IN VITRO CONSERVATION, ECOLOGICAL MAPPING WITH CHEMICAL AND MOLECULAR CHARACTERIZATION OF GARCINIA SPECIES OCCURRING IN MAHARASHTRA

A THESIS

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By

NITASHA SINGH

RESEARCH GUIDE
DR. (MRS.) S.R. THENGANE

PLANT TISSUE CULTURE DIVISION,

NATIONAL CHEMICAL LABORATORY

PUNE-411008

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Date : (Ms.Nitasha Singh)
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CERTIFICATE

This is to certify that the work incorporated in the thesis, **"In vitro conservation, ecological mapping with chemical and molecular characterization of Garcinia species occurring in Maharashtra"** submitted by **Ms. Nitasha Singh,** for the Degree of **Doctor of Philosophy,** was carried out by the candidate under my supervision in Plant Tissue Culture Division, National Chemical Laboratory, Pune – 411008, India. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. (Mrs.) S.R. Thengane
(Research Supervisor)

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **"In vitro conservation, ecological mapping with chemical and molecular characterization of Garcinia species occurring in Maharashtra"** Submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me under the guidance of **Dr. (Mrs.) S.R. Thengane** and has not formed the basis for the award of any degree, diploma, associate ship, fellowship, titles in this or any other university or other institute of higher learning.

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

August 2009 (Ms. Nitasha Singh)

Key to abbreviations

°C	Degree Celsius
v/v	Volume/volume (concentration)
w/v	Weight/ volume (concentration)
ANOVA	Analysis of variance
sd	Standard deviation
$HgCl_2$	Mercuric Chloride
MS	Murashige and Skoog medium (1962)
GR	Plant Growth Regulator
BAP	6-Benzyl amino purine
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
NAA	α-Naphthaleneacetic acid
GA_3	Gibberellic acid
IAA	Indole acetic acid
IBA	Indole butyric acid
2,4-D	2,4-di chlorophenoxy acetic acid
Taxim	Cefotaxime (antibiotic)
PPM	Plant Preservative Mixture
TBA	Tertiary butyl alcohol (2-methyl propan-2-ol)
DPX-4 mountant	[189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)]

ABSTRACT

Rationale of the study

The genus *Garcinia* – belonging to the family Clusiaceae is represented by over 400 species, out of which 35 are endemic to India. Of these, 31 species are found in India the rest are introduced and cultivated in gardens. They are evergreen glabrous trees and shrubs found in the evergreen and semi-evergreen forests of the tropical ever wet zone, or in areas with a relatively mild monsoon climate. They are usually found below an altitude of 1000m, though some species may be found up to 1830m. In the forests the *Garcinias* appear as symmetrical, medium-sized, straight-stemmed trees with horizontal branches. They produce new leaves in flushes at intervals (Holttum, 1954).The *Garcinias* are one of the slowest growing trees known in the tropics. Bark, leaves, fruits and seeds of *Garcinia* are used in Ayurvedic system of medicine for the treatment of different diseases e.g. to improve appetite and is anthelmintic, cardio tonic, useful in bleeding piles, dysentery, tumour, pain and heart diseases. The genus contains xanthones, depsidone, biflavanoids, flavanoids and terpenoids. Xanthones are reported to exhibit anti-microbial and anti-malarial properties. Biflavanoids were found to possess anti-HIV properties. In *in-vitro* studies, xanthone derivative subelliptenone F and the reported compounds have shown inhibitory effect against topoisomerase I and topoisomerase II enzymes. The xanthones of Guttiferae are considered to be prospective lead molecules for the development of anticancer drugs. The fruit rind contains two acids viz. garcinic acid and (–)-HCA (hydroxycitric acid) along with yellow pigment garcinol and a dark red coloured anthocyanin pigment which is a mixture of two major anthocyanins, cyanidin-3-glucoside and cyanidin-3-cambubioside. (-)–HCA is reported to possess some important biological activities, most important of which is inhibition of lipogenesis. It is a powerful inhibitor of the enzyme ATP dependent citratelyase, which is involved in mammalian lipogenesis.

Garcinia is represented by 4 species in Maharashtra – *Garcinia indica, Garcinia talbotii, Garcinia spicata* and *Garcinia xanthochymus*. Two out of these four i.e. *Garcinia talbotii* and *Garcinia indica* are listed as endangered species. On account of the destruction of forests due to human activities the need for conservation as well as reintroduction of this genus becomes important, for which knowledge of the ecological

and genetic diversity of this plant is needed. For reintroduction of this plant an efficient regeneration system is required which can produce propagules round the year. We can also go for the improvement of this plant through genetic manipulation when a protocol for regeneration is available

Objectives

1. It is proposed to do detailed ecological diversity studies in these species and their mapping along the Western Ghats of India .

2. For the conservation *in vitro* techniques will be applied for propagation via organogenesis /embryogenesis.

3. Chemical studies will be undertaken to evaluate %HCA among the species

4. Genetic diversity studies will be done in this species using ISSR/RAPD primers.

Chapter 1. General Introduction

This chapter will consist of a general introduction on medicinal plants, the need for conservation of medicinal plants and the role of biotechnology in medicinal plant research. It will contain a review of literature on the use of *in vitro* techniques for propagation, use of cell culture for production of desired metabolite and importance of transgenic etc. for medicinal plants. An introduction to the concept of ecological mapping, the use of molecular markers for the genetic diversity studies in plants and an introduction to the genus *Garcinia*

Chapter 2. Materials and Methods

The materials used and various general techniques adopted for *in vitro* plant propagation, ecological mapping, genetic diversity studies and (-)HCA estimation of *Garcinia* species will be dealt with.

Chapter 3. Biodiversity studies in *Garcinia* sps.

This chapter will be divided into three sections

- **Ecological mapping of *Garcinia* species**

This chapter will give an introduction to the four species of *Garcinia* found in Maharashtra and their therapeutic uses. It will also contain the geographical distribution of these species in Maharashtra and the environmental conditions of the locations.

- **Genetic diversity in *Garcinia* species**

The genetic diversity of species at the population level using ISSR markers as well as the genetic diversity between the four species is dealt with in this chapter.

- **Analysis of (-)-HCA content in *Garcinia* species**.

This chapter will deal with the estimation of (-)HCA content in the fruits of the four species collected from different locations in Maharashtra.

Chapter 4. *In vitro* studies in *Garcinia* species.

This chapter will be divided into two sections

- ***In vitro* Germination studies**

It will consist of the different parameters studied for the *in vitro* germination studies in *Garcinia indica, Garcinia talbotii* and *Garcinia xanthochymus*

- ***In vitro* regeneration by direct Organogenesis in *Garcinia* species**

This chapter will deal with *in vitro* regeneration by apical/ axillary shoot proliferation in *Garcinia indica* and *G. talbotti* from mature explant and direct organogenesis in all the four species from seed explant.

Chapter 5. *In vitro* regeneration by Somatic Embryogenesis in *Garcinia* species

This chapter will consist of *in vitro* regeneration in *Garcinia talbotii, Garcinia spicata* and *Garcinia xanthochymus* by somatic embryogenesis

Chapter 6. Summary and Conclusion

This section will contain salient findings and conclusions of the present study.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Since the beginning of human civilization man was dependent on nature for the medication to combat various ailments. The beginning of the 20th century saw the dawn of antibiotics in the west - the wonder drugs that were an answer to everything that ailed mankind and then the later part of the century was marked by awareness of the side effects of these drugs. The growing evidence that some of the side effects were more serious than the disorders themselves caused a sense of disillusionment with the current medical research and practices. Due to this many turned to alternative and relatively unknown indigenous systems of medicines like Ayurveda, Tibetan, Unani, Homeopathy along with Yoga, Naturopathy etc. Alternative is a misnomer as these indigenous systems were mainstream and were in vogue for centuries in some countries and to this day are widely practiced. Historically, plant medicines were discovered by trial and error. Our ancestors noticed that aches and pains went away when they drank tea made from the bark of a willow tree. Later, scientists found that willow bark contains salicylic acid, the active ingredient in aspirin. Thus, people who use traditional remedies may not understand the scientific rationale behind their medicines, but they know from personal experience that some medicinal plants can be highly effective if used at therapeutic doses. Today we have a better understanding of how the body functions; we are in a better position to understand the healing powers of plants and their potential as multi-functional chemical entities for treating complicated health conditions.

Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or synergistically to improve health. The World Health Organization estimates that up to 80% of the world's population relies mainly on herbal medicine for primary health care (Fakim 2006). Over the time, the practice of herbal medicine has grown more complex. Science has enabled us to process natural substances into pills, tinctures and powders. Some 40–50% of current medicines are derived from natural product sources (Koeh and Carter 2005, Newman et al. 2003). For oncology and anti-infectives the figures go higher, towards 70–80%. Of the 877 small molecules or NCEs (new chemical entities) brought to market between 1982 and 2000, 61% can be traced back directly or indirectly to natural products. Of the 24 chemical scaffolds used for

drug development since 1955, the great majority are natural products specifically from plant origin (Koeh and Carter 2005, Newman et al. 2003).

Three approaches to the development and use of plant-based pharmaceuticals may be discerned:

- Components of plants used directly as therapeutic agents

These comprise some 6% of all prescribed drugs and cover a range of therapeutic applications from analgesics to anti-cancer agents and contraceptive preparations. While in many cases alternative routes of chemical synthesis have been developed, none have provided a viable commercial alternative to direct use from the plant

- Semi-synthetic production from scaffolds derived from plants

These use a core chemical scaffold derived from plants but which are modified to improve such properties as lower toxicity, greater efficacy or enhanced delivery. These comprise some 27% of all prescription drugs and, as before, are used to treat a variety of therapeutic indications.

- Total synthesis but where the plant-derived molecule has provided the original insight into the structure possessing therapeutic properties.

These are essentially natural product mimics and comprise some 23% of natural product derived or inspired drugs. In the area of total synthesis, to a large degree emphasis has been on the development of plant based drugs for application in oncology and, to lesser extent, anti-infectives.

Over 25% of the new drugs approved in the last 30 years are based on molecules of plant origin, and about 50% of the top selling chemicals derive from knowledge on plant secondary metabolism (Terryn et al. 2006). Increased interest in plants as a source of novel pharmacophores recognizes their chemical diversity and versatility, not matched by synthetic chemistry libraries. In spite of the surge of activity in synthetic chemistry over the last 20 years or so, almost half of the some 850 small molecules introduced as drugs were derived from plant sources. Over 100 small molecules derived either directly or indirectly from plants are currently at some point in the clinical trials process (Fowler 2006). Apart from their high level of structural diversity, naturally derived structures distinguish themselves in other ways when compared to synthetic libraries; for example,

they tend to have a higher degree of chirality and increased structural steric complexity compared with synthetic molecules. They also have a broader distribution of molecular activity.

The great diversity of chemical structures observed in plants and other organisms is a result of millions of years of evolution and selection pressures resulting from interactions between the organism and its environment and challenges to the organism's survival. Many of those challenges will have been from pathogens. In such circumstances it is perhaps hardly surprising that plants should have developed a diverse chemistry in response to such challenges and that many structures have arisen which exhibit anti-pathogenic or in human terms therapeutic properties, from which a range of potent plant-derived drugs have been developed.

Most medicinal plants are not cultivated; rather they are collected from the wild. At least 175 plants native to North America are for sale in the non-prescription medicinal market in the U.S. Many of these are collected from the wild in large quantities (hundreds of thousands of plants) as techniques to cultivate these plants on a commercial scale have not been developed. For example, during the last few years, about 65 million goldenseal plants and 34 million ginseng plants have been harvested from the wild in the forests of the eastern United States on an annual basis. In the United States, the market for medicinal herbs is worth more than $3 billion. In the past, quantities needed to meet demand were relatively low; however, increasing commercial demand is fast outpacing the supply. Currently between 4,000 and 10,000 medicinal plants are on the endangered species list and this number is expected to increase (Canter et al. 2005).

It is argued that the present use of plant-derived drugs and remedies only scratches the surface of what is a major reservoir of untapped potential, the level of biological and chemical diversity possessed by plants. Of the estimated 250,000 plant species on earth, only 2% have been thoroughly screened for chemicals with potential medicinal use. Because native plant habitats are destroyed almost daily, many medicinally valuable plants will be gone before scientists can even investigate them. Most of these plants are found in the tropical rain forests of the world. Tropical rainforests are incredibly rich ecosystems

that play a fundamental role in the basic functioning of the planet. Rainforests are home to probably 50 percent of the world's species, making them an extensive library of biological and genetic resources. However, these precious systems are among the most threatened on the planet. Although the precise area is debated, each day at least 80,000 acres (32,300 ha) of forest disappear from Earth's surface or at least another 80,000 acres (32,300 ha) of forest are degraded. Along with them, the planet looses as many as several hundred species, the vast majority of which have never been documented by scientists.

The rich biodiversity regions on earth have been termed as 'biodiversity hotspots'. There are about 25 biodiversity hotspots on earth out of which India harbors two i.e. the Western Ghats and the Eastern Himalayas. The Western Ghats runs north to south along the western edge of the Deccan Plateau, and separates the plateau from a narrow coastal plain along the Arabian Sea. The range starts near the border of Gujarat and Maharashtra, south of the River Tapti, and runs approximately 1600 km through the states of Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala ending at Kanyakumari, at the southern tip of India. About sixty percent of the Western Ghats are located in the state of Karnataka.

Fig 1.1: Map showing the Western Ghats of India

These hills cover 60,000 km² and form the catchment area for a complex of river systems that drain almost 40% of India. The average elevation is around 1,200 m. The area is one of the world's ten "Hottest biodiversity hotspots" and has over 5000 species of flowering plants, 139 mammalian species, 508 bird species and 179 amphibian species. At least 325 globally threatened species occur in the Western Ghats. Climate in the Western Ghats varies with altitudinal gradation and distance from the equator. The climate is humid and tropical in the lower reaches tempered by the proximity to the sea. Elevations of 1,500 m (4,921 ft) and above in the north and 2,000 m (6,562 ft) and above in the south have a more temperate climate. Average annual temperature here is around 15 °C (60 °F). In some parts frost is common, and temperatures touch the freezing point during the winter months. Mean temperature range from 20 °C (68 °F) in the south to 24 °C (75 °F) in the north. It has also been observed that the coldest periods coincide with the wettest.

During the monsoon season between June and September, the unbroken Western Ghat´s chain acts as a barrier to the moisture laden clouds. The heavy, eastward-moving rain-bearing clouds are forced to rise and in the process deposit most of their rain on the windward side. Rainfall in this region averages 3,000–4,000 mm (120–160 in) with localised extremes touching 9,000 mm (350 in). The eastern region of the Western Ghats which lie in the rain shadow, receive far less rainfall averaging about 1,000 mm (40 in) bringing the average rainfall figure to 2,500 mm (150 in). Data from rainfall figures reveal that there is no relationship between the total amount of rain received and the spread of the rain. Areas to the north in Maharashtra state receive the heaviest rainfall, but are followed by long dry spells, while regions closer to the equator receive less annual rainfall, with rain spells lasting almost the entire year.

The Western Ghats are home to four tropical and subtropical moist broadleaf forest ecoregions – the North Western Ghats moist deciduous forests, North Western Ghats montane rain forests, South Western Ghats moist deciduous forests, and South Western Ghats montane rain forests.

The northern portion of the range is generally drier than the southern portion, and at lower elevations makes up the North Western Ghats moist deciduous forests ecoregion,

with deciduous forests made up predominantly of teak. Above 1,000 meters elevation are the cooler and wetter North Western Ghats montane rain forests, where evergreen forests are characterized by trees of family *Lauraceae*.

The evergreen Wayanad forests of Kerala mark the transition zone between the northern and southern ecoregions of the Western Ghats. The southern ecoregions are generally wetter and more species-rich. At lower elevations are the South Western Ghats moist deciduous forests, with *Cullenia* the characteristic tree genus, accompanied by teak, dipterocarps, and other trees. Above 1,000 meters are the South Western Ghats montane rain forests, also cooler and wetter than the surrounding lowland forests, and dominated by evergreen trees, although some montane grasslands and stunted forests can be found at the highest elevations. The South Western Ghats montane rain forests are the most species-rich ecoregion in peninsular India; eighty percent of the flowering plant species of the entire Western Ghats range are found in this ecoregion.

The area is ecologically sensitive to development and was declared an ecological hotspot in 1988 through the efforts of ecologist Norman Myers. Though this area covers barely five percent of India's land, 27% of all species of higher plants in India (4,000 of 15,000 species) are found here. Almost 1,800 of these are endemic to the region. The range is home to at least 84 amphibian species, 16 bird species, seven mammals, and 1,600 flowering plants which are not found elsewhere in the world.

