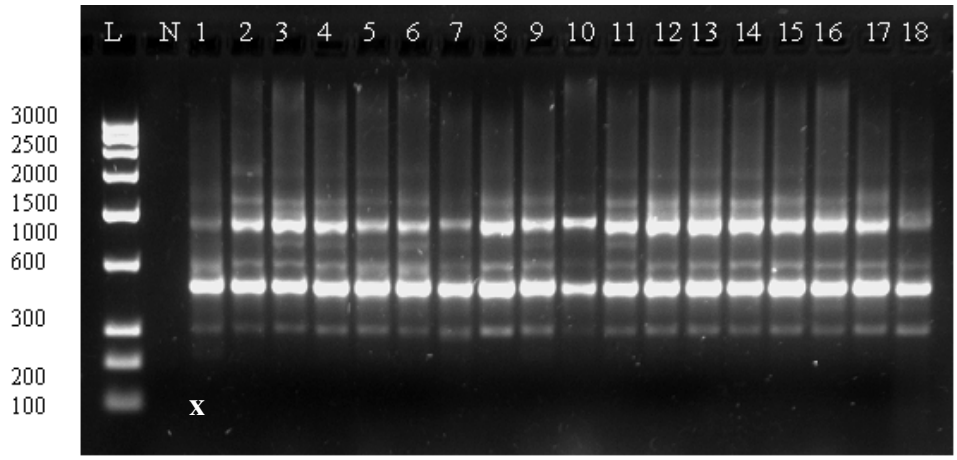


Fig. 4A x. Agarose gel electrophoresis pattern of amplification products of DNA trees (1-18) of Gillagada (AP) location by using the primer 834 L: Low range DNA ruler (3kb). N: Negative control.

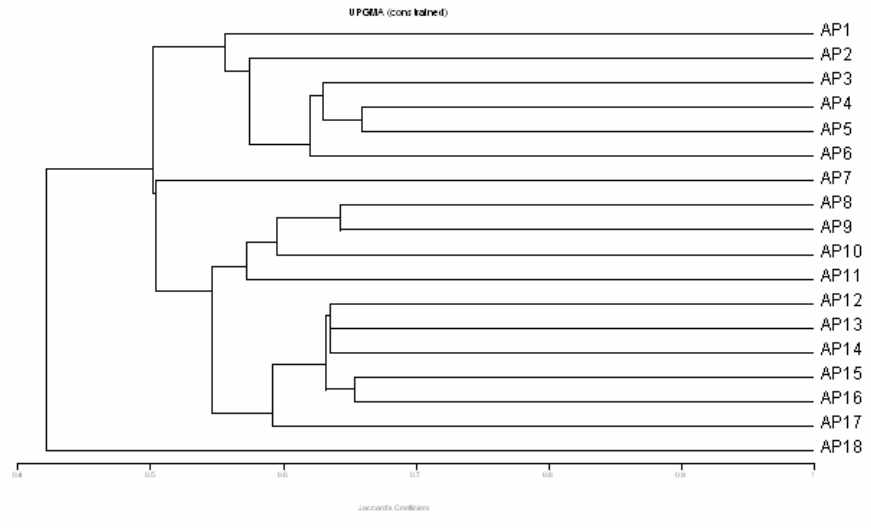


Fig. 4A y. UPGMA dendrogram analysis of plants of Gilladada (AP) location based on Jaccard's coefficient.

DNA amplification using ISSR primers yield variable number of bands in trees of different locations. The percentage of polymorphic loci with in population ranges from 78% in trees from Narayanpur to 95% in trees of Gillagada (**Table 4A.3**).

Table 4A.3 Genetic diversity of *Semecarpus anacardium* L. detected by ISSR analysis from different regions.

Plants from different location	No. of Plants	Band size(bp)	No. of Bands	No. of polymorphic bands	Percentage polymorphism
Mulshi (MH)	20	202-3000	194	160	82.4%
Katraj (MH)	13	200-3313	146	129	88.3%
Sinhagad (MH)	15	193-2223	178	156	87.6%
Narayanpur (MH)	12	197-3235	147	115	78.3%
Gillagada (AP)	18	196-3243	191	182	95.2%

The genetic diversity among the population in different location appears to be very high. When data from of all the location was compared 100% polymorphism was observed in the primers studied. Trees from all the location show large genetic difference with less genetic similarity. The simple sequence repeats, which are the basis for primer sites of ISSRs are known to have a high rate of gaining and losing repeat units due to DNA slippage (Schlo¨tterer, 1998), and may account for the ISSR variations detected. Mutation and chromosomal structural rearrangements have also been suggested sources of ISSR variation (Wolfe and Liston, 1998). In *Semecarpus anacardium* genetic variation level was high in between the populations compared to within the population. This observation was in relation to high level of genetic diversity found in *Gmelina arborea*, from 8 different geographical regions (Naik *et al.* 2009).Un-weighted pair group method with arithmetic averages (UPGMA) analysis used for preparation of dendrogram (**Fig. 4Az**) for all locations produce one minor cluster (A) and major cluster (B). The minor cluster

comprising tree 14 and 15 is from Sinhagad location only. The major cluster (B) can be divided in to 4 clusters. Cluster 1 contains plants from Mulshi dam location. Cluster 2 can again be divided in to 2 sub-clusters I with trees of Katrajghat location and II with trees of Narayanpur location. Cluster 3 contains trees from Gillagada (AP) location. The fourth cluster contains trees from Sinhagad region except tree 14 and 15. Similarity index between the plants varies from 0.200-0.862.

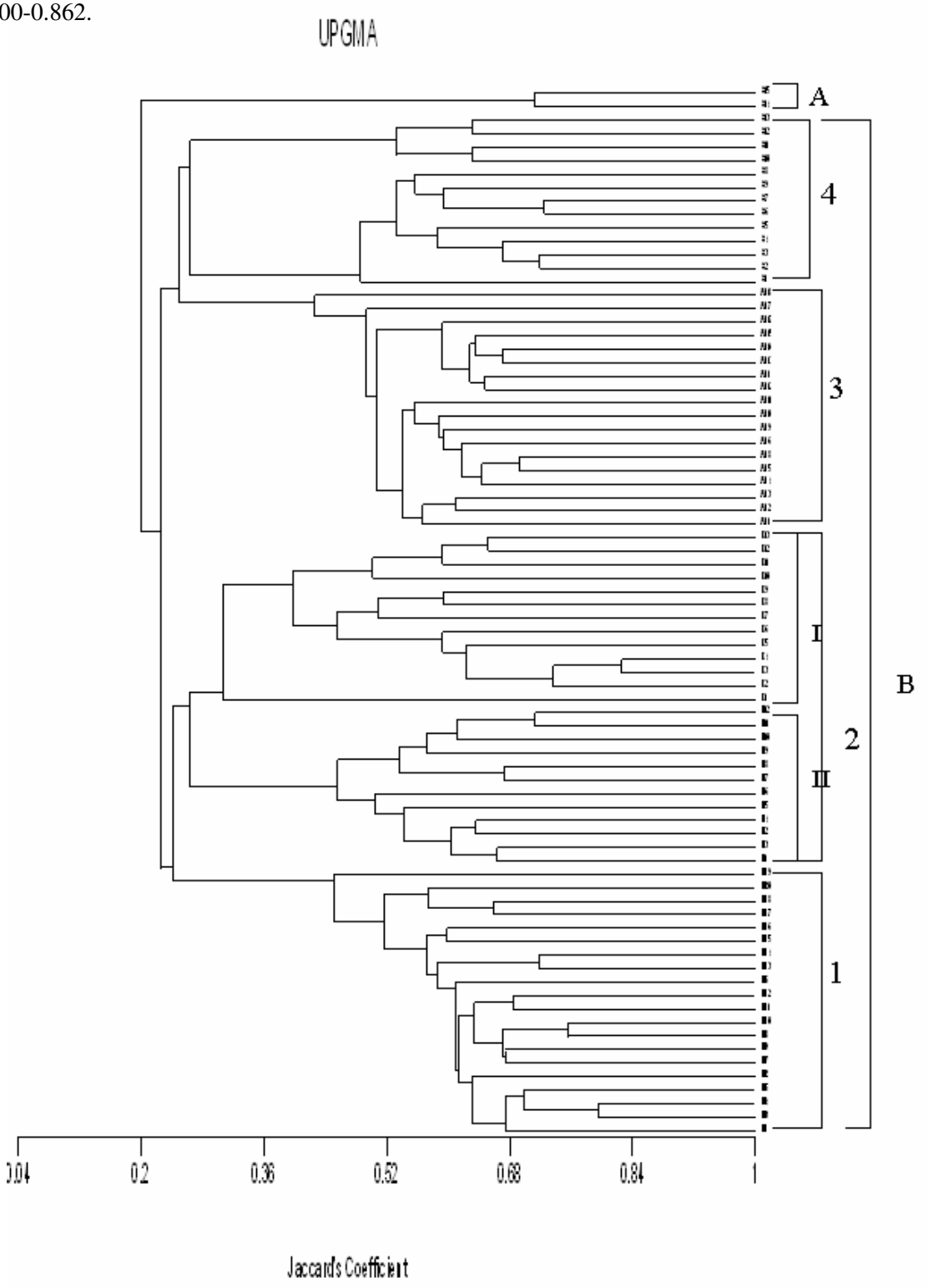


Fig. 4A z. UPGMA dendrogram analysis of plants of all the 5 location based on Jaccard's coefficient.

Cluster analysis revels that all individuals of the population were arranged in population specific clusters where as two population from nearest location did not tend to cluster together. Similar pattern of genetic variation was observed in *Elephantopus scaber* from different locations of south china (Wang *et al.* 2006)Based on the present analysis (% polymorphic bands, gene diversity) the populations were found to be relatively more diverse, and could be candidate for conservation. The high genetic variability among population in the plant may be a consequence of cross pollinated sexual reproduction, mutations of somatic cells, selection, gene flow, genetic drift and influence of environment factors as observed in case of wild *Cymbidium goeringii* (Gao and Yang 2006).

4A. 4 CONCLUSION

The high genetic diversity among the plants of *Semecarpus* states the need for an appropriate and effective conservation strategy. The information about population genetic diversity represents population adaptation to environments, which conditions on the level of its adaptive evolution. It is also of critical importance to the conservation and management of plants, including the assessment of the conservation value and status of special populations (Bawa and Ashton 1991). The high genetic diversity maintained within populations of *Semecarpus anacardium* is encouraging. In addition, the plant possesses great potential for medicinal compounds. It is necessary to protect existing natural populations and its habitat in order to preserve as much genetic variety as possible.

4B. MICROPROPAGATION OF *SEMECARPUS ANACARDIUM* L. FROM MATURE TREE DERIVED NODAL EXPLANTS

4B.1 INTRODUCTION

Propagation of S*emecarpus anacardium* by seed was difficult due to low germination frequency and poor viability (Panda and Hazra 2009). *S. anacardium* is a highly outbreeding species, in which cross-pollination takes place primarily by insects and air. This leads to high variations in characters like shape and size of nut, maturation time, oil content etc. This necessitates standardization of a protocol for clonal propagation of this species using mature tree derived nodal explants. The literature on this species is restricted to chemical and medicinal aspects only.

A major problem encountered with the *in vitro* culture is the occurrence of somaclonal variation amongst sub-clones of one parental line, as a direct consequence of *in vitro* culture of plant cells, tissues or organs (Venkatachalam *et al*. 2007). Thus, clonal fidelity is one of the most important pre-requisites in the micropropagation of any plant species. Morphological variation generated in tissue culture could be visible only at the later stages of maturity (Pietsch and Anderson 2007). Consequently, early detection of variations among the tissue culture raised plants using molecular techniques is desirable. Clonal uniformity based on morphological and phenological traits is not precise (Rahman and Rajora 2001; Guo *et al*. 2006). However, genomic stability of these regenerated plants can be determined using molecular markers. To standardize a protocol for micropropagation of *S. anacardium* from mature tree derived explants and to test the fidelity of the propagules, following experiments were carried out to:

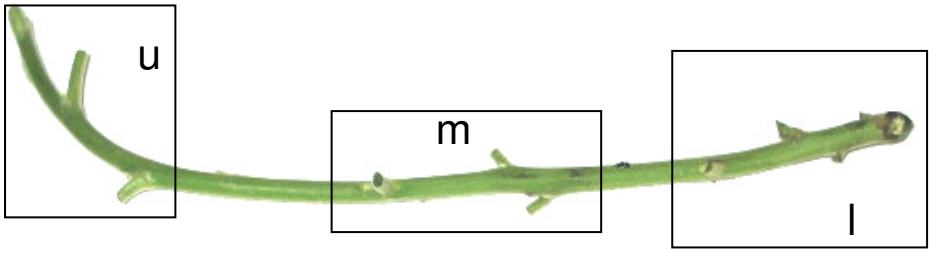
1. Standardize a surface sterilization procedure to get microbe free, mature tree derived nodal explants and optimum sprouting of the meristems.
2. Identify the media additives and growth regulators for optimum sprouting frequency of the mature tree derived axillary buds. Grow the sprouted buds to establish shoot cultures.
3. Rooting, hardening and acclimatization of the *in vitro* raised shoots.
4. Testing the *in vitro* raised clones for genetic uniformity using molecular markers (ISSR).

4B.2 MATERIALS AND METHODS

A method for clonal propagation of *S.anacardium* using the *in vitro* raised, seedling derived explants is described in the previous section of this chapter (Chapter 3, Section B). Some of the experiments described in the previous section were extended to test the viability of the methods for the mature tree derived nodal buds. Some of the modifications made to suit the mature buds are also described.

Surface sterilization procedure:

Mature buds of *Semecarpus anacardium* were collected from trees growing naturally around Pune, Maharashtra. The surface sterilization procedure established for *Pongamia pinnata* (Sujatha and Hazra 2007) was extended to mature tree derived single nodal (SN) buds of *S. anacardium*. In brief, nodal explants of approximately 2cm in length were excised and washed thoroughly. Explants were treated with 1% Bavistin (Carbendazim 50%WP, India) containing a few drops of detergent for 1hr on a gyratory shaker, followed by multiple washings with sterile, distilled water and treatment with 4%(v/v) Savlon (liquid antiseptic, Johnson and Johnson Ltd., Mumbai, India) for 10min. The explants were then surface sterilized with 0.1% $HgCl_2$ for 8min followed by four times rinsing with sterile water. This surface sterilization protocol was not effective in getting microbe free culture in *S. anacardium*. Mature tree derived twigs containing 6-8 nodes (20-25cm) were collected in different months (April, May and June) from the trees. Leaves and shoot tips were removed. The defoliated twigs were washed thoroughly by keeping them in 2-litre beaker under running tap water for 11-12 hr. The mature tree derived defoliated twigs (MTDDT) (**Fig. 4Ba**) were treated with four different surface sterilization procedures to get aseptic culture.



a

Fig. 4B a. Mature tree derived defoliated twigs of S. *anacardium.* The twig is divided into three equal parts. The middle buds (m) are more responding than upper (u) and lower (l).

(A) The explants (MTDDT) were washed in few drops of liquid detergent for 10min, followed by Bavistin 2% for one hour. Washing several times with sterile distilled water eliminated Bavistin. This was followed by 15min of Savlon (6%) treatment and finally with sodium hypochlorite (4%) for 30min was given. Several washings with sterile distilled water followed each treatment.

(B) Few drops of liquid detergent were added along with Bavistin (2%) and the explants (MTDDT) were treated for one hour followed by treatments of Savlon and Sodium hypochlorite as described in (A).

(C) The explants (MTDDT) washed with detergent were treated with antibiotics Cefotaxime (Alkem, India) 400mg/lit for 30min, before Bavistin treatment; while other treatments were similar to procedure (A).

(D) In this protocol Bavistin (2%), liquid detergent (1%) and Cefotaxime (400mg /lit) were given in combination for one hour with intermittent shaking and then washed thoroughly (4-5)

times in sterile distilled water. This was followed by treatments of Savlon and Sodium hypochlorite as in (A). In all the experiments 5-6 washes with sterile water was given to remove Sodium hypochlorite in the final decontamination step. Surface sterilization procedure from Bavistin treatment in all the experiments was done aseptically. Sterile measuring cylinder was used for surface sterilization, which has the advantage of height to accommodate the defoliated twigs (20-25cm).

In another experiment the MTDDT were cut into single nodal explants **(Fig. 4Bb)** and subjected to surface sterilization procedure as in (D) to compare the frequency of microbe free culture in both types of explants. Further experiments were carried out using single nodal buds from surface sterilized MTDDT explants only.

Fig. 4B b. Single nodal buds from freshly sprouted twigs of *S. anacardium.*

Seasonal influence and in vitro response:

The MTDDT nodal buds of *Semecarpus anacardium* were collected in April, May and June from different locations around Pune, Maharashtra, India. The plant passes through different physiological stages in these months, i.e., pre-sprouting/just sprouted, post-sprouting/pre-flowering and bloomed/flowered. These explants were cultured in WPM medium containing 2% sucrose and 0.2% phytagel to evaluate the response of the buds *in vitro*. Frequencies of explants responding and number of microbe free cultures were noted after 4 wks of culture.

Position of explants in the twig:

In another experiment MTDDT were surface sterilized following the procedure (D). The twigs were cut into three equal parts designated as upper, middle and lower (**Fig. 4Ba**) consisting of 1-3 nodes in each part. Single nodal explants were isolated from each of these three parts and were cultured separately to assess the potential of *in vitro* response of the buds with respect to its position in the twig.

Effects of basal media formulations:

Single nodal buds were isolated aseptically from surface sterilized MTDDT explants and cultured in four basal media formulations including MS (Murashige and Skoog 1962), WPM (Loyd and Mc Cown 1980), half strength WPM, SH (Schenk and Hilderbrandt 1972) and B5

(Gamborg *et al*. 1968) to identify the basal medium suitable for optimum sprouting of mature tree derived nodal buds.

Effects of different carbon sources and optimization of sucrose concentration:

To identify the appropriate carbon source, different carbohydrates including fructose, glucose, maltose and sucrose were incorporated at the concentration of 2% in WPM basal medium. Thereafter optimum requirement of sucrose for sprouting of nodal explants was determined by culturing the mature tree derived nodal buds in WPM media containing 2, 4, 6 and 8% sucrose.

Effects of gelling agents:

Phytagel (0.2%) and Agar (0.7%) were tested for optimum sprouting response of the buds in cultures.

Effects of PPM:

Plant preservative mixture (PPM) 2ml/lit was added aseptically in WPM medium with 2% sucrose and 0.2% phytagel to control the microbial contamination that appeared in mature tree derived cultures.

Effects of growth regulators on sterility and sprouting:

Single nodal buds were isolated from the surface sterilized MTDDT aseptically and cultured in WPM medium with different growth regulators. To control the endogenous bacterial contamination in later stages of culturing the explants, cefotaxime 400 mg/lit was incorporated in the media. Cultures were initiated in 150x20mm test tubes containing 20ml of the medium. Growth regulators, Benzyl aminopurine (BAP) (2.22, 4.44, 8.88, 13.32 and 22.20µM), Kinetin (KN) (2.32, 4.64, 9.28, 13.92 and 23.20µM), BAP and KN combination (BAP 2.22+ KN 2.46, BAP 4.44 + KN 4.64, BAP 4.44 + KN 4.64, BAP 4.44 + KN 9.28, BAP 4.44 + KN 16.92 and BAP 4.44 + KN 23.20µM), Thidiazuron (TDZ) (0.45, 2.27, 4.54, 9.08 and 13.62µM), Zeatin (ZE) (2.28, 4.56, 9.12, 13.68 and 22.80µM) and Gibberellic acid (GA_3) (0.29, 1.44, 2.89, 5.78 and 8.67µM) in WPM basal medium were tested to get optimum sprouting *in vitro*.

Frequency of sprouting and number of sterile explants were scored after 4wk of culture. Responding buds from TDZ incorporated medium were cultured in GR free WPM medium with antibiotics Cefotaxime 400mg/lit for four passages to control bacterial contamination. These bacterial growths were evident upon transferring to antibiotics free medium. Buds sprouted in other GRs are very few compared to TDZ containing medium and were not cultured further. Sprouted buds were cultured in 250ml Erlenmeyer flask after third passage (12wk) to accommodate the growing shoot buds. After four passages, the sprouted buds were transferred to charcoal incorporated WPM medium. Number of buds and shoot length was scored every 4wks

during transfer to GR free medium up to five passages. After five passages numbers of shoots produced from each explant were recorded for two more passages of four wks each.

Shoots approximately 1-2cm were used for rooting. Explants were cultured in tubes containing half strength WPM liquid medium with IBA at 2.46µM, 4.92µM, and 7.38µM concentration. Filter paper bridges were used to support the explants. Observations from all the cultures were taken after 4wks. All the cultures were incubated in culture room maintained at temperature of 25±2°C in 16hr photoperiod with an irradiance of 50µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Phillips). The pH of all media was adjusted to 5.8 before sterilization by autoclaving at 121°C for 20min at 1.06 kg cm^{-2}. Phytagel (0.2%) was used for gelling the semi-solid medium. Antibiotics, GA_3, Zeatin and IBA were added aseptically to the medium after autoclaving. Rooted plantlets were transplanted in plastic cups filled with autoclaved soil sand (1:1) mixture. The plants were kept in 24hr light, covered with transparent polythene bags to maintain high humidity. After 4wks, hardened plants were transferred to green house for further acclimatization.

All the experiments were repeated three to five times with 10 explants in each repeat. The experiments with varying parameters were conducted simultaneously as the sprouting season in *S. anacardium* was of short duration and nodal explants in large number from mature trees are required. The data generated were subjected to Analysis of Variance (ANOVA).

Clonal fidelity:

To test the clonal fidelity, leaf material was taken from 15 *in vitro* raised hardened plants after hardening. Deoxyribose nucleic acid (DNA) was extracted by protocol described by Khanuja *et al.* (1999), with minor modifications as described in (Chapter 2). The DNA was quantified by UV–Vis spectrophotometer and finally diluted to a concentration of 20ng/ml and stored at -20ºC for further use. PCR amplification was carried out using ISSR primers by the method described in (Chapter 2). Amplification products were separated on 2% agarose gel using 0.5X Tris-acetic acid EDTA buffer and stained with 3µl of Ethidium bromide (10mg/ml). Electrophoresis was carried out at 50mA for 2hr and 30min. The gels were photographed under UV using a gel documentation system. Consistent, well-resolved fragments, in the size range of 100bp to 3kb were scored manually. Scoring of bands was done on the basis of their presence (‘1’) or absence (‘0’) in the gel. The data was presented in excel sheet. This data was subjected to the cluster analysis of un-weighted pair group method with arithmetic averages (UPGMA) using MVSP software. The genetic associations were evaluated by calculating Jaccard’s similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by primers.

4B.2 RESULTS AND DISCUSSION

Tree propagation *in vitro* has been a difficult proposition compared to other plants. The longer life span of trees may add to the problem of contamination *in vitro* by the symbiotic association of microorganisms. The important step in establishing aseptic cultures of mature plants is to eliminate microorganisms from the explants (Perez-tornero and Burgos 2007). The major constraint encountered in establishment of *S.anacardium in vitro* culture was to eliminate the microbial infection (Panda and Hazra 2009). Extensive efforts were made in every step starting from collection of buds to culturing in medium. Buds cultured in different seasons and from different locations were followed for several passages in culture for microbe free response. Washing the explants prior to surface sterilization was done in various ways to remove the contaminants and phenolics. In *Ficus carica* L. (Pasqual and Ferriera 2007) keeping the mature nodal buds in running tap water eliminates dust particles, other contaminants and removes some of the inhibitory phenolics substances, which cause oxidation of the explants.

Surface sterilization treatments:

The surface sterilization procedure tested for *Pongamia pinnata* (Sujatha and Hazra 2007) was ineffective for *S. anacardium* buds. From the four surface sterilization methods tested, the method (D) was effective in controlling contamination and 61% of the cultures remained aseptic. Similar method of surface sterilization was reported in bamboo and rose (Thakur and Sood 2006), in which the multinodal explants were treated with Tween-20, Bavistin and Tetracycline in measuring cylinder for effective surface sterilization. Cultures initiated following the other methods (A, B, C) of surface sterilization were lost due to fungal contamination. The surface sterilization procedure (D) was more effective in MTDDT **(Fig. 4Ba)** than single nodal explants from MTDDT **(Fig. 4Bb)**.

In single nodal explants from MTDDT the percentage of non-contaminated explants was 3.3% compared to 66% in MTDDT explants (**Table 4B.1**).The sprouting frequency in MTDDT explants was around 33% whereas it was 17% in single nodal explants from MTDDT. Cut ends of single nodal explants from MTDDT serves as sites for harboring microbes and penetration of sterilants. It is assumed that contaminants enter deep inside the tissue, thereby, escaping the surface sterilization procedure. These contaminants grow and proliferate when the explants are cultured in medium.

Table 4B.1 Effect of explant types on microbe free culture establishment and sprouting.

Explant type	**Microbe free cultures (mean± sd) %**	**Sprouting (mean± sd) %**
Single nodal	3.33 ± 5.77	16.66 ± 5.77
Multi nodal	66.66 ± 5.77	33.33 ± 5.77
ANOVA	S 1%	S 5%

50 explants in 5 repeats

Seasonal variation on sprouting and sterility:

In *S. anacardium* the sprouting of axillary buds in the tree was more in the month of May. During this time young sprouts were in abundance and the floral bud development just initiated. Buds collected in the month of April were just sprouted. In the month of June the sprouted buds started flowering. The percentage of aseptic cultures in the month of May was 60%, which is significantly higher from buds collected in April (8%) and June (10%) however, the sprouting frequency and number of non-contaminated explants were drastically reduced. Sprouting frequency in the month of May was 37.5±5.0%, compared to 5±5.77% and 12±8.16% in April and June respectively **(Table 4B.2)**. In the month of June the environment was hot and humid with occasional rain which adds to more endogenous bacterial and fungal contamination. Sanjaya *et al*. (2006) reported the season of explant collection influence shoot culture establishment in *Santalum album*, where sprouted buds that were collected from November-January showed optimum *in vitro* response. The survival of mature tree-derived explants *in vitro* was highly dependent on season, with satisfactory survival in sprouting season only. The difference in seasonal responses of buds *in vitro* might be ascribed to the physiological states as well as their growth conditions as has been reported in *Taxus mairei* (Chang *et al*. 2001).