1.2 Conservation of medicinal plants

The more we use medicinal plants on a commercial scale the more important it is to ensure that they come from sustainable sources, so that these plants will continue to exist. To counter over-exploitation of natural resources and consequent threats to biodiversity, sustainable practices have been recommended and several worldwide organizations have established guidelines for collection and sustainable cultivation of medicinal plants (Klingenstein et al. 2006). Legislation to protect biodiversity is in place in very few countries and conservation efforts are minimal even though awareness is increasing in a number of countries, e.g., Costa Rica.

- *In situ* conservation of medicinal plants:

For *in situ* conservation public awareness is required so that overexploitation of medicinal plants do not take place leading to extinction of the species. Certain regions having rich biodiversity have been declared as reserves and strict laws have been made to conserve these regions. Reintroduction of the plants can be done for the endangered species.

- *Ex situ* conservation of medicinal plants:

The medicinal plants have been grown in Botanical gardens so that even if the plant becomes extinct in the wild it is conserved in the botanical garden and can be used for reintroduction of the species.

Cultivation of medicinal plants:

Cultivation of medicinal plants has conservation advantages: however, costs are frequently prohibitive because of their slow growth rate and the fact that many tropical plants are very difficult to cultivate in a commercial setting. Despite such difficulties, several compelling advantages call for serious efforts in the sustainable cultivation of endangered medicinal plants. Some of these advantages include authoritative and reliable identity of particular plant species and populations, reduction in genotypic and phenotypic variability, and control of the cultivation process to allow accurate prediction of the levels and purity of the principal ingredient(s). Breeding programs targeting selection of high yielding populations and certification programs to allow better quality control can also be implemented under cultivation.

1.3 Role of biotechnology in medicinal plants

Medicinal plants are of great interest in biotechnology; the study of methods by which the productive potential of living cells can be used in industrial processes, and the production of materials in agriculture, forestry, horticulture and medicine can be profitable. By the use of tissue culture, various problems in plant biotechnology, such as micropropagation, biosynthesis and biotransformation of biologically active compounds, storage of plant cells and organs, and genetic engineering of higher plants can be solved (Reinert and Bajaj 1977).

Micropropagation: It is the *in vitro* propagation of the plant in artificial media under controlled culture conditions.

Plant regeneration *in vitro* can be achieved in three different ways;

1. Regeneration from existing meristems
2. Regeneration from adventitious meristems
3. Regeneration via somatic embryogenesis

In some species, existing meristems spontaneously develop whilst for other species a weak cytokinin treatment is required to stimulate meristem development. The advantages are that there seem to be minimal risks for physiological after-effects and genetic changes. The disadvantage with the method is that, in some species, only a limited number of plants are produced and there can be problems with the rooting of the plants. This method is used for propagating forest tree species like *Betula* (McCown and McCown 1987), *Populus* (Gupta and Mascarenhas 1987) and *Eucalyptus* (Mascarenhas et al. 1988), and medicinal plants like *Stereospermum personatum* (Shukla et al. 2008), *Asparagus racemosus* (Bopana and Saxena 2008) and *Desmodium gangeticum* (Ahuja et al. 2008)

Adventitious meristems are stimulated to differentiate by a cytokinin treatment. A relatively high number of plants can be produced but there can be problems with rooting and some risks for physiological after-effects. This method is used for propagating *Pinus radiata* in New Zealand (Gleed 1992), *Centaurium erythrae* (Subotić et al. 2009), *Arnebia euchroma* (Bo et al. 2005), *Caesalpinia bonduc* (Kannan et al. 2008), *Tylophora indica* (Chaudhuri et al. 2004).

Somatic embryogenesis is a process where somatic cells differentiate into somatic embryos. Somatic embryogenesis has several advantages compared to the other techniques of micropropagation. Somatic embryos can be propagated on a large scale in bioreactors and in unlimited numbers. A high yield of plants can be obtained in a short time and the embryos have already a root meristem. Artificial seeds can be formed which makes the handling cheaper. Somatic embryos can also be cryopreserved for germplasm storage. Somatic embryogenesis has been used in the propagation of medicinal plants like

Azadirachta indica (Murthy and Saxena 1998, Shrikhande et al. 1993), *Terminalia chebula* (Anjaneyulu et al. 2004), *Tylophora indica* (Chandrashekhar et al. 2006) etc.

Micropropagation of medicinal plants has the following benefits:

- Increase in the propagation rate of plants
- Multiplication of the plant with elite characters and their protection against segregation or mutation
- Conservation of germplasm
- Production of medicinal plants can be carried out round the year irrespective of season.
- The multiplication is comparatively rapid, requires less space and is less expensive.
- Production of uniform clones from highly heterozygous plants
- Plant improvement by regeneration technique in conjunction with *in vitro* cell manipulation

Table 1.1: Reports on the micropropagation of medicinal plants

Plant	Explant used	Method of regeneration	Reference
Acacia catechu	Nodal explants from seedlings	Shoot proliferation	Sahini and Gupta 2002
Aegle marmelos	Nodal explants from mature trees	Shoot proliferation	Ajithkumar and Seeni 1998
Aquilaria agallocha	Shoots from seedlings	Direct organogenesis	Meng-ling et al. 2005
Azadirachta indica	Mature seeds	Direct and indirect somatic embryogenesis	Murthy and Saxena 1998
Azadirachta indica	Immature cotyledons	Indirect somatic embryogenesis	Shrikhande et al. 1993
Calophyllum inophylum	Seeds	Shoot proliferation	Thengane et al. 2006
Catharenthus roseus	Different explants	Direct organogenesis and embryogenesis	Dhandapani et al. 2008
Cephalis ipecacuanha	Leaves	Indirect somatic embryogenesis	Rout et al. 2000
Cinnamomum camphora	Axillary and apical buds from mature trees	Shoot proliferation	Babu et al. 2003

Cornus mas	Axillary buds from mature trees	Shoot proliferation	Durkovicˇ 2008
Crataeva nurvala	Seedling explants	Direct or indirect organogenesis	Walia et al. 2003
Curcuma longa	Apical buds from seedlings	Direct organogenesis	Prathanturarug et al. 2005
Garcinia indica	Immature seeds	Direct somatic embryogenesis	Thengane et al. 2006
Garcinia indica	Mature seeds	Direct organogenesis	Malik et al. 2005
Garcinia mangostana	Leaves from seedlings and mature trees	Direct organogenesis	Goh et al. 1990
Garcinia mangostana	Leaves from seedlings	Direct organogenesis	Goh et al. 1994
Garcinia mangostana	Leaves from seedlings	Indirect organogenesis	Te-chato and Lim 2000
Garcinia mangostana	Seeds	Direct organogenesis	Normah et al. 1995
Hoslundia opposita	Nodal explants	Shoot proliferation	Prakash and Van Staden 2007
Maytenus canariensis	Axillary and apical buds from seedlings	Shoot proliferation	Gutiérrez-Nicolás et al. 2008
Pimpenella tirupatiensis	Hypocotyls of seedlings	Indirect somatic embryogenesis	Prakash et al. 2001
Pittosporum napaulensis	Axillary buds from mature trees	Shoot proliferation	Dhar et al. 2000
Phellodendron amurense	Leaves from seedlings	Direct and indirect organogenesis	Azad et al. 2005
Pterocarpus marsupium	Cotyledonary nodes of seedlings	Shoot proliferation	Anis et al. 2005
Stereospermum personatum	Shoot buds from seedlings	Shoot proliferation	Shukla et al. 2008
Terminalia arjuna	Nodal explants from mature trees	Shoot proliferation	Pandey et al. 2006
Terminalia arjuna	Cotyledonary nodes	Shoot proliferation	Pandey and Jaiswal 2002
Tylophora indica	Mature leaves	Direct somatic embryogenesis	Chandrasekhar et al. 2006
Vitex negundo	Axillary buds of mature trees	Shoot proliferation	Ahmad and Anis 2007

Tissue culture can also be used for the large scale production of pharmaceutically active compounds without destroying the natural population of the plant. Tissue culture techniques like cell suspension cultures can be used for large scale production of the

secondary metabolites. The major advantages of cell culture over conventional cultivation of whole plants are:

- Useful compounds can be produced under controlled conditions independent of climate changes or soil conditions
- Cultured cells would be free of microbes and insects
- Automated control of cell growth and rational regulation of metabolic processes would reduce labor costs and improve productivity
- Organic substances are extractable from callus cultures.

Table 1.2: Bioactive secondary metabolites from plant tissue culture

Plant name	Active ingredient	Culture medium	Culture type	Reference
Allium sativum	Alliin	MS+IAA(11.4µM), NAA(10.8µM), Kinetin(9.3µM), Coconut water(15%)	Callus	Malpathak and David 1986
Aloe saponaria	Tetrahydroanthracene glucosides	MS+2,4-D(1ppm), Kinetin(2ppm)	Suspension	Yagi et al. 1983
Bupleurum falcatum L	Saikosaponins	B5+IBA(8mg/l), Sucrose(1-8%)	Root	Kusakari et al. 2000
Calophyllum inophyllum	Inophyllum B&P	WPM+IBA(2mg/l), WPM+IBA(2mg/l) +BAP(1mg/l)	Callus	Pawar et al. 2007
Camellia sinensis	Theamine, γ-glutamyl derivatives	MS+IBA(2mg/l), Kinetin(0.1mg/l), Sucrose(3%), Agar(9g/l)	Suspension	Orihara and Furuya 1990
Canavalia ensiformis	L-Canavanine	LS+NAA(1.8mg/l), 2,4-D(0.05mg/l), BA(4.5mg/l), Picloram (0.05mg/l)	Callus	Ramirez et al. 1992
Capsicum annum L.	Capsaicin	MS+2,4-D(2mg/l), Kinetin(0.5mg/l), Sucrose(3.0%)	Suspension	Johnson et al. 1990
Catharanthus roseus	Catharanthine	MS+NAA(2 mg/ L), IAA (2 mg/ L), Kinetin (0.1 mg/L), Sucrose (3 %)	Suspension	Zhao et al. 2001

Cephaelis ipecacuanha A. Richard	Emetic alkaloids	MS+NAA(1mg/l)or IAA(3.0mg/l)	Root	Teshima et al. 1988
Chrysanthemum cinerariaefolium	Pyrethrins	MS+2,4-D(2.0mg/l), Kinetin(5.0mg/l), Sucrose(3.0%)	Callus	Rajasekaran et al. 1991
Citrus sp	Naringin, Limonin	MS+2,4-D(0.66mg/l), Kinetin(1.32mg/l), Coconut milk(100ml)	Callus	Barthe et al. 1987
Digitalis purpurea L.	Cardenolides	MS+BA(1mg/l), IAA(1mg/l), Thiamine HCL(1mg/l)	Suspension	Hagimori et al. 1982
Eucalyptus tereticornis SM	Sterols and Phenolic compounds	MS+2,4-D(2.0mg/l)	Callus	Venkateswara et al. 1986
Ephedra spp.	L-Ephedrine D-Pseudoephidrine	MS+ Kinetin(0.25μM), 2,4-D or NAA(5.0μM)+ Sucrose(3.0%)	Suspension	O´ Dowd et al. 1993
Ginkgo biloba	Ginkgolide A	MS+NAA (1.0 mg/ L), Kinetin (0.1 mg/L), Sucrose (3 %)	Suspension	Carrier et al. 1991
Glycyrrhiza echinata	Flavanoids	MS+IAA(1mg/l), Kinetin(0.1mg/l)	Callus	Ayabe et al. 1986
Hyoscyamus niger	Tropane alkaloids	LS+NAA(10^{-5}M), BA(5×10^{-6}M)	Callus	Yamada and Hashimoto 1982
Lithospermum erythrorhizon	Shikonin derivatives	LS+IAA(10^{-6}M), Kinetin(10^{-5}M)	Suspension	Fujita et al. 1981
Mentha arvensis	Terpenoid	MS+BA(5mg/l), NAA(0.5mg/l)	Shoot	Phatak and Heble 2002
Panax ginseng	Saponins and sapogenins	MS(without glycine)+2,4-D(1mg/l)	Callus	Furuya et al. 1973
Papaver somniferum	Morphine, Codeine	MS+2,4D(0.1mg/l), Cysteine HCl(2.5mg/l), Kinetin(2mg/l), Sucrose(3.0%)	Suspension	Siah and Doran 1991

Papaver somniferum L.	Alkaloids	MS(without Glycine)+Kinetin (0.1mg/l)	Callus	Furuya et al. 1972
Rauwolfia serpentina× Rhazya stricta hybrid plant	3-Oxo-rhazinilam	LS medium	Callus	Gerasimenko et al. 2001
Taxus spp	Taxol	B5 medium+2,4-D(0.2mg/l), BA(0.5mg/l), Casein hydrolysate (200mg/l), Sucrose(3.0%)	Suspension	Wu et al. 2001
Withania somnifera	Withaferin A	MS+BA(1mg/l), Sucrose(3%)	Shoot	Ray and Jha 2001

In order to obtain high yields suitable for commercial production, efforts have focused on exploiting the biosynthetic capacity of cultured cells, achieved by optimizing the cultured conditions, selecting high producing strains and employing precursor feeding, transformation methods and immobilization techniques (Dicosmo and Misawa 1995). *In vitro* cell culture offers an intrinsic advantage for foreign protein synthesis in certain situations since they can be designed to produce therapeutic proteins, including monoclonal antibodies, antigenic proteins that act as immunogens, human serum albumin, interferon etc. (Hiatt et al. 1989**,** Manson and Arntzen 1995, Doran 2000)

Several secondary metabolites of pharmaceutical interest are accumulated in plant roots (Flores et al. 1999). However, harvesting roots is destructive for the plants and hence there has been a growing interest to generate hairy roots from medicinal plant species. Hairy roots develop as the consequence of the interaction between *Agrobacterium rhizogenes*, a Gram-negative soil bacterium, and the host plant. Hairy roots are characterized by high growth rate independent of any source of exogenous hormones, absence of geotropism and high branching; furthermore, they often produce secondary metabolites for a long period of time, unlike natural roots. Elicitation, precursor feeding, cell permeabilization and trapping of the molecules released in the liquid medium are efficient ways to increase the productivity of hairy roots. Several elicitors have been successfully tested to increase the production or the secretion of secondary metabolites in

hairy roots from different plant species. For example, the stress hormone methyl jasmonate (MeJa), which often has a role as secondary messenger in elicitor transduction pathways, is also efficient in inducing or increasing the production of valuable secondary metabolites in hairy root cultures (Palazon et al. 2003, Yaoya et al. 2004, Komaraiah et al. 2003). Sometimes the biosynthesis of a valuable metabolite by hairy roots is limited by the availability of its precursor. One solution to address this problem is to add the precursor to the culture medium, but this can be costly if the precursor is difficult to synthesize or to obtain from other natural sources. In this context, the co-culture system has proved to be a judicious alternative for the production of the anti tumor drug podophyllotoxin by *Podophyllum hexandrum* cell suspensions, synthesis of which is limited by the availability of coniferin. When *Podophyllum hexandrum* cells are cultured together with *Linum flavum* hairy roots, which produce and secrete coniferin, the production of podophyllotoxin was increased by 240% in the dual shaker flask system. Metabolic engineering strategies are used to circumvent problems linked to precursor availability or negative regulatory loops (Memelink 2005). Overexpressing genes encoding key enzymes of the tropane alkaloid biosynthesis pathway increased the alkaloid production in *Duboisia* hybrid, *Datura metel* and *Hyoscyamus muticus* hairy root (Palazon et al. 2003, Moyano et al. 2003). Transgenic hairy root cultures have revolutionized the role of plant tissue culture in secondary metabolite production. Using this method a wide range of chemical compounds has been synthesized (Shanks and Morgan 1999, Giri and Narasu 2000).