Table 4B.2 Effect of physiological status of MTDDT on sterility and sprouting

Month of collection	**Microbe free cultures (mean± sd) %**	**Sprouting (mean± sd) %**
April	7.5 ± 5	5.0 ± 5.77
May	60 ± 8.16	37.5 ± 5.0
June	10 ± 8.16	12 ± 8.16
ANOVA	S1%	S1%

40 explants in 4 repeats.

The dominance of seasonal effect on establishment of aseptic cultures was noticed in tree species such as *Crataeva adansonii* (Sharma *et al*. 2003), *Ziziphus spina-christi* (Sudhersan and Hussain 2003) in which sprouting of buds *in vitro* was correlated with sprouting *in vivo*. Earlier reports suggest that *in vitro* response was more when buds were collected in actively growing season (Dhar and Upreti 1999). Similar response of mature buds of *S. anacardium* was noticed *in vitro*.

Explant position:

The buds isolated from the MTDDT responds differently depending on its position in the twig. A gradient in the morphogenetic response was noticed in nodal stem segments of *S. anacardium* having nodes from different positions. Percentage of aseptic culture was more in upper and middle part as compared to lower part **(Table 4B.3; Fig. 4Ba)**.

Table 4B.3 Relationship of position of bud on the twig with sterility and sprouting.

Position of buds	**Microbe free cultures (mean± sd) %**	**Sprouting (mean± sd) %**
Apical	57.5 ± 9.57	10 ± 8.16
Middle	60 ± 8.16	35 ± 5.77
Basal	5 ± 5.77	2.5 ± 5.0
ANOVA	S1%	S1%

30 explants in 3 repeats

The nodal buds isolated from the upper portion often turned brown with poor sprouting response. The upper explants were quite tender to tolerate the harsh surface sterilization procedure resulting in poor sprouting response. Similar response has been reported in *Azadirachta indica* (Arora *et al*. 2009) where tender explants from the first nodes were difficult to establish in culture. Nodal buds isolated from the middle region from MTDDT of *S. anacardium* were more responsive. Sprouting frequencies in nodal buds of upper, middle, and lower region were 10, 35 and 2% respectively **(Fig. 4Bc, Table 4B.3)**.

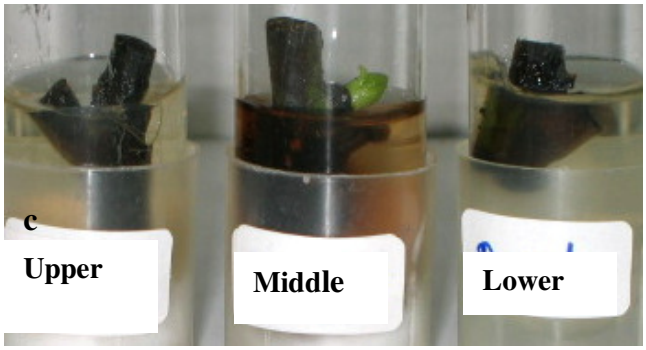


Fig. 4B c. The middle buds (m) are more responding than upper (u) and lower (l).

Optimum bud break in middle order nodal explants has been observed in cultures of *Azadirachta indica* (Arora *et al*. 2009). In basal explants of *S.anacardium* the internodal distances was short which may leaves some of the contaminating microbes escapes the surface sterilization procedure which leads to high contamination in culture. It has been demonstrated in *Robinia pseudoacacia* (Han *et al*. 1997) that the shoot-tip explants showed poorer proliferation than nodal or basal explants reflecting different physiological states of the buds at the different positions.

Basal media on sprouting and sterility:

From the basal media tested for sprouting of MTDDT nodal explants of *S.anacardium*, WPM was most favorable. The frequency of microbe free culture ranged from 55-65% irrespective of basal media tested (**Table 4B.4**). A significant difference was noticed in spouting frequency of the explants. In full strength WPM medium-sprouting frequency was 38% compared to 18.75% in half strength WPM and 5% in MS medium. Media formulation of SH and B5 did not support sprouting of buds **(Fig. 4Bd and Table 4B.4).**

Table 4B.4 Effect of basal media on sprouting frequency of MTDDT derived single nodal buds.

Basal medium	**WPM**	**1/2WPM**	**MS**	**SH**	**B5**	**ANOVA**
Sprouting (mean± sd) %	33 ± 8.5	18.75 ± 2.5	5.0 ± 4.1	00 ± 00	00 ± 00	S1%

50 explants in 5 repeats.

Fig. 4B d. Effect of different basal salt formulation on *in vitro* sprouting of MTDDT derived single buds *S. anacardium*.

In cashew (*Anacardium occidentale*), a close relative of *S.anacardium*, MS was found to be medium of choice among five different basal media tested (Boggetti 1999; Jha and Das 2004). WPM medium having lower concentration of Potassium, Iodine, Chlorides, Cobalt and Sodium salts may be responsible for higher sprouting frequency. As weaker salt formulation reported to promotes axillary bud differentiation in forest trees (McCown and Sellmer 1987). But the concentration of salts in half strength WPM may not be enough to support sprouting.

Effects of carbon sources, sucrose concentration:

Influence of sucrose and different carbon source on proliferation and growth of shoot cultures in seedling-derived explants was described in Chapter 3. Based on the observation of seedling derived nodal explants, an experiment was designed to optimize the sucrose concentrations for the protocol in which the seedling-derived buds are substituted with MTDDT derived nodal buds. Similar to the seedling derived explants, MTDDT derived nodal buds shows significantly higher number of sprouting frequency in sucrose containing medium (35%). Sprouting frequencies were less in explants cultured in other carbon sources (**Table 4B.5**). Sucrose is the most widely used carbohydrate source in plant tissue culture media and numerous studies have stated that sucrose is the optimum carbon source (Smeekens 2000).

Table 4B.5 Effects of different carbon source on sprouting of MTDDT derived single nodal buds.

Carbon source	**Sucrose**	**Glucose**	**Fructose**	**Maltose**	**ANOVA**
Sprouting (mean± sd) %	35 ± 5.77	5 ± 5.77	5 ± 5.77	17.5 ± 5	S1%

30 explants in 3 repeats.

Out of the four different sucrose concentrations tested maximum sprouting of 36% was noted in explants cultured in 2% of sucrose, other concentrations of sucrose (4, 6 and 8%) induces less sprouting frequency (**Table 4B.6**). Exogenous carbohydrates play an important role in plant tissue culture. They have essential function in plant metabolism since they serve as carbon and energy sources (Thorpe 1974) and acts as osmotic agent (Brown *et al*. 1979). Sensor proteins sense the sugar status of plant cells. Sugar sensing is the interaction between a sugar molecule and a sensor protein in such a way that a signal is generated. The signal then initiates signal

transduction cascades that result in cellular responses such as altered gene expression and enzymatic activities. Sugars as signaling molecules affect the plants at all stages of growth starting from seed germination to seed development. Sugars, like hormones, can act as primary messengers and regulate signals that control the expression of various genes involved in sugar metabolism (Smeekens 2000). In guinean cashew genotypes of *Anacardium occidentale* in spite of the concentration it is the type of carbohydrate that influences the response shoots in culture. Therefore, the carbohydrate requirements have to be optimized for a specific species/genotype and explants (Gemas and Bessa 2006).

Table 4B.6 Effects of different Sucrose source concentration on sprouting of MTDDT derived single nodal buds.

Sucrose concentration	**2%**	**4%**	**6%**	**8%**	**ANOVA**
Sprouting (mean± sd) %	36 ±7.07	25 ±7.07	15 ±7.07	10 ±00	S 5%

30 explants in 3 repeats each.

Effects of gelling agents:

In accordance with the result obtained in seedling derived nodal buds of *S. anacardium*, in which phytagel was found to be growth enhancing. Sprouting was higher (33%) in phytagel than agar (25%) gelled medium in the MTDDT derived nodal explants **(Table 4B.7)**. This gelling agent was found to be ideal for culture initiation in cashew (Thimmappaiah *et al*. 2002).

Table 4B.7 Effects of gelling agents on sprouting of MTDDT derived single nodal buds.

Gelling agent	**Agar**	**Phytagel**	**ANOVA**
Sprouting (mean± sd) %	25 ± 8.16	32.5 ± 12.58	NS

30 explants 3 repeats

Effects of PPM on establishment of aseptic culture:

Addition of PPM in the medium did not improve the percentage of microbe free culture. More over percentage of sprouting in nodal explants of *S. anacardium* was 32% without addition of PPM, which decreases to 20% in PPM incorporated medium **(Table 4B.8)**. Use of PPM for controlling microbial contamination in mature nodal explants of *Pongamia in vitro* (Sujatha and Hazra 2007) and in Bamboo (Ogita *et al*. 2008) has been reported. However, in *S. anacardium* PPM was found to be ineffective in controlling contamination.

Table 4B.8 Effects of PPM on microbe free culture and sprouting of MTDDT derived single nodal buds.

Media	**WPM without PPM**	**WPM with PPM**	**ANOVA**
Microbe free culture (mean± sd) %	58±5.77	56±5.77	NS
Sprouting (mean± sd) %	32.5±5	20±8.16	S5%

30 explants 3 repeats.

***Effects of cytokinins on sprouting of the explants*:**

To induce sprouting and shoot multiplication various PGR were tested. Like earlier cultures percentage of microbe-free explants varied from 50-70%. In WPM medium without GR the sprouting frequency was 32%. Out of the 5 GR tested single or in combination significant response was noted in media containing TDZ **(Table 4B.9).** Cytokinins (BAP, KN, ZEA) and GA_3 are very less effective in inducing sprouting in the axillary meristems of MTDDT derived nodal buds. Optimum frequency response (38%) was attained in nodal buds cultured in TDZ 2.27µM containing medium. The responding buds from TDZ containing medium were shifted to fresh growth regulator free medium along with non-responding ones. However, the non-responding explants from TDZ and other cytokinins containing medium remain as such, these explants turn black and necrotic later during subsequent transfer to plant growth regulator free medium.

Table 4B.9 Effect of growth regulators on microbe free culture and Sprouting frequency of MTDDT derived single nodal buds of *S. anacardium.*

Concentration of GRs in µM	**Microbe free cultures (mean± sd) %**	**Sprouting (mean± sd) %**
WPM	60 ± 00	32±8.36
BAP 2.22	63±7.07	6±5.47
BAP 4.44	50±00	8±8.36
BAP 8.88	57±7.07	12±4.47
BAP13.32	58±7.07	8±10.95
BAP 22.20	50±00	10±7.07
KN 2.32	55±7.07	6±8.94
KN 4.64	55±7.07	2±4.47
KN 9.28	56±14.14	00±00
KN 14.92	55±7.07	0.4±0.89
KN 24.20	57±7.07	4±5.4
ZEA 2.28	52±0	00±00
ZEA 4.56	54±7.07	6±8.94
ZEA 9.12	50±00	10±7.07
ZEA 13. 68	65±7.07	12±16.43
ZEA 22 .80	60±00	6±7.07
BAP 2.22 + KN2.46	60±14.14	5±7.07
BAP 4.44 + KN4.64	50±00	5±7.07
BAP 8.88 + KN9.28	56±14.14	10±00
BAP 4.44 + KN9.28	55±7.07	10±14.14
BAP 4.44 + KN14.92	50±00	10±00
BAP 4.44 + KN24.20	53±00	15±7.07
GA_3 0.29	60±14.14	6±8.94
GA_3 1.44	50±00	14±20.73
GA_3 2.88	60±00	16±18.16
GA_3 5.76	55±21.21	16±15.16
GA_3 8.64	65±7.07	10±17.32
TDZ 0.45	60±14.14	36±11.40
TDZ 2.27	70±00	38±20.73
TDZ 4.54	60±00	35±13.41
TDZ 9.08	50±00	35±19.23
TDZ13.62	55±00	34±15.16
ANOVA	NS	S1%

40 explants in 4 repeats.

Conspicuous swelling of the axillary meristem was observed in all the concentrations of TDZ tested **(Fig. 4Be)**, similar finding was reported in *Pongamia pinnata* (Sujatha and Hazra 2007), in Soyabean (Shan *et al*. 2005) and in TDZ treated axenic shoot derived nodal explants of *S. anacardium* (unpublished data). The swelling was more pronounced in higher concentration of TDZ tested. Only few bud differentiated in presence of TDZ at lower concentration (0.45µM). After 4wks of culture, the TDZ treated explants were shifted to fresh GR free WPM medium with antibiotics for 4 passages. Differentiation of the nodal buds was obvious when transfer to GR free medium.

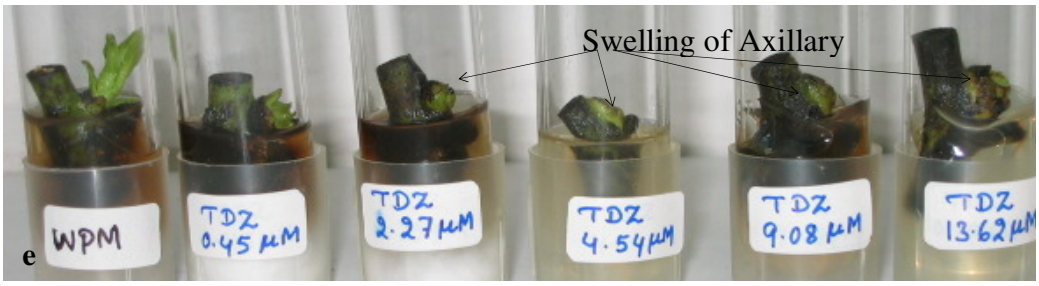


Fig. 4B.e. Effects of different concentration of TDZ on mature nodal explants after 4 wks. The meristematic region in all the concentration of TDZ showing conspicuous swelling.

After 4wk of cultured in WPM medium TDZ treated explants at lower concentration of 0.45µM shows elongation and bud differentiation from the swollen axillary meristem **(Fig. 4Bf).**whereas explants pre-cultured in TDZ 2.27µM containing medium shows bud differentiation only **(Fig. 4Bg).**

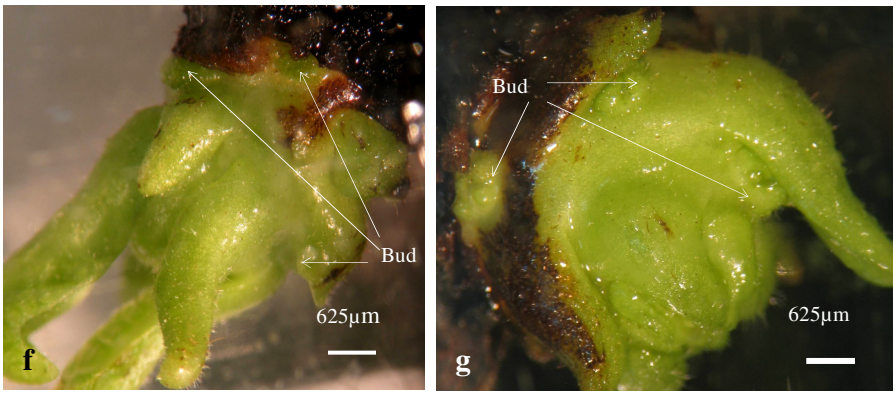


Fig. 4B f. Opening of leaf primordial with few differentiated buds in nodal explants cultured in 0.45µM of TDZ**; g.** Compact meristematic region with number of differentiating buds in nodal explants after 4wks in WPM medium, these explants were pre-cultured for 4weeks in 2.27µM of TDZ.

The explants pre treated in TDZ 4.45µM did not show any differentiation at all. In these explants the axillary meristem was cover by swollen compact leaf primordia **(Fig. 4B h)**.

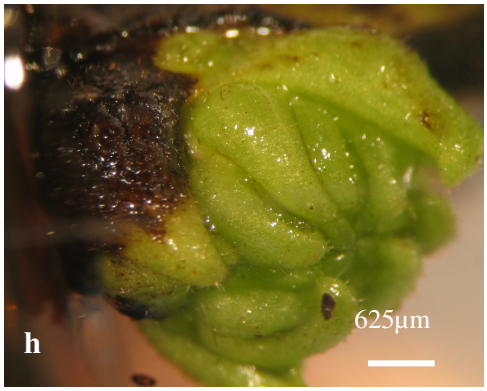


Fig. 4B h. Compact swollen overlapping leaf primordial without any differentiation in the axillary bud of nodal explants after 4wk in WPM medium, pre-cultured for 4weeks in 4.54µM of TDZ.

In concurrence with the earlier reports (Mehta *et al*. 2004; Sujatha and Hazra 2007; Ahmed and Anis 2007), differentiation of shoot buds in *S.anacardium* was triggered on withdrawal of the GR and more number of buds developed and sprouted with every passage in GR free medium. In *S.anacardium* the TDZ treated explants on repeated transfers in medium without GR differentiated to produce more shoot primordial **(Fig. 4B i)**. All the explants treated with TDZ were associated with profuse basal callusing and large amount of phenolics leaching into the medium **(Fig. 4B i).** Basal callusing in cultures of mature explants has been reported in silver maples at higher concentration of TDZ (Huettman 1988; Preece *et al*.1991) and in cocoa (Traore *et al*. 2003).

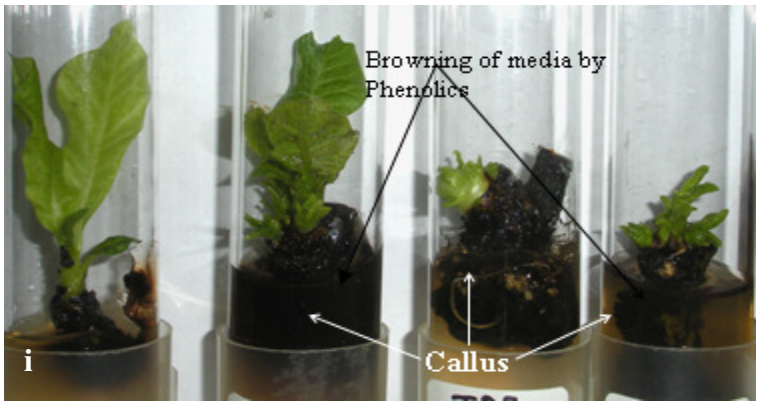


Fig. 4B i. Shoot bud differentiation in explants cultured in WPM for 8wks, initially cultured in TDZ containing medium for 4wks.

Explants treated with TDZ 4.54µM produces higher number of multiples (11.3±0.91) after five passages in WPM GR free medium **(Table 4B.10)**. Number of multiples produced in TDZ 0.45µM and 2.27µM was 6.2±0.6 and 9.6±1.6 respectively. When shoot elongation was compared in the TDZ treated explants, in lower concentration the elongation was more compared to higher concentrations. Thidiazuron induced inhibition of shoot elongation has been reported in several woody species (Huetteman and Preece 1993; Lu 1993). In higher concentration of TDZ decreased in shoot length has been observed in *Oroxylum indicum* (Dalal and Rai 2004) and in *Holarrhena antidysenterica* (Mallikarjuna and Rajendrudu 2007).

Table 4B.10 Effect of TDZ on multiplication of MTDDT derived single nodal buds of *Semecarpus anacardium* after culturing in GR free medium.

Conc. of TDZ in µM	No of multiple buds(mean± sd)	No of multiple buds, In WPM GR free medium (mean ±sd)				
	4 wk In TDZ	4 wk	8 wk	12 wk	16 wk	20 wk
0	1 ±0	1±0	1±0	1±0	1±0	1±0
0.45	1.33±0.6	2.6±0.6	4±0.4	5.3±0.41	5.5±0.5	6.2±0.6
2.27	1.33±0.6	4.8±1.2	6.33±1.2	8±0.87	8.9±1.4	9.6±1.6
4.54	1±00	2.0±0.4	3.33±0.4	4.9±0.57	8.3±0.6	11.3±1
9.08	1±00	1.5±0.2	1.5±0.3	1.5±0.23	1.5±0.23	1.5±0.3
13.62	1±00	1±0	1±0	1±0	1±0	1±0
ANOVA	NS	S1%	S1%	S1%	S1%	S1%

3 repeat 8 explants in each repeat.

Elongation of *S.anacardium* nodal bud was 2.07±0.20 cm in 2.27µM of TDZ after five passages of four weeks in WPM basal medium. In higher concentration of TDZ (4.54µM) tested, the elongation was1.46±0.11 **(Table 4B.11)**.

Table 4B.11 Effect of TDZ on shoot elongation of MTDDT derived single nodal buds of *Semecarpus anacardium* after culturing in GR free medium.

Conc. of TDZ in µM	Mean shoot length	Mean shoot length in WPM (mean ±sd) cm				
	4 wk in TDZ (mean± sd)	4 wk	8 wk	12 wk	16 wk	20 wk
0	0.46 ±0.11	0.78±0.07	1.06±0.11	1.12±0.12	1.17±0.14	1.21±0.12
0.45	0.29±0.05	0.39±0.09	0.57±0.09	0.85±0.07	1.30±0.09	1.45±0.16
2.27	0.23±0.03	0.34±0.07	0.52±0.08	0.77±0.05	1.24±0.09	1.93±0.23
4.54	0.23±0.04	0.34±0.06	0.49±0.07	0.80±0.07	1.09±0.15	1.46±0.11
9.08	0.25±0.03	0.29±0.04	0.33±0.09	0.38±0.04	0.38±0.04	0.38±0.04
13.62	0.22±0.03	0.29±0.06	0.30±0.09	0.30±0.09	0.30±0.04	0.30±0.04
ANOVA	S1%	S1%	S1%	S1%	S1%	S1%

3 repeats 8 explants in each repeats.

The swollen axillary meristem formed in increasing concentrations of TDZ (9.08µM and 13.62µM) fails to differentiate and remain as such or turn brown and necrotic. Necrosis of tissue in higher concentration of TDZ has been reported in Flex (Bretagne *et al*. 1994). The cluster of bud developed in the axillary meristematic region of mature nodal buds of *S.anacardium* **(Fig. 4Bj)** elongates to form shoots. A strong dominance phenomenon of the elongated shoots was noted in all explants pretreated with TDZ, only one bud differentiated into shoot and rest of the buds remain stunted **(Fig. 4Bk)**. After removal of the elongated shoot, other buds resume elongation. Similar apical dominance in tissue culture of tree species has been observed in cocoa (Traore *et al*. 2003) where removal of apical shoot promotes elongation of basal axillary buds.

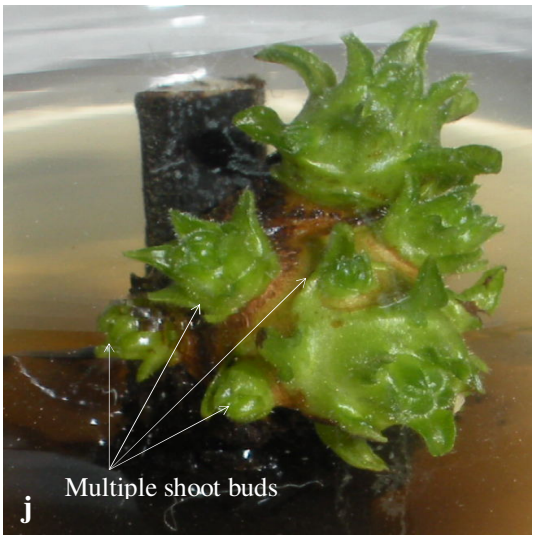


Fig.4B j. Cluster of shoot buds in the meristematic region after 16 weeks of culture, initially cultured in 4.54µM of TDZ. Brownness of the media was due to phenolics.

Fig. 4B k. Elongated shoot from the multiple buds on culturing in WPM with charcoal incorporated medium. Apical dominance of the elongated bud can be observed as rest of the induced buds remains stunted.

Rapid differentiation and elongation of the axillary buds occurs when the cluster of shoot primordia separated from primary explants and cultures in WPM medium with charcoal **(Fig. 4Bl)**. The stump left after removal of cluster of buds sporadically develops additional caulogenic shoots buds upon re-culturing in WPM medium with charcoal (0.2%) **(Fig. 4Bm)**. Caulogenic bud formation from the stumps of mature nodal buds of *Pongamia pinnata* on reculturing has been reported (Sujatha and Hazra 2007). In most of the buds of *S.anacardium* it was observed that, the cluster of buds attached to the primary explants adds contamination into the culture during further sub-culturing **(Fig. 4Bm)**. More number of multiple buds differentiated into shoots on further culturing in WPM medium without growth regulators after separating from the primary explants **(Fig. 4Bn)**.

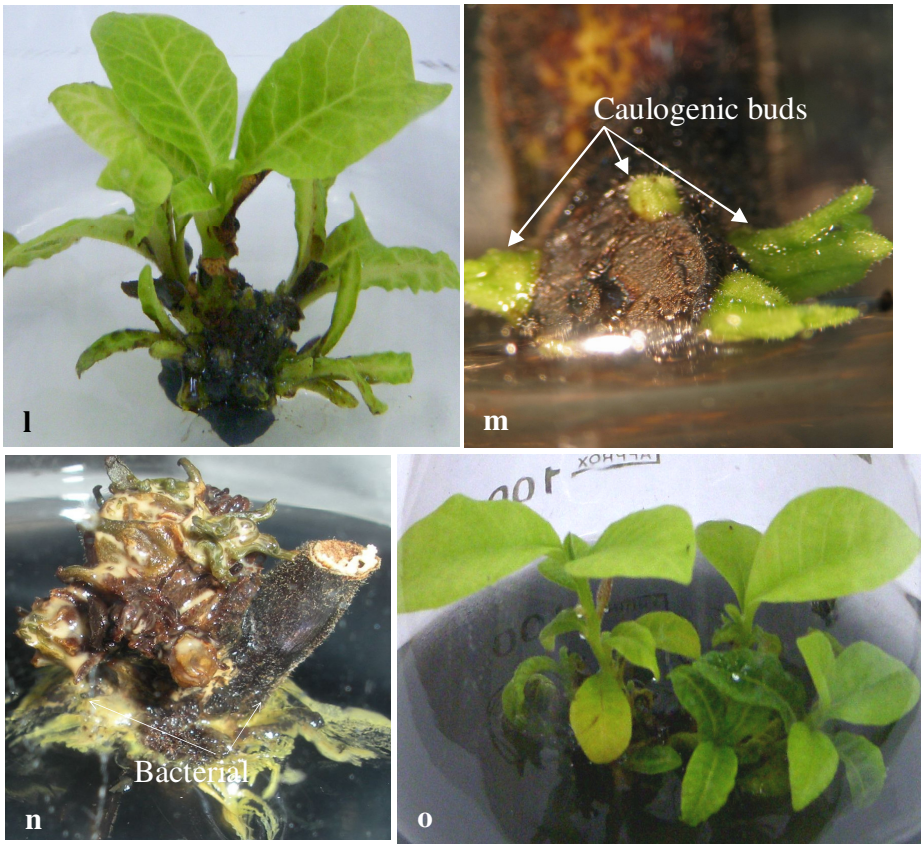


Fig. 4B l. Cluster of shoot buds showing elongation when separated from mother explants and cultured in GR free medium**; m.** Occasional appearance of caulogenic buds developed from the cut end of mother explants after removal of cluster of buds; **n**. On culturing the cluster of shoot bud with the mother explants often causes bacterial contamination**; o.** Elongated shoots from cluster of buds induced in TDZ maintained in WPM medium containing charcoal.

After culturing for 20 wks in WPM, medium number of shoots produced from each explants were isolated for three passages of 4 wks each **(Table 4B.12)**. Number of shoot produced after 28 wks was (7.43±0.79) in explants pre-cultured for 4 wks in 2.27µM of TDZ. Shoot produced from 0.45µM and 4.54µM of TDZ was 4.49 and 6.09 respectively. This type of repeated sub culturing and isolation shoot produces more plantlet for mass-propagation of this tree species. Adventitious rooting of woody plants is usually induced by auxin. Lower strength medium has been found suitable for the rooting in tree species such as *Cercis canadensis* (Mackay *et al*. 1995) and in *Robinia ambigua* (Guo *et al*. 2006). Low concentration of inorganic salts elongated root lengths to a considerable extent and enhances the rate of transplantation (Dalal and Rai 2004). The beneficial effects of low concentrations of media with a combination of auxin supplementation have been recorded in inducing roots in shoot explants of *Bauhinia valii* (Bhatt and Dhar 2000). Among the auxins IBA is found to be the most effective in root induction in tree species as reported in Cashew (Mneney *et al*. 2001; Ananthakrishnan *et al*. 2002), Pistachio (Tilkat *et al*. 2005) in *Ulmus minora* (Conde *et al*. 2008) and in *Balanites aegyptica* L. (Del.) (Siddique and Anis 2009).

Table 4B.12 Mean number of shoots produced per explants pre-cultured for 4wks in TDZ containing medium.

Conc. of TDZ in µM	20 wk (mean± sd)	24 wk (mean± sd)	28 wk (mean± sd)
0	0.79±0.11	0.52±0.13	0.19±0.07
0.45	2.99±0.81	5.07±1.31	4.49±0.91
2.27	4.57±0.41	7.08±0.47	7.43±0.79
4.54	2.49±0.10	3.78±0.30	6.05±0.07
9.08	00±00	00±00	00±00
13.62	00±00	00±00	00±00
ANOVA	S1%	S1%	S1%

3 repeats 8 explants in each repeats.

Rooting and hardening:

Rooting was observed in 15% of the shoots of *S.anacardium* when cultured in half strength liquid WPM medium with filter paper. All the rooted when cultured in liquid medium containing 7.36µM of IBA **(Table 4B.13, Fig. 4Bp)**. Rooting frequency was (90%) in shoots of *S.anacardium* cultured in half strength liquid medium with 2.46µM of IBA without filter paper support **(Fig. 4Bq)** where as it was 55% in explants cultured with filter paper. However in medium without filter paper support the roots were associated with callus.

Table 4B.13 Rooting of *S.anacardium* shoots after 4 weeks in half strength liquid medium with different concentration of IBA.

Conc. of IBA in µM	Rooting (%)	Mean root number	Mean root length
0	15	0.2 ±0.08	0.3 ±0.21
IBA2.46 without filter paper	90	2.25±0.48	2.38±0.3
2.46	55	1.05 ±0.07	2.15 ±0.07
4.92	85	2.4 ±0.05	2.35 ±0.35
7.36	100	4.3 ±0.56	2.51 ±0.19
ANOVA	S 1%	S1%	S1%

30explants in 3 repeats

Fig. 4B p. Rooting of micro shoot in half strength WPM medium containing IBA with the help of filter paper support**; q.** Rooting of shoot directly in half strength liquid medium with 2.46µM of IBA without filter paper support.

This method of direct rooting is simple and beneficial in saving time, resources and will be cost effective for root induction *in vitro* in large number of shoots. The rooted plants were hardened in soil-sand mixture in 1:1 ratio before transferring to green house **(Fig. 4Br)**. All the plants hardened and survived. These hardened plants well acclimatized and growing in green house **(Fig. 4Bs)**.

Fig. 4B r. Hardened plantlets in cups containing sand soil mixture 1:1**; s.** Growing acclimatized plants in green house.

Clonal fidelity:

In this study 12 ISSR primers were used to test the clonal fidelity of *in vitro* raised plants of *S.anacardium* for analysis based on prior amplification data (Chapter 4A). The number of bands per primer ranged from 4 (**UBC841**) to 10 (**UBC821**) with an average of 6.92 bands per primer. The band sizes ranged from 250 bp to 2060 bp **(Table 4B.14)**.

Table 4B.14 Selected primer with primer sequences number of distinct bands scored annealing temperature and similarity index.

UBC Primers	Primer sequences	Number of bands	Annealing temperature	Similarity %
811	GAGAGAGAGAGAGAGAC	9	44ºC	100
812	GAGAGAGAGAGAGAGAA	7	50ºC	100
816	CACACACACACACACAT	8	56ºC	100
821	GTGTGTGTGTGTGTGTT	10	56ºC	100
825	ACACACACACACACACT	6	52ºC	100
827	ACACACACACACACACG	5	47ºC	100
834	AGAGAGAGAGAGAGAGYT	7	53ºC	100
835	AGAGAGAGAGAGAGAGYC	8	56ºC	100
841	GAGAGAGAGAGAGAGAAYC	4	53ºC	100
842	GAGAGAGAGAGAGAGAAYG	7	53ºC	100
861	ACCACCACCACCACCACC	7	56ºC	100
862	AGCAGCAGCAGCAGCAGC	5	56ºC	100

Banding pattern of different ISSR marker, UBC825 **(Fig. 4Bt),** UBC811 (**Fig. 4Bu),** UBC 835 (**Fig. 4Bv),** UBC812 **(Fig. 4Bw)** and UBC834 **(Fig. 4Bx)** show similar amplification with the mother plant.

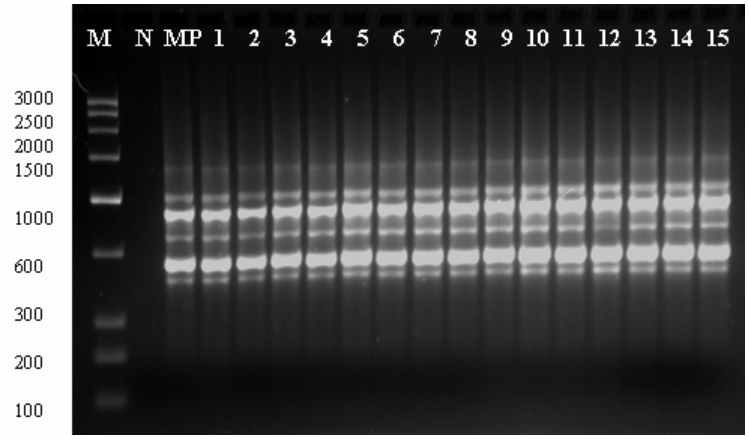


Fig. 4B t. PCR amplification products obtained using the primer UBC-825.M-Low range DNA ruler (3kb). N- Negative control of PCR amplification MP-Mother Plant. 1-15 are *in vitro* raised clones.

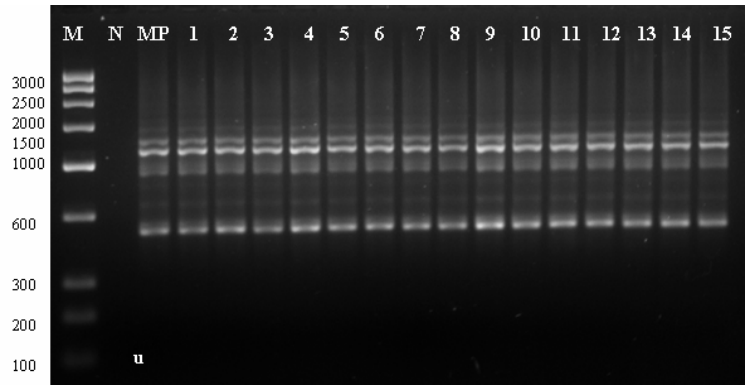


Fig. 4B u. PCR amplification products obtained using the primer UBC-811.M-Low range DNA ruler (3kb). N- Negative control of PCR amplification MP-Mother Plant. 1-15 are *in vitro* raised clones

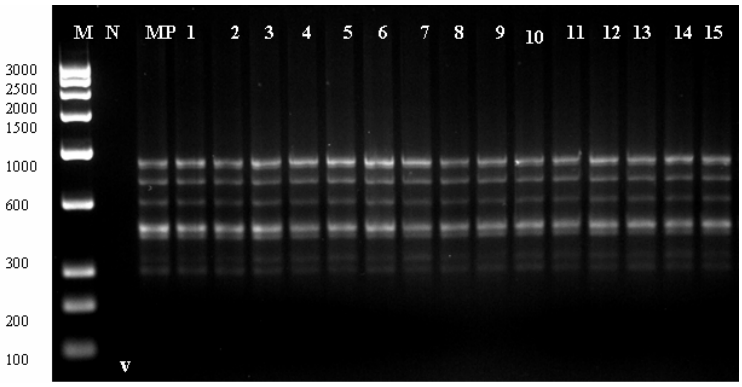


Fig. 4B v. PCR amplification products obtained using the primer UBC-834.M-Low range DNA ruler (3kb). N- Negative control of PCR amplification MP-Mother Plant. 1-15 are *in vitro* raised clones.

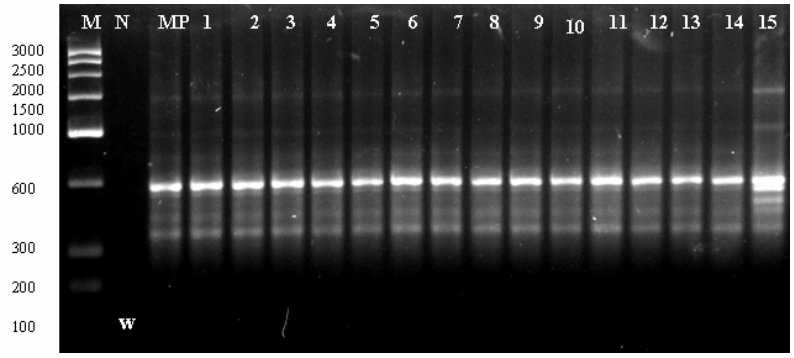


Fig. 4B w. PCR amplification products obtained using the primer UBC-812.M-Low range DNA ruler (3kb).N- Negative control of PCR amplification. MP- Mother Plant. 1-15 are *in vitro* raised clones.

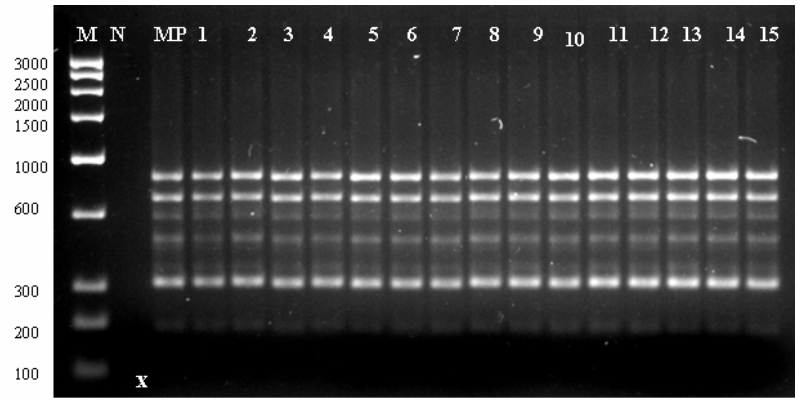


Fig. 4B x. PCR amplification products obtained using the primer UBC-835.M-Low range DNA ruler (3kb). N- Negative control of PCR amplification MP-Mother Plant. 1-15 are *in vitro* raised clones.

All other primers tested revels uniform banding pattern for each primers. There was no polymorphism among the micropropagated plants. Genetic similarity indices among all the individuals are 1.00. The pair wise matrix of genetic similarities as shown in **(Table 4B.15)** depicts indices of similarities among *in vitro* raised plants with the mother plant. Clonal fidelity of *in vitro* raised plants of Almond (Martins *et al*. 2004), *Ocheinauclea missionis* (Chandrika and Rai 2009), *Vitis* Spp (Alizadeh and Singh 2009) by ISSR markers has been reported. In these plants the in vitro raised plants are genetically uniform with the mother plants. This study in *S.anacardium* confirms the fact that axillary multiplication is the safest mode of micropropagation to produce true to type progeny.

Table 4B.15 UPGMA Jaccard's Coefficient (C3-Mother plant, C4-C18 plants from *in vitro* raised cloned) Similarity matrix

	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
C3	1															
C4	1	1														
C5	1	1	1													
C6	1	1	1	1												
C7	1	1	1	1	1											
C8	1	1	1	1	1	1										
C9	1	1	1	1	1	1	1									
C10	1	1	1	1	1	1	1	1								
C11	1	1	1	1	1	1	1	1	1							
C12	1	1	1	1	1	1	1	1	1	1						
C13	1	1	1	1	1	1	1	1	1	1	1					
C14	1	1	1	1	1	1	1	1	1	1	1	1				
C15	1	1	1	1	1	1	1	1	1	1	1	1	1			
C16	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
C17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
C18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18

4B.4 CONCLUSION

Method for clonal propagation of *Semecarpus anacardium* using single nodes from surface sterilized MTDDT derived axillary meristem is described. A surface sterilization method consisting of bavistin, liquid soap and antibiotics were effective in controlling contamination up to 60% in single nodes from surface sterilized MTDDT. Young sprouts collected from the mature trees before flowerings are the best explants for response in *vitro* culture. Percentage of sprouting and sterility was more in surface sterilized MTDDT derived single nodal explants than surface sterilized single nodal explants of the same plant. Woody plant medium was superior to other formulations for sprouting. Plant preservative mixture was in effective in controlling the contaminants. Thidiazuron (TDZ) in all the tested concentration stimulates the meristem activity in axillary nodes. At the tested concentrations of TDZ, sprouting of the axillary buds is suppressed and meristematic region become swollen. Shoot primordia develop from the swollen meristem on withdrawal of TDZ and differentiate into shoots after repeated culture on medium devoid of GR. For the buds pre-cultured on higher concentrations of TDZ (9.08µM, 13.62µM) the swollen meristematic region fails to differentiate and turns necrotic on further culturing. Differentiation and elongation is slower in the buds induced on higher TDZ concentration. Concentration of 0.45µM TDZ is optimum for elongation and shoots differentiation of *Semecarpus* shoot cultures. Thidiazuron at 4.45µM was optimum for multiple inductions. Reculturing of original explants occasionally produces caulogenic shoot buds but in most of the case these explants were infected contaminated by bacterial contamination. Sprouted axillary shoots being harvested in each cycle. These shoots were used as cuttings for propagation. The described protocol will be useful as a part of isolation stage in establishing the standard shoot culture protocol but also a system to study some of the processes of TDZ induced morphogenetic activity. Repeated proliferation of buds from the same meristematic bud may find application in rescue of endangered germplasm. Genetically uniform among the clone proved by ISSR markers

from the *in vitro* raised plants validate the micropropagation protocol. This is the first report of any *in vitro* regeneration protocol for mature tree derived nodal explants for *Semecarpus anacardium*. This *in vitro* technique also has the advantage of establishing long term proliferating shoot cultures with a high multiplication. Since direct establishment of *in vitro* regeneration protocol from mature tree derived nodal explants from members of *Anacardiaceae* is lacking, the above protocol may be extended to other members also.

CHAPTER 5

DE NOVO MORPHOGENESIS IN COTYLEDON EXPLANTS OF *SEMECARPUS ANACARDIUM* L.

5.1 INTRODUCTION

Micropropagation techniques of plants were developed in order to multiply desired genotypes as clones, and through time these techniques have been further developed to provide plant materials suitable for genetic transformation (Merkle and Dean 2000). The more current systems used for the regeneration of tree species are via somatic organogenesis and somatic embryogenesis.

De novo morphogenesis is 'the *de novo* production of plant organs (buds, shoots, roots) or somatic 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 multiplication methods is a potential for higher frequency plantlet production in a shorter period of time. Direct or indirect *de novo* morphogenesis is more desirable for genetic transformation studies. Advantage of these systems over the axillary shoot proliferation methods lays in the amenability to different gene delivery techniques. Transformation by *Agrobacterium*-mediated transformation in cells of pre-existing meristem is often unsuccessful or inefficient, perhaps due to the low number of attachment sites for *Agrobacterium* in the cell walls (Mattysse and Gurlitz 1982).

Unlike methods based on axillary bud multiplication, *de novo* morphogenic methods especially those requiring intermediate callus, may be associated with the production of significant amounts of somaclonal variations in the regenerated plantlets, which are often heritable. It is recognized (Jain 2001) that somaclonal variations could be useful for improvement of plants and might be a valuable source for introduction of new traits. The *de novo* morphogenic methods have promising application in studies on isolation of somaclonal variants. Frequency of variation in cells could be increased, by treating the explants with mutagenic agents. 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 tree species **(Table 1.3)**.

A. De novo organogenesis

The distinction between *de novo* organogenesis and shoot proliferation from pre-existing meristems is that *de novo* organogenesis requires a re-determination of existing genetic programs expressed within a cell. Like micropropagation from pre-existing 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; Joy and Thorpe 1999).

There are reports of shoot *de novo* organogenesis from the explants of mature trees like cambial explants in *Dalbergia 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 *Albizzia.lebbeck, Cassia fistula,* and *C. siamea* (Gharyal and Maheswari 1990), mature leaves of *Paulownia tomentosa* (Corredoira *et al.* 2008), mature leaves of *Pistochio vera* (Tilkat and Onay 2009). Organogenesis systems from mature genotypes of sweetgum (Merkle and Battle 2000; Merkle *et al.* 2003), Holm oak (Mauri and Manzanera 2004), English oak (Toribio *et al.* 2004), *Gentiana* spp (Fiuk and Rybczyński 2008), *Eucalyptus globulus* (Pinto *et al.* 2009) and in other tree species has been established.

Expression of organogenic potential is greatly influenced by the source of explant, age, physiological status, size, type, orientation, genotype of explants and the culture conditions (Prakash and Gurumurthi 2009). Most frequently used explants that have led to successful regeneration have been the seed or seedling parts including cotyledons, hypocotyl, cotyledonary nodes, epicotyl and embryonic axis. Cotyledons were used as explant in many species like *Sesbania bispinosa* (Kapoor and Gupta 1986) *Albizzia falcataria* (Sinha and Mallick 1993), *Sesbania grandiflora* (Detrez *et al.* 1994), *Dalbergia sissoo* (Chand *et al.* 2002), *Lens culinaris* (Khawar *et al.* 2004), *Macadamia tetraphylla* (Mulwa and Bhalla, 2006) *Myrciaria aureana* (Motoike *et al.* 2007) *Pongamia pinnata* (Sujatha *et al.* 2008) etc.

Morphogenic responses within the cotyledon explant vary with different regions of cotyledon. Proximal portion of the cotyledon (portion close to node) is generally more responsive than the remaining parts in many species. Ainsley *et al.* (2001), Chand *et al.* (2002), Sujatha *et al.* (2008) demonstrated high regeneration capacity of the proximal segment of cotyledon in Prunus, Dalbergia and Pongamia, respectively. Response in explant varies with its orientation on medium. In *Vigna unguiculata* (Kulothungan 1997) and in cashew nut (Ananthakrishnan *et al.* 2002) organogenic differentiation in proximal end of cotyledon was noted when the distal ends are in contact with medium. It is suggested that proximal region of the cotyledons might be the source of high regenerative cells (Murthy *et al.* 1995). 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) and *Pongamia pinnata* (Sujatha *et al.* 2008) etc.

Embryo axis explants are known to have high regenerative potential and has been used for morphogenetic studies in several species (Giri *et al.* 2004). Non-meristematic regions of the

zygotic embryo axis were used for the induction of *de novo* organogenesis in tamarind (Mehta *et al.* 2000) and *Fraxinus pennsylvanica* (Du and Pijut 2008).

The GRs most commonly used for induction of shoot primordia are cytokinins. Among the GRs, TDZ has proved to have potential in elucidating *de novo* organogenic response in various woody species (Huetteman and Preece 1993; Lu 1993; Murthy *et al*. 1998; Chenong and Pooler 2003; Mehta *et al*. 2004). Effectiveness of TDZ in inducing organogenic response is described (Chapter 3 and 4). This GR has been reported to induce *de novo* organogenic response in tree species like *Acacia manginum* (Xie and Yang 2001), Chestnut (Sanchez *et al*. 2001), *Tamarindus indica* (Mehta *et al*. 2004 and 2005), *Embelia ribes* (Raghu *et al.* 2006), *Pongamia pinnata* (Sujatha *et al.* 2008), *Fraxinus pennsylvanica* (Du and Pijut 2008), *Paulownia tomentosa* (Corredoira *et al.* 2008) etc. Reports on successful regeneration via *de novo* organogenesis (both direct and indirect) in tree species are listed (**Table 1.3**).

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. Somatic embryo resembles zygotic embryo. They are bipolar and bear typical embryonic organs. However, they originate via a different pathway. Rather than developing from a zygote after fusion of the gametes, somatic embryos can theoretically be derived from cells within any type of tissue. They not only occur *in vitro* but can also be widely found in nature. 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. Since then number of plants from different taxonomic groups has been induced to form somatic embryogenesis (Arnold *et al*. 2002).

Unlike organogenesis, which may either be *de novo* or from existing meristem, SE is always *de novo* in origin. It is the unique expression of totipotency of plant cells. The choice of donor tissue is critical, and is usually determined empirically. For many patterns of SE embryonic or highly juvenile tissues have to be used as explants. Juvenile tissues are sometimes the only practical choice when culturing woody plants, in which the transition from juvenile to mature phases is associated with lignification. Haploid embryogenesis of many plant species has been reported (Litz and Gray 1995; Germana` 2006). Juvenile tissues like cotyledon, embryo axis and immature seeds appear to be more suitable for induction of somatic embryos (Tulecke 1987; Giri *et al*. 2004). Cotyledons from semi mature green pods produced somatic embryos in woody trees like *Sesbania sesban* (Shanana and Gupta 2002), *Dalbergia sissoo* (Singh and Chand 2003), *Myrciaria aureana* (Motoike 2007), *Elaeagnus angustifolia* L. (Karami and Piri 2009). The developing zygotic

embryo derived explants generally showed a high potential for SE (Williams and Maheswaran 1986). In tree species, most reports of SE are from zygotic embryo particularly in *D. latifolia* (Muralidhar and Lakshmi Sita 1996), *Acacia mangium* (Xie and Hong 2001), *Hardwickia binata* (Chand and Singh 2001), *Acacia arabica* (Nanda and Rout 2003), *Terminalia chebuta*, (Anjaneyulu *at al*. 2004), *Melia azedarach* L. (Vila *et al*. 2007).

Developmental stage of explant is critical for expression of embryogenesis in woody species (Arya *et al*. 2000; Mauri and Manzanera 2003). Zygotic embryos of *Cercis Canadensis,* 96 and 110 days post anthesis produced somatic embryos at optimum frequency (Trigiano *et al.* 1988). In trees it is difficult to ascertain the age of immature zygotic embryos of many woody species since often the time of pollination is not determined. Instead, size of the immature embryo is referred to decide the potential criterion of explants. In tamarind, zygotic embryo of 1-10 mm size and approximate age of 10-12 weeks were successfully used as explants for embryogenesis (Mehta and Hazra 2008). The process of SE 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 of auxins or in presence of a lowered concentration of auxin. Embryogenesis was mostly induced by use of auxins like 2,4-D, NAA, 2,4,5-T, etc. Another herbicide with auxin-like properties is picloram, which was proved to be effective for maintenance of embryogenic calli and plant regeneration of peach palm, *Bactris gasipaes* Kunth., (Steinmacher *et al.* 2007), *Fragaria ananassa* L. (Kordestin and Karami 2008). Dicamba, also a herbicide with auxin like property is shown to induced somatic embryos in *Gentiana kurroo* (Fiuk and Rybczyn´ski 2007) and in *Tamarindus indicus* (Mehta and Hazra 2008). Although a large number of crop plants have been regenerated via somatic embryogenesis, woody horticultural and forest species continues to present a major challenge (Litz and Gary 1995).

Morphogenesis in Semecarpus anacardium:

Until recently, the literature on this species was restricted to chemical and medicinal aspects only. *In vitro* germination of the plant was reported recently (Chapter 3, Section A, Panda and Hazra 2009). Plant regeneration through organogenesis and somatic embryogenesis has been reported in some members of Anacardiaceae including *Mangifera indica* (Litz *et al*. 1989; Ara *et al*. 1999; Rivera- Dominguez *et al*. 2004), *Schinopsis balansae* (Sansberro *et al*. 2003), *Anacardium occidentalis* (Mneney *et al*. 1998; Boggetti *et al*. 1999; Cardoza and D'Souza 2002; Gogate and Nadgauda 2003; Martin 2003; Aliyu *et al*. 2005), *Pistachio vera* (Onay 2000; Onay 2003; Tikat *et al*. 2009; Benmahioul 2009) etc. However, till date, there is no report on *de novo* morphogenesis in *Semecarpus anacardium*. Experiments designed and carried out to establish a regeneration system via *de novo* morphogenesis are described in this chapter.