The genetic manipulation of plants together with the establishment of *in vitro* plant regeneration systems facilitates efforts to engineer secondary product metabolic pathways. There are many reasons for wishing to alter the levels and profile of secondary metabolites in plants which include:

- improving agronomic traits through the expression of a metabolite that might protect the plant against insect or pathogen attack
- lowering levels of noxious or antinutritional factors in food and feed crops
- increasing levels of beneficial or desirable components in food and feed crops
- increasing the production of pharmaceutically active compounds in medicinal plants

Several excellent reviews describe approaches for achieving some or all of these objectives (Verpoorte et al. 2000, Oksman-Caldentey and Inze 2004). Several approaches can be considered to increase levels of a valuable molecule in a target plant that makes trace amounts of the molecule in question. These include: increasing flux to the target molecule, overcoming rate limiting steps, reducing flux through competing pathways, over-expressing regulatory genes or transcription factors that induce the pathway, inhibit or limit catabolism of the molecule or increase the number of specialized cells producing the compound in question. To make a novel compound in a plant, precursors might be formed by the introduction of appropriate heterologous genes. To reduce or eliminate levels of undesirable compounds, flux in the pathway leading to such compounds can be reduced or redirected to competing pathways. Particular steps in the pathway leading to such undesirable compounds can be blocked by antisense, cosuppression or RNA interference (RNAi) methods. Similar results might be achieved by suppressing genes that upregulate the pathway or by increasing catabolism. Early experiments targeting the genetic transformation of medicinal plants were carried out using *Agrobacterium rhizogenes* to obtain hairy root cultures, or *Agrobacterium tumefaciens* to produce transformed cells to be maintained in culture or to regenerate whole plants. Even though the establishment of transgenic cell lines and hairy root cultures is relatively simple, such systems have inherent drawbacks limiting their usefulness in terms of developing efficient production systems for target molecules. Key limitations include the high cost of bioreactors and instability of cell lines, which often lose their capacity to produce target molecules over the time.

Table 1.3: Transgenic cultures and plant systems used to produce secondary plant products from medicinal plant species

Species	Use	Transformation method	Marker gene used	Resulting transgenic tissues	References
Acacia mangium	Emulsifiers	*A. tumefaciens*	gusA, npt-II	Plants	Xie and Hong 2002
Aesculus hippocastanum	Varicose veins, edema, sprains	*A. rhizogenes*	gusA	Hairy roots–plants	Zdravkovic-Korac et al. 2004

Echinacea purpurea	Treatment of colds, immunostimulant, and anti-inflamatory	*A. tumefaciens*	gusA, npt-II	Plants	Wang and To 2004
Eritrichium sericeum	AntiHIV and antiallergic	*A. rhizogenes*	rolC	Cell and root cultures	Bulgakov et al. 2005
Lathyrus sativus	Neurotoxic	*A. tumefaciens*	npt-II, gusA	Plants	Barik et al. 2005
Ruta graveolens	Plant defense and drugs	*A. tumefaciens*	npt-II, gusA	Plants	Lievre et al. 2005
Saussurea involucrata	Inhibits tumorigenesis, traditional chinese medicine	*A. rhizogenes*	No marker gene used	Hairy roots	Fu et al. 2005 Fu et al. 2006 Li et al. 2006
Taraxacum platycarpum	Choleretic, diuretic and anticarcinogenic	*A. tumefaciens*	gusA	Plants	Bae et al. 2005
Tylophora indica	Important medicinal plant in India	*A. rhizogenes*	No marker gene used	Callus–hairy roots	Chaudhuri et al. 2005

1.4 Ecological mapping

Ecological mapping is concerned with mapping of organisms, assemblages and habitats (whether it is from the ground, air or space) but it can also be concerned with mapping fungi on a forest floor or microbes in the soil. Ecological mapping became a sophisticated science in the Netherlands as long ago as 1970, where the identification and monitoring of areas for conservation and ecological importance were undertaken. Ecological mapping of coastal zones to enable the identification of ecologically sensitive areas was also one of the early developments. That information has been used for clean up operations after oil spills so that areas in greatest need of protection were given priority. Today apart from the above uses ecological mapping is used to understand the shift in the vegetation due to global climatic changes.

Historically ecological mapping was done by field observation. The distribution of the species in a particular region was mapped. The environmental conditions of that region

were then studied. This gave the distribution of the species as well as the prospective locations where the species could be found or could be introduced. The method used earlier had its own limitations as the area under the study was generally very small and the method was very costly.

With the advancement in ecological research new technologies were introduced for ecological mapping. The new techniques with their advantages and limitations are listed in Table 1.4

Table 1.4: Characteristics of field, GIS and RS data sources used for ecological mapping and monitoring

Data collection	Benefits	Limitations
Field observation	Fine spatial scale Detailed information Direct observation	Limited temporal extent Incomplete spatial coverage Expensive Subjective
GIS(Geographic information system)	Associated with potential distributions Can be used for species level mapping Can be used to derive direct and resource gradients	Limited spatial resolution Unknown accuracy Cannot be frequently updated Pixel values usually result from interpolation Indirect gradients most readily available
RS(Remote sensing)	Associated with actual distributions Allows data collection in remote areas Synoptic perspective Systematic measurement for every pixel; Complete spatial coverage Enable larger study areas Multitemporal; high temporal resolution Cost-effective for large extents	Atmospheric obstructions possible Expensive for fine spatial scales Less detailed information Processing methods intimidating (to untrained users) Usually represent indirect or functional gradients
GIS and RS (integrated)	Data can be upscaled More consistent and objective databases Can provide updated	Compounding of quantitative–positional errors Lack of automated methods to aid integration

	environmental data Data are readily available Direct, resource, indirect, and functional gradients can be combined	Paucity of faster GIS data with fine spatial resolution (to match RS data)

Field observation provides the most detailed and fine-scale information, although the spatial coverage is not continuous. Field data are also expensive and time-consuming to collect, and many of the observations are relatively subjective or suited for a narrow purpose. GIS data can provide continuous spatial coverage (usually through interpolation methods), albeit at coarser spatial resolution and lower or unknown positional accuracy. GIS data, particularly digital elevation models (DEM), have been used to derive complex environmental variables that are more ecologically relevant (e.g. topographic moisture index, potential solar radiation). Remote sensing facilitates data collection in difficult- or impossible-to-reach areas and provides an important synoptic and multitemporal perspective. Remote sensing also provides a systematic value for each pixel and spatially continuous coverage. However, fine spatial resolution imagery consistent with the scale of field observation is expensive, some amount of processing is required, and atmospheric obstructions can be problematic. The integration of GIS and remotely sensed data improves upon many of their individual limitations.

For information at the species level field observation is the only method. It helps in locating the ecologically threatened regions for the species that needs immediate attention. It also helps to understand the habitat of the particular species and where else the plant can be expected or can be introduced. It helps in recognizing the rare and endangered plants and the immigrant plants. It gives information on the type of threat to the particular plant-whether biotic or abiotic.

1.5 Genetic Diversity studies

A study of the genetic diversity is required to understand the future prospects of the species. A large diversity will not only give a better adaptability to the changes in the environment but will also give a wealth of genetic material for genetic manipulations of the species. Estimates of genetic relationship are important in designing crop improvement

programs. Information on genetic diversity is also valued for the management of germplasm and for evolving conservation strategies At the genus level genetic diversity helps to distinguish morphologically similar species. Molecular markers are used to study the genetic diversity in a species.

Most molecular markers for genetic diversity studies fall into one of two basic categories of techniques that use either hybridization or are based on the PCR:

1. Hybridization based (non-PCR) techniques: It includes restriction fragment length polymorphism (RFLP) where DNA is first digested with restriction enzymes. The resultant fragments are separated by gel electrophoresis and transferred onto filters by southern blotting and then sequence specific probes are used to detect polymorphism.
2. Arbitrarily primed PCR and other PCR based multi-locus profiling techniques: The development of the PCR removed the necessity for probe hybridization steps. In this category we can group together all PCR based techniques, which use arbitrary or semi arbitrary primers for amplification of DNA. A common feature of these techniques is the lack of requirement for sequence information from the genome under investigation. Arbitrarily chosen primers are used in PCR conditions in random amplified polymorphic DNA (RAPD) analysis. The primers will initiate synthesis even when the match with the template is imperfect. In this case each amplified product will be derived from a region of the genome that contains short segments which share sequence similarity to the single primer and which are on opposite strands and sufficiently close together for the amplification to work. The PCR products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light.

In a second subgroup the primers used are semi arbitrary in that they are based upon restriction enzyme sites or sequences that are interspersed in the genome, such as repetitive elements, transposable elements and microsatellites. The use of primers based on restriction sites is the basis of the technique of amplified fragment length polymorphism (AFLP). In AFLP, DNA is restricted with two restriction enzymes, adaptors are ligated, and then PCR is carried out with primers which comprise a common part corresponding to the adaptors and restriction site and a unique part corresponding to selective bases.

Because of the high mutability of simple sequence repeats (SSR), there are many versions in which microsatellites are used as primers.

1. SSR, which is very similar to RAPD but the primers are SSR based.
2. ISSR, in which two SSR primers anchored at the 5' or 3' end are used. This technique is more reproducible than SSR and RAPD.

Table 1.5: Reports on genetic diversity studies using molecular markers

Plant	Genetic diversity study	Molecular marker used	References
Andrographis paniculata	genetic diversity between plants	RAPD	Lattoo et al. 2008
Digitalis obscura	genetic diversity between population	RAPD	Nebauer et al. 1999
Dioscorea opposita	genetic variation in Cultivar	ISSR	Zhou et al. 2008
Echinacea species	species diversity	RAPD	Kapteyn et al. 2002
Eurycoma longifolia	genetic diversity between plants	SNP	Osman et al. 2003
Gardenia jasminoides	genetic diversity between population	AFLP	Han et al. 2007
Hesperozygis ringens Benth.	genetic diversity between population	RAPD and AFLP	Fracaro and Echeverrigaray 2006
Matricaria chamomilla	genetic diversity between plants	RAPD	Solouki et al. 2008
Prunus africana,	genetic diversity between population	RAPD	Dawson and Powell 1999
Sarcandra glabra	genetic diversity between population	ISSR	Ni et al. 2008
Stevia rebaudiana	genetic diversity between population	ISSR	Heikal et al. 2008
Tribulus terrestris	genetic diversity between population	AFLP,SAMPL, ISSR and RAPD	Sarwat et al. 2008
Vitex rotundifolia	genetic diversity between populations	ISSR	Hu et al. 2008

1.6 *Garcinia*

The genus *Garcinia* – belonging to the family Clusiaceae (formerly Guttiferae) is represented by over 400 species, out of which 35 are endemic to India. Of these, 31 species are found in India and the rest are introduced. They are evergreen glabrous trees and shrubs found in the evergreen and semi-evergreen forests of the tropical ever wet zone, or in areas with a relatively mild monsoon climate. They are usually found below an altitude of 1000 m, though some species may occur upto 1830 m.

Taxonomic classification of *Garcinia*:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Malpighiales
Family: Clusiaceae
Genus: *Garcinia*

In the forests the, *Garcinias* appear as symmetrical, medium-sized, straight-stemmed trees with horizontal branches. They produce new leaves in flushes at intervals (Holttum 1954). The *Garcinias* are one of the slowest growing trees known in the tropics. They are also very slow in coming into bearing. The wood is moderately hard or hard, close grained, yellowish white, red or grey. Exudate often yellow, resinous. Branches usually opposite, horizontal or pendulous. Flowers polygamodioecious or pseudohermaphrodite, the female or pseudohermaphrodite always in lesser number, axillary or terminal, solitary or in cymes, small or medium sized, hypogynous. Sepals 4, decussate or 5, imbricate, rarely 2. Petals 4, alternate with the sepals, imbricate, seldom 5. Male flowers: stamens indefinite, rarely few, free or united surrounding a rudimentary pistil. Female flowers: staminodes minute, various free or united. Ovary superior, bi- to multilocular; ovule solitary. Fruit is a berry enclosing several large seeds. Embryo a solid homogeneous mass (tigellus); cotyledons absent or minute. (Maheshwari 1964). Bark, leaves, fruits and seeds of *Garcinia* are used in Ayurvedic system of medicine for the treatment of different diseases e.g. to improve appetite and is anthelmintic, cardio tonic, useful in bleeding piles, dysentery, tumour, pain and heart diseases. The genus contains

xanthones, depsidone, biflavanoids, flavanoids and terpenoids. Xanthones are reported to exhibit anti-microbial and anti-malarial properties. Biflavanoids were found to possess anti-HIV properties. In *in-vitro* studies, xanthone derivative subelliptenone F has shown inhibitory effect against topoisomerase I and topoisomerase II enzymes. The xanthones of Guttiferae are considered to be prospective lead molecules for the development of anticancer drugs. The fruit rind contains two acids viz. garcinic acid and (–)-HCA (hydroxycitric acid) along with yellow pigment garcinol and a dark red coloured anthocyanin pigment which is a mixture of two major anthocyanins, cyanidin-3-glucoside and cyanidin-3-cambubioside. (–)-HCA is reported to possess some important biological activities, most important of which is inhibition of lipogenesis. It is a powerful inhibitor of the enzyme ATP dependent citratelyase, which is involved in mammalian lipogenesis.

Garcinia is represented by 4 species in Maharashtra – *Garcinia indica, Garcinia talbotii, Garcinia spicata* and *Garcinia xanthochymus*. Two out of these four i.e. *Garcinia talbotii* and *Garcinia indica* are listed as endangered. *Garcinia spicata* is rare in Maharashtra while *Garcinia xanthochymus* is an introduced species.

The work was carried out with the following aim:

1. Detailed ecological diversity studies in the species and their mapping along the Western Ghats
2. For the conservation *in vitro* techniques were applied for development of propagation protocols via micropropagation/embryogenesis.
3. Chemical studies were undertaken to evaluate %(-)-HCA among the species
4. Genetic diversity studies were done in the species using ISSR primers

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

This chapter describes the materials and general techniques routinely practiced in plant tissue culture (2.2), in isolation and amplification of DNA (2.3) and in chemical analysis by HPLC method (2.4). The materials and methods, specific to particular experiment, are dealt in details in respective chapters.

2.2 Plant Tissue Culture

2.2.A Materials

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

Glasswares and Plastic wares:

Test tubes (25x150 mm), conical flasks (2000, 1000, 500 and 250 ml capacity), screw cap bottles (250 ml), pipettes (0.1, 0.2, 1, 2, 5, 10 ml capacity) and measuring cylinders (25 ml, 100 ml, 250 and 1000 ml capacity) of Borosil, India were used for culturing the tissues and for preparation of media. Autoclavable, screw cap bottles (100, 250 & 500 ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (60 ml capacity), slides (Blue Star, India) and cover slips (Micro-Aid, India).

Plasticwares including sterile disposable plastic petriplates of 55 & 85 mm diameter were procured from Laxbro, Pune. Klin wrap, used for sealing the petriplates was obtained from local supplier. Micropipette of different precision measurements (1000 µl, 200 µl, 50 µl, 20 µl and 10 µl) and microtips were procured from Gilson (India) and Tarson (India) respectively.

Chemicals:

Chemicals used for surface sterilization procedures were Bavistin® (BASF, India), savlon (Johnson and Johnson Limited, India) and mercuric chloride (Qualigens Fine Chemicals, India). A media additive sterilant, Plant Preservative Mixture (PPM) was procured from Sameer Science Laboratory, India. An antibiotic, cefotaxime (Alkem,

India) was often used for controlling the growth of the contaminating bacteria in plant tissue culture medium.

Inorganic salts and vitamins used for preparation of culture media and for other experiments were of AnalaR grade (Hi-Media and Qualigens Fine Chemicals, India). Sucrose, fructose, glucose, maltose were procured from Hi-Media (India) and Qualigens Fine Chemicals (India). Agar agar (bacteriological grade), used as gelling agent in the semisolid culture medium was procured from Hi-Media (India). Gelling agent Phytagel™ (Gelrite) used in some of the experiments was procured from Sigma Chemicals Co. (U.S.A.). Activated charcoal was procured from Merck, Mumbai.

All growth regulators like kinetin (KN), 6-benzyl amino purine (BAP), N-phenyl-N'-1, 2, 3-thidiazol-5-ylurea (Thidiazuron, TDZ), α-naphthalene acetic acid (NAA), 3-indole acetic acid (IAA), 3-indole butyric acid (IBA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were obtained from Sigma-Aldrich (U.S.A.).

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

Equipments:

pH meter: pH is the negative logarithm of hydrogen ion concentration. The 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 emf or voltage difference causing current flow. The current intensity gives the value of pH.

Electronic Balance: A manual top loading balance (Contech, India) was used for quick weighing and for analytical purposes. This is a single pan balance capacity 100-200 g,

sensitivity 0.1 mg operating on 230 V 50 Hz AC mains. Precision of ± 0.005 g, weighing range 0.1-200 g, digital read out was used for making stock solutions of growth regulators and for other fine weighing.

Autoclave: The autoclave (Nat Steel Equipment Private Limited Bombay) was used for sterilization of media, glassware, water, dissecting instruments etc and for decontamination of contaminated cultures in culture vessels. It is based on application of steam under pressure. Autoclaving was carried out at 121°C temperature under 15 lb/in^2 pressure. Except culture media, all other materials were autoclaved for 45 min. The culture media were autoclaved for 20 min.