5.2 MATERIALS AND METHODS

Green semi-mature nuts (prior to ripening) of *Semecarpus anacardium* L. were collected in early December from trees growing wild around Pune, India. The pseudo-fruits were removed and the nuts were washed several times with distilled water to remove the dust and other adhering contaminants. These were treated for one hour with 2% Bavistin solution and few drops of liquid soap (Exalin, Merck) on a shaker at 100rpm. Thereafter, the nuts were washed aseptically with sterile distilled water 4-5 times to remove residual Bavistin sticking to the nuts. This was followed by treating the nuts with 6% aqueous solution of Savlon for 10min. Adhering Savlon was removed by washing the nuts aseptically three times with sterile distilled water. Finally, the surface of the nuts was decontaminated with sodium hypochlorite (Merck) for 30min. These were washed aseptically with sterile water for 5-6 times to remove the traces of sodium hypochlorite. With the help of secator, the seeds were broken and the cotyledon pieces were isolated carefully. This process was done on piece of sterile filter paper. The nuts are filled with large amount of fluid, which spills during cutting and often cause allergy on the skin. To avoid allergy, sterile rubber gloves were used while cutting the nuts. Inspite of precautions taken during separation of the cotyledons, it breaks into pieces of different shapes and sizes. The embryo axis was removed and the cotyledon pieces were cultured in 90mm Petridishes on WPM medium with varying concentrations of growth regulators. In each Petridish 5-6 cotyledon pieces of different sizes ranging from 2-4mm were cultured randomly. The orientation of the explants could not be determined as the cotyledons were broken into pieces during isolation. The experiment was repeated thrice with 30 replicates (explants) per repeat. The different GRs tested for induction of morphogenic response include BAP (2.22, 4.44, 8.88, 13.32, 22.20, 26.64µM), KN (2.32, 4.64, 9.28, 13.92, 23.20µM), TDZ (0.45, 2.27, 4.54, 9.08, 13.62 and 22.70µM), GA_3 (0.29, 1.44, 2.89, 5.78, 8.67), 2,4-D (2.26, 4.52, 13.6, 22.6 and 45.2µM), Picloram (2.07, 4.14, 12.4, 20.7 and 41.4µM), NAA (2.69, 5.38, 10.76µM), IAA (2.85, 5.70, 11.40µM) and IBA (2.46, 4.92, 9.84µM). After 4wks in the GR containing medium the cotyledon pieces were transferred to GR free medium containing charcoal (0.2%). The response in all the explants in the form of swelling, morphogenic structures, rooting and callusing was noted before reculturing the explants in fresh GR free medium. The frequency of response was determined.

Morphogenic response in the form of shoot organogenesis and embryogenic mass formation was obvious in explants cultured in TDZ containing medium. Hence these explants were carried for further culturing in GR free medium containing charcoal 0.2%. Number of morphogenic structures appeared on the surface of the cotyledon pieces was scored under microscope. The number of morphogenic structures are approximate figures as the size of the explants were not uniform and the structures appeared on the surface were often too dense and some were indistinguishable due to close origin and uniformity in color (green). Similar morphogenic

response was noted in all the concentrations of TDZ tested. Observations were noted after fourth week in GR containing medium and subsequently after four wks of culturing in GR free medium. After 8wks in GR free medium the cotyledonary explants were transferred to 250ml Erlenmeyer flasks for differentiation and growth. Explants with morphogenic response were repeatedly cultured in GR free WPM medium with charcoal for 4-5 passages of 4wks each. Shoots of 2-3cm length were excised and after removal of these elongated shoots the cotyledon explants were recultured in GR free WPM medium for elongation of more shoots. The excised shoots were cultured for 4wks, in half strength WPM liquid medium containing 2.46µM of IBA in tubes with filter paper support for rooting.

Some of the cotyledonary explants cultured in TDZ containing medium the morphogenic response was in the form of embryogenic mass formation. The explants with embryogenic mass formation were noted and number of embryogenic mass per explants was scored under microscope. These explants were maintained for 6-8 passages in GR free medium containing 0.2% of charcoal. In addition to organogenic and embryogenic response callusing was also observed in some of the explants on culturing in GR free medium containing 0.2% charcoal for 6-8 passages. These explants were pre-cultured in TDZ containing medium for 4wks. These calluses are compact and morphogenic in nature. The callus on further culturing gave rise to somatic embryo like structure. This callusing response and subsequent embryo like structure formation could not be scored due to less number of explants.

All media used in the above experiments contained 2% sucrose and were gelled using phytagel 0.2%. The pH of the media was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2} for 20min at 121ºC. Cultures were incubated in cool white light at irradiance of 50µ$molm^{-2}s^{-1}$ with 16hr photoperiod at 25±2ºC. Rooted plantlets were hardened on transferring to autoclaved sand soil mixture 1:1 in cups and covered with polyethylene bags to maintain high humidity for 4wks. Hardened plants were transferred to green house for further acclimatization. All data were subjected to statistical analysis (ANOVA).

Histological Techniques:

Sections were prepared for histological studies following the methods described (Ruzin 1999). The cotyledon explants with morphogenic buds 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. These were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (mp 58-60°C). Serial sections of 10µM were cut from the paraffin blocks using a rotary microtome (Reichert-Jung 2050, Germany). Sections were double stained with haematoxylin-eosin and mounted with DPX (Loba Chemie, Mumbai, India). The stained sections were studied under a research/compound microscope (Leica).

5.3 RESULTS AND DISCUSSION

Inspite of repeated trials with various surface sterilization procedures, there was high contamination frequency in the cotyledon explants isolated from mature seeds. These explants released abundant amount of phenolics causing darkening of the medium within a few hours. These hindrances were partially controlled by substituting the cotyledon of mature nuts with nuts prior to complete ripening and maturation. These nuts although not mature were hard and carried the dark brown fluid which caused allergy in form of blisters on the skin. To avoid the contact of the fluid the dissection of the nuts had to be done with precaution. Thus the breaking of the nuts was in irregular pieces and the surfaces of the explants or its origin (proximal, middle or distal) could not be distinguished during culture. The cotyledon pieces cultured in WPM media without GR (**Fig. 5.3a**) did not show any response. Type of response in the cotyledons varied with the GR in the medium. In medium with BAP or KN individually, the explants turned brown within 4wks and did not show any morphogenic response. Some of the explants (**Table 5.1**) pre-cultured in medium with combination of BAP and KN (**Fig. 5.3b**) or with GA_3 (**Fig. 5.3c**) singly, swelled and small globular structures appeared on the explants on culturing for 4wks in GR free WPM medium. These structures neither differentiated to give rise to organ nor proliferated to produce more structures. Instead, these structures become black and turn necrotic after culturing for 3-4 passages in GR free WPM medium. In Auxins (IAA, IBA and NAA) containing media rooting was noted in 73-95% of the explants. Other explants did not show any morphogenic response or callusing. Formation of loose callus was noted in the explants cultured in media containing PIC or 2,4-D.

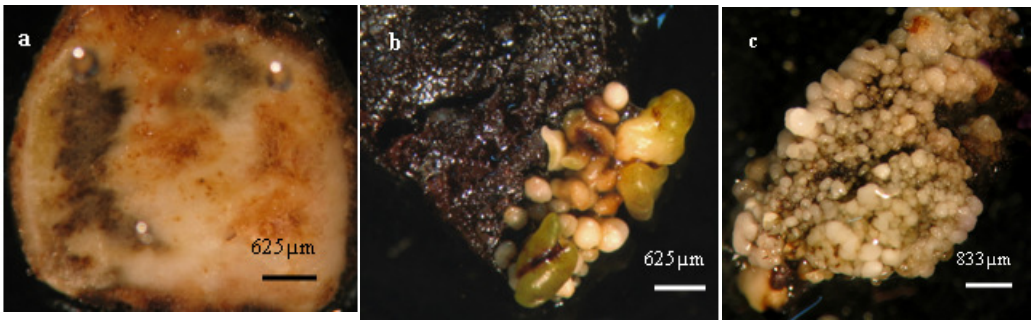


Fig. 5.3 a. Cotyledonary explant becomes brown after 4wks of culture in WPM medium; **b.** Small globular structures appeared on the surface of cotyledon explants after 4wks of cultures in GR free medium which are pre-cultured for 4wks in media containing (BAP 8.88µM + KN 9.28µM); **c.** Small globular structures appeared on the surface of cotyledon explants after 4wks of cultures in media containing GA_3.

Organogenic, embryogenic and callusing responses were observed in the cotyledon explants pre-cultured in all the media containing TDZ. Responding explants turned green with expansion in size of the cotyledon pieces and non-responding explants turns brown after culturing. Regeneration of shoot primordia was evident from the protrusions that appeared on the surface of

the cotyledonary explants (**Fig. 5.3d**). The protrusions appeared all over the surface of the responding explants in all the concentrations of TDZ tested **(Fig. 5.3 e and f**).

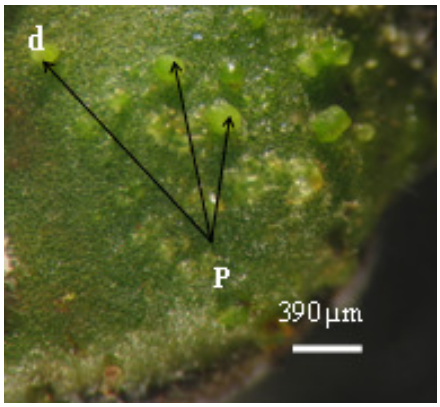


Fig. 5.3 d. Small bud like protrusion coming out from the surface of the cotyledon cultured in 0.45µM of TDZ after 4wks.

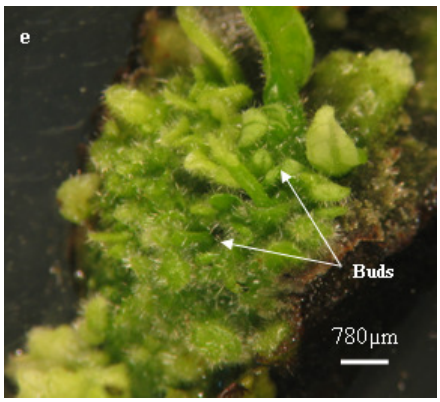


Fig. 5.3 e. Formation of in numerable buds all over the surface of cotyledon in explants cultured in 4.54µM of TDZ for 4wks.

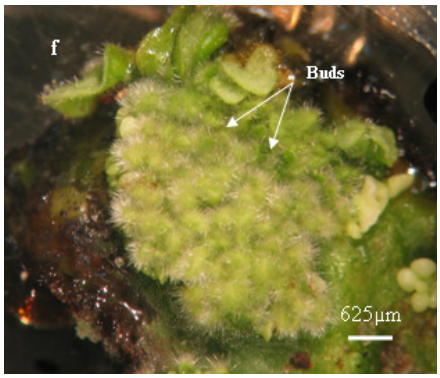


Fig. 5.3f. Shoot primordia formation on the surface of cotyledon cultures in 9.08µM of TDZ for 4wks.

The part of cotyledon in contact with the medium turned brown or black**.** Thidiazuron is identified as the most active cytokinin-like substance, which induces greater *in vitro* responses than many other cytokinins in number of plant species (Khawar *et al*. 2004, Amutha *et al.* 2006). There are several reports describing TDZ induced caulogenesis and embryogenesis in a number of species including many woody plants (Huettemam and Preece 1993; Murthy *et al.* 1998; Mulwa and Bhalla 2006; Jones *et al*. 2007; Chhabra *et al*. 2008; Kong *et al*. 2009). Concentration of TDZ is critical in shoot organogenesis (Lu 1993). The frequency of response increased with concentration of TDZ. Optimum response was 100% in the form of organogenesis, callusing or embryo like structures in TDZ (4.54-22.72µM) (**Table 5.1**).

Table 5.1 Response of cotyledonary explants in different growth regulator after 4wks.

Concentration of GRs in µM	% of response	Type response
WPM	00(41)	Browning of tissue
BAP2.22	00(46)	Browning of tissue
BAP4.44	00(54)	Browning of tissue
BAP8.88	00(40)	Browning of tissue
BAP13.32	00(37)	Browning of tissue
BAP22.2	00(42)	Browning of tissue
BAP26.64	00(55)	Browning of tissue
BAP35.52	00(60)	Browning of tissue
BAP44.4	00(49)	Browning of tissue
KN2.32	00(44)	Browning of tissue
KN4.64	00(49)	Browning of tissue
KN9.24	00(49)	Browning of tissue
KN14.92	00(52)	Browning of tissue
KN24.20	00(55)	Browning of tissue
BAP 2.22 + KN2.46	4±2.5(53)	Swelling
BAP 4.44 + KN4.64	11±5.3(52)	Swelling
BAP 8.88 + KN9.28	13±1.5(65)	Globular structures
BAP15.32+KN14.92	12±1.5(62)	Globular structures
GA_3 0.29	13±1.5(44)	Globular structures
GA_3 1.44	14±4(45)	Globular structures
GA_3 2.88	17±2(48)	Globular structures
GA_3 5.76	18±2(54)	Globular structures
GA_3 8.64	11±2(56)	Globular structures
IAA 2.85	84±2(51)	Rooting
IAA 5.7	91±1(53)	Rooting
IAA 8.55	76±2(53)	Rooting
IBA 2.46	94±2((42)	Rooting
IBA 4.92	95±0.5(48)	Rooting
IBA 9.84	89±1.5(44)	Rooting
NAA 2.69	81±1.5(46)	Rooting
NAA 5.38	85±2.5(51)	Rooting
NAA 5.07	73±1.5(52)	Rooting
PIC 4.14	81±1.5(45)	Loose callus
PIC 12.42	82±5(49)	Loose callus
PIC 20.7	95±2(54)	Loose callus
PIC 41.4	100±0(55)	Loose callus
2.4-D 4.52	77±2(47)	Loose callus
2,4-D 13.56	92±3(57)	Loose callus
2,4-D 22.60	100±0(54)	Loose callus
2,4-D 45.20	100±0(55)	Loose callus
TDZ 0.45	73±2(60)	Bud like structures/ callusing
TDZ 2.27	90±2(53)	Bud like structures/ callusing
TDZ 4.54	100±0(40)	Bud like structures/ callusing
TDZ 9.08	100±0(52)	Bud/globular structures/ callusing
TDZ 13.27	100±0(54)	Bud/ globular structures/ callusing
TDZ 22.7	100±0(52)	Bud/ globular structures/ callusing
ANOVA	S1%	

Number in parenthesis indicates number of sterile replicates.

Organogenic response in the form of shoot primordia and leafy structures were 68±6 % was noted in the explants cultured in 9.08µM of TDZ after 4wks of culturing in TDZ containing medium (**Table 5.2**).

Table 5.2 Organogenic response of cotyledonary explants cultured in TDZ.

Conc. of TDZ in µM	**Percentage of explants showing organogenesis after 4 wks in TDZ containing medium (mean± sd)**	**Number of Buds/explants after 4 wks in TDZ containing medium (mean± sd)**	**Number of buds/explants after 4 wks in WPM (mean± sd)**
0	00±00 (61)	00±00	00±00
0.45	35±5.5(57)	23±3.5	43±4.7
2.27	43.6±5.5(55)	30±3.7	49±1.5
4.54	61.6±1.5(54)	41±6.8	58±5.6
9.08	68±6 (69)	**51±4.3**	**72±5.5**
13.62	62.3±6(63)	48±5.0	59±5.1
22.70	62±5.6(64)	49±5.5	56±1.5
ANOVA	S1%	S1%	S1%

Number in parenthesis indicates number of replicates.

Optimum number of shoot buds 51±4.3 was scored in 9.08µM TDZ containing medium (**Table 5.2**) after 4wk. Incorporation of charcoal in GR free WPM medium enhanced the bud formation in the cotyledons. The explants pre-cultured for 4wks in 9.08µM of TDZ and subsequent culturing for 4 wks in GR free WPM medium with charcoal produced 72±5.5 buds (**Table 5.2**). The caulogenic buds induced on the surface of explants in TDZ containing medium differentiated on repeated cultures in WPM medium without GR (**Fig. 5.3g**). These shoot buds differentiated and elongated to form shoots (**Fig. 5.3h**), on repeated transfer of the cotyledon explants with buds to GR free medium with charcoal for 4-5 passages of 4wks each.

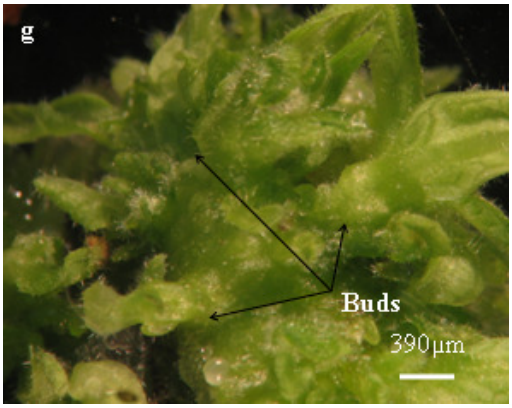


Fig. 5.3 g. Differentiation of buds on the surface of the cotyledon cultured in WPM GR free medium for 8wks these explants were pre cultured for 4wks in 4.54µM of TDZ containing medium

Fig. 5.3 h. A fully differentiated bud to shoot after 12wks of culture in WPM medium with out growth regulators, the cotyledon was pre cultured in 4.45µM of TDZ for 4wks.

Differentiation of the shoot buds on withdrawal of TDZ from the medium confirms the inhibitory role of the growth regulator on proliferation of shoots. In *Tamarindus indica* (Mehta *et al.* 2005), the existing axillary meristems of the embryo axes were exposed to TDZ, due to which profuse bud induction was noted in the axils, which differentiated to shoots only on withdrawal of TDZ from culture medium**.** In *Semecarpus anacardium* the caulogenic bud differentiation to shoot primordial was some times associated with callusing and blackening of the cotyledonary explants (**Fig. 5.3i**). Shoot differentiation from the induced buds was asynchronous. Few buds elongated to form shoots whereas rest of the shoot primordia remained as such **(Fig. 5.3j)**. Although buds were induced on the whole surface of cotyledon, most of them turned necrotic and brown, only 1-2 buds elongated to form shoot **(Fig. 5.3k)**.

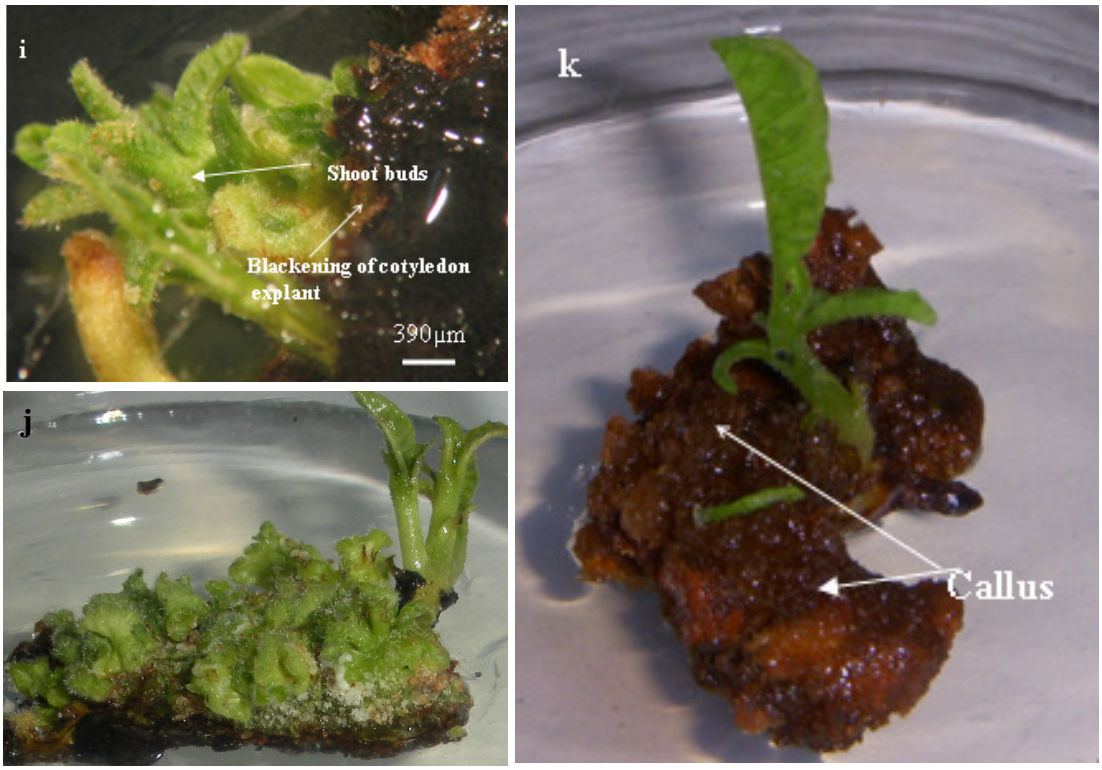


Fig. 3 h. Cluster of shoot buds arising from the cotyledonary explants, the cotyledon explants turning black; **i.** Shoot buds started elongating on WPM medium the elongation was asynchronous as only few buds elongating where as other buds still remains dormant; **j.** In some of the cotyledonary explants except 1-2 buds, rest of the buds became callused.

Similar observations were noted in *Tamarindus indica* (Mehta *et al*. 2005) and in *Pongamia pinnata* (Sujatha *et al.* 2008). The caulogenic buds on cotyledonary explants differentiated and elongated to form shoots on culturing for several passages in WPM medium with charcoal and devoid of growth regulators (**Fig. 5.3l**). Some of the elongated shoots rooted spontaneously in WPM medium with charcoal (**Fig. 5.3m**).

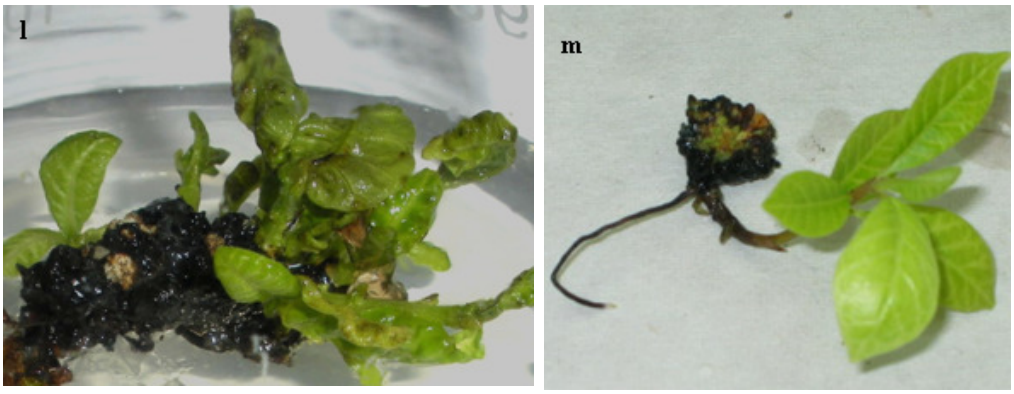


Fig. 5.3 l. Cluster of shoot buds after differentiation and elongation at different stages after 4 passages of 4wks in WPM medium, Pre-cultured for 4wks in TDZ incorporated medium; **m.** Elongated shoot buds from the cotyledons, which rooted spontaneously in WPM medium containing 0.2% charcoal.

All the elongated shoots rooted in half strength WPM liquid medium with 2.46µM of IBA as described in Chapter 3B (**Fig. 5.3n**). The rooting was induced at the base of the shoots whereas some callus appeared in the region above the cut end. This part of the shoot was in contact of the liquid medium containing IBA. Rooted plantlets survived (90%) in soil sand (1:1) mixture and hardened in 4wks (**Fig. 5.3o**). Hardened plants acclimatized and were maintained in green house.

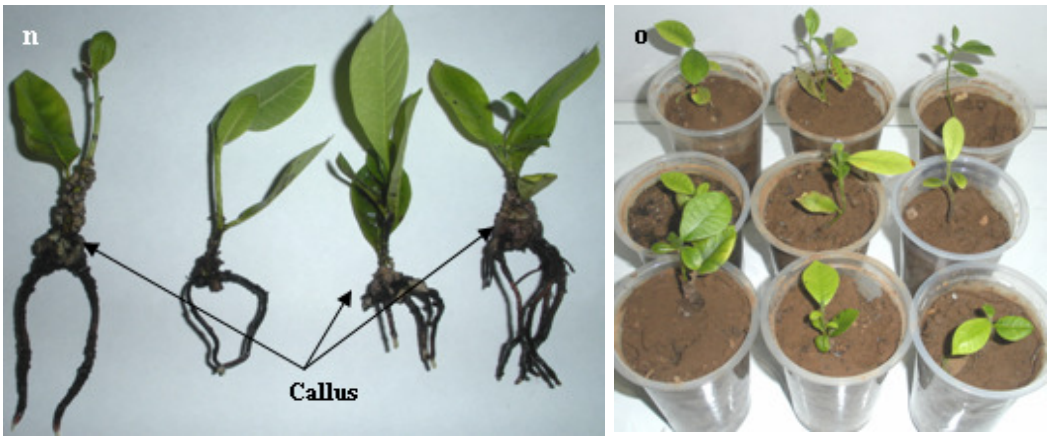


Fig. 5.3 n. Rooted explants in half strength WPM liquid medium containing 2.46µM of IBA, with callusing at the base of the shoots; **o.** Hardened plants in cups containing soil sand (1:1) mixture for after 4wks of hardening.

Histological studies demonstrated the emergence of morphogenic buds and shoot primordia from the cotyledon explants without intervention of callus (**Fig.5.3 p, q**). The induction of caulogenesis was asynchronous. Thus buds at different stages of development were noted in the same explant (**Fig. 5.3p**). At some sites on the surface of cotyledon only morphogenic outgrowth was noted, whereas in some others there was development of distinct shoot primordia with leaf opening (**Fig. 5.3q**).

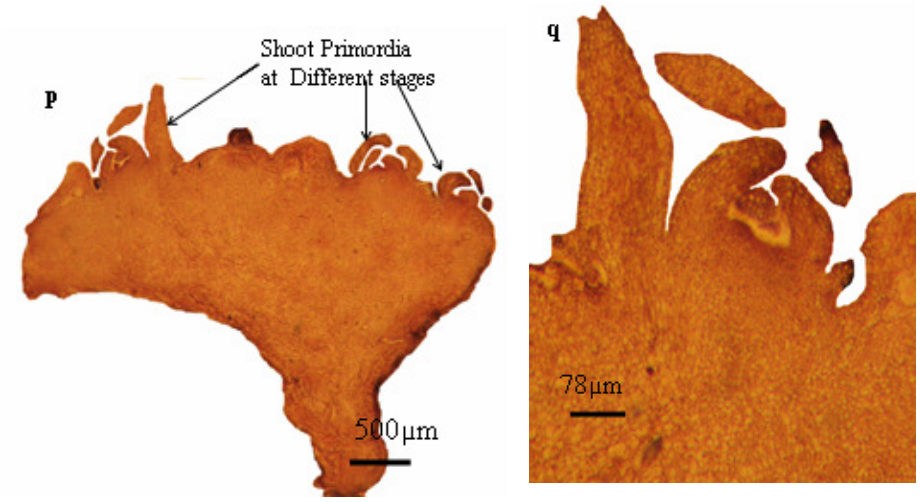


Fig. 5.3 p. Histology of cotyledonary explants cultured in 2.27µM of TDZ for 4wks. Number of shoot primordia at different stages of development was visible on the surface of cotyledon; **q.** A magnified portion of **Fig.5.3 p**, shoot primordia with opened leaves.

5.3.1 *Embryogenic response:*

In addition to organogenic response globular structures resembling embryos and callusing was also observed in the cotyledonary explants of *Semecarpus anacardium*. This type of TDZ induced morphogenesis suggesting different mode of action of TDZ. Jones *et al.* 2007a observed formation of shoot and somatic embryos from the leaf explants in TDZ containing medium in *Echinacea purpurea*. Direct induction of globular embryogenic mass was observed in WPM semi solid media containing 4.45-22.7µM of TDZ (**Fig. 5.3.1a**). The embryogenic mass was associated with callus occasionally (**Fig. 5.3.1b**). Such mixed type of response by organogenesis, callusing and embryogenic mass formation was also observed in immature cotyledonary explants of *Macadamia tetraphylla* (Mulwa and Bhalla 2006) in leaf explants of *Echnacea puepurea* (Jones *et al.* 2007a) and in immature zygotic embryos of *Cinnamomum pauciflorum* (Kong *et al.* 2009) in response to TDZ.

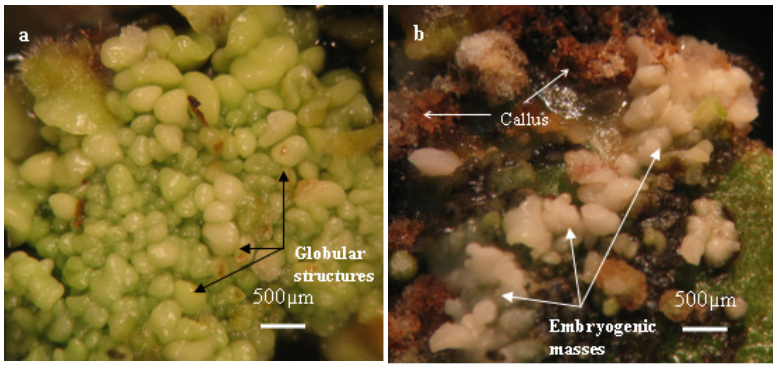


Fig. 5.3.1 a Cotyledonary explants cultures in 22.7µM of TDZ for 4wks with globular embryo like structures; **b.** Formation of embryogenic mass associated with callus after 4wks of culture in WPM medium, pre-cultured for 4wks in 13.2µM of TDZ.

Optimum response of embryogenic mass formation was noted in cotyledon pieces of *S. anacardium* cultured in 9.08µM of TDZ containing medium. In this medium 13±1.1% of explants showed embryogenesis after 4wks of culture. On culturing the explants for 4wks in WPM medium with charcoal (0.2%) without GR embryogenic response increased to 21.6±3.2 %. In lower concentration of TDZ (0.45 and 2.27µM) containing medium the cotyledon explants did not exhibit any embryogenic response after culturing for 4wks. Embryogenic response was noted in all the concentrations of TDZ, after withdrawal of TDZ from the media and subsequent culturing in WPM GR free medium with charcoal. The number of embryogenic masses noted per individual explants showed a similar pattern (**Table 5.3**).

Table 5.3 Embryogenic responses of cotyledonary explants cultured in TDZ.

Concentration of TDZ in µM	**Percentage of explants showing embryogenic mass formation (mean±sd)**		**Number of embryogenic mass /explants (mean±sd)**	
	4wks in TDZ containing medium	**4wks in WPM GR free medium**	**4wks in TDZ containing medium**	**4wks in WPM GR free medium**
0	00±00(61)	00±00	00±00	00±00
0.45	00±00(57)	16.6±9.0	00±00	4.5±3.0
2.27	00±00(55)	19.3±1.5	00±00	10±0.9
4.54	11±1(54)	19±1	8.49±0.34	24±1.0
9.08	13±1.1(69)	21.6±3.2	15.25±1.08	38±1.13
13.62	16±1.8(63)	20±1.7	23.88±0.4	34±2.05
22.70	17±1.5(64)	16±4.5	30.18±1.95	26±1.3
ANOVA	S1%	S%	S%	S%

Number in parenthesis indicates number of replicates.

The embryogenic mass gave rise to embryo like structures on further culturing for 2-3 passages of 4 wks each in WPM GR free medium with charcoal. Different stages of embryos could be identified (**Fig. 5.3.1 c, d and e**). These embryos occasionally germinated in the same medium which demonstrated emergence of root (**Fig. 5.3.1 e**). However, these embryos did not convert into plantlet and in GR free medium they continued to proliferate forming more embryos. Somatic embryo induction by TDZ has been reported in immature zygotic embryos of trees like *Melia azedarach* L. (Vila *et al*. 2003, 2007) and *Cinnamomum pauciflorum* (Kong *et al.* 2009).

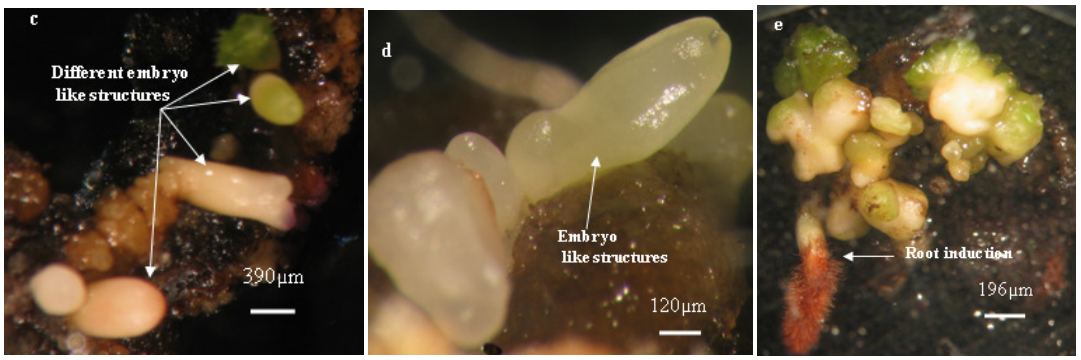


Fig. 5.3.1 c. d and e Different stages of embryo like structures appearing on the surface of the cotyledons. In **Fig. e** rooting of the embryogenic structures in WPM basal medium containing 0.2% charcoal.

On histological examination of these structures different cell type was evident (**Fig. 5.3.1 f and g**). In **Fig. 5.3.1 f** cells in the central region are more distinct like vasculature. Angular compact cells were visible in the central region of the globular structure (**Fig. 5.3.1g**). A torpedo shaped embryo showing bipolar structure was seen (**Fig. 5.3.1h**). These embryo like structures continued division and re-division to form secondary embryo like structures from the bases (**Fig. 5.3.1i**). The proliferations of the embryogenic mass continued further on culturing them in WPM medium with charcoal. Conversion of these structures could not be achieved either in GR containing or GR free medium. Further experiments are in progress for successful germination and conversion of these embryos like structures to plantlets.

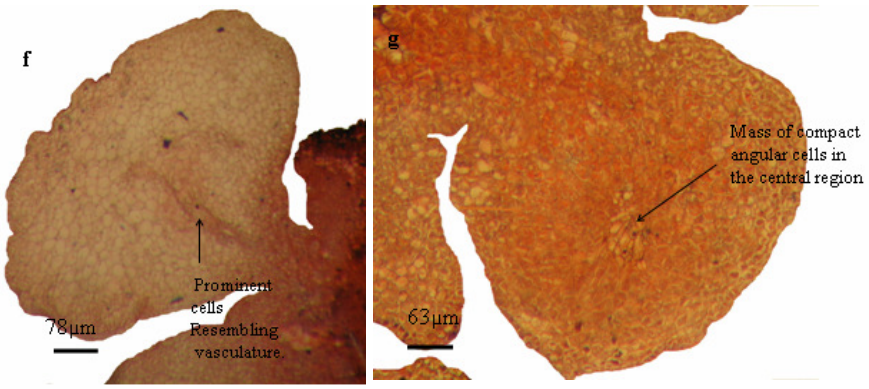


Fig. 5.3.1 f. Globular structure arising from the cotyledon, distinct cell in the central region resembling vasculature; **g.** Structure resembling globular embryo having compact angular cells at the central region.

Fig. 5.3.1 h. Bipolar torpedo shaped embryo; **i.** Embryo like structure showing secondary embryo like development from the base.

5.3.2 *Callusing*

In few cases different type of response in the form of callusing was noted from the cotyledonary explants cultured in WPM containing charcoal for 7-8 passages of 4wks each. These explants were pre-cultured for 4wks in TDZ containing medium. These calluses were compact and morphogenic in appearance. Emergence of small embryo like structures was obvious from the callus (**Fig. 5.3.2a**). Some of these structures are heart shaped (**Fig. 5.3.2b**), bipolar showing clear distinction of shoot poles and root poles (**Fig. 5.3.2 c, d)** and cotyledonary shaped embryos (**Fig. 5.3.2e**). In few cases these embryo like structures formed in cluster with different shapes at different stages of development (**Fig. 5.3.2f**). Secondary structures often appeared from them (**Fig. 5.3.2 f** and **g**).

Occasionally germination was also visible in some of these structures (**Fig. 5.3.2h**). Greenness of the apical region and germination in basal region of the structures further supports that these structures are embryos (**Fig. 5.3.2i**). In the present study, both organogenesis and embryogenic structures were observed from the cotyledon explants. Thidiazuron is capable of fulfilling both cytokinin and auxin requirement of various regeneration responses suggesting different mode of action (Jones *et al*. 2007a). This growth regulator can affect meristem formation, promotes shoot development from pre-existing meristems and induce adventitious bud formation in number of recalcitrant woody plants (Cuenca *et al*. 2000 and Bunn *et al*. 2005). This finding in *Semecarpus* was similar to other reports where both organogenesis and somatic embryogenesis has been observed simultaneously (Jones *et al*. 2007a; Chhabra *et al*. 2008).

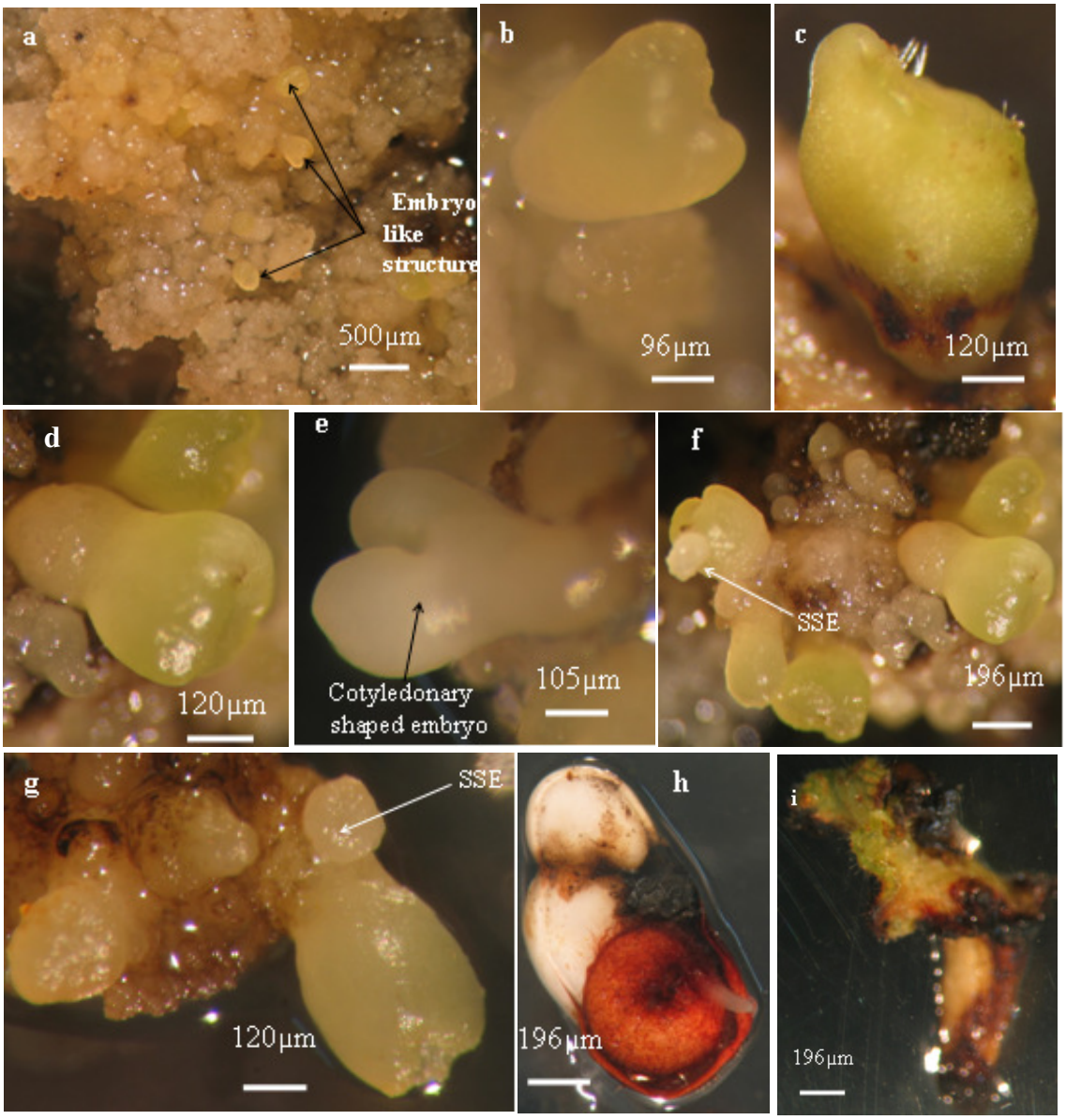


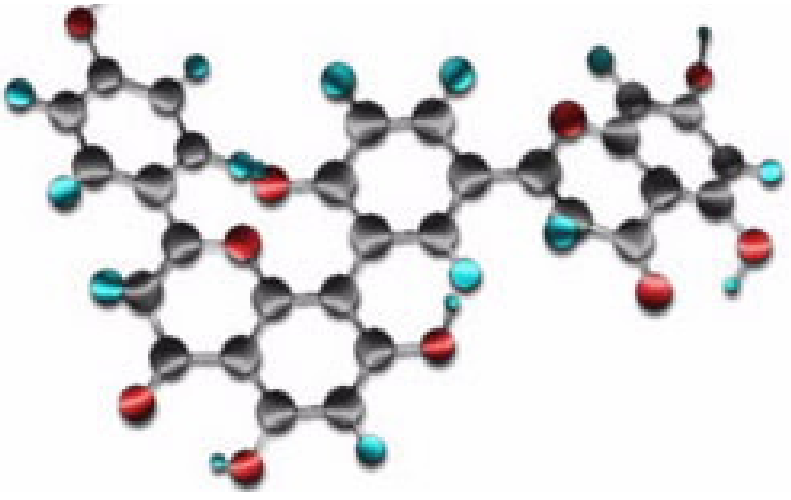
Fig. 5.3.2 a. Compact callus induction from cotyledon after several passages in WPM medium containing charcoal, showing small embryo like structures; **b.** Torpedo shaped embryo arising from the callus; **c.** Embryo showing clear distinction of shoot and root pole; **d.** Cup shaped embryo differentiating from the callus; **e.** A cotyledonary shaped embryo; **f.** Differentiation of callus into cluster of embryos of various shape and sizes; **f and g.** Embryo like structure showing secondary embryo like development. **h**. Occasional rooting of the globular structures. **i.** Rooting in embryo like structures in WPM medium with charcoal.

5.4 CONCLUSION

In the present study effect of TDZ on morphogenic response of cotyledonary explants was observed. Thidiazuron in all the concentration tested elicited organogenic as well as embryogenic response. The organogenic buds differentiated in WPM medium without growth regulators containing charcoal. Elongated shoots were rooted in half strength liquid medium with 2.46µM of IBA. Plants were successfully hardened and acclimatized. However, the embryogenic mass resulted in embryo like structure directly or indirectly failed to germinate in most of the cases and continued to proliferate into embryo like structure and callus in GR free medium.

CHAPTER 6

IDENTIFICATION OF BIOLOGICALLY ACTIVE COMPOUNDS IN *IN VITRO* CULTURED TISSUES



6.1 INTRODUCTION

Forests have been an invaluable source of medicinal plants since the time humans realized the preventive and curative properties of plants. Even when no synthetic medicines existed, our ancestors had been depending on herbs and medicinal plants and their derivatives to cure common ailments. There is a continuous unbroken tradition of the use of medicinal plants for over four millennia, and medicinal plant use is still a living tradition. This is borne around millions of indigenous and village-based carriers of herbal medicine. There are specialized herbal healers, birth attendants, bonesetters and visha vaidyas besides millions of women and elders who have traditional knowledge of herbal remedies and of food and nutrition. The Indian system of medicines, namely, ayurveda, siddha and unani predominantly use plant-based raw materials for their preparations and formulations (Singhal 2005). Medicinal plants are natural, non-narcotic, having no side effects and cost effective for achieving the goal of 'Health for All'. As a result, demands for medicinal plants are increasing both in developing and developed countries. With the changing perception of traditional medicines there has been increased recognition and use of medicinal plants for primary health care by rural as well as urban populations. Even today, 75% of the world's population relies on plants for traditional medicine (Sharma *et al*. 2009). This has resulted in twin pressures from traditional practitioners, as well as from modern pharmaceutical sources. In spite of this threat, medicinal plants continue to provide health security to millions of rural people all over the world (Gupta 1988; Pandey and Bisaria 1998). Plants will continue to provide novel products as well as chemical models for new drugs in the coming centuries. The chemistry of the majority of plant species is yet to be characterized (Cox and Balick 1994). Despite advancements in synthetic chemistry, we still depend upon biological sources for a number of secondary metabolites including pharmaceuticals (Pezzuto 1995). Plant secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes in plants, but they do have an important role in the interaction of the plant with its environment. They mostly have an ecological role as attractants of pollinating insects or in defense mechanisms against predators. The distribution of secondary metabolites in plants is far more restricted than that of primary metabolites; a compound is often only found in a few species, or even within a few varieties within a species. The production of these compounds is often low (less than 1% DW), and it depends greatly on plant species and plant's physiological and developmental stage (Namdeo 2007). Moreover, secondary metabolites often accumulate in the plant in specialized cells or organs. Many of these secondary metabolites are unique to the plant kingdom and are not produced by microbes or animals. Many plants containing high-value compounds are difficult to cultivate (Rates 2001). At the same time, the chemical synthesis of plant-derived compounds is often not economically feasible because of their highly complex structures and specific stereo-chemical characteristics. The production of

valuable secondary metabolites in plant cell cultures is an attractive alternative to the extraction of the whole plant material. Biotechnology offers an opportunity to exploit the cell, tissue, organ or entire organism by growing them *in vitro* and to genetically manipulate them to get desired compounds in increased quantities (Rao and Ravishankar 2002).

Number of secondary compounds with proven medicinal importance like amentoflavone (Ishratullaha *et al*, 1977), Tetra-hydroamentoflavone (Selvam *et al*. 2004), Butein and 7,3',4'-trihydroxyflavone (Selvam *et al*. 2004), 1,2-dihydroxy-3-pentadecaenyl-8'-benzene and 1,2-dihydroxy-3-(pentadecadienyl-8',11') benzene (Young *et al*. 1999), 3-(8(Z),11-(Z)-pentadecadienyl) Catechol (Nair *et al*. 2009) were isolated from different parts of *Semecarpus anacardium.* Cytotoxicity of these compounds was demonstrated against human cancer cell lines. Curative properties of different ayurvedic preparation from the nuts of *Semecarpus anacardium* against variety of diseases were described in the introduction (**Chapter 1**). In the present investigation Amentoflavone (**Fig. 6a**) has been chosen as a model to assess its *in vitro* production pattern with respect to different plant organs.

The rationale behind selecting this compound is,

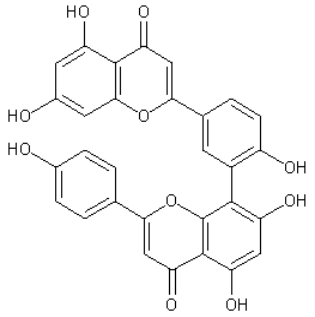


Fig. 6 a. Molecular structure of Amentoflavone.

- This compound is present in leaves which can be obtained from *in vitro* shoot cultures.
- Amentoflavone, a biflavonoid, with potential anticancerous, neuroprotective, antiviral, anti HIV agent and is also recognized for its anti-inflammatory activity (Lee *et al*. 2009).
- The chemical synthesis of this compound was not reported.

Semi-synthetic preparation of amentoflavone was reported from biflavones isolated from autumnal *Ginkgo biloba* leaves (Hanrahan *et al*. 2003). *In vitro* studies on *Hypericum perforatum* indicated that shoot cultures are excellent sources for hypericins and hyperforin production (Kirakosyan *et al*. 2004). When medicinal compounds are synthesized in leaves, the use of shoot culture in production of secondary metabolite may be advantageous. Microbe free shoot culture can be grown in culture in abundance, producing plant organs in aseptic condition for production of medicinal compounds may have advantages in production and long term storage. Shoot cultures are genetically stable, have good capacity to produce secondary metabolites and provide a system to co-relate between growth and production of secondary metabolite (Bourgoud *et al*. 2001; Matwoski 2008).

This chapter deals with experiment conducted to identify and quantify amentoflavone in different *in vitro* raised plant parts and comparison with *ex vitro* leaves of *Semecarpus anacardium*. Different carbon sources influence the *in vitro* morphogenesis in plants (Fuentes *et al*. 2000). Therefore, the influence of carbon sources on production of Amentoflavone by the shoot cultures was studied. Amentoflavone was reported to be produced in leaves. Different organs viz., roots, stem of the *in vitro* raised plants and callus were analyzed to see whether amentoflavone can be produced in *in vitro* system which provides a unique environmental condition to the respective tissues.

6.2 MATERIALS AND METHODS

Plant Material:

Established shoot cultures described in (**Chapter 3B**) were grown in WPM medium containing 2% of different carbon sources viz., Sucrose, Glucose, Fructose and Maltose. In another experiment shoots were grown in WPM medium containing 2, 4, 6 and 8% of sucrose. After 8 wks the shoots were harvested and washed with distilled water. Leaves and stems were separated and dried at room temperature. In order to get higher biomass of leaf and stem tissues, the plant materials were cultured for 8 wks. Roots were isolated from shoot cultures maintained in WPM medium with 0.2% charcoal during sub-culturing. These roots were washed with distilled water and dried at room temperature. Callus from cotyledon tissues induced in 2, 4-D containing medium were harvested, washed with distilled water and dried. The extracts of these plant parts were used for identification and quantification of Amentoflavone. All the cultures were incubated in culture room maintained at temperature of 25±2°C under 16hr photoperiod with an irradiance of 50µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Phillips). The pH of all media was adjusted to 5.8 before sterilization by autoclaving at 121°C for 20 minutes at 1.06kg cm^{-2}. Phytagel (Sigma) (0.2%) was used for gelling the medium. Leaves from naturally growing mature plants were collected and air-dried at room temperature. These leaves serve as control for comparison between the *in vitro* and *ex vitro* conditions.

Methods:

Stock solution (5mg/ml) of the authentic amentoflavone was prepared by dissolving in HPLC grade methanol. The working standard solutions of different concentrations (0.01-0.08mg) were prepared by serial dilution of the original stock with mobile phase (methanol: water (75: 25): 0.1% acetic acid). HPLC analyses of these concentrations of authentic amentoflavone were carried out. The standard curve was derived from the peak area of each concentration (**Fig. 6b**). Identification of Amentoflavone in different sample was performed by comparing its retention time with the authentic standards (**Fig. 6c**). Amount of amentoflavone present in the extract was

calculated manually by comparing the area of authentic standards with that of the sample. All the experiments of plant material harvesting, extraction and HPLC were repeated twice.