Laminar airflow ultra clean unit: All aseptic manipulations were carried out on 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.

Apart from these instruments magnetic stirrer (Remi, India), steamer (Ultradent, India), temperature controlled oven (Pathak Electricals, India), light microscope (Carl-Zeiss, Germany), microtome (Reichert Jung, Germany), camera (Nikon, Japan/Zeiss, Germany), disposable membrane filter sterilizing unit (Laxbro, Pune) etc. were also used.

Source of Explants:

The plant material was collected along the Western Ghats of Maharashtra. Seeds were exised from the fruits collected in the month of Feb-May and used as explant for embryogenesis and organogenesis. For clonal propagation actively growing shoots were collected and apical and axillary buds were used as explant.

2.2.B Methods

Preparation of glassware and instruments:

Glassware used in our studies was cleaned by boiling in a solution of sodium bicarbonate for 1 hr followed by washing in tap water. These were then immersed in 30%

nitric acid solution for 30 min and were washed thoroughly with tap water. After rinsing with double distilled water these were allowed to dry on a draining rack.

Tubes and flasks were plugged with non absorbent cotton (Safe surgical industries, Beawar, India). All dissecting instruments were wrapped singly with paper and put in autoclavable plastic bags for sterilization by autoclaving. Ordinary grade filter paper pieces of approximately 10x20 cm were kept in stack alternatively with brown paper pieces of similar size. These were packed in autoclavable plastic bags as stack of 20-25 pieces and autoclaved. Dissection and transfer of explants were carried out on these 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 1 h at 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), B5 (Gamborg 1968), SH (Shenk and Hildebrandt 1972), WPM (Lloyd and McCown 1980) etc. are now available. In the present studies WPM, MS and B5 were used. Concentrations of the macro and microelements, salts and organic constituents of the different media used in the present studies are listed in Table 2.1. 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

Table 2.1: Composition of basal medium mg/lit

Ingredients	MS medium	B5 Medium	WPM medium
Macronutrients			
KNO_3	1900	2528	400
NH_4NO_3	1650	-	-
$CaCl_2.2H_2O$	440	150	96
$MgSO_4.7H_2O$	370	246	370
KH_2PO_4	170	-	170
K_2SO_4	-	-	990
$(NH4)_2$ SO4	-	134	-
Ca $(NO_3)_2.4H_2O$	-	-	556
Micro-nutrients			
$MnSO_4.4H_2O$	22.3	10	22.3
$ZnSO_4.7H_2O$	8.6	2.0	8.6
H_3BO_3	6.2	3.0	6.2
KI	0.83	0.75	-
$CuSO_4.5H_2O$	0.025	0.025	0.25
$Na_2MoO_4.2H_2O$	0.25	0.25	-
$CoCl_2.6H_2O$	0.025	0.025	-
$FeSO_4.7H_2O$	27.8	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.3	37.3
Vitamins			
Myo-inositol	100	100	100
Thiamine-HCl	0.1	10.0	0.1
Nicotinic acid	0.5	1.0	0.5
Pyridoxine-HCl	0.5	0.1	0.5
Glycine	2.0	-	2.0

Stock solutions of growth regulators (GR) were prepared by adding few drops of appropriate solvent in the required amount of growth regulator to dissolve. After dissolution, the required concentration was made by the addition of double distilled water

and stored in refrigerator in sterilized bottles. Table 2.2 describes the list of solvents and diluents, stock concentration of growth regulators used.

Table 2.2: Preparation and Concentration of Growth Regulators

Growth Regulator	Molecular weight	solvent	Diluent	Stock Concentration
BA	225.3	1N NaOH	H_2O	10 mg/100mL
KN	215.2	1N NaOH	H_2O	10 mg/100mL
TDZ	220.2	DMSO	H_2O	10 mg/100mL
2,4-D	221	1N NaOH	H_2O	10 mg/100mL
NAA	186.2	1N NaOH	H_2O	10 mg/100mL
IBA	203.2	1N NaOH	H_2O	10 mg/100mL
IAA	175.2	1N NaOH	H_2O	10 mg/100mL
GA_3	346.4	EtOH	H_2O	10 mg/100mL

Preparation of Cefotaxim stock: Cefotaxim is available in the market in powder form in packs of 500 mg or 1000 mg with 2.5 ml / 5 ml sterile water. The sterile water is mixed with the powder in laminar air flow unit. Then calculated amount (200-400 mg per litre of media) of this stock is added to autoclaved media by micropipette.

Plant preservative media: It is added directly to autoclaved media in laminar air flow unit (usually 1-2 ml of this solution per litre of media).

For media preparation calculated amount of aliquots were added from the stock solutions. Carbohydrate (Sucrose, Fructose, Glucose or Maltose) was weighed and added in required quantity (2%, 4%, 6%, 8%) and allowed to dissolve. The volume was made up with double distilled water. Unless mentioned, pH of all the media was adjusted to 5.6-5.8 using 1N NaOH or 1N HCl after mixing all the constituents except the gelling agent. The media were solidified by using either agar agar (0.7-0.8%) or phytagel (0.2%). In case of charcoal containing medium 0.2% charcoal was added to the media. The media were then heated on water bath or steamed for the agar to melt. Molten medium was dispensed into sterile culture tubes (20 ml)/ flasks (100 ml) or bottles (80 ml) after thorough mixing. Semisolid medium containing agar was used in most of the studies unless otherwise mentioned. All the culture media were autoclaved for 20 min. at 121°C and 15 lbs/(inch)^2. For culturing in petridishes, autoclaved molten medium was poured in sterile petridishes in

the laminar air flow unit before it solidifies, as and when required. Some heat labile substances like GA_3, PPM (Plant Preservative Medium) and antibiotic like taxim (cefotaxime) were added aseptically to the autoclaved semisolid medium before solidifying. In case of charcoal containing medium individual tubes were shaken after autoclaving and before setting of medium for uniform distribution of charcoal.

Preparation of explants:

The explants (fruits or nodal buds) were washed under running tap water for one hour. They were then cleaned with a few drops of detergent (Labolene, Qualigens) and washed with distilled water. They were treated with 10% savlon (chlohexidine gluconate solution I.P. 1.5% v/v and cetrimide I.P. 3% w/v), Johnson and Johnson, India for 3-5 min followed by washings with distilled water. Explants were then treated with 1% Bavistin (carbendazim 50%WP, BASF, India) for an hour, on a gyratory shaker followed by rinsing three times with distilled water to remove Bavistin. Hereafter, the tissues were manipulated under aseptic condition in laminar air flow bench. The explants were rinsed with 70% ethanol followed by thorough washing with sterile distilled water. This was followed by 0.05-0.1% mercuric chloride treatment for specified time period depending on the nature of the explant (0.05% for 10 min. for fruits, and 0.1% for 7 min. for nodal buds). Adhering mercuric chloride was removed by washing the explants repeatedly with sterile distilled water.

Initiation of cultures:

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

Culture conditions:

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

Histological Techniques:

Sections were prepared for histological studies following the methods described by Sharma and Sharma, 1980. The tissues were cut into small pieces (approx 3 x 4 mm) and fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48 h at room temperature. Tissues were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (mp 58-60°C). Serial sections of 7 μm were cut using a rotary microtome (Reichert-Jung 2050, Germany). Sections were double stained with 0.5% haematoxylin- 1% eosin and mounted with DPX (Qualigens, India) for studies under microscope.

Preparation of haematoxylene: 0.5 g haematoxylene is dissolved in 10 ml of 96% alcohol and the volume is made up to 100 ml by distilled water. The stain is then filtered and allowed to ripen for 6-8 weeks.

Preparation of eosin: 1 g of eosin is dissolved in 100 ml of alcohol.

Statistical Procedures:

Statistics was used for comparison of treatment means during optimization of the parameters for micropropagation using both immature and mature explants, and for morphogenic response. Completely Randomized Designs were used. The data was subjected to analysis of variance (ANOVA) and treatment means were compared. The differences among the treatment means were subjected to Tukey test at 1% and 5% probability level, wherever applied. The data was analyzed using the statistical function given in Origin pro 8 software.

Microscopy and Photography:

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

2.3 Molecular characterization

2.3.A Materials

Glasswares and Plasticwares:

Micropipette of different precision measurements (1000, 200, 100, 20, 10 & 2µl) was procured from Gilson medical Electronics, France. Micro tips, appendorff tubes (0.05, 1.5 & 2 ml) and microtip-boxes were procured from Axygen scientific Pvt. Ltd. (India). while centrifuge tubes (50ml) were purchased from Tarson products Pvt. Ltd, (Kolkata, India).

Reagents and Chemicals:

- Tris-HCl pH 8.0 (1 M); EDTA pH 8.0 (0.5 M); NaCl (5 M); CTAB (20%); Chloroform:Iso-amylalcohol (24:1 v/v); β-mercaptoethanol; cold isopropanol and ethanol (70%)
- Extraction buffer: 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.5 M NaCl, 2.0% CTAB, 0.2% β-mercaptoethanol (v/v) (added immediately before use).
- TE buffer: 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA.
- Agarose (Sigma, USA)
- Electrophoresis buffer: Tris-borate-EDTA (1x)
- Loading buffer: Bromophenol blue (0.25%) and glycerol (30%)
- Fluorescent dye: Ethidium bromide (10 µg/mL)
- Marker: Low range DNA ladder (3 Kb) (Bangalore Genei, India)
- Enzymes: RNAase A (10 mg/mL) and Taq DNA Polymerase (Bangalore Genei, India)
- Buffers: *Taq* DNA Polymerase buffer with $MgCl_2$ (Bangalore Genei, India)
- Nucleotides: dNTPs (G, A, T, C) (Bangalore Genei, India)
- Primers: ISSR Primer Set #9 (801…900) obtained from University of British Columbia, USA was used (Table 2.3)

Equipments:

Milli-RO water system (Millipore, USA): The instrument is used for obtaining deionized water. There are series of ion exchanging columns. When ordinary water is passed through these columns, it is made free of salts and minerals that are naturally present in tap water.

It is important to use deionized water for preparation of reagents as the presence of minerals and salts may alter the final composition.

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

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

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

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

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

PCR Robocyler (Stratagene, USA): It is microprocessor controlled for block laboratory instrument utilizing a robotic arm to quickly move from one temperature block to another based on user defined program. This system has four separate anodized aluminum temperature blocks (3-heating block &1 cold block) containing 96 precision cut wells that remain at set temperature, where thermal cyclers have a single block that changes

temperature during each cycle. In the robocycler tubes are moved from one block to next by the robotic arm. The four-block design decreases cycling time by up to 30% and achieves a well-to-well temperature uniformity of ± 0.1 °C for amplification process.

Table 2.3: List of ISSR Primers

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

Source of plant material:

For each species of *Garcinia* leaves were collected from different locations in Maharashtra. The locations were usually 100 km apart. From each location five plants were sampled.

2.3.B Methods

DNA isolation protocol: DNA was isolated using the standard protocol of Lodhi et al. 1994. The protocol is as follows:

- Collected young leaves were stored in -70°C until used. 2.0 g of leaves were ground using mortar and pestle in the presence of liquid nitrogen.
- Added 20.0 mL of extraction buffer to the ground leaves and mixed in the mortar.
- Poured the slurry into clean 50 mL polypropylene centrifuge tubes, rinsed the mortar and pestle with 1 mL of extraction buffer and added to the original extract. 2% (v/v) β-mercaptoethanol was added to the mixture and mixed gently.
- Incubated at 60°C for 60 minutes and cooled to room temperature.
- Added equal volume of 24:1 (v/v) chloroform:isoamyl alcohol and mixed gently by inverting the tubes 20 to 25 times to form an emulsion.
- Spinned at 1000 rpm at room temperature.
- Transfered the top aqueous phase to a new 50 mL centrifuge tube with a wide-bore pipette tip. A second chloroform:isoamylalcohol extraction was performed.
- Added 0.5 volume of 5M NaCl to the aqueous solution recovered from the previous step and mixed well.
- Added equal volume of cold (-20°C) isopropanol and cooled at 4 to 6°C for 15-20 minutes or until DNA strands begin to appear.
- DNA was spooled out and washed with cold (0 to 4°C) 70% ethanol. Ethanol was then completely removed without drying the DNA pellet by leaving the tubes uncovered at 37°C for 20 to 30 minutes.
- Dissolved the DNA in 200 to 300 µL TE.
- Treated with 1 µL RNAaseA per 100 µL DNA solution and incubated at 37°C for 15 minutes.
- Extracted with equal volume of choloroform: isoamyl alcohol (24:1).

- Transferred the aqueous layer to a fresh 1.5 ml microfuge tube and added equal volume of isopropanol.
- Spinned at 10,000 rpm for 10 min at 25-30ºC.
- Washed the pellet with 70% ethanol. Ethanol was then completely removed without drying the DNA pellet by leaving the tubes uncovered at 37°C for 15 to 20 minutes.
- DNA was dissolved in 200 µl of sterile milliQ water and stored at 4 ºC until required.
- DNA concentrations were determined either by running aliquots of DNA samples on a 0.8% agarose gel electrophoresis or by taking the absorbance at 260 nm. The ratio between 260 and 280nm provided an estimate of the purity of the DNA sample. DNA samples with a ratio of approximately 1.8 under spectrophotometer and producing an intact single band without smear on 0.8% Agarose gel electrophoresis were considered as good quality DNA.

PCR protocol using ISSR primers:

- The PCR protocol described by Mullis (1986) was followed and carried out in a total volume of 25 µl containing following components: 4 µl of 10 ng/µl genomic DNA; 2.5 µl of 10X *Taq* buffer without $MgCl_2$; 2.5 µl of 25 mM $MgCl_2$; 5 µl of 1.0 mM dNTPs ; 0.6 µl of 1 U/µl Taq Polymerase ; 4 µl of 1.5 µM/µl Primer and 6.9 µl of sterile milliQ water.
- PCR amplifications were performed on a Stratagene RoboCycler under the following conditions (Fig. 2.1).
- Negative controls, containing all PCR components except DNA were also set up and run with each set of reactions.
- A low range DNA ladder (3 Kb) was used for band sizing.
- The ISSR products were loaded on 1.5% agarose gel stained with ethidium bromide for electrophoresis in 1.0X TBE at a constant current 60 mA, < 150 V for 2 h. Visualization and photography of the gel was done in Gel Documentation system.

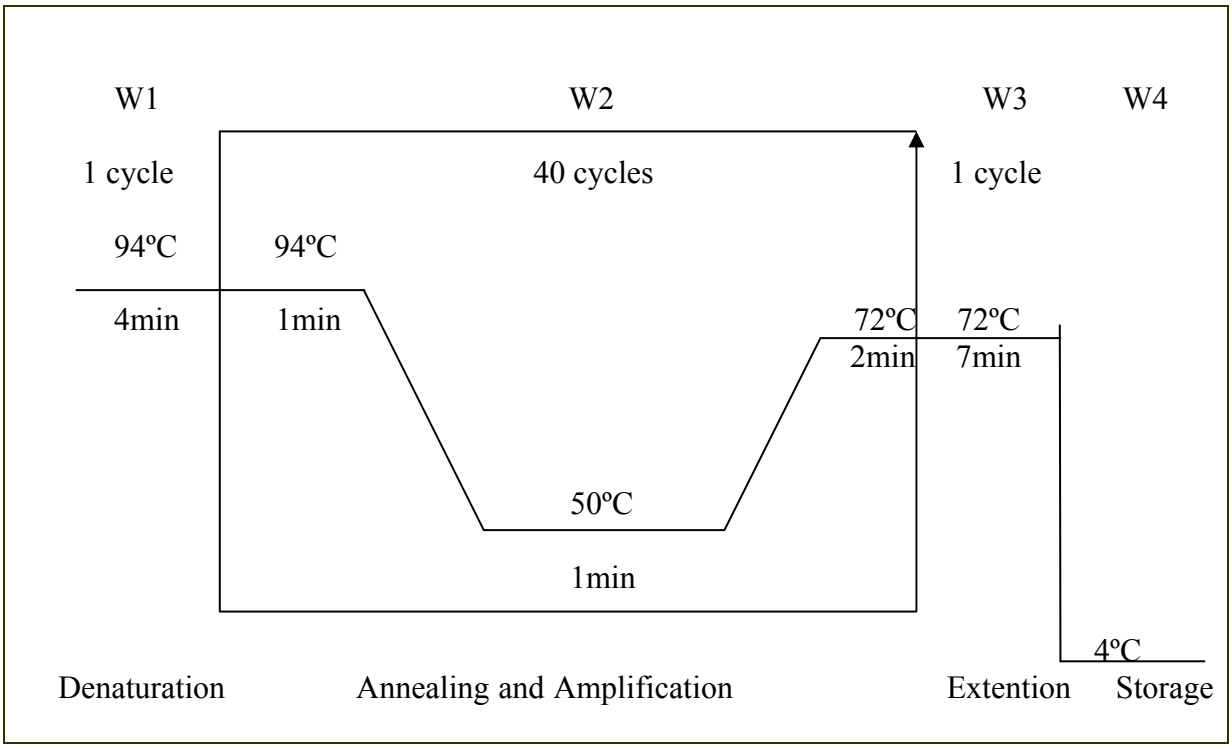


Fig 2.1: Schematic diagram of PCR reaction

Data Analysis:

Presence/absence of each scorable fragment was recorded in a binary data matrix and the frequency of each band in sample was determined. Wind Dist software program in NTSYS format was used to produce similarity matrix. Dendrogram was prepared using the matrix.