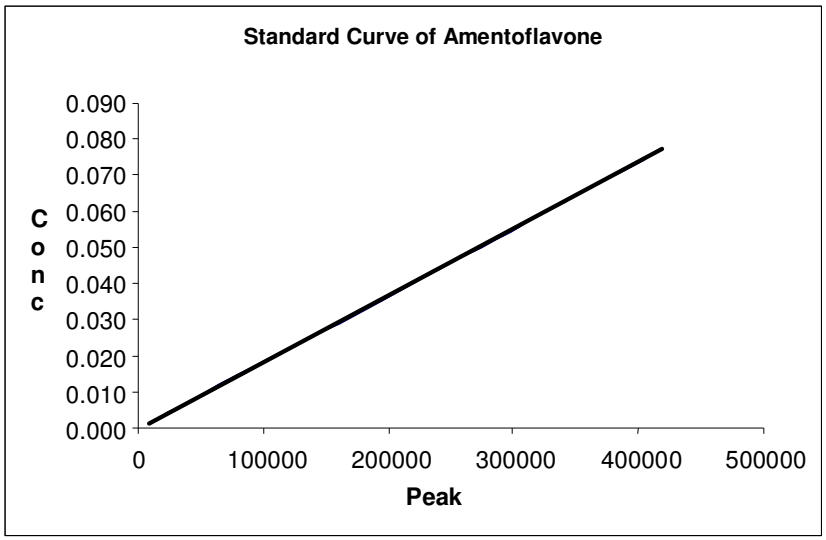


Fig. 6 b. Standard curve of amentoflavone dissolved in methanol and mobile phase.

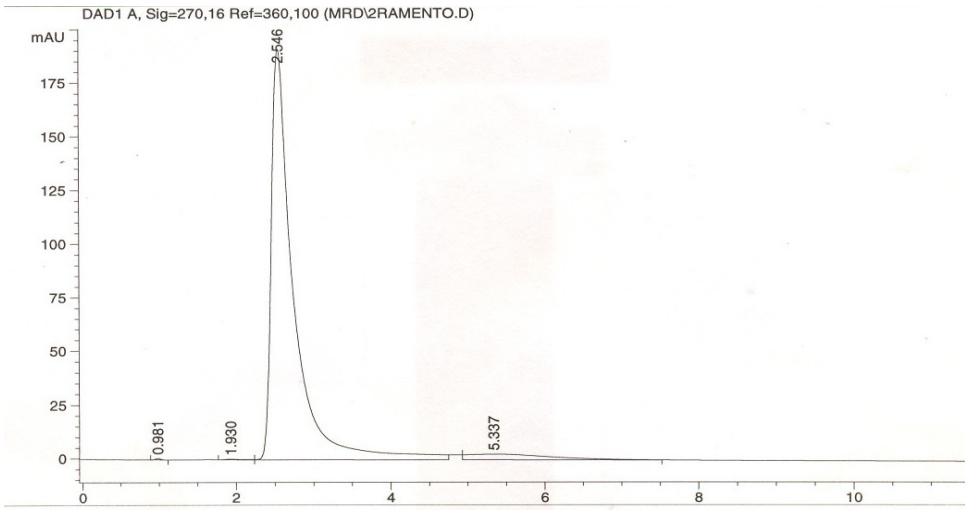


Fig. 6 c. HPLC chromatogram of standard amentoflavone.

Extraction procedure:

Extraction of amentoflavone from different tissues was carried out by the procedure of Jung *et al.* (2007), with minor modifications. Approximately 10gms of air-dried samples of *ex vitro* leaves and in *vitro* raised leaves, stem and callus were ground to fine powder in liquid nitrogen with the help of motor and pestle. The powdered samples were soaked in 100ml of GR grade methanol (Merck, India) for 6-8hr. The methanol extract was filtered using Whatman-1 filter paper and the filtrate was separated. The residue was again soaked in 100ml of methanol and filtered after 6-8hr. This extraction procedure was repeated three times for complete extraction of the chemical compounds. This process of extraction was carried out for all the samples separately at room

temperature. The methanol extracts of each samples was pooled and evaporated in rotavapour (Buchi, Germany) at 50ºC. The dried methanol extract was dissolved in 100ml sterile milli Q water and successively partitioned in separating funnel. Initially, 100ml of petroleum ether was added to the crude extract and shaken well to separate fatty/oily substances, pigment etc. This step was repeated three times for complete removal of the non-polar substances. Then the aqueous extract was partitioned with ethyl acetate three times successively for complete isolation of amentoflavone. The ethyl acetate (Merck, India) extract were pooled, concentrated to dryness in room temperature. The dried ethyl acetate extracts of all samples were dissolved in 10ml each of HPLC grade methanol (Merck, India) and analyzed for the presence of amentoflavone.

Condition for HPLC:

The amounts of Amentoflavone in the extracts were analyzed on Shimadzu HPLC system consisting of LC 10 AT VP pump, a Rheodyne injector, a SPD 10 AVP UV Visible detector and Class-AVP HPLC software. The analytical column used to achieve chromatographic separation was Atlantis RP-18 column (250x4.6 mm with internal diameter 5µm particle size). The peak purity was determined by comparing with authentic standard Amentoflavone. The mobile phase consisted of methanol: water (75: 25): 0.1% acetic acid. Injections were carried out by auto sampler with 20µl of sample at room temperature (25±2ºC) and the flow rate was 1.0 ml/ min. Detection was performed at 270 nm.

6.3 RESULTS AND DISCUSSION

Several tissue culture reports refer to the influence of the carbon source on the *in vitro* morphogenesis of different plant species. Carbon source was found to be a significant factor in plant cell metabolism (Zhong 2001). Among the many available carbon sources, sucrose has been the major one (Petersen *et al*. 1999; Fuentes *et al*. 2000). Out of the four carbon sources tested in the medium for growing shoot of *Semecarpus anacardium*, leaves from plants grown in media containing 2% maltose yields 102.2µg of amentoflavone (**Table 6.1; Fig. 6d**). Leaves from fructose and glucose containing media yield 90.6 and 67.4µg of amentoflavone, respectively. Carbon sources other than sucrose influences secondary metabolite accumulation *in vitro* (Bourgoud *et al*. 2001). In callus cultures of *Commiphora wightii* production of guggulsterone was 16µg/gm of dry weight cultured in 4% sucrose containing medium, which increased to 59µg/gm when the callus was cultured in 4% maltose. In the same experiment guggulsterone content was 21.3µg/gm in glucose (4%) containing medium. In *Glycyrrhiza uralensis*, fructose was superior to sucrose for callus growth and flavonoid formation, and the optimum concentration was 2%. The flavonoids content was 2 times higher than that of sucrose as carbon sources (Yang *et al*. 2006). Four carbon sources sucrose, fructose, and glucose tested *in vitro* to determine optimum growth, morphogenesis, and the production of secondary metabolites

in *Nepeta rtanjensis* shoots. Out of these carbon sources glucose proved to be the most efficient energy source and for production of nepetalactone (Misic *et al*. 2003). Increase in production of amentoflavone in leaves of *S.anacardium* shoot cultured in medium containing Glucose, Fructose and Maltose may be due to decrease in pH. Significant decrease in pH of medium by addition of these sugars had been previously reported (Neto *et al*. 2003).

Table 6. 1 Quantity of Amentoflavone extracted from tissues of *Semecarpus anacardium*.

Type of tissue	**Concentration of amentoflavone /10 gm of dry weight of tissue.**
2%Sucrose (*In vitro* leaves)	48.22µg
2% Glucose (*In vitro* leaves)	67.4µg
2% Fructose (*In vitro* leaves)	90.6µg
2% Maltose (*In vitro* leaves)	102.2µg
2% Roots (*In vitro*)	Not detected
2% Callus (*In vitro*)	40.6µg
2% Stem (*In vitro*)	Not detected
Ex vitro Leaves	220µg

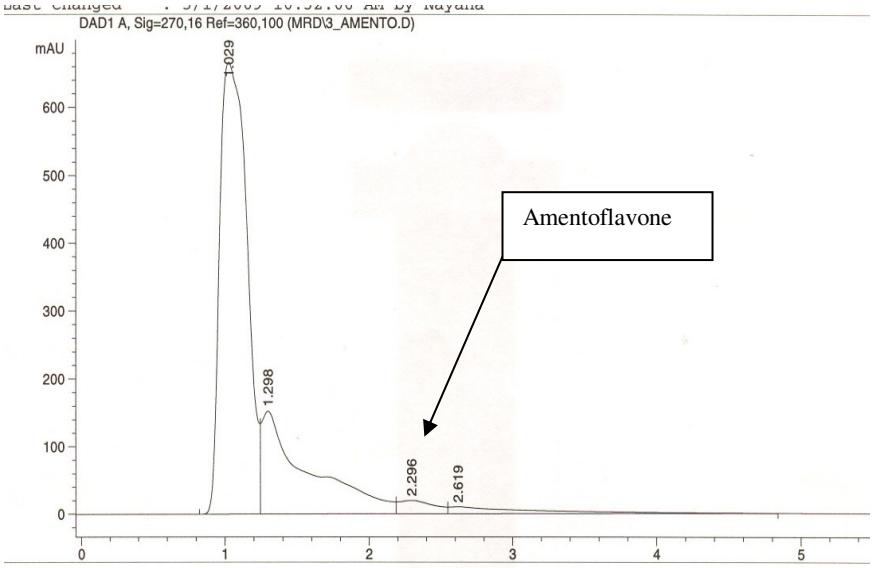


Fig. 6 d. HPLC chromatogram of Leaf extracts of *Semecarpus anacardium* cultured in 2% Maltose.

Amentoflavone was not detected in *in vitro* raised stem shoots and roots **(Table 6.1)** by HPLC that may be due to presence of the compound lower then detectable limit. The callus culture obtained from cotyledons yielded 40.6µg of amentoflavone. Cell suspension cultures were mostly used for production of secondary metabolites *in vitro* (Smetanska 2008). Increased production of viblastine, ajamalicine, catharanthine, vincristine and vindoline in callus and

suspension culture of *Catharanthus roseus* has been extensively reviewed (Pietrosiuk *et al*. 2007). The leaves of mature plant of *S.anacardium* contained 220µg of amentoflavone.

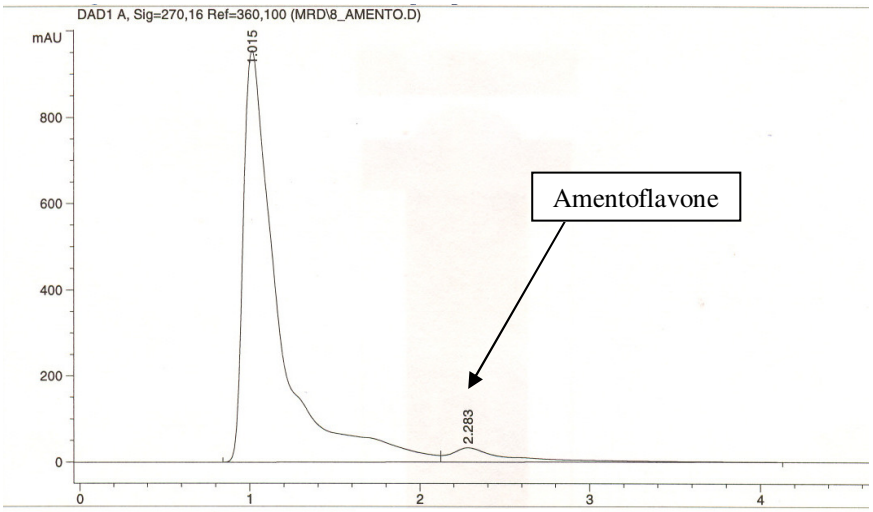


Fig. 6 e. HPLC chromatogram of leaf extracts of *Semecarpus anacardium* collected from mature trees.

A large number of carbon sources support growth of cell cultures with highest growth rate is usually obtained with sucrose and glucose. Nevertheless sucrose, has been proved to be preferable for metabolite production (Kurz 1982). Even though amount of amentoflavone was more in leaves of shoot culture grown in maltose (2%) containing medium but the growth of shoot culture was not optimum (Chapter 3B). In an alternative the shoot cultures of *Semecarpus anacardium* were grown in different concentration of sucrose containing medium to examine the production of amentoflavone. Presence of amentoflavone at varying concentration was detected in leaves of shoot culture grown in WPM medium supplemented with different concentration of sucrose (**Table 6.2**). In WPM medium-containing 2% sucrose the concentration of amentoflavone was found to be 48.22µg per 10gm of dry weight of the tissue (**Fig. 6f**). Optimum concentration of sucrose for amentoflavone production was found to be 4% (**Fig. 6g**). Leaves of shoot culture growing in 4% of sucrose yield maximum 94.34µg of amentoflavone. Further higher concentration of sucrose (6 and 8%) tested produced less amount of amentoflavone in comparison to that in 4% sucrose (**Table 6.2**). Several tissue culture reports refer to the influence of the carbon source on the *in vitro* morphogenesis of different plant species (Fuentes *et al*. 2000). Sucrose is the most popular carbon source for plant tissue culture and the level of the sugar also affects the secondary metabolite productivity (Misawa 1985; Merkil *et al*. 1997). In shoot culture of *Ruta graveolens* optimum production of furanocoumarins is reported in B5 medium containing 1% of sucrose (Massot *et al*. 2000). Similarly higher concentration of sucrose (6%) increases the production of amarogentin significantly in root cultures of *Swertia chirata*

(Kiel *et al*. 2000). Optimum pyranocoumarins production from hairy root cultures of *Angelica gigas* was achieved at 4% of sucrose after culturing for 3 weeks in SH medium (Xu *et al*. 2009). Production of amentoflavine in *S.anacardium* leaves of shoot cultured in 4% sucrose containing medium may be due to dual role of the sugars as carbon source and also as osmoticum in the medium. Dual role of sucrose as carbon source and osmotic agent was proposed for increase amarogentin production in hairy root cultures of *Swertia chirata* (Kiel *et al*. 2000). Yields of benzophenanthridine alkaloids from suspension cultures of *Eschscholtzia californica* were increased 10-fold to around 150mg/l by increasing the sucrose concentration from 2% to 8% (w/v) (Berlin *et al*. 1983). Nordihydroguaiaretic acid and Quercetin contents of *in vitro* shoots cultures of *Larrea divaricata* was optimum when grown in 5% sucrose supplemented medium compared to that containing 3 and 4% of sucrose (Placio *et al*. 2008). Similarly 4% sucrose in suspension cultures of *Panax notoginseng* is optimum for production of ginseng saponin (secondary metabolite) and polysaccharide (primary metabolite) (Zhang *et al*. 1996).

Table 6.2 Quantity of Amentoflavone extracted from leaves of *Semecarpus anacardium* grown in WPM medium with different concentrations of sucrose.

Type of tissue	**Concentration of amentoflavone /10gm of dry weight of tissue.**
2% Sucrose (*In vitro* leaves)	48.22µg
4% Sucrose (*In vitro* leaves)	94.34µg
6% Sucrose (*In vitro* leaves)	77.70µg
8% Sucrose (*In vitro* leaves)	43.90µg

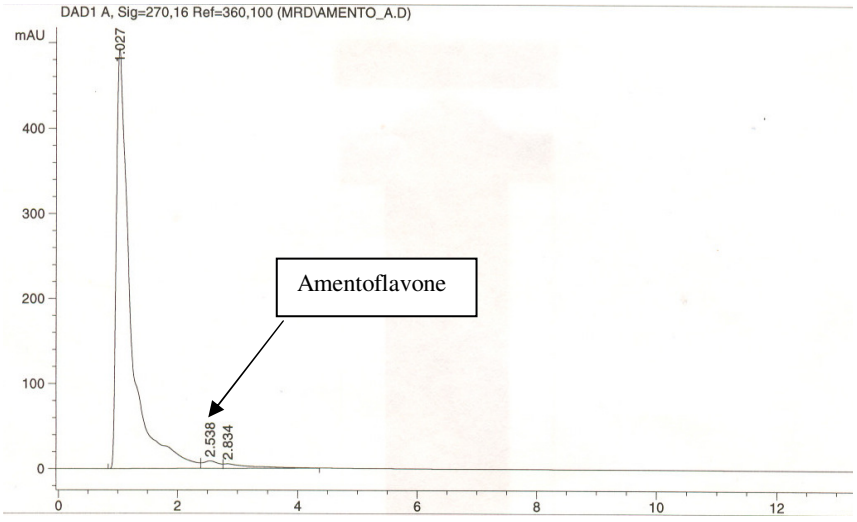


Fig. 6 f. HPLC chromatogram of Leaf extracts of *Semecarpus anacardium* cultured in 2% sucrose.

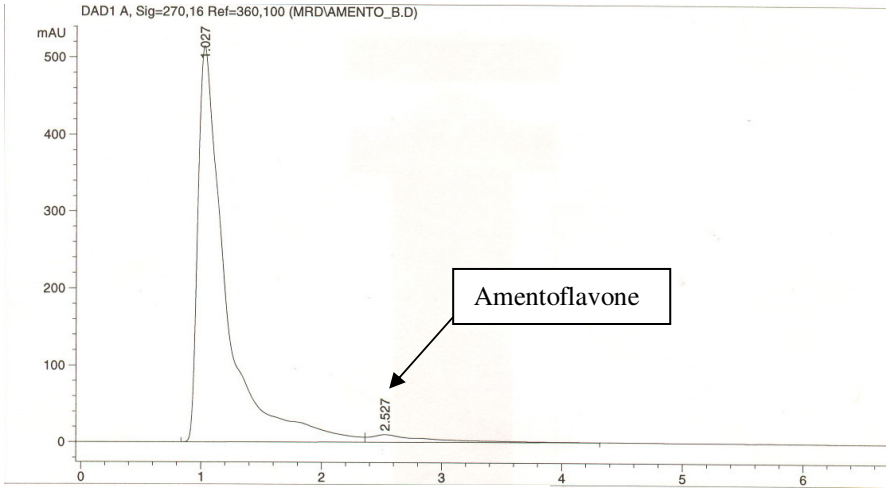


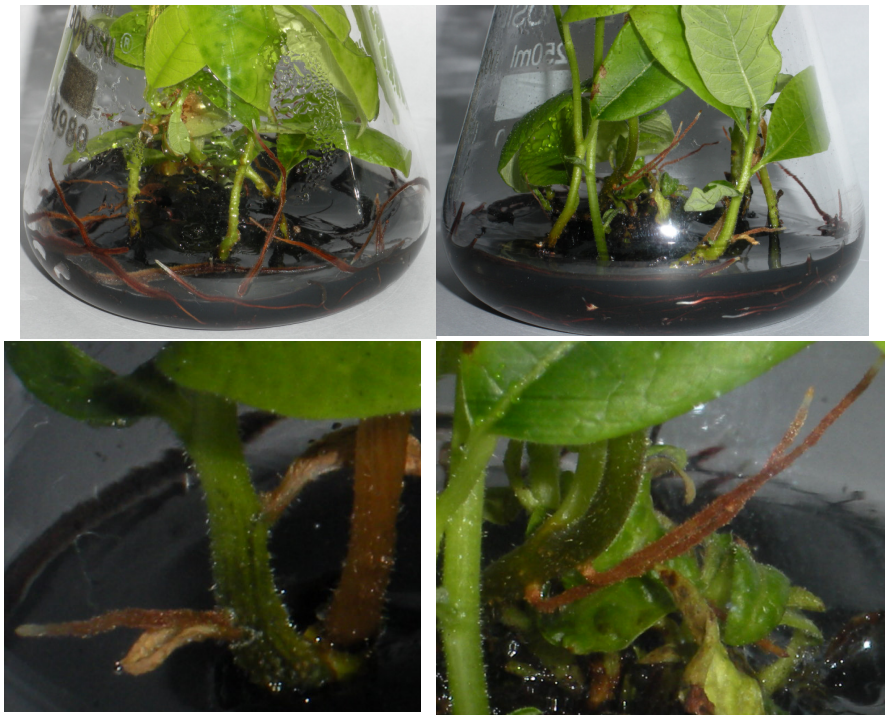
Fig. 6 g. HPLC chromatogram of Leaf extracts of *Semecarpus anacardium* cultured in 4% sucrose.

6. 4 CONCLUSION

In this study we could detect the presence of amentoflavone in the leaves of shoot cultures of *in vitro* raised plant at varying quantities by adding Maltose, glucose, fructose and sucrose at different concentrations to the media. These are some of the preliminary finding of *in vitro* secondary metabolites production in leaves derived from *in vitro* shoot cultures of *Semecarpus anacardium*. Further studies need to be carried out for large-scale production of this compound and other secondary metabolites from this plant.

CHAPTER 7

GENETIC TRANSFORMATION OF *SEMECARPUS ANACARDIUM* USING *AGROBACTERIUM RHIZOGENES*



7.1 INTRODUCTION

Agrobacterium rhizogenes a gram-negative soil bacterium, causes a plant disease known as hairy root. When the bacterium infects the plant, the T-DNA between the T_R and T_L regions of the Ri-plasmid in the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces a valuable by-product, hairy root, which form at or near the site of infection. In addition, opines are produced and serve as specific nutrition for the bacterium (Chilton *et al*. 1982). Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone free medium. The transformed root is highly differentiated and can cause stable and extensive production of secondary metabolites. Other plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesize very low levels of useful secondary metabolites (Rhodes *et al.* 1990; Merkli *et al*. 1997; Kittipongpatana *et al*. 1998; Hu and Du 2006).. In addition to the Ri plasmid, over half these plants have been transformed with foreign genes, including agronomically useful traits (Christey 2001). The natural host range of *A. rhizogenes* appears to be limited to a small number of plant species: apple, cucumber, tomato or melon etc. (Weller *et al*. 2004). However, under laboratory conditions, more than 450 different species of plants are found to be susceptible to infection by *A. rhizogenes* including diverse range of dicotyledonous and monocotyledonous plant families, some gymnosperms several endangered medicinal plants, affording opportunities to produce important phytochemicals and proteins in eco-friendly conditions (Veena and Taylor 2007). Most plant tissues and organs, including the hypocotyl, leaf, stem, stalk, petiole, shoot tip, cotyledon, protoplast, storage root, and tuber are susceptible to be infected and genetically transformed by *A. rhizogenes*, with the resulting production of hairy roots (Giri *et al*. 2000; Kuzovkina and Schneider 2006). However, the response varies depending upon the *A. rhizogenes* strain and its interaction with the plant species and tissue type (Schmidt *et al.* 2007; Saxena *et al*. 2007).

An important feature of *A. rhizogenes*-induced roots is their unique ability to grow *in vitro* in the absence of exogenous plant growth regulators (Rao and Ravishankar 2002). The use of *A. rhizogenes*-mediated transformation enables the development of transgenic plants via marker-free selection through use of hairy root morphology as the primary indicator of transformation (Christey 2001; Christensen *et al*. 2008). Due to high rates of co-transformation the genes of interest on the binary vector are likely to also be present. GUS and GFP reporter genes were used (Puddephat *et al*. 2000, Hughes *et al*. 2002; Azlan *et al*. 2002) to select transgenic root cultures, eliminating the need to use antibiotic resistance or herbicide resistance as marker genes. *A. rhizogenes* is confined to specific uses with potential applications for plant breeding and plant improvement including: root system alteration, use of *A. rhizogenes* and *rol* genes for altered phenotype, and the introduction of desirable foreign genes (Christey 2001). Transgenic plants, produced from genetic transformations using wild-type *A. rhizogenes* exhibited the distinct

phenotypes of curled leaves, shortened internodes, reduced apical dominance, reduced fertility, and plagiotropic roots, and were characterized as "hairy-root phenotype" (Tepfer 1984).

In some species, the difficulty in regenerating transgenic plants has been circumvented by development of rapid and efficient transformation protocols using *A. rhizogenes* to produce hairy roots on "composite" plants. Composite plants are plants with transformed root system, which produces hairy roots, the shoot portion remains untransformed. These composite plants have been used in studies focused on root characteristics such as nodulation in *Lotus japonicus* (Stiller *et al*. 1997; Martirani *et al*. 1999), soybean (Narayanan *et al*. 1999), and in barrel medic (Boisson-Dernier *et al*. 2001). Binary and co-integration vectors introduced into *A. rhizogenes* strains have been used in transformation of several plant species, including melon (Toyoda *et al*. 1991), petunia (Kiyokawa *et al*. 1992b), *Eustoma grandiflorum* (Handa 1996) and in *Pisum sativum* L. (Svabova and Griga 2008).

Semecarpus anacardium is a tree species which produces secondary metabolites of medicinal importance. The roots of this plant have been used in folk medicine as anti-fertility agent for women. There is no report on chemical aspect of the compounds present in roots. Induction of hairy root and establishment of hairy root culture could lead to development of a system to study the production of and identification of chemical compounds from *in vitro* raised roots in controlled environments. The present study was conducted to optimize conditions for establishment of hairy root culture of *S.anacardium*. Experiments were planned with the following objectives.

Experiments:

1. Establishment of hairy root culture using leaf explants and *A. rhizogenes* ATCC15834.
2. Testing virulence of *Agrobacterium rhizogenes* strains (A4 and LBA9402) and susceptibility of different explants leaves, stem (shoot without leaves) and shoot for optimization of compatibility of bacterial strain and explant type.
3. Confirmation of transformation by PCR using *rol* specific primers.

7.2 MATERIALS AND METHODS

Bacterial strains:

Three wild-type agropine strains of *A. rhizogenes*, ATCC15834 (harboring pRi15834), A4 (pRiA4) and LBA9402 (pRi1855), used for transformation. *A.rhizogenes* ATCC15834 were kindly provided by Prof. Sujata Bhargava (Department of Botany, University of Pune) for preliminary investigation of transformation procedure. *A. rhizogenes* A4 and LBA9402 were

later obtained from Prof. Sumita Jha (Center of Advanced Studies in Cell and Chromosome Research, Department of Botany, University of Calcutta, Kolkata).

Bacterial media:

Yeast Mannitol Broth (YMB) medium was used for maintenance of the bacterial culture.

Composition of YMB medium

Mannitol	10 g/l
Yeast extracts	0.4g/l
Sodium Chloride	0.1g/l
Magnesium Sulfate	0.2g/l
Potassium dihydrogen phosphate	0.5 g/l

pH of the medium was adjusted to 7.2 before autoclaving.