2.4 Chemical analysis:

2.4.A Materials

Glasswares:

Autoclavable bottles (250 ml) with screw able caps, measuring cylinders (100 ml, 50 ml), 500 ml round bottomed flask, funnels and test tubes were purchased from Borosil Glass Works Ltd (Mumbai, India). HPLC autosampler vials (1.5 ml) were purchased from Perkin Elmer (Mumbai, India). For filtering the HPLC solvents, filtration unit (1 L capacity) was purchased from Supelco Pvt. Ltd. (India).

Chemicals:

MiliQ water, H_2SO_4 (AnalaR, Merck), rectified spirit, activated charcoal (Merck, India), standard HCA sample (Cipex, India)

Equipments:

Rotary evaporator: For concentration of the extracts, a rotary evaporator (Buchi, Germany) was used. A rotary evaporator consists of (1) a motor unit which rotates the evaporation flask or vial containing one's sample; (2) a vapor duct which acts both as the axis for sample rotation, and as vacuum-tight conduit for the vapor being drawn off the sample; (3) a vacuum system, to substantially reduce the pressure within the evaporator system; (4) a heated fluid bath, generally water, to heat the sample being evaporated; (5) a condenser with either a coil through which coolant passes, or a "cold finger" into which coolant mixtures like dry ice and acetone are placed; (6) a condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses; and (7) a mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

High performance liquid chromatography (HPLC) **(**Perkin Elmer, series 200, USA**):** HPLC method for the analysis of bioactive compounds was developed on Perkin-Elmer's Series 200 HPLC system. This system was equipped with a quaternary gradient pump, an autosampler and diode array detector. The column used in the present study was bondclone 10 C18 (phenomenex) of diameter 3.90 X 300 mm.

2.4.B Methods

Estimation of % (-)-HCA in the fruits of *Garcinia* species:

Fruits of *Garcinia* species were collected from different locations along the Western Ghats of Maharashtra. The locations were about 100 km apart. From each location effort was made to collect fruits from 5 plants. For the extraction of (-)-HCA from the fruits of *Garcinia* species the method reported by Sakariah and Jayaprakasha 1998 was used with slight modifications. 25 g of the fruit rind was autoclaved at 15 lbs/inch^2 with 100 ml of distilled water for 20 min and filtered. Autoclaving and filteration was repeated twice for complete extraction of the acid. The combined coloured extract was decolorized

using activated charcoal and filtered. The decolorized extract was concentrated to 25 ml on rotary vapour and was treated with 50 ml of ethanol to remove pectinaceous material and centrifuged. The supernatant was concentrated under reduced pressure to 5 ml and stored at 4°C until further use. 5 ml of this solution was dissolved in 20 ml of 10 mM H_2SO_4 and centrifuged. 1 ml of this solution was filtered and used for HPLC analysis. The mobile phase used was 10 mM H_2SO_4. The column used for (-)-HCA estimation was C18. The flow rate was set to 0.7 ml/min. The chromatogram was recorded at 214 nm. For quantification external standard method was used. The standard (-)-HCA used for quantification was procured from Cipex, India.

CHAPTER 3

BIODIVERSITY STUDIES IN *GARCINIA* SPECIES.

3.1 Introduction

"The variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems." is the definition of biodiversity included by the United Nations in the Convention on Biological Diversity (UNEP 1992). Diversity within species is the genetic diversity, between species is the species or taxonomic or organismal diversity; and of ecosystems is the ecological or habitat diversity.

The biodiversity found on Earth today is the result of approximately 3.5 billion years of evolution. Until the emergence of humans, the earth supported more biodiversity than any other period in geological history. However, since the dominance of humans, biodiversity has begun a rapid decline, with one species after another suffering extinction.

The maintenance of biodiversity is important for the following reasons:

- Ecological stability

Each species performs a particular function within an ecosystem. They can capture and store energy, produce organic material, decompose organic material, help to cycle water and nutrients throughout the ecosystem, control erosion or pests, fix atmospheric gases, or help regulate climate.

Ecosystems provide support of production and services without which humans could not survive. These include soil fertility, pollinators of plants, predators, decomposition of wastes, purification of the air and water, stabilisation and moderation of the climate, decrease of flooding, drought and other environmental disasters.

Research show that the more diverse an ecosystem the better it can withstand environmental stress and the more productive it is. The loss of a species thus decreases the ability of the system to maintain itself or to recover in case of damage. There are very complex mechanisms underlying these ecological effects.

- Economic benefits to humans

Most people see biodiversity as a reservoir of resources to be drawn upon for the manufacture of food, pharmaceutical, and cosmetic products. Thus resource shortages may be related to the erosion of the biodiversity.

Some of the important economic commodities that biodiversity supplies to humankind are:

Food: crops, livestock, forestry, and fish

Medication: Wild plant species have been used for medicinal purposes before the beginning of recorded history. For example, quinine (used to treat malaria) comes from the bark of the Amazonian cinchona tree; digitalis from the foxglove plant (chronic heart trouble), and morphine from the poppy plant (pain relief).

According to the National Cancer Institute of the USA, over 70 % of the promising anti-cancer drugs come from plants in the tropical rainforests. Animal may also play a role, in particular in research. It is estimated that of the 2, 50,000 known plant species, only 5,000 have been researched for possible therapeutic applications.

Industry: fibres for clothing, wood for shelter and warmth. Biodiversity may be a source of energy (such as biomass). Other industrial products are oils, lubricants, perfumes, fragrances, dyes, paper, waxes, rubber, latexes, resins, poisons and cork can all be derived from various plant species. Supplies from animal origin are wool, silk, fur, leather, lubricants, waxes. Animals may also be used as a mode of transportation.

Tourism & Recreation: biodiversity is a source of economical wealth for many areas, such as many parks and forests, where wild nature and animals are a source of beauty and joy for many people. Ecotourism in particular, is a growing outdoor recreational activity.

- Ethical reasons

The role of biodiversity is to be a mirror of our relationships with the other living species, an ethical view with rights, duties, and education. If humans consider species have

a right to exist, they cannot cause their extinction voluntarily. Besides, biodiversity is also part of many cultures' spiritual heritage.

But we are fast losing this natural heritage due to factors that include habitat destruction through changes in land use; pollution, for example by nitrogen deposition; the invasion of ecosystems by non-native plant and animal species (biotic exchange); and the biological consequences of increased levels of carbon dioxide in the atmosphere. In the short to- medium term, human-induced fragmentation of natural habitat and invasive species are particular threats to biodiversity. In the long term climatic changes are a threat to biodiversity.

3.2 Biodiversity can be measured at three levels:

- **Genetic diversity:**

Genetic diversity provides the core differences that divide life into its major types, especially important when visible structure and shape provide no reliable guide. This genetic diversity is a witness not only to the deepest division of life but also some shared characteristics, stretching across huge taxonomic distances.

Methods for Measuring genetic diversity

Protein electrophoresis: Widely used since 1960s, this technique analyses the different proteins which in turn reflect the different alleles in an individual.

Restriction site mapping: A resent advance relying on specific bacterial enzymes that cut the DNA at specific sequences. Analysis of these cut points is called restriction mapping.

DNA and RNA sequencing: Allows analysis of DNA. A frequent alternative is RNA found in ribosomes. Ribosomes are cell organelles that read genetic data and manufacture proteins based on these instructions. 16s rRNA has been particularly important.

- **Species diversity:**

It refers to the variety of species of animals, plants or microorganisms found on the earth. Biodiversity term is mostly considered as a synonym to species diversity. It is very important level of biodiversity since it is easier to work with it and the species can be seen with the naked eyes unlike genetic diversity that can be worked out only in the

laboratories. There is a wide difference in the various estimates for the total number of species found on this earth (this varies from 5 to 100 million). However, so far nearly 1.7 million species have actually been described. Species diversity can be measured in a number of ways. Most of these ways can be classified into three groups of measurements - species richness, species abundance and taxonomic diversity. Measures of species richness count the number of species in a defined area. Measures of species abundance sample the relative numbers among species. A typical sample may contain several very common species, a few less common species and numerous rare species. Measures of species diversity that simplify information on species richness and relative abundance into a single index are in extensive use. Another approach is to measure taxonomic or phylogenetic diversity, which considers the genetic relationships between different groups of species. These measures are based on analysis which results in a hierarchical classification usually represented by a 'tree' that depicts the branching pattern which is thought to best represent the phylogenetic evolution of the taxa concerned.

Different measures of taxonomic diversity emphasise various taxic characteristics and relationships. The species level is generally regarded as the most appropriate to consider the diversity between organisms. This is because species are the primary focus of evolutionary mechanisms and therefore are relatively well defined.

On a broad scale species diversity is not evenly distributed across the globe. The single most obvious pattern in the global distribution of species is that overall species richness is concentrated in equatorial regions and tends to decrease as one moves from equatorial to polar regions. In general, there are more species per unit area in the tropics than in temperate regions and far more species in temperate regions than there are in polar regions. In addition, diversity in land ecosystems generally decreases with increasing altitude. Other factors which are generally believed to influence diversity on land are rainfall patterns and nutrient levels. In marine ecosystems, species richness tends to be concentrated on continental shelves, though deep sea communities are also significant. At the global level, an estimated 1.7 million species have been described to date; current estimates for the total number of species in existence vary from five million to nearly 100 million.

- **Ecosystem diversity:**

It includes various types of ecosystems and the diversity of habitats and ecological processes occurring therein. Examples of various ecosystems are coral reefs, tropical rain forests or temperate rain forests and these are based on the major communities. The measurement of biodiversity within an ecosystem is a difficult task because of their complex nature. It is harder to define ecosystem diversity than species or genetic diversity because the 'boundaries' of communities (associations of species) and ecosystems are more fluid. Since the ecosystem concept is dynamic and thus variable, it can be applied at different scales, though for management purposes it is generally used to group broadly similar assemblages of communities, such as temperate rainforests or coral reefs. A key element in the consideration of ecosystems is that in the natural state, ecological processes such as energy flows and water cycles are conserved.

The classification of the Earth's immense variety of ecosystems into a manageable system is a major scientific challenge, and is important for management and conservation of the biosphere. At the global level, most classification systems have attempted to steer a middle course between the complexities of community ecology and the oversimplified terms of a general habitat classification.

Generally these systems use a combination of a habitat type definition with a climatic descriptor; for example, tropical moist forest, or temperate grassland. Some systems also incorporate global biogeography to account for differences in biota between regions of the world which may have very similar climate and physical characteristics.

The measurement of ecosystem diversity is still in its infancy. Nevertheless, ecosystem diversity is an essential element of total biodiversity and accordingly should be reflected in any biodiversity assessment.

3.3 Ecological mapping for monitoring biodiversity at the species and ecosystem level:

Mapping of ecological variables using a range of techniques is called ecological mapping. Ecological mapping is concerned with mapping of organisms, assemblages and habitats (whether it is from ground, air or space) but it can also be concerned with mapping

fungi on a forest floor or microbes in the soil. Ecological mapping became a sophisticated science in the Netherlands as long ago as 1970 where the identification and monitoring of areas for conservation and ecological importance were undertaken. Ecological mapping of coastal zones to enable the identification of ecologically sensitive areas was also one of the early developments. That information has been used for clean up operations after oil spills so that areas in greatest need of protection were given priority.

Sources of data for monitoring land use and land cover can be either secondary (e.g. Ordnance Survey maps) or primary and collected primarily for the purpose of monitoring. Primary data has previously been obtained from ground surveys, aerial photography and satellite imagery. Ground surveys or inspection of the land on foot potentially allow us much land to be examined in as much detail as time and facilities permit. Such systematic recording has considerable advantages in terms of accuracy but may be costly in terms of time and effort. Aerial photography has long been used to study changes in landscape / land use and this method has been well researched.

In practice aerial photography requires careful interpretation and by itself cannot provide detailed information about ecological attributes such as interior structure of a woodland or species composition of grassland. The technique is suitable for less complex vegetation like coastal dune vegetation. Remote sensing techniques with satellite imagery have greatly improved the accuracy of recording and monitoring land use change, nevertheless the success of satellite imagery is dependent on acquisition of good ground reference data.

Spatial distribution data have traditionally been presented in printed map form only but this becomes cumbersome to handle especially when several categories of information (such as soil characteristics, water characteristics, current land uses, land capability) are being analysed. Recent advances in the sophistication of computers and GIS software has greately influenced the way data is handled, stored, analysed and presented.

3.4 Maharashtra

Maharashtra is the 3rd largest state of India located between 16° N to 22° N latitudes and 72 -80° E longitudes.

On the basis of geographical features the state is divided into 3 natural regions

1. Konkan comprising the coastal area
2. Sahyadri hill ranges known as Western Ghats
3. The Deccan plateau

Major portion of the state is semi arid with three distinct season of which rainy season comprises of July to September. There are large variations in the quantity of rainfall within different parts of the state. The Western Ghat and coastal districts receive an annual rainfall of 2000 mm but most part of the state lies in the rain shadow belt of the Western Ghat with an average of 600 to 700 mm.

Fig 3.1: Agro Climatic zones of Maharashtra

The state has been divided into 9 agro-climatic zones based on rainfall, soil type and the vegetation as mentioned below

1) South Konkan coastal zone
2) North Konkan coastal zone
3) Western Ghat zone
4) Transition zone - 1
5) Transition zone - 2
6) Scarcity zone
7) Assured rainfall zone
8) Moderate rainfall zone
9) Eastern Vidarbha zone

Table 3.1a: South Konkan coastal zone - Very high rainfall zone with laterite soil

Geographical location	Geographical spread of the zone/ Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
15° 30´ to 18° 50´ N Latitude 72° 45 to 74° 50 E Longitude	Comprises mainly of Ratnagiri and Sindhudurg districts Total area of the zone is 13.20 lakh ha.area under culivation 3.5 lakh ha.	Daily temp. above 20° C. through out the year. May hottest above 33° C. Rainfall due to S-W monsoon from June to Sept.	3105 mm in 101 days	Laterite. pH 5.5-6.5 acidic, poor in phosphorous rich in nitrogen and potasscium

Table 3.1b: North Konkan coastal zone - Very high rainfall zone with non lateritic soils

Geographical location	Geographical spread of the zone/ Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
17° 52´ to 20° 20´ latitude 70° 70´ to 73° 48´ E longitude	Comprises of Thane & Raigad districts.Total area 16.59 lakh ha. Net sown area 4.69 lakh ha. With forest zone about 3%. 32% of land is under forest.	Avg. daily temp 22° to 30°C.Mini. temp17° to 27° C. Humidity 98%in rainy season & winter-60%	2607 mm in 87 days. Maximum rain received in July i.e.41%	Coarse & shallow. pH 5.5 to 6.5, acidic Rich in nitrogen, poor in phosphorus & potash.

Table 3.1c: Western Ghat zone

Geographical location	Geographical spread of the zone/ Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
Narrow strip extending from north to south along the crest of Sahyadri ranges	It includes hilly high lying terrains of Kolhapur, Satara, Pune, Ahmednagar & Nasik districts & small area of Sindhudurg district. Altitude varies from 1000- 1900m	Maximum temp. ranges from 29-39° C. Minimum temp ranges from 13-20° C.	3000 to 6000 mm. Rainfall recorded in different places of the zone viz Igatpuri, Lonawala, Maha baleshwar, & Radhanagari.	'Warkas' i.e. light laterite & reddish brown. Distinctly acidic, poor fertility low phosphorous & potash content.