Bacterial growth:

Fresh bacterial culture was initiated from glycerol stock. The bacteria were streaked on nutrient YMB agar (15g/l) medium and incubated for 48hr at 27°C. Single colony of the *A. rhizogenes* was picked up and inoculated in 50ml of YMB nutrient broth. The culture was incubated in dark at 27°C on rotary shaker at 180–200rpm. The OD of the culture was checked intermittently using Spectrophotometer, till the OD_{600} was approximately one. These cultures were used for infecting different explants of *S.anacardium*.

Plant material:

Tissues from seedling derived *in vitro* shoot cultures of *S.anacardium* (Chapter 3) were used as explants. Isolated leaves, stems (shoots without leaves) and whole shoots were tested for co-cultivation with different strains of *A. rhizogenes* ATCC15834, A4 and LBA9402.

Infection and co-cultivation of explants:

Leaves, stems and shoots, were isolated from the axenic cultures and maintained in WPM medium with charcoal. The explants were transferred aseptically to 90mm petridishes containing 20ml of bacterial suspension. For experimental control culture the bacterial suspension was substituted with YMB medium. All the explants were pricked with the help of sterile hypodermic syringe needle. In the first experiment *A. rhizogenes* ATCC15834 was used to optimize infection time and period of co-cultivation with leaf explants. In second experiment, *A. rhizogenes* A4 and LBA9402 were tested along with *A. rhizogenes* ATCC15834 in conjunction with different explants including leaves, stems and shoots. The wounded explants were removed from suspension and blotted on filter paper. These were transferred to half strength semi solid WPM basal medium in petridishes, and co-cultivated in light for different periods of time ranging from 1 to 7 days. These explants from various time periods were then washed in antibiotic solution of

cefotaxime (Alkem, India) (400mg/l) to eliminate *A.rhizogenes* adhering to the surface of the explants. The explants were then blotted on filter paper to remove excess antibiotic solution and transferred to half strength WPM basal medium containing 400mg/l cefotaxime. Explants from the control cultures were also treated similarly even though there was no bacterial growth. Ten to fifteen explants were inoculated per plate with 3-4 plates per replicate. All the experiments were done in triplicate. Observations were recorded after 8wks of culture. Concentration of cefotaxime in semi solid media was halved each week and finally, cultures free of *A. rhizogenes* were transferred to half strength WPM basal medium without antibiotics. Explants with single root or root cluster were noted for transformation. The frequency of infection was also determined. DNA isolated from untransformed roots that were induced from shoot cultures as described earlier (Chapter 3) served as negative control for the putative transformed roots.

All cultures were incubated at 25±2ºC temperature in 16hr photoperiod under diffuse cool white fluorescent lights (50µmolm^{-2}s^{-1}). The observed data was subjected to Analysis of Variance (ANOVA). Graphs were plotted using Origin Version 6.1 software.

Stock solutions for plasmid DNA Isolation:

Solution A: 50mM Glucose, 10mM EDTA, 0.1% Triton X-100, 25mM Tris- HCl pH 8.0.

Solution B: 0.2M NaOH, 1% SDS (w/v).

Solution C: 5M Potassium acetate pH 4.8 was prepared by adding glacial acetic acid to 5M Potassium acetate until pH 4.8.

3M Sodium acetate pH 5.2: Prepared by dissolving 40.8gm sodium acetate in 100ml water and the pH was adjusted with 3M Acetic Acid.

RNAase A: Stock 10mg/ml.

DNA isolation:

Genomic DNA were extracted from the putatively transformed and untransformed roots (from shoot cultures) following DNA extraction protocol (Khanuja *et al.* 1999) described in Chapter 2.

Plasmid DNA isolation:

Plasmid DNA from the *A. rhizogenes* strains was isolated using standard alkaline lysis method (Sambrook *et al*. 1989).

1. About 1.5ml of overnight culture (16h) was taken in to 1.5ml eppendorf tube and pelleted out at 10,000rpm for 1min.
2. Supernatant was discarded and the pellet was re-suspended in 100µl of solution A by vortexing for five seconds.
3. The mixture was incubated in ice for 10min.

4. To the mixture, 200µl of freshly prepared solution B was added and the contents were mixed by gentle inversion. Again, the mixture was incubated on ice for 5min.
5. To this, 150µl of 5M Potassium acetate pH 4.8 was added and the contents were mixed by vortexing.
6. The mixture was centrifuged at 10,000 rpm for 5min and the supernatant was retained in another eppendorf.
7. To the above mixture 3µl of RNAase was added and the mixture was incubated at 37°C for 15min.
8. To this, 500µl of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added, the contents were mixed by inversion and centrifuged at 10,000rpm for 10min.
9. The supernatant was collected in another eppendorf and 40µl of 3M Sodium acetate pH 5.2 and 1.0ml of cold (-20°C) absolute ethanol were added. The contents were mixed and incubated at -20°C for 1h.
10. The mixture was centrifuged at 12000 rpm for 15min and the pellet was washed with 70% cold (-20°C) ethanol and air dried at room temperature.
11. Pellet was re-suspended in 100µl of sterile deionized water.

For visual estimation of quantity, plasmid DNA (5µl) was loaded in a 0.8% agarose gel. Quantitative estimation was also done with spectrophotometer by reading absorption at 260nm. Purity of DNA was tested by ratio of absorption values at 260nm / 280nm.

Primers used for screening of rol genes:

Transformants were screened for presence of *rol A, B* and *C* (genes using the sequence specific primers. The primers were synthesized by MWG-Biotech, Bangalore, India.

For r*ol A* gene: For- 5′-CAGAATGGAATTAGCCGGACTAA-3′
Rev- 5′-CGTATTAATCCCGTAGGTTTGTTT-3′

For *rol B* gene: For- 5′-ATGGATCCCAAATTGCTATTCCCCCACGA-3′
Rev- 5′-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3′

For *rol C* gene: For- 5′- CATTAGCCGATTGCAAACTTG -3′
Rev- 5′- ATGGCTGAAGACGACCTG -3′

PCR condition:

The PCR reactions were carried out in a total 50µl volume and consisted of 40ng of DNA, 10pm/µl primer, 200mM dNTP, 1U of Taq DNA polymerase, 1X PCR buffer and 1.5mM $MgCl_2$. DNA amplifications were performed in a thermal cycler (Veritti thermal cycler, Applied biosystems) using the programme: initial denaturation at 94ºC for 5min, followed by 38 cycles of 94ºC for 1min, 52.5ºC (for *RolB* gene)/62ºC (for *RolA* and *C* gene) for 1.5min and 72ºC for 2min

and a final extension at 72ºC for 10min. The amplification products were visualized on 1.5% w/v agarose gel stained with ethidium bromide (0.5µg/ml).

7.3 RESULTS AND DISCUSSION

The factors that influence the successful transformation of a plant tissue and hairy root induction include the genotype, species, age, type of plant tissue (Sevon and Oksman-Caldentey 2002), the type of *Agrobacterium* strain and the density of the bacterial suspension (Park and Facchini 2000). A number of chemicals may also promote these processes, e.g., acetosyringone (Joubert *et al*. 2002).

7.3.1 Infection of leaf explants with *A. rhizogenes* ATCC15834.

Optimum transformation frequency of 61% was noted in the explants infected for 30 minutes in the bacterial suspension **(Table 7.1)**. Explants with 10 minutes of infection did not show any transformation except on 4th day of co-cultivation. Varying percentages of transformation was observed in 20 minutes of infection, but was less in comparison to 30 minutes of incubation **(Table 7.1)**. Different researchers have reported effect of infection time on transformation frequency using *A. rhizogenes*. It varies from plant to plant. Five minutes of infection of the wounded explants was effective in inducing hairy roots in *Datura tatula* L. (Peng *et al*. 2008), *Papaver bracteatum* Lindl. (Rostampour *et al*. 2009) and in *Echinacea sp* (Romero *et al*. 2009). Whereas, in *Silybum marianum* (Rahnama *et al*. 2008) ten minutes of infection was optimum. In *Artemisia annua* it was 20 minute (Giri *et al*. 2001) and in *Glycine max* 45 minute of infection was required (Liu *et al*. 2008) to get successful transformation.

Table 7.1 Effect of infection time on transformation frequency (%) of *Agrobacterium rhizogenes* ATCC15834 with leaves as explants.

Days of Co-cultivation	**Transformation frequency (mean ±sd) %**		
	Time duration of Infection.		
	10min.	**20min.**	**30min.**
1	00±00 (135)	00 (115)	00±00 (130)
2	00±00(125)	23±1.4(106)	35±2.12(140)
3	00±00(98)	28±4.12(106)	49±3.5(128)
4	7±00(122)	39±2.5(123)	61±2.8(110)
5	00±00(140)	25±2.8(90)	53±2.12(95)
6	00±00(102)	15±2.12(145)	42±4.2(145)
7	00±00(110)	7±1.4(120)	29±2.12(116)
ANOVA	------	S1%	S1%

Figures in parenthesis ( ) Indicates number of replicates.

In *Semecarpus anacardium* varying transformation frequency was observed when the leaf explants were co-cultivated for different periods. Optimum transformation was achieved after 4 days of co-cultivation on growth regulator free medium **(Table 7.1)**. In *Rhmannia glutinosa*

(Hwang 2009) the co-cultivation period was one day to achieve 46.7% transformation. Two days of co-cultivation was effective to obtained 59% of transformants in *Saponaria vaccaria* L. (Schmidt *et al*. 2007), 77% in apple root stock Jork 9 (Pawlicki-Jullian *et al*. 2002), and 75% of transformants in *Arachis hypogaea* L. (Kim *et al*. 2008). *Silybum marianum* (Rahnama *et al*. 2008) *Tylophora indica* (Chaudhuri *et al*. 2005) *Gmelina arborea* Roxb (Dhakulkar *et al*. 2005) needed three days of co-cultivation to get optimum transformation frequency of 30, 60 and 32% respectively using *Agrobacterium rhizogenes* strains. In the present study there was no root formation in control leaf explants treated with bacterial free YMB medium (**Fig. 7a)**. However, the leaf explants co-cultivated with *Agrobacterium rhizogenes* for 3-4 days showed successful root induction after 25-30 days of culture in antibiotic supplemented medium over 3 passages of 7 days each **(Fig. 7b)**.

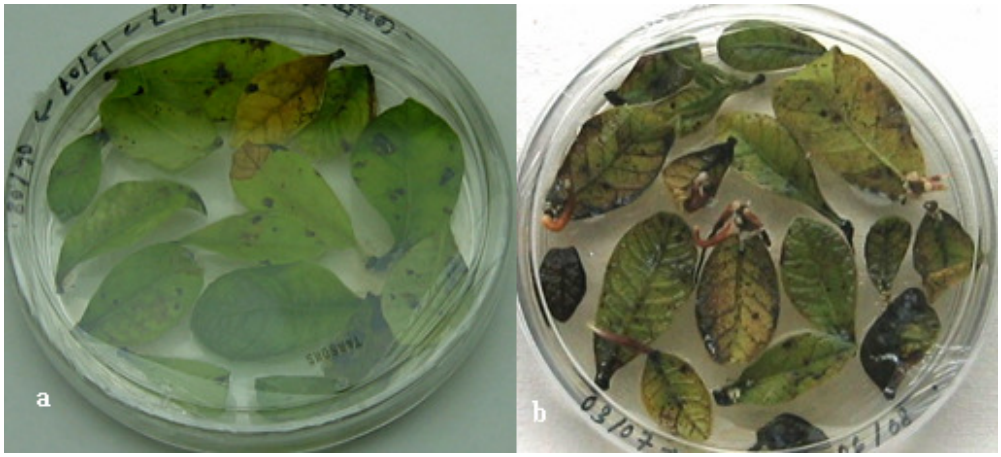


Fig. 7 a. Control leaves explants in half strength WPM medium without any root development after 8wks of culturing in antibiotic incorporated medium; **b.** Induction of root in leaves infected with *Agrobacterium rhizogenes* after 3-4wks of culturing in half strength WPM medium containing antibiotics.

Root induction was mostly observed in leaves of *Semecarpus* injured along the midrib. Differentiation of roots from the wounded sites in midrib region of leaf was associated with callusing (**Fig. 7c)** after 30 days of infection.

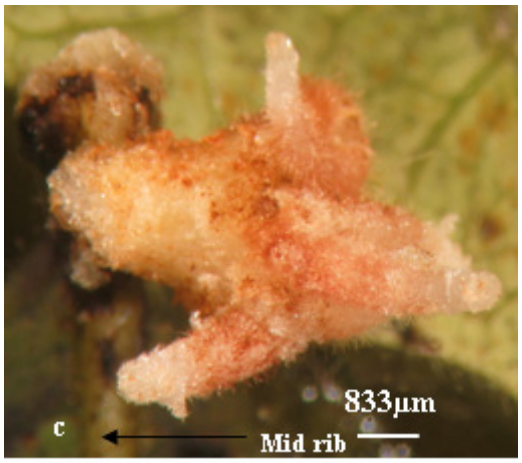


Fig. 7 c. Initiation of hairy root like structure originating from the mid rib region of leaf explants in half strength WPM medium containing antibiotics.

Callusing response from the wounded sites of seedling explants of California poppy (Park and Facchini 2000) and nodes/internodal explants in *Tylophora indica* has been previously reported (Chaudhri *et al*. 2005). Hairy root development was confined to the wounded site of different explants of *Rubina tinctorum* (Ercan *et al*. 1999), *Papaver somniferum* (Park and Facchini 2000) and in *Arachis hypogaea* (Kim *et al*. 2008). It has been reported that cell division in the host target explants tissue is a prerequisite for successful *Agrobacterium* transformation (Binns and Thomashow 1988). Roots with hairy structures elongated and callusing at the base of the roots was observed in some of the cultures **(Fig. 7d)**. Thin slender roots with root hairs developed from the clusters of roots on culturing in GR free half strength WPM medium **(Fig. 7e)**. Transformed roots grew in cluster from the midrib region of the leaves after culturing in half strength WPM basal medium without antibiotics for 12wks **(Fig. 7f)**.

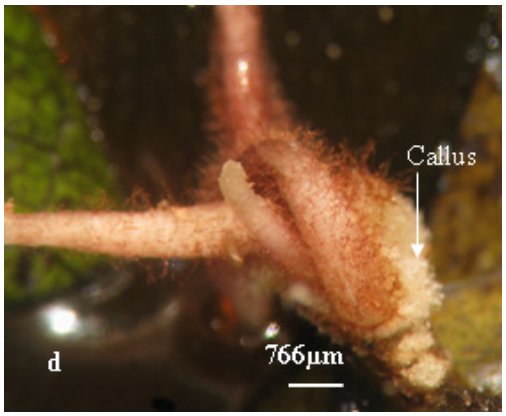


Fig. 7 d. Cluster of roots with fine root hairs arising from the leaf explants in half strength WPM medium containing antibiotic**;**

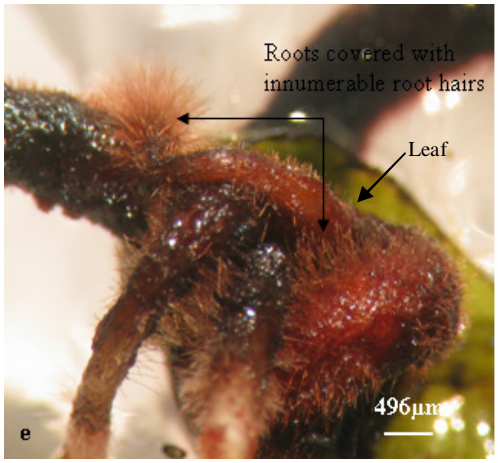


Fig. 7 e. Magnified view of hairy roots initiated from leaf.

Fig. 7 f. Cluster of hairy roots showing growth and elongation from leaf explants infected with *Agrobacterium rhizogenes* cultured in half strength WPM medium.

7.3.2 Virulence of different *Agrobacterium* strains (A4, ATCC15834 and LBA9402) and susceptibility of explants (Leaf, stem and shoot).

Experiment was extended to determine the optimum co-cultivation period and efficacy of different bacterial strains to induce hairy roots in different explants. Varying root induction with different period of co-cultivation was observed in leaf explants infected with the three strains of *Agrobacterium rhizogenes.* In the leaf explants of *S.anacardium* optimum rooting frequency was 62±2.12%, infected with strain ATCC15834 and co-cultivated for 4 days. This result is in accordance with earlier observation of transformation in leaves as explants. Strains A4 and LBA9402 induce rooting in 59±4.94% and 42±12.02% of explants respectively **(Table 7.2)**.

Table 7.2 Effects of co-cultivation duration and bacterial strains on transformation frequency (%) of different explants.

Bacterial Strains	**Transformation frequency (mean ±sd) %**						
	Days of co-cultivation						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Leaf explants							
Control	00±00 (96)	00±00 (112)	00±00 (110)	00±00 (133)	00±00 (123)	00±00 (98)	00±00 (129)
A4	00±00 (98)	32.5±11 (120)	47±8.9 (123)	58.5±5 (128)	46.5±9 (112)	30±4.24 (120)	27±4.3 (142)
ATCC15834	00±00 (96)	30±4 (116)	51.5±2.2 (148)	61.5±2 (132)	53±2 (134)	40±2.82 (143)	28±2.8 (114)
LBA9402	00±00 (98)	31±11 (125)	41.5±11 (120)	41.5±12 (147)	35.5±4 (125)	31±9.19 (150)	23±9.89 (114)
ANOVA		NS	S5%	S1%	S1%	S1%	S1%
Stem explants							
Control	00±00 (96)	00±00 (106)	00±00 (112)	00±00 (132)	00±00 (95)	00±00 (96)	00±00 (124)
A4	00±00 (90)	31±18 (103)	32.5±11 (113)	45±7 (134)	52±3 (96)	40±3 (95)	39±1.4 (123)
ATCC15834	00±00 (93)	35±3 (123)	43.5±4 (120)	52±3 (122)	60±9 (103)	44±3 (116)	37±5.6 (143)
LBA9402	00±00 (92)	32±10 (121)	29.5±12 (94)	39±2 (110)	42±1 (107)	35±4 (112)	29±8.4 (134)
ANOVA		NS	S5%	S1%	S1%	S1%	S1%
Shoot explants							
Control	00±00 (112)	00±00 (116)	00±00 (128)	00±00 (134)	00±00 (102)	00±00 (122)	00±00 (136)
A4	00±00 (122)	20±10 (110)	37±28.8 (135)	49±3 (121)	39±11 (112)	41±17 (127)	44±3.6 (98)
ATCC15834	00±00 (124)	32±3 (123)	43±15.3 (138)	65±10 (118)	67±3 (134)	63±12 (146)	58±8 (95)
LBA9402	00±00 (113)	17±8 (116)	27±15.3 (121)	36±5.7 (118)	33±7.6 (126)	32±8 (133)	27±12 (104)
ANOVA	-----	S1%	S1%	S1%	S1%	S1%	S5%

Figure in parenthesis indicates number of replicates

Induction of hairy roots in stem explants was lower (52%) compared to leaves (62%) after 4 days of co-cultivation. The rooting frequency increased to 60±9% in stem explants after 5 days of co-cultivation. Like leaf explants, stem explants also exhibited higher rooting as well as the transformation frequency when infected with strain ATCC15834. Transformed roots originated

in cluster from stem explants cultured in half strength WPM medium without GR (**Fig. 7g**). These roots grew slowly and covered the surface of solid media in petriplates **(Fig. 7h)**.

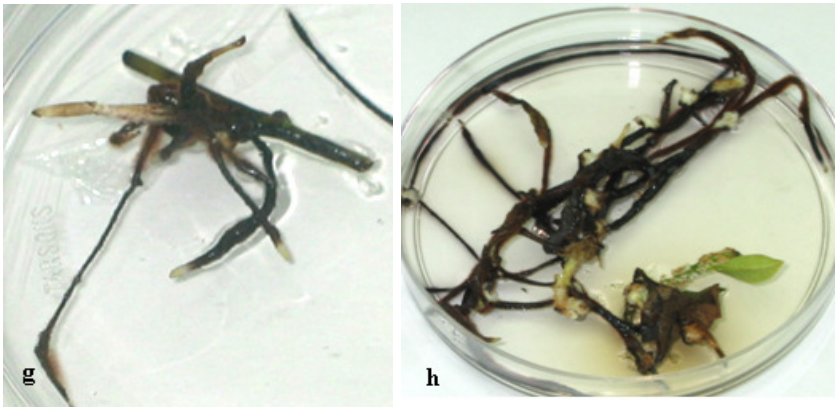


Fig. 7 g and **h**. Initiation and growth of hairy roots from stem explants cultured in half strength WPM medium.

Optimum hairy root induction of 67±2.3 % in shoot explants was noted after 5 days of co-cultivation with *A. rhizogenes* strain ATCC15834. Whereas, 65% of root initiation was observed in cultures co-cultivated for 4 days. Transformation frequency with A4 and LBA9402 strains was 49±2.8% and 36±5.7% respectively in shoots co-cultivated for 4 days. *A. rhizogenes* strain ATCC15834 was found to be more virulent in all the three explant types when compared to other strains. Roots in clusters **(Fig. 7 i, j and k)** arose from the wounded sites of intact leaves of shoots. The root induction sites were mainly concentrated in the midrib region of leaves **(Fig. 7k).** Such rooting from the veins in leaf of *Pueraria phaseoloides* infected with *A. rhizogenes* ATCC15834 was previously reported (Shi and Kintzios 2003). *A. rhizogenes* strain ATCC15834 was more potent in infecting cotyledon explants of *Rubia tinctorum* (Ercan *et al*. 1999) in comparison to strains 2628, R1000 and 9365.

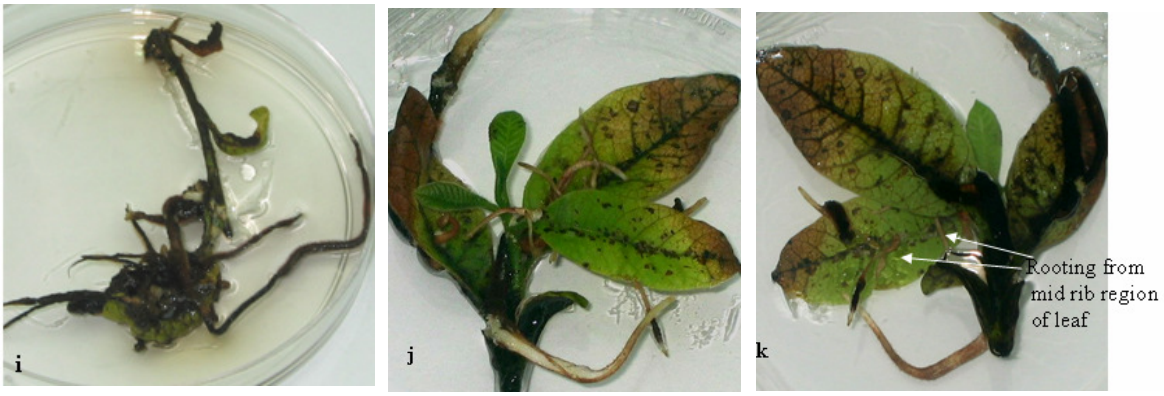


Fig. 7 i, j. Hairy roots in stem explants, these roots originate mostly from leaves attached to shoot explants infected with *A.rhizogenes* in half strength WPM medium**; k.** Emergence of hairy roots from the mid rib region of leaf explants attached to shoot.

To summarize the data (**Fig.71**) generated in this experiment using three explants and the three *A.rhizogenes* strain, it is obvious that ATCC15834 is the best strain and the shoot is the best explant.

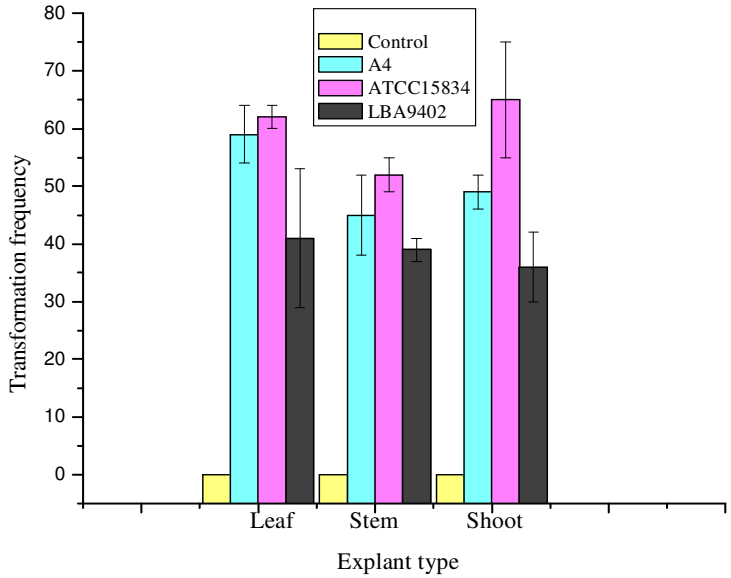


Fig. 7 l. Comparison of different *A. rhizogenes* strain and explant type after 4 days of co-cultivation periods on transformation frequency of *Semecarpus anacardium.*

The optimum transformation frequency in the shoot explants may be ascribed to the presence of intact leaves in addition to the stem. Shoots are the explants of choice for hairy root induction by *A. rhizogenes* in apple rootstock Jork 9 (Pawlicki-Jullian *et al*. 2002), *Papaver bracteatum* Lindl. (Rostampour *et al*. 2009), and in *Tylophora indica* (Chaudhuri *et al*. 2005). Virulence or infectivity of *Agrobacterium* strains varies among plant hosts and explant type (Hobbs *et al*. 1989; Bush and Pueppke1991; Baranski *et al*. 2006). In *Gmelina arborea* Roxb optimum hairy root induction was observed in cotyledon explants from 5-day-old aseptically germinated seedling, co-cultivated with *A. rhizogenes* ATCC15834 (Dhakulkar *et al*. 2005). Saxena *et al*. 2007 noticed high frequency of hairy root induction in leaf explants compared to internodes and petioles of *Pelargonium sp*. The transformation efficiency of plant species can vary between different bacterial strains (Godwin *et al*. 1991; Hu and Alfermann 1993; Kuzovkina) and optimum hairy root initiation was reported in shoot tips of *Artemisia annua infected* with *A. rhizogenes* strain LBA9402 than strain A4, ATCC15834, 9365 and 9340 (Giri *et al*. 2001). Similarly, in *Saponaria vaccaria* L. *A. rhizogenes* strain LBA9402 found to be the potent strain than ATCC15834 in infection leaf explants (Schmidt *et al*. 2007). The strain *A. rhizogenes* A4 is reportedly most potent for dicotyledonous plants (Kuzovkina and Schneider 2006). In leaf explants of *Pelargonium sp* 100% transformation frequency was reported with strain A4 (Saxena *et al*. 2007). In all the cultures roots thickened and dedifferentiated in to callus on further

culturing in semi-solid medium **(Fig. 7 m, n and o)**. Shorter and thicker root development has been observed in apple rootstock jork9 with *A. rhizogenes* ATCC15834 (Pawlicki-Jullian *et al*. 2002).

Fig. 7 m, n and o. Hairy roots dedifferentiated in to callus during culturing after limited growth in solid half strength WPM medium.

The hairy roots on transferring to half strength liquid medium elongated. But the elongation rate was very slow in thicker roots (**Fig. 7p**). After 3-4 passages in liquid medium thin hairy roots started differentiating slowly which were separated from the original explants (**Fig. 7q**). Visible growth of the roots was observed in liquid medium after 2-3 months of culturing in liquid WPM medium (**Fig.7 r and s**). The slow growth could be due to presence of excess growth inhibitory phenolics in the culture or due to slow growth of *Semecarpus anacardium* L. *in vitro*.

Fig. 7 p. Cluster of hairy roots elongating slowly after culturing in half strength liquid WPM medium, roots are still attached to leaf explant**; q.** Hairy roots growing slowly in liquid media without the original explant.

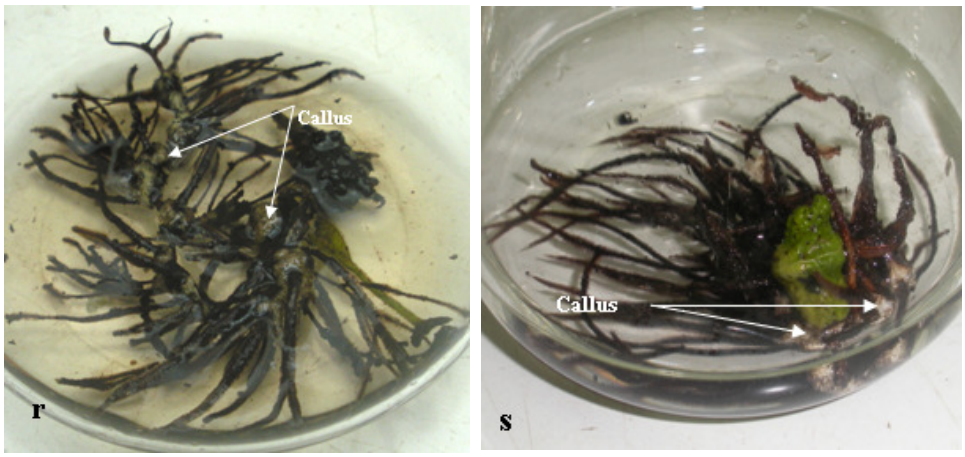


Fig. 7 r. Profuse branched hairy roots in liquid medium with callusing and leaching of phenolics in to the medium**; s.** Hairy roots culture in liquid medium, the original leaf explant was still attached, with dedifferentiation of the roots in to callus.

The browning of the culture medium was indicative of leaching of phenolics **(Fig. 7t)**. Further studies are required to reveal the nature of phenolics compounds. Callus formation from the roots in liquid medium was noted, similar differentiation of the roots was already observed in semisolid medium. Callusing in hairy roots cultured in GR free medium was reported in *Datura tatula* L. (Peng *et al*. 2008) and was assigned due to high capacity of the tissue to dedifferentiate resulting in callus formation. Large numbers of *A.rhizogenes* mediated transformants were produced in *Semecarpus anacardium* L. but only very few of those gave rise to hairy root structures.

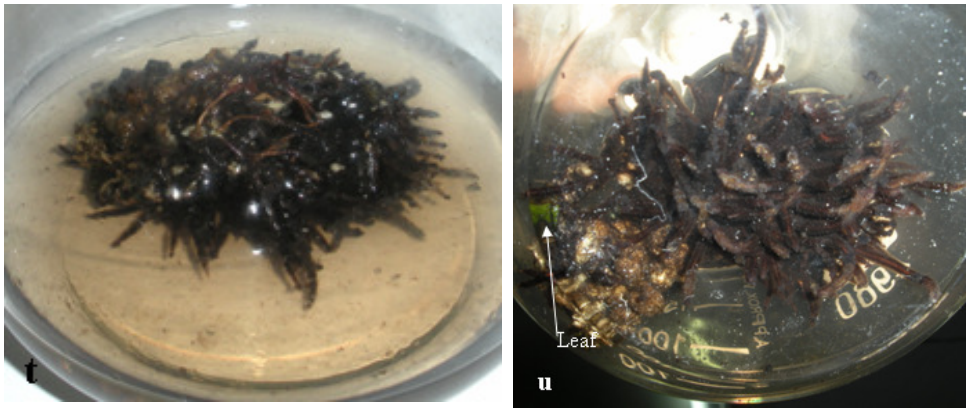


Fig. 7 t. Clump of hairy roots cultured in liquid medium, the medium become brown due to leaching of phenolics compound**; u.** Profuse callusing can be observed from the origin of hairy roots, the original leaf explant was still attached.

7.3.3 *Confirmation of transgenic status*

However, due to the plasticity of the plant cells roots could be induced in explants in condition other than that of transformation. Therefore, efforts were made to confirm the presence of *rol* genes using PCR. Confirmation of transgenic status of the tissue was done by the PCR amplification of the DNA isolated from the hairy roots using forward and reverse primers of *rol*

genes (*rolA*, *rolB* and *rolC*). Plasmids from A. *rhizogenes* served as the positive control and DNA from the non-transformed roots of shoot culture, served as the negative control. All transformants (infected with different strains) showed presence of the *rolA* (300bp) **(Fig. 7u)**, *rolB* (780bp) **(Fig. 7v)** and *rolC* (590bp) **(Fig. 7w)** in DNA amplified product confirmed the transgenic nature of the hairy root lines.

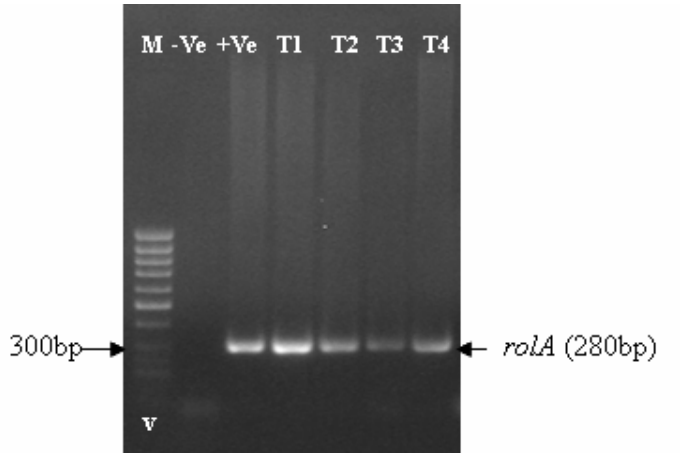


Fig. 7 v. PCR amplification of 280bp fragment of the *rolA* gene. Lane 1 = molecular weight marker (100bp ladder); lane 2 = DNA from non-transformed roots (negative control); lane 3 = *A. rhizogenes* plasmid DNA (positive control); lane 4 = sample 1; lane 5 = sample 2; lane 6 = sample 3; lane 7 = sample 4. Samples 1, 2, 3 and 4 were DNA of transformed roots obtained after *A. rhizogenes* infection.

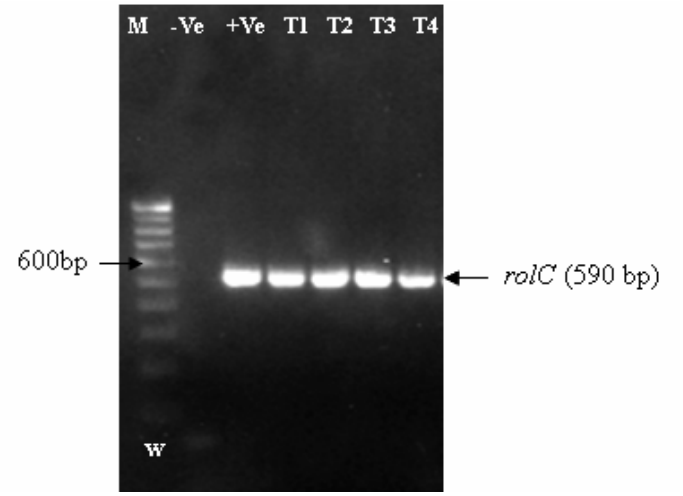


Fig. 7 w. PCR amplification of 590bp fragment of the *rolC* gene. Lane 1 = molecular weight marker (100bp ladder); lane 2 = DNA from non-transformed roots (negative control); lane 3 = *A. rhizogenes* plasmid DNA (positive control); lane 4 = sample 1; lane 5 = sample 2; lane 6 = sample 3; lane 7 = sample 4. Samples 1, 2, 3 and 4 were DNA of transformed roots obtained after *A. rhizogenes* infection.

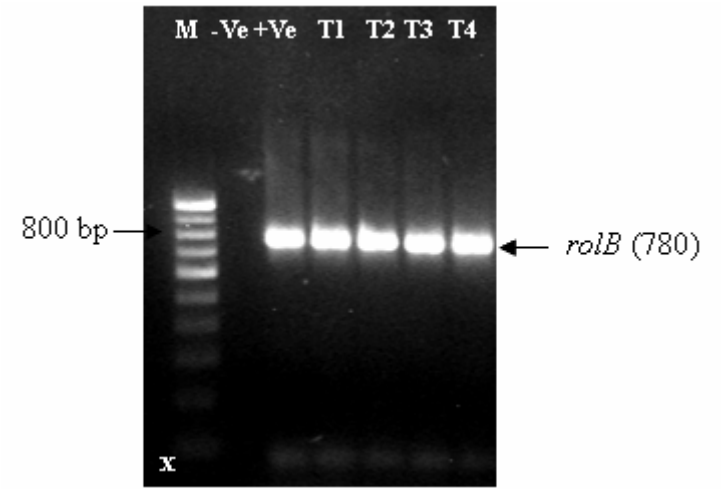


Fig. 7 x. PCR amplification of 780bp fragment of the *rolB* gene. Lane 1 = molecular weight marker (100bp ladder); lane 2 = DNA from non-transformed roots (negative control); lane 3 = *A. rhizogenes* plasmid DNA (positive control); lane 4 = sample 1; lane 5 = sample 2; lane 6 = sample 3; lane 7 = sample 4. Samples 1, 2, 3 and 4 were DNA of transformed roots obtained after *A. rhizogenes* infection.

7.4 CONCLUSION

In the present investigation hairy root cultures were established from an important medicinal woody tree *Semecarpus anacardium*. Induction of hairy root occurs at frequency of 67% and 62% in shoot and leaf explants respectively. There was a significant difference in hairy root induction with respect to the type of explants and bacterial strains used. Shoot was found to be the explant of choice for hairy root induction compared to stem and leaves. Among the bacterial strains tested ATCC15834 was found to be the most virulent than A4 and LBA9402. These are some of the preliminary findings regarding hairy root culture for *Semecarpus anacardium*. Further research needs to be done for isolation, identification and scaling up of novel secondary metabolites in hairy root cultures.

SUMMARY

Summary

Semecarpus anacardium L. is a tree species described in Ayurveda as a potent source of drug against variety of ailments (Nadkarni, 1976). The fruit nut of *Semecarpus* contains number of alkaloids, flavonoids, biflavonoids, and other compounds (Rao *et al*., 1973). Various formulations of the nut are used against many diseases like atherosclerosis adjuvant arthritis and hepatocellular carcinoma. Antitumour activity of nut extract has been proven. Other proven uses of *S.anacardium* L. nut are, its cytotoxic activity in cancer, anticholesterol activity, antihelminthic action, syphilis and a cure for neurological disorders. Exudates of stem bark are valuable for scrofulous, veneral and leprous infection. The resinous liquid from nut can be used in lacquers, varnishes, enamels, tanning materials and ion exchange resins (Wealth of India Vol. IX, 1999). The kernel of nut contains 20-25% sweet semidrying non-edible oil (Database of oil yielding plants, BSI 2004). Oil used as wood preservative and as lubricant. The suitability of this oil for production of biofuel is not explored. The kernel of the seed contains a phenolic glycoside known as anacardioside. Seeds of *Semecarpus* have poor viability, in terms of percentage of germination and loss of viability on storage (Wealth of India, Vol. IX 1999).Available literature on *S.anacardium* L. is restricted to chemical and medicinal aspects and there is no literature related to *in vitro* regeneration in this plant. There is need for *in vitro* regeneration protocols for micropropagation, *in vitro* conservation, chemo-biodiversity assessment, and genetic manipulation of this medicinally important oil producing plant. *S.anacardium* L., which represent a substantial variability in phenotype as well as chemo type properties for the oil and its components, need to be studied for establishing phylogenetic relationships and unique marker profiles at DNA level. Till date, there is no literature available regarding molecular studies of this tree.

Objectives of the study:

"Studies on *S.anacardium* L. for *in vitro* regeneration and identification of biologically active compounds" was taken up with the following objectives:

(1) Standardization of protocol for clonal propagation from seedling explants.
(2) Optimization of conditions for micropropagation of mature trees.
   (a) Genetic evaluation of mature trees in natural stands.
   (b) Micropropagation of trees and genetic fidelity of the propagules.
(3) Standardization of reproducible protocols for *de novo* morphogenesis.
(4) Protocols for genetic transformation using *Agrobacterium rhizogenes* to establish hairy root culture.
(5) Studies on isolation, purification and quantification of some of the chemical compounds.

Microbe-free seedlings *of S. anacardium* L. could not be raised *in vitro* due to poor seed viability, acute seed-borne microbial contamination, and leaching of phenolics. Seed viability

(30-35%) reduced with time and was completely lost in 6 months. Soaking seeds in concentrated sulfuric acid (H_2SO_4) helped in eliminating the pigmented layer from the surface of the seeds thereby making way for the phenolics to be released from seeds in washings. Thus leaching of phenolics by the seeds in culture medium was reduced by treating them with H_2SO_4 prior to surface sterilization with NaOCl. Changes in the seed surface following acid treatments for varying periods (10, 20, 30, 40 min) were studied microscopically. Concentrated H_2SO_4 treatment for 20 min helped to increase the seed coat permeability and excretion of phenolics from the seeds. It also acted as a surface sterilant to a limited extent. Frequency of germination was increased to 63% when seeds were treated with H_2SO_4 for 20 min while exposure for 10 min was ineffective in controlling contamination whereas longer exposures (30 and 40 min) injured the embryos. Elimination of sucrose in the medium improved germination from 50 to 63%. A gradual loss of seed viability from 33 to 4% following storage for 5 months was demonstrated **(Seedling culture of *Semecarpus anacardium* L. - Bhuban Mohan Panda and Sulekha Hazra (2009) *Seed Science and Biotechnology* 3(2) 54-59).**

A protocol for *in vitro* regeneration of this plant has been standardized using nodal buds of seedling derived axenic culture. Proliferation of shoots from axillary meristem was achieved in WPM medium supplemented with BAP and KN. Factors including basal salt formulation, carbon source, gelling agents, antioxidants and culture vessels, were identified for optimum growth of shoots. *In vitro* raised shoots were rooted in half strength WPM liquid medium with auxins. Indole butyric acid (IBA) at 2.46µM gives 100% rooting. Survival percentage of the plants in green house was 91%. *Ex vitro* rooting was achieved in 80% of the explants treated with Seradix B mixture **(*In vitro* regeneration of *Semecarpus anacardium* L. from axenic seedling derived nodal explants. - Bhuban Mohan Panda and Sulekha Hazra – Revised and submitted).**

In order to establish an efficient protocol for clonal propagation of this species, axenic shoot culture derived nodal explants were cultured in media with thidiazuron (TDZ). Shoot differentiation from axillary meristem was limited and the meristem swelled to form a meristematic mass in higher concentrations of TDZ. Swelling of the meristem was attributed to proliferation of the meristematic cells. Development of shoots from the meristematic mass on withdrawal of TDZ from medium indicated inhibitory influence of TDZ on shoot differentiation. Additional shoots appeared and elongated on repeated transfer of the TDZ pre-treated explants in medium devoid of growth regulator. The meristematic mass developed in TDZ, gave rise to additional shoots in every passage of 4 weeks each in growth regulator free medium. Total number of shoots was optimum in explants pre-cultured in TDZ 2.27µM. The study confirms the stimulatory influence of TDZ on proliferation of meristematic cells and inhibitory influence on shoot differentiation. All elongated shoots, rooted in medium with IBA and hardened plants

survived in soil (***In vitro*** **regeneration of** ***Semecarpus anacardium*** **L. from seedling derived axenic culture using Thidiazuron. - Bhuban Mohan Panda and Sulekha Hazra - Revised and submitted).**

Mature trees of *S.anacardium* L. were identified from 5 locations. Variation in structure of pseudo-fruits, maturation time of nut and shape of nut was noticed. Keeping in mind these variations may be linked to the genetic make up of the plant, experiments using ISSR primers was conducted for molecular characterization of the plants. DNA was isolated from the tender leaves of these plants and PCR amplification of the DNA was performed using 100 ISSR primers. Fifteen primers show good amplification and polymorphism. Analysis of the ISSR-PCR data demonstrated high degree of variation within the trees. This observation advocates the need for reliable regeneration protocol for rapid propagation of elite genotypes.

Mature tree derived nodal buds were collected during the month of April, May and June. Like in seeds, the major constraint encountered in mature tree derived tissues was to obtain microbe free explants for culture. After extensive trials, a procedure involving fungicide, antibiotic and sodium hypochlorite treatments was standardized to obtain 50-70% microbe free explants. Mature tree derived defoliated twigs containing 6-8 nodal buds were surface sterilized and aseptically cut into single nodal explants and cultures in different growth regulators containing medium. Percentage of microbe free culture was very less when single nodal explants were surface sterilized by the above procedure. Four basal media formulations including MS, SH, B5 and WPM were tested. WPM basal media was superior for mature bud culture of *S.anacardium.* Bud-break in mature plant derived nodal cuttings was observed after 7-15 days of culture in WPM medium and with growth regulators singly and in combinations. Media containing TDZ were more effective. Explants pre-cultured in TDZ, sprouted producing multiples buds on repeated culturing in growth regulator free WPM medium containing 0.2% of charcoal. These buds differentiated and elongated in growth regulator free WPM medium containing antibiotics for 4 passages of 4wks each. Similar to the seedling culture derived shoots (Chapter 3), rooting was induced in half strength liquid WPM medium with IBA. Rooting was 100% in medium with 7.38 µM IBA. Rooted shoots survived in sand: soil mixture and the hardened plants were transferred to green house. Fifteen clonally propagated plants from a single mature tree were analyzed for clonal fidelity using the selected ISSR primers as described in section (a). All the clones show similar banding pattern. **The data generated is being compiled for publication.**

Surface sterilized nuts were cut and the cotyledons were separated. These were cultured in WPM basal medium supplemented with varying concentrations of growth regulators. Caulogenic buds and embryogenic mass like structures appeared on the surface of the cotyledons in TDZ containing media. The caulogenic buds differentiated to form shoot primordia and shoot in GR

free WPM with charcoal (0.2%). Differentiation of the buds to shoot was slow and needed 4-5 passages of four weeks each to achieve the height of 3-4cm. The shoots rooted in half strength liquid WPM with IBA. Plantlets were hardened and transferred to green house. Histological studies confirmed the *de novo* origin of the caulogenic buds.

In TDZ containing medium, caulogenic response in the cotyledon explants was associated with formation of well-defined globular cell masses and calli. Bipolar and heart shaped embryo like structures appeared from these globular masses on culturing in WPM medium with charcoal for several passages. Callus formed on the cotyledon explant was compact and gave rise to small embryo like structures after several passages in WPM medium with charcoal. Efforts made to convert these embryo-like structures to plantlets remained futile. These structures either dedifferentiated into callus or gave rise to more embryos like structures in each passage in growth regulator free medium. **Manuscript being compiled for publication.**

Presence of Amentoflavone in the mature leaves of *S.anacardium* L. has been reported (Ishratulla *et al*., 1977). Amentoflavone, a biflavonoids having potential anticancerous, neuroprotective, antiviral and anti-inflammatory activity was detected in the leaves of the plants maintained *in vitro*. Cotyledon-derived callus tissue also produces Amentoflavone. The biflavnoid was not detected in roots and stems of *in vitro* raised plants. Increased Amentoflavone was detected in the shoot cultures maintained in 4% sucrose containing medium. Among the carbon sources tested, maltose at 2% produced more amentoflavone.

Experiments were conducted to optimize parameters for *Agrobacterium rhizogenes* mediated transformation. Three *Agrobacterium rhizogenes* strains (ATCC15834, LBA9402, A4) were tested for transformation of *S.anacardium* L. Experiments were conducted using leaves, internodal segments and *in vitro* raised shoot explants. Surface of the explants was pricked repeatedly with a sterile needle, keeping the explant in bacterial suspension. Rhizogenesis was observed in explants co-cultured with strain ATCC15834 for 30 minutes for infection followed by co-cultivation for 4 days. Root induction was noted after 25-30 days of culture in antibiotic containing medium. Roots developed singly/cluster from the site of infection. Shoots were more susceptible to *Agrobacterium rhizogenes*. DNA was isolated from the roots. Integration of *rol* genes (A, B and C) was confirmed by PCR. The growth of the hairy roots was slow.

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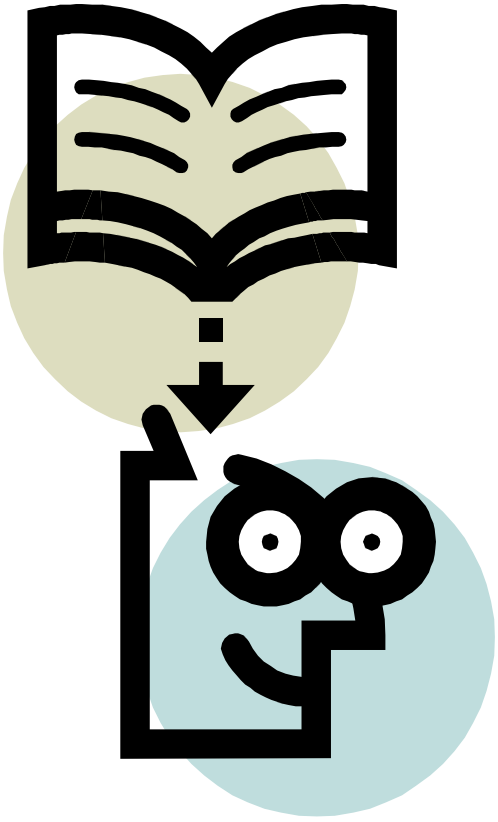

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AUTHOR'S PUBLICATIONS

RESEARCH WORK PUBLISHED / COMMUNICATED

1. *In vitro* regeneration of *Pongamia pinnata* via *de novo* organogenesis. K. Sujatha, **B.M. Panda** and Sulekha Hazra (2005). 'National Symposium on Plant Biotechnology: New Frontiers', November 18-20, 2005 held at CIMAP, Lucknow. Presented as poster. Full length paper published in the Proceedings pp. 312-315.

2. *De novo* organogenesis and plant regeneration in *Pongamia pinnata,* oil producing tree legume. K. Sujatha, **B.M. Panda** and Sulekha Hazra. ***Trees- structure and function*** (2008) 22:711–716.

3. Seedling culture of *Semecarpus anacardium* L. **Bhuban Mohan Panda** and Sulekha Hazra.***Seed Science and Biotechnology* (2009) 3(2) 54-59.**

4. *In vitro* regeneration of *Semecarpus anacardium* L. from axenic seedling derived nodal explants. **Bhuban Mohan Panda** and Sulekha Hazra, **revised and submitted.** ***Trees-structure and function.***

5. *In vitro* regeneration of *Semecarpus anacardium* from seedling derived axenic culture using Thidiazuron. **Bhuban Mohan Panda** and Sulekha Hazra. **revised and submitted.** ***Canadian Journal of Forest Research*.**

POSTERS / ORAL PRESENTATIONS

1. K. Sujatha, **Bhuban M. Panda** and Sulekha Hazra (2005) "*In vitro* regeneration of *Pongamia Pinnata* via *de novo* organogenesis". Poster presented and Abstract published during '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.

2. K. Sujatha**, Bhuban M. Panda** and Sulekha Hazra (2006) "*In vitro* regeneration of *Pongamia pinnata* via *de novo* organogenesis". A poster presented on National Science Day at National Chemical Laboratory, Pune.

3. **Bhuban M. Panda** and Sulekha Hazra (2007) "*In vitro* regeneration of *Semecarpus anacardium*". A poster presented on National Science Day at National Chemical Laboratory, Pune.

4. **Bhuban M. Panda** and Sulekha Hazra. Oral presentation on Tissue culture of *S. anacardium* (2007*)* In National Seminar on New trends in Biotechnology, 11-12 January 2007, Organised by Department of Biotechnology at Dhempe College of Arts and Science, Goa. Abstract published in Proceedings.

5. **Bhuban M. Panda,** K. Sujatha, Urmil J. Mehta and Sulekha Hazra (2008) Exploring oil producing plants having potential in production of Biodiesel, A poster presented on National Science Day at National Chemical Laboratory, Pune.

6. **Bhuban M. Panda** and Sulekha Hazra. (2009) Micropropagation of *S.anacardium* from seedling derived axenic cultures using Thodiazuron. Poster presented and Abstract published during "National Symposium on plant propagation, conservation, modification and characterization in the 30th annual meeting of Plant tissue culture association (IHBT) Palampur, HP.