Table 3.1d: Transition Zone -1 (Sub Montane Zone)

Geographical location	Geographical spread of the zone/ Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
Located on eastern slopes of Sahyadri ranges	Spreads over 19 tahsils of five districts viz, Nasik, Pune, Satara, Sangli & Kolhapur. The area of the zone measures 10,289 Sq Km	Average maximum temperature is between 28-35°C and minimum 14-19° C	700-2500 mm.Rains received mostly from S-W monsoon.	Soils are reddish brown to black tending to lateritic. pH 6-7.Well supplied in nitrogen but low in phosphorous & potash

Table 3.1e: Transtion Zone-2 (Western Maharashtra Plain Zone)

Geographical location	Geographical spread of the zone/ Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
It is a wider strip running parallel to eastern side of Sub Montane Zone.	This zone includes tehsils of Dhule, Ahmednagar, Sangli & central tahsils of Nasik, Pune, Satara & Kolhapur districts. Geographical area 17.91 lakh ha.Net area sown is 8.86 Lakh ha.	Water availability ranges from 120-150 days .Maximum temperature 40°C & minimum 5° C.	Well distributed rainfall 700 to 1200 mm.	Topography is plain. Soils greyish black .Moderately alkaline 7.4- 8.4, lowest layer is 'Murum' strata. Fair in NPK content. Well drained & good for irrigation.

Table 3.1f: Scarcity Zone

Geographical location	Geo graphical spread of the zone/ Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
Western Maharashta	This zone covers geo graphical area of 73.23 lakh ha. The gross & net cultivated area is 58.42 and 53.0 lakh ha respectively.	Suffers from very low rainfall with uncertainty & illdistribution. Occurance of drought is noted once in three years. Dry spell varies from 2-10 weeks. Water availability 60-140 days. Which is affected due to 1) delayed onset of monsoon 2) early cesation of monsoon. Maximum temperature 41º C minimum 14-15º C	Less than 750mm in 45 days. Two peaks of rainfall., 1) June/ July 2) september. Bimodal pattern of rainfall.	General topography is having slope between 1-2%. Infiltration rate is 6-7 mm/hr.The soils are vertisol. Soils have Montmorilonite clay. Poor in nitrogen, low to medium in phosphate & well supplied in potash.

Table 3.1g: Assured Rainfall Zone

Geographical location	Geographical spread of the zone/ Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
Central Maharashtra Plateau region	Comprises parts of Aurangabad, Jalna beed & Osmanabad districts. Major parts of Parbhani & Nanded & complete Latur Buldhana & parts of Akola, Amravati, Yavatmal, Jalgaon, Dhule & Solapur. Area accounts to 75 lakh ha. Gross cropped area is 67.8 lakh ha. Forest accounts to 9.90 % of geographical area.	Maximum temperature 41°C Minimum temperature 21° C	700 to 900 mm. 75 % rains received in all districts of the zone.	Soil colour ranges from black to red. Type- 1) vertisols 2) entisols & 3) inceptisols PH 7-7.5

Table 3.1h: Moderate Rainfall Zone

Geographical location	Geographical spread of the zone/Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
Central Vidarbha	There are five sub- zones of central Vidarbha zone based on climate soil & cropping pattern. The zone includes entire Wardha, major parts of Nagpur, Yavatmal, 2 tahsils of Chandrapur & parts of Aurangabad, Jalna, Parbhani & Nanded districts. Largest agro climatic zone encompassing 49.88 lakh ha geographical area & 35.73 lakh ha net cropped area.	Maximum temperature 33-38° C Minimum temperature 16-26° C. Average daily humidity 72 % in rainy season, 53 % in winter & 35% in summer.	1130 mm.	Black soils derived from basalt rock. Medium to heavy in texture alkaline in reaction. Low lying areas are rich and fertile.

Table 3.1i: Eastern Vidarbha Zone - High Rainfall Zone with Soils derived from parent material of different crops. There are four subzone based on climate, soils and crop pattern

Geo graphical location	Geo graphical spread of the zone/Districts and tahsils included	Climatic conditions	Average annual rainfall	Soiltype
Eastern Vidarbha	Includes entire Bhandara & Gadchiroli and parts of Chandrapur and Nagpur districts. Geographical area is 32.7 lakh/ha. And with almost 50% under forest. Gross crop area 10.8 lakhs/ha.	Mean Maximum temperature varies from 32° to 37° C. Minimum temperature 15° to 24° C. Daily humdity 73% for rainy season 62 for winter & 35 for summer	950 to 1250 mm on western side. 1700 mm on extreme east side No of rainy days 59.	Soils derived from parent rock granite, gneisses, and schists. Brown to Red in colour. pH 6 to 7

3.5 *Garcinia spicata* Hook

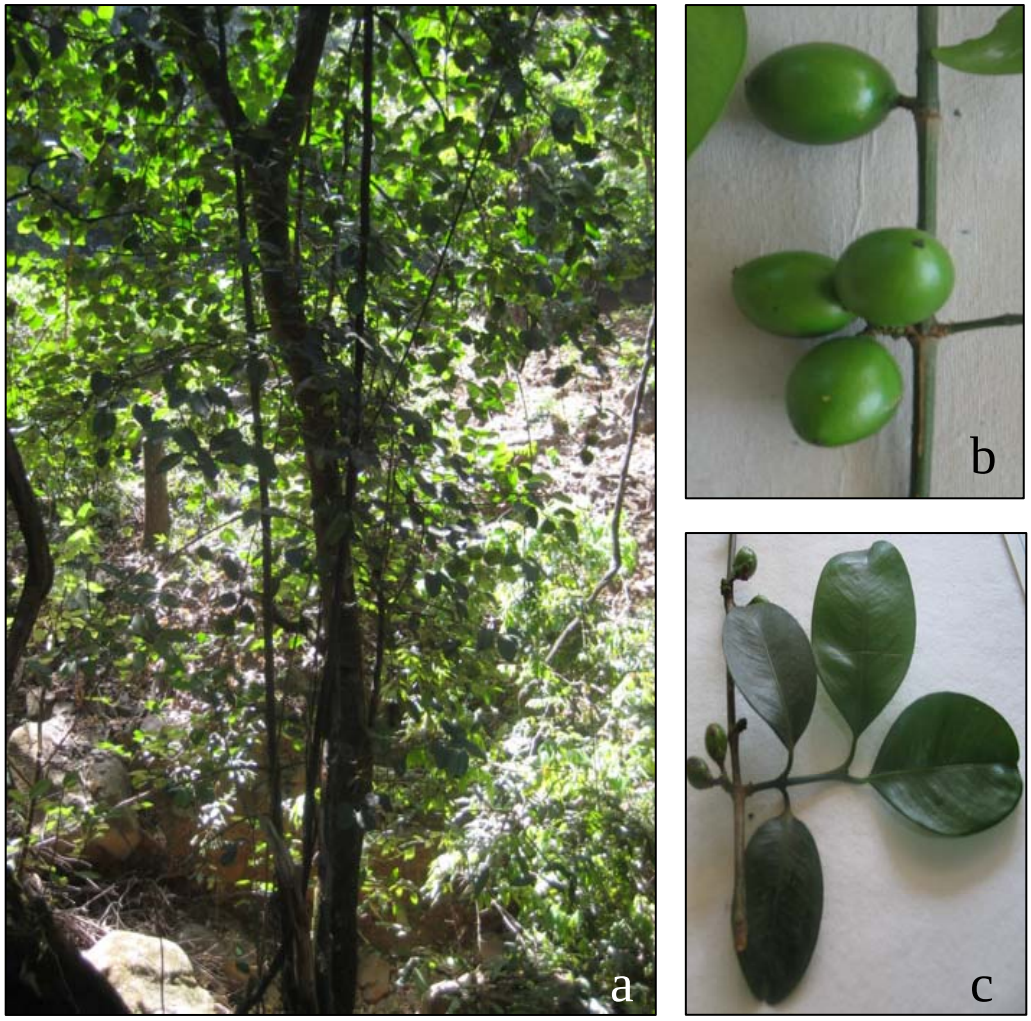


Fig 3.2: a-habit of *Garcinia spicata*, b- fruits, c-leaves

Medium sized trees, 6-12 m high distributed in the Western Ghats from Konkan southwards to Kerala at low elevations and in the Eastern Ghats from Ganjam southwards to Pudukkotti in Tamil Nadu. Bark thick, smooth, olive-green or brownish. Branches often 6 ribbed. Leaves 9-22 x 4-8 cm, ovate, ellipticoblong or lanceolate or suborbicular, rotundate or often emarginated, rarely acute, base rotundate; midrib strong, prominent below, laterals slender, about 18, prominent on both sides; petiole 1cm long. Male flowers: about 10mm in diameter, pedicellate. Sepals 4 or 5, half as large as the petals. Petals 5, concave. Stamens in 5 long-clawed spathulate fascicles, opposite petals; anthers didymous; filaments short, free. Female flowers: often more and usually on much longer pedicels than

the males. Staminodes 5, small with weak anthers. Ovary globose, 3 to 4-locular; style short; stigma 5 lobed to the middle. Fruit is a berry broadly oblong, size of a walnut, dark green containing 1-3seeds.

Chemical constituents: Presence of xanthochymol and isoxanthochymol as major and minor compounds respectively, has been detected in stem bark; macluraxanthone, friedelin and sitosterol has also been isolated (Waterman and Crichton 1980); while friedelin, friedelan-3β-ol, sitosterol, biflavanones GB-1, GB-1a and Morelloflavone has been isolated from leaves (Gunatilaka et al. 1984).

Uses: The wood is a strong timber useful for general construction purposes, also suitable for wattle and daub buildings. The fruit is eaten and the pulp of young fruits affords a chrome-yellow pigment. The bark is reported to contain the colouring matter Fukuji, used as a mordant dyestuff in Japan. (Maheshwari 1964)

3.6 *Garcinia talbotii* Raizada

Fig 3.3: a-habit of *Garcinia talbotii*, b-fruits of *Garcinia talbotii*

This species is closely allied to *Garcinia spicata* Hook. F. and is mainly distinguished by its obtuse leaves; large male flowers, 18-27 mm in diameter and anthers 8-12 in a bundle. It is easily confused with *Garcinia xanthochymus* Hook. F., from which it can be distinguished by the number of stigmatic lobes: *Garcinia talbotii*: 3, *Garcinia xanthochymus*: 5-6. From the leaves alone it is not possible to distinguish these two species. *Garcinia talbotii* Raizada is a medium sized tree of 6-15 m height endemic to the Western Ghats of India, North Kanara southwards (Maheshwari 1964). It has horizontal opposite branches with simple oppositely arranged leaves. The leaves are obtuse. The flowers are found in axilllary umbels on old wood. Male flowers vary in colour from creamy-white, white to greenish-yellow. Fruit is a berry about 5cm in diameter containing 2 to 4 seeds.

Chemical constituents: Talbotaflavone and morelloflavone have been isolated from roots and characterized by Joshi et al. 1970. Morelloflavone is known to inhibit lipid peroxidation with IC_{50} value in the micromolar range and is also known as scavenger of superoxide (Sanz et al. 1994). They also inhibit human secretory phospholipase A2 and thus have anti-inflammatory properties (Gil et al. 1997). Morelloflavone shows significant antiviral activity against HIV-1 (strain LAV-1) in phytohemagglutinin stimulated primary human peripheral blood mononuclear cells at an EC_{50} value of 6.9 μM and a selectivity index value of approximately 10 (Linn et al. 1997). Talbotaflavone shows anti-tumour activities and can be used for the formation of anticancerous drugs (Ito et al. 1999).

3.7 *Garcinia xanthochymus* Hook

Fig 3.4: a- habit of *Garcinia xanthochymus*, b- fruits, c-flowers

Medium sized or tall trees distributed in India and Burma. It has a straight trunk with drooping branches. Bark blackish or dark grey, exfoliating in small round scales;

when cut the exudate is milky, which turns yellow later. Leaves 12-35 x 4-10 cm, linear-oblong or oblong-lanceolate, acute and acuminate, base cuneate, midrib prominent below, laterals irregular, subparallel, arched, anastomosing at the apex; petiole 1-2.5 cm long. Male flowers: white, about 1.5 cm in diameter, in 4-10 flowered fascicles, axillary; pedicels thickened, about 2.5cm long. Sepals 5, rarely 4. Petals 5, about 8mm long, alternating with sepals. Stamens in 5 broad bundles of 3-5 each, antipetalous, alternating with 5 fleshy glands; anthers bilocular. Female flowers: like the males. Staminodes few, complanate. Ovary 5 locular, stigmatic rays 5. Fruit is a berry about 6.5 cm in diam; subglobose, pointed, dark yellow containing 1-4 seeds.

Chemical constituent: Xanthochymol and isoxanthochymol have been isolated from fruits (Rama Rao et al. 1980); volkensiflavone, morelloflavone, biflavones GB-I and GB-la, maclurin, 1, 5-dihydroxyxanthone and 1, 7-dihydroxyxanthone have also been isolated from its fruits (Baslas and Kumar 1979). Three hydroxylated xanthones with prenyl or geranyl substituents, were isolated from the twig bark of *Garcinia xanthochymus*, along with the four known compounds 1,4,5,6-tetrahydroxy-7,8-diprenylxanthone, 1,3,5,6-tetrahydroxy-4,7,8-triprenylxanthone, garciniaxanthone E, and 6-prenylapigenin. Their structures were elucidated by extensive spectroscopic analysis, including 1D- and 2D-NMR as well as HR-MS experiments. All compounds showed moderate cytotoxicities against breast cancer (MDA-MB-435S) and lung adenocarcinoma (A549) cell lines, but lacked antifungal activity against *Candida albicans* (Han et al. 2007).

Uses: The acidic fruits are used to make sherbets, medicaments, jams and preserves. The gumresins from the fruits and stem make good water colour.

3.8 *Garcinia indica* Choisy

Fig 3.5: a- habit of *Garcinia indica,* b- flowers, c- fruits

Slender trees with drooping branches upto 8 m high endemic to the Western Ghats of India from Konkan southwards. Bark light brown, leaves 6.5-11 x 1.5-4 cm, lanceolate or obovate-oblong, acute or acuminate, base contracted in petiole, midrib prominent below, lateral veins slender, few (7-18), prominent on both sides; petiole 5-10 mm long, slender. Male flowers: small 4-8 in axillary and terminal fascicles; pedicels 6 mm long. Sepals 4, yellowish-orange to pinkish orange, outer ones 3-4.5 mm long, inner ones 4.5-5 mm long. Petals thick larger than sepals. Stamens numerous, forming a short capitate column; filaments short; anthers oblong. Rudimentary pistil absent or a few equaling stamens. Female flowers: solitary, terminal, pedicels 3 mm long, thick. Sepals and petals as in male flowers. Staminodes 10-18, in 4 unequal, 2-3 seriate phalanges alternating with petals, 1-3

mm long. Ovary 4-8 locular, stigma 4-8 rayed. Fruit cherry shaped about 30 mm in diameter, purple or wine brown, surrounded by persistent calyx containing 5-8 seeds.

Uses: The fruits of *Garcinia indica* are a rich source of hydroxycitric (HCA), an important biologically active plant metabolite used as an antiobesity and anticholesterol drug (Sullivan et al. 1977, Mathias et al. 1981, Pullinger and Gibbons 1983, Leonhardt and Langhans 2002). Two polyisoprenylated phenolic pigments- garcinol and isogarcinol have been isolated from the fruit rind and their structures proposed (Krishnamurthy et al. 1981). Garcinol exerts anti-inflammatory effect and is a neuroprotectant. Cyanidin-3-glucoside and cyanidin-3-sambubioside have been isolated from fruits. Kokum butter prepared from the fruit is a remedy for dysentery and diarrhea. Fruits act as demulcent and emmolient (Watson et al. 1969). Fruit rind extracts also have antifungal and antioxidant properties (Selvi et al. 2003). The fruits are used in culinary preparations in konkan region of India. They are also used to prepare a beverage that has bilious action (Kirtikar and Basu 1995). Two polyisoprenylated benzophenones, xanthochymol and isoxanthochymol have been isolated from fruits of *Garcinia indica*. The two compounds possess cytotoxic activity against human cancer cell lines (Kumar et al. 2007). The fat extracted from the seeds is used in cosmetics as emmolient.

According to Maheshwari (1964) the different species in this genus can be distinguished on the following basis.

a. Sepals and petals 5, rarely petals 4:

b. Rudimentary pistil in male flowers absent or if occurring non-fungi form:

c. Ovary 3-4 locular; branches of the tree twiggy, not thick:

d. Leaves obtuse, often emarginated; flowers about 10 mm in diameter

G. spicata

d. Leaves obtuse; flowers larger, 18-27 mm in diam.; anthers 8-12 in a fascicle

G. talbotii

c. Ovary 5-locular, rarely 2-4 locular; branches of the tree somewhat thick, compressed, 4-6 gonous or on drying winged

e. Pedicels about 2.5 cm long; sepal tips ciliate; petals expanded; berry rather large, about 6.5 cm in diam. *G. xanthochymus*

a. sepals and petals 4

f. flowers fasciculate, paniculate or solitary; stamens mono-to polyadelphous; ovary bi-to multilocular:

g. anthers bilocular, dehiscence by 2 vertical clefts; rudimentary pistil frequently absent; ovary 3-plurilocular; stigma frequently lobed or radiate, rough or glandular :

h. male and female flowers solitary or in axilllary and terminal fascicles:

i. male flowers several, in terminal and axilllary fascicles:
ii. berry smooth
iii. ovary 4 to 7-locular; stamens numerous, inserted on a hemisphere-subquadrate torus. *G. indica*

3.A Ecological mapping of *Garcinia* species

Ecological mapping of *Garcinia* species was done to evaluate the current distribution pattern of this genus. To find out the agroclimatic conditions suitable to the plant and to locate the ecologically threatened locations of this plant.

Methods:

For ecological mapping frequent trips were made to the Western Ghats of Maharashtra and datasheets were prepared. The plants were identified and their geographical locations were noted down by means of GPS (Global Positioning System). The name of the village and district in which the plant occurred was noted. The soil conditions- pH and type of that

region was noted down. The population structure – whether the plant exists singly or in population was also noted down.

3.A.1 Ecological mapping of *Garcinia indica*

Results:

Fig 3.A.1: Map showing the distribution of *Garcinia indica* in Maharashtra

Table 3.A.1: Ecological data of the different locations of *Garcinia indica* in Maharashtra

S. No.	Location	District	Latitude (N)	Longitude (E)	Altitude (m)	Single/ population	Summer temp.range	Winter temp.range	Rainfall (cm)
1	Amboli	Kolhapur	16°04.769′	74°06.027′	679	population	22- 40	10- 30.	750
2	,,	,,	16°01.174′	74°03.200′	682	population	,,	,,	,,
3	,,	,,	15°56.026′	73°58.541′	818	population	,,	,,	,,
4	,,	,,	15°56.11′	73°54.053′	39	Population	,,	,,	,,
5	Patgaon	,,	16°08.683′	73°57.699′	595	single	24-35	12-32	750
6	,,	,,	16°07.616′	73°54.228′	642	single	,,	,,	,,
7*	Shivdav	,,	16°14.36′	73°57.560′	--	single	--	--	--
8	Fonda Ghat	,,	16°22.402′	73°51.608′	532	population	22-40	12-32	378
9	,,	,,	16°21.141′	73°44.956′	110	Population	,,	,,	,,
10	,,	,,	16°21.302′	73°50.319′	110	population	,,	,,	,,
11	Radhanagri	,,	16°24.601′	73°59.975′	602	single	24-36	10-32	381
12	Radhanagri	,,	15°56.110′	73°54.530′	38	single	,,	,,	,,
13	Gaganbavda	,,	16°33.120′	73°50.747′	588	single	24-35	12-32	378
14	,,	,,	16°33.408′	73°51.474′	559	single	,,	,,	,,
15	,,	,,	16°33.296′	73°51.938′	566	single	,,	,,	,,
16	,,	,,	16°33.550′	73°52.030′	582	single	,,	,,	,,
17	Savantavadi	Sindhudurg	15°53.310′	73°49.579′	104	population	24-32	10-32	248.2
18	Bhedshi	,,	15°43.404′	74°01.787′	041	single	24-32	10-32	248.2
19	Danoli	,,	15°55.981′	73°54.728′	060	single	22- 40	10- 30.	235
20	,,	,,	15°55.981′	73°52.382′	046	single	,,	,,	,,
21	Achara	,,	16°13.888′	73°28.016′	002	single	22-40	12-33	227.5
22	Kankauli	,,	16°29.436′	73°22.707′	084	population	24-30	10-32	230
23	,,	,,	16°19.26′	73°43.021′	70	population	,,	,,	,,
24	,,	,,	17°34.015′	73°17.080′	64	population	,,	,,	,,

25	Deogad	,,	16°29.696´	73°22.707´	00	Single	25-32	20-32	224.7
26	,,	,,	16°01.397´	73°38.498´	020	Single	,,	,,	,,
27	,,	,,	16°01.397´	73°38.498´	020	Single	,,	,,	,,
28	Malvan	,,	15°53.280´	73°34.955´	160	single	22-40	12-33	227.5
29	Khanoli	,,	16°28.014´	73°34.955´	160	single	22-35	12-33	235
30	Vengurla	,,	15°51.459´	73°39.143´	014	population	24-33	18-32	238
31	Ratnagiri	Ratnagiri	17°01.452´	73°17.557´	02	Population	25-32	19-32	378.7
32	,,	,,	16°59.057´	13°17.033´	03	Population	,,	,,	,,
33	,,	,,	16°59.057´	73°17.033´	40	Population	,,	,,	,,
34	Nevre	,,	17°06.062´	73°17.080´	78	Population	25-38	14-32	318.8
35	Malgund	,,	17°09.963´	73°16.104´	025	Single	25-38	14-32	318.8
36	Pirandavane	,,	17°04.381´	73°11.793´	30	Single	25-38	14-32	318.8
37	Pavas	,,	16°51.844´	73°19.775´	00	Population	25-32	19-32	378.7
38	,,	,,	16°51.844´	73°19.775´	00	Population	,,	,,	,,
39	,,	,,	16°52.023´	73°19.035´	00	population	,,	,,	,,
40	Savarde	,,	17°23.032´	73°31.067´	105	Population	24-39	14-32	318.8
41	Lanja	,,	16°54.054´	73°31.094´	128	Single	24-39	14-32	287.4
42	Sukondi	,,	17°51.35´	79°07.700´	135	Population	24-39	14-32	287.4
43	Guhagar	,,	17°29.043´	73°13.077´	80	Population	25-34	18-32	280.0
44	Rajapur	,,	16°29.090´	73°31.025´	00	Population	25-32	19-32	378.7
45	Mhasala	Raigad	18°15.018´	73°04.583´	98	Population	22-38	12-33	350
46	Shrivardhan	,,	18°25.915´	73°00.844´	05	Population	22-40	12-33	249.2
47	,,	,,	18°03.06´	73°00.78´	05	Population	,,	,,	,,
48	Diveagar	,,	18°10.238´	72°59.377´	05	Population	22-40	12-33	300
49	,,	,,	18°19.082´	72°57.41´	07	population	,,	,,	,,
50	Dabhol	,,	17°34.155´	73°11.395´	24	single	22-40	12-33	250
51	Alibag	,,	18°36.866´	72°54.259´	02	single	22-40	12-33	249.2

52	Murud	,,	16°51.844´	73°19.775´	00	population	22-40	12-33	217.7
53	,,	,,	18°19.085´	73°57.38´	09	population	22-40	12-33	217.7
54	Panvel	,,	18°51.568´	73°06.017´	33	single	22-40	12-33	250
55	Kelshi	,,	17°46.095´	73°11.93´	09	population	22-38	12-33	399.6
56	Chaul	,,	18°36.866´	72°54.259´	02	population	22-40	12-33	249.2
57*	Khandala	,,	18°3'	74°1'	666	population	22-36	12-30	450
58*	Shivthar	,,	18°09.715´	73°36.836´	115	--	22-40	12-33	250
59	Mulshi	Pune	18°28.079´	73°27.023´	610	Population	20-38	6-28	72.2
60	,,	,,	18°27.825´	73°26.114´	616	Population	,,	,,	,,
61	,,	,,	18°28.410´	73°27.023´	610	Population	,,	,,	,,
62	,,	,,	18°28.247´	73°24.929´	600	Population	,,	,,	,,
63	,,	,,	18°25.482´	73°24.161´	541	Population	,,	,,	,,
64	,,	,,	18°28.235´	73°24.888´	580	population	,,	,,	,,
65	Varandha Ghat	,,	18°06.387´	73°37.494´	811	population	22-36	10-28	450
66	Pratapgad	Satara	17°57.216´	73°34.646´	696	population	16-29	5-24	500
67*	Ghodbunder	Bombay	18°16´	72°55´	11	--	25-35	15-25	220
68*	Victoria gardens	,,	18°55´	72°54´	11	--	,,	,,	,,
69*	Matheran	,,	18°58.60´	73° 16'	677	--	20-30	12-25	524

*Data collected from literature

Discussion:

Garcinia indica is endemic to the Western Ghats of India mainly found in northern part of Central Sahyadri and extends up to South Konkan Coast. They are under story trees found in disturbed evergreen forests to semi-evergreen forests up to a height of 700m. In Maharashtra the plant is distributed in the Konkan region and the Western Ghat region covering 6 districts-Raigad, Ratnagiri, Sindhudurg, Kolhapur, Satara and Pune. The plant is also cultivated on a small scale in this region so even though the plant is listed as endangered the coastal region can be seen densely populated with this species. The soil found in this region is red laterite soil/ non laterite soil having a pH of about 6.18**.** The soil is acidic having poor fertility. The rainfall in this region varies from 72-750cm.

3.A.2 Ecological mapping of *Garcinia talbotii*

Results:

Fig 3.A.2: Map showing the geographical distribution of *Garcinia talbotii* in Maharashtra

Table 3.A.2: Ecological data of the different locations of *Garcinia talbotii* in Maharashtra

S No.	Location	District	Latitude (N)	Longitude (E)	Altitude (m)	Single/ population	Summer temp. range(ºC)	Winter temp. range(ºC)	Rainfall (cm)
1	Hiranyakeshi	Kolhapur	15º57.313´	74º01.655´	817	population	22- 40	10- 30.	750
2	,,	,,	15º57.314´	74º01.654´	818	population	,,	,,	,,
3	Dajipur	,,	16º22.338´	73º55.342´	795	population	22-40	12-33	700
4	,,	,,	16º22.304´	73º51.861´	599	population	,,	,,	,,
5	,,	,,	16º20.066´	73º53.915´	767	population	,,	,,	,,
6	,,	,,	16º22.297´	73º52.097´	600	population	,,	,,	,,
7	Fonda Ghat	,,	16º21.790´	73º51.168´	531	single	22-40	12-32	378
8	Amboli	,,	15º57.266´	73º59.913´	749	population	22- 40	10- 30.	750
9	Chaukul	Sindhudurg	15º56.640´	74º00.011´	790	--	22-40	10-33	250
10	Baparde	,,	16º26.732´	73º29.036´	023	--	25-32	20-32	224.7
11	Mulshi	Pune	18º28.252´	73º25.013´	607	population	20-38	6-28	72.2
12	,,	,,	18º28.242´	73º24.929´	600	population	,,	,,	,,
13	,,	,,	18º27.676´	73º24.806´	567	population	,,	,,	,,
14*	Varandha Ghat	,,	16º06.396´	73º37.479´	821	--	22-36	10-28	450
15	Bhimashankar	,,	19°04.0´	73°31.60´	945	population	22-40	10-30	600
16	Pratapgad	Satara	17º56.165´	73º35.082´	835	single	16-29	5-24	500
17	Ratnagiri	Ratnagiri	17° 08'	73° 19'	03	population	25-32	19-32	378.7
18*	Khandala	Raigarh	18° 30'	74° 1'	666	single	22-36	12-30	450
19*	Shirgaon	Bombay	18° 49'	72° 56'	--	--	25-35	15-25	220

*Data collected from literature

Discussion:

Garcinia talbotii is endemic to the Western Ghats of India, North Kanara southwards. They are subcanopy trees in low elevation wet evergreen forests upto 800m. Since the plant is endangered not many accessions of this plant could be collected in Maharashtra. In Maharashtra the plant is distributed in the konkan and Western Ghat region covering 6 districts- Raigad, Ratnagiri, Kolhapur, Sindhudurg, Satara and Pune. It was observed that the locations are similar to that of *Garcinia indica* except that they are fewer in number. Except for the population found at Ratnagiri, *Garcinia talbotii* is found in dense forests at high altitudes. Moreover, at the same location for e.g. in Mulshi we found *Garcinia indica* at lower altitudes in the residential area of the local people, while *Garcinia talbotii* was found at higher altitudes away from the disturbance by man. The plant prefers black soil. The pH of the soil where the plant grows was noted as 5.67. Thus we may infer that deforestation has led to the rarity of this species.

3.A.3 Ecological mapping of *Garcinia spicata*

Results:

Fig 3.A.3: Map showing the distribution of *Garcinia spicata* in Maharashtra

Table 3.A.3: Ecological data of the different locations of *Garcinia spicata* in Maharashtra

S. No.	Location	District	Latitude (N)	Longitude (E)	Altitude (m)	Single/ population	Summer temp. range(ºC)	Winter temp. range(ºC)	Rainfall (cm)
1	Suleran	Kolhapur	16º04.769´	74 º 06.027´	676	single	22- 40	10- 30.	750
2	Hiranyakeshi	,,	15 º 57.314´	74 º 01.654´	818	population	,,	,,	,,
3*	Ajra	,,	16°12´	74°2´	695	--	,,	,,	,,
4*	Chandgarh	,,	15°56´	74°12´	--	--	,,	,,	,,
5	Savantwadi	Sindhudurg	15°54´	73°52´	610	single	22-32	10-30	248.2
6	Mulshi	Pune	18 º 28.252´	73 º 25.013´	607	single	20-38	6-28	72.2

*Data collected from literature

Discussion:

The plant is distributed in the Western Ghats from Konkan southwards to Kerala at low elevations and in the Eastern Ghats from Ganjam southwards to Pudukottah in Tamil Nadu. The plant is rare in Maharashtra. It is mainly found in the Kolhapur and Sindhudurg district of Maharashtra. The plant is found in the high altitude region from 600-800 m. The plant prefers shade to direct sunlight and grows on black soil. The pH of the soil where the plant grows was noted as 7.62. Thus we found that even though the plant is very similar to *Garcinia talbotii* morphologically, its ecological distribution is very different from it. The distribution of this plant seems to be governed by soil conditions, and the plant prefers 'Warkas' i.e. light laterite & reddish brown soil which is found in Kolahapur, Satara, Pune, Ahmednagar & Nasik districts & small area of Sindhudurg district. So we can think of introducing this species in these districts of Maharashtra.

3.A.4 Ecological mapping of *Garcinia xnthochymus*

Results:

Fig 3.A.4: Map showing the distribution of *Garcinia xanthochymus* in Maharashtra

Table 3.A.4: Ecological data of the different locations of *Garcinia xanthochymus* in Maharashtra

S.No.	Location	District	Latitude (N)	Longitude (E)	Altitude (m)	Single/ population	Summer temp. range(°C)	Winter temp. range(°C)	Rainfall (cm)
1*	Mumbai	Raigarh	18°55´	72°54´	8	--	25-35	15-25	220
2*	Khandala	,,	18°30'	74°1'	666	--	22-36	12-30	450
3	Diveargar	,,	18°10.238´	72°59.377´	05	single	22-40	12-33	300
4	Dapoli	Ratnagiri	17°45.222´	73°10.932´	162	single	24-36	14-32	325
5	Ganpatipule	,,	17°09.737´	73°16.141´	06	single	24-38	14-32	318.8
6*	Ramghat	Kolhapur	15°50´	74°7´	--	--	24-35	12-32	378
7*	Gaganbawda	,,	16°33´	73°50´	588	--	24-35	12-32	378
8	Vengurla	Sindhudurga	15°51.459´	73°39.143´	014	single	24-33	18-32	238

*Data collected from literature

Discussion:

Garcinia xanthochymus although distributed in the four districts of Maharashtra-Kolhapur, Sindhudurg, Raigad, Ratnagiri is found rare in this state. We did not find any natural population of the species. In most of the places it appears that the plant is introduced. The plant is mostly found near streams in semi evergreen to wet deciduous forests. They are found at low altitudes usually upto 200m. The plant prefers red laterite soil. The pH of the soil where the plant was growing luxurienltly was noted as 6.

3.B Genetic Diversity studies in *Garcinia* species

The availability of a variety of DNA markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and intersimple sequence repeat (ISSR) has enabled researchers to investigate genetic diversity among various plant species across natural populations (Archak et al. 2003, Deshpande et al. 2001). Local populations of traditional cultivars provide a valuable resource for plant breeding as well as for the preservation of genetic diversity (Kollicker et al. 2003). Because of this the exploration, evaluation, and conservation *in situ* and *ex situ* of genetic diversity in natural populations is imperative to guarantee sustainable development (Nevo 1998). The use of molecular markers is contributing to the speeding up of plant breeding and clarifying, confirming or even reformulating the systematic taxonomy of several groups of organisms. The molecular markers can be used to identify different genotypes (Heikal et al. 2008).

Microsatellites or simple sequence repeats (SSR) are short DNA sequence stretches consisting of motifs of one to six bases that are tandemly repeated. Owing to their ubiquity, hypervariability, abundance and genome-wide distribution, SSR loci represent a new generation of powerful genetic markers for eukaryotes. Use of this marker system, however, is hampered by the requirement for sequence information from flanking regions, from which primers are designed for polymerase chain reaction (PCR) amplification. Discovery and characterization of a large number of SSRs is therefore time-consuming and expensive for many taxa.

A recently developed modification of SSR-based marker systems, i.e. ISSR (intersimple sequence repeat) analysis, circumvents this requirement for flanking sequence information, and thus has found wide applicability in a variety of plants. Popularized largely by A. Wolfe and his colleagues (Wolfe et al. 1998), ISSR analysis involves PCR amplification of genomic DNA using a single primer that targets the repeat *per se*, with 1-3 bases that anchor the primer at the 3' or 5' end. In addition to freedom from the necessity of obtaining flanking genomic sequence information, ISSR analysis is technically simpler

than many other marker systems. The method provides highly reproducible results and generates abundant polymorphisms in many systems.

The ISSR technique is similar to that for RAPD, except that ISSR primers consist of a di- or trinucleotide simple sequence repeat with a 5' or 3' anchoring sequence of 1–3 nucleotides. Compared with RAPD primers, the ISSR primers sequence is usually larger, allowing for a higher primer annealing temperature, which results in greater band reproducibiliy than RAPD markers (Culley and Wolfe 2000). They have been successfully used to assess genetic variation in plants such as citrus (Fang and Roose 1997) *Viola pubescens* (Culley and Wolfe 2000), potato (Prevost and Wilkinson 1999), and *Oryza* (Qian et al. 2001). It is a powerful technique to assess genetic diversity among closely related species and to detect similarities between and within species levels (Ghariani et al. 2003, Moreno et al. 1998, Pasakinskiene et al. 2000, Zietkiewicz et al. 1994).

In the present study ISSR primers were used to study the genetic diversity at the population level both within and among the species of *Garcinia*.

3.B.1 Genetic diversity studies in *Garcinia indica*

Five populations were used for the genetic diversity studies in *Garcinia indica*. Five plants from each population were selected. 100 ISSR primers were screened for the study. 28 primers gave amplification. 6 primers were used to make the dendrogram.

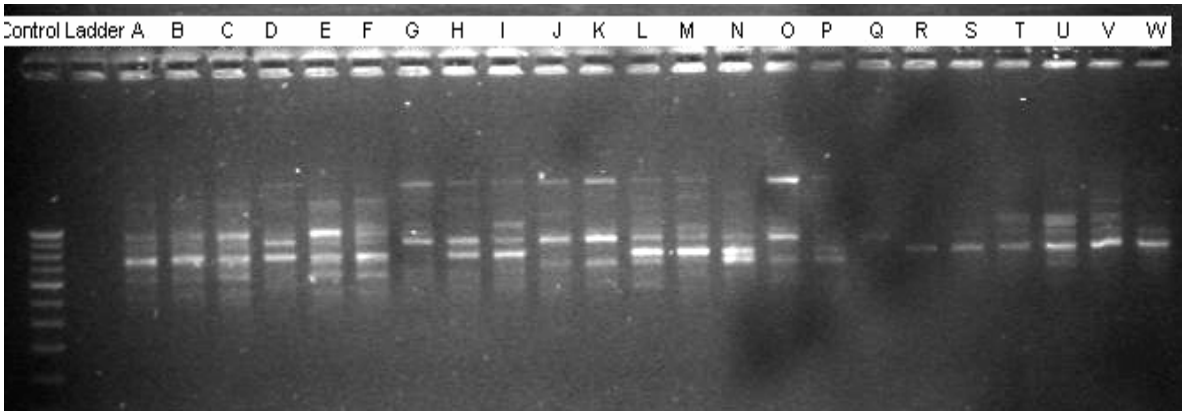
Fig 3.B.1: Map showing the locations of the five populations used in genetic diversity studies of *Garcinia indica*

Table 3.B.1: Geographical data of the five populations of *Garcinia indica*

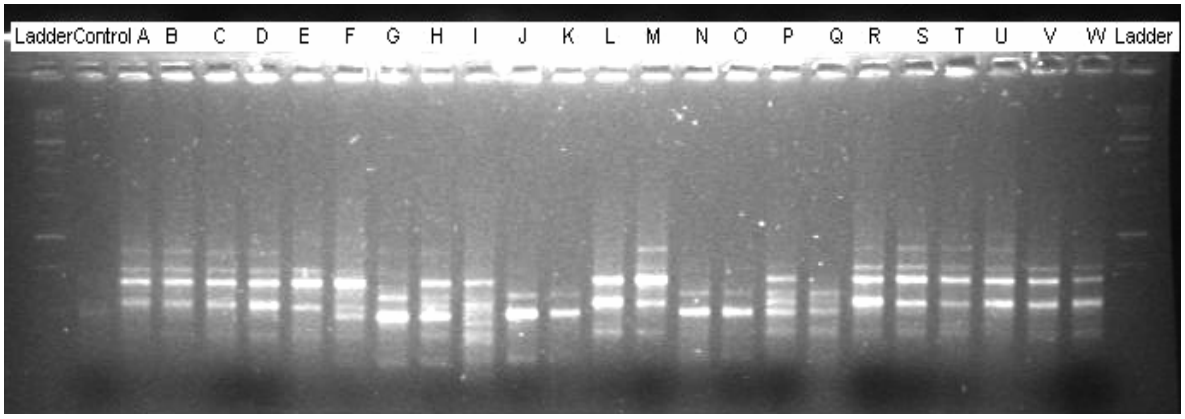
Location	District	Latitude	Longitude	Altitude(m)
Diveagar	Raigarh	18º 19.082´ N	72º 57.41´ E	7
Guhagar	Ratnagiri	17º 51.35´ N	73º 13.077´ E	135
Nevre	Ratnagiri	17º 06.062´ N	73º 17.080´ E	78
Rajapur	Ratnagiri	16º 29.090´ N	73º 31.025´ E	0
Amboli	Sindhudurg	15º 56.11´ N	73º 54.053´ E	39

Table 3.B.2: Primers used for the Genetic diversity studies in *Garcinia indica*

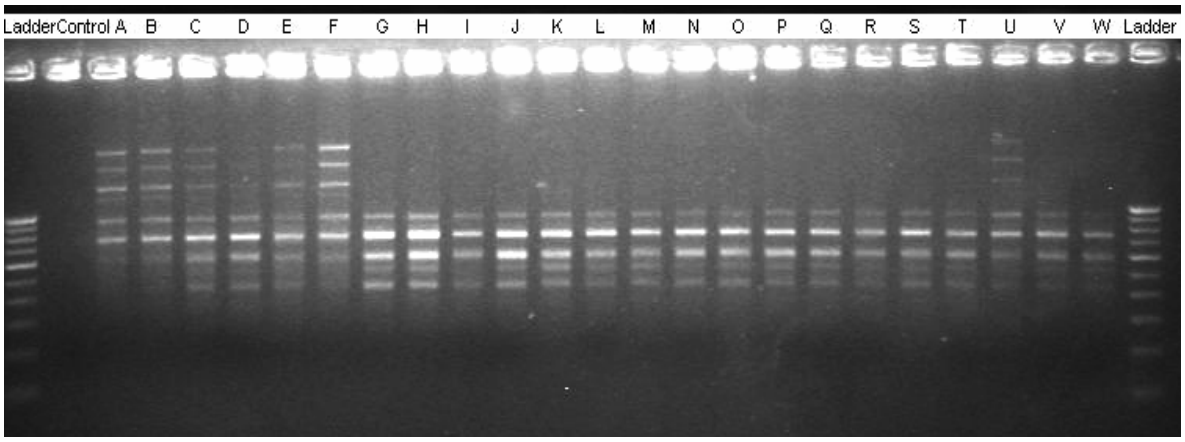
Primer no	Sequence	Annealing Temperature	No of scorable bands	No of Polymorphic bands	% Polymorphic bands
UBC 11	GAG AGA GAG AGA GAG AC	52	11	11	100
UBC 17	CAC ACA CAC ACA CAC AA	52	4	2	50
UBC 36	AGA GAG AGA GAG AGA GYA	51	17	17	100
UBC 41	GAG AGA GAG AGA GAG AYC	52	26	26	100
UBC 80	GGA GAG GAG AGG AGA	50	16	16	100
UBC 86	VDV CTC TCT CTC TCT CT	53	34	34	100



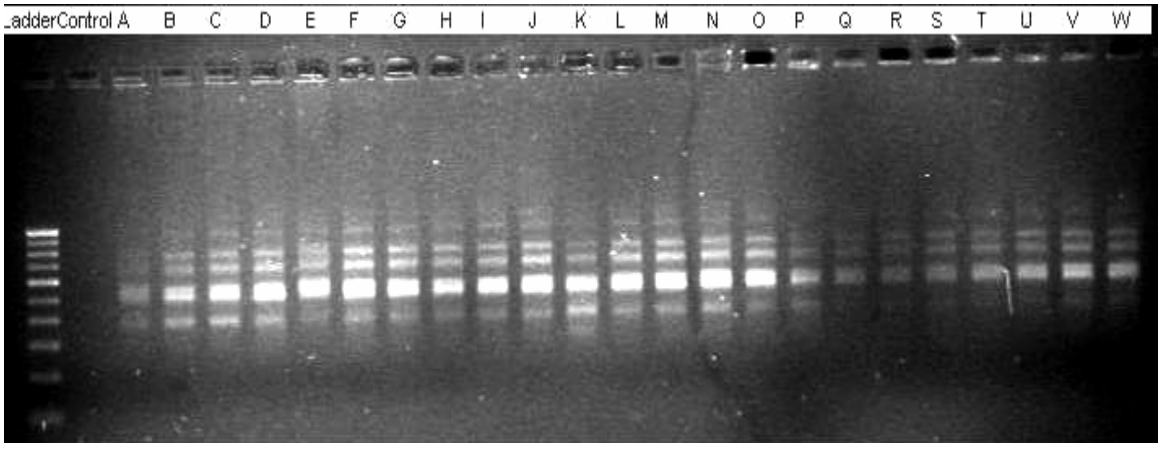
Primer: UBC 811



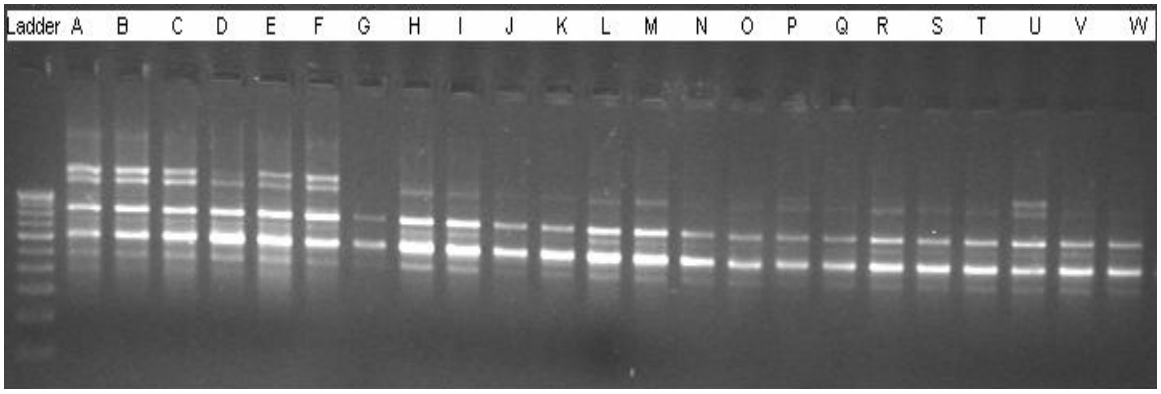
Primer: UBC 817



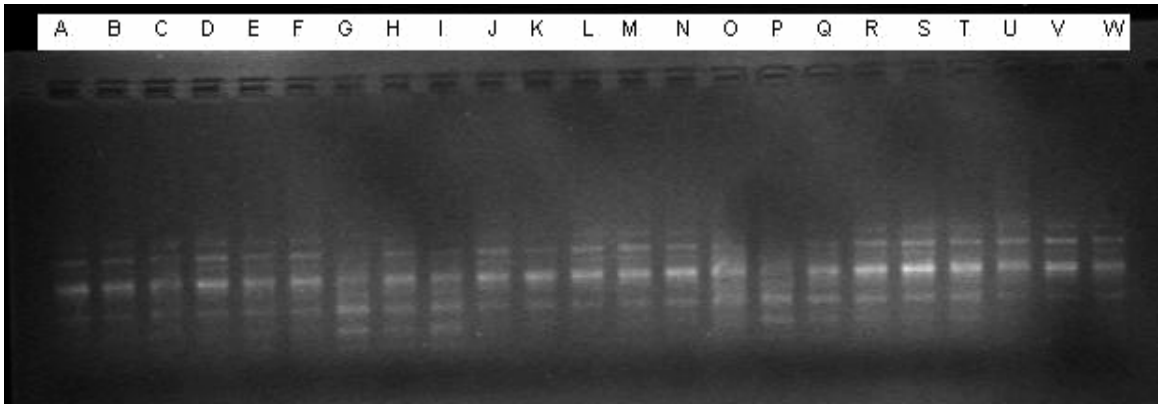
Primer: UBC 836



Primer: UBC 841



Primer: UBC 880



Primer: UBC 886

Fig 3.B.2: Banding pattern of the five populations of *Garcinia indica.*

A-D-Guhagar,

E-I-Nevre,

J-M-Rajapur,

N-R-Amboli,

S-W-Diveargar

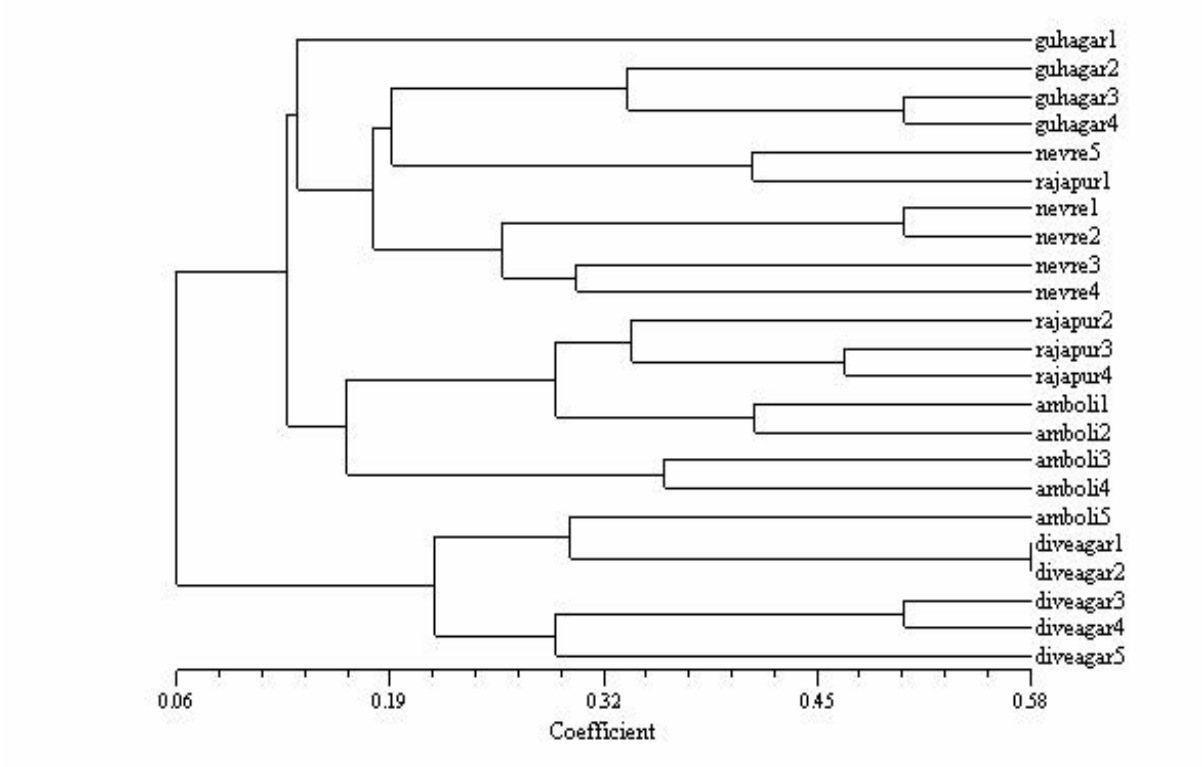


Fig 3.B.3: Dendrogram of the five populations of *Garcinia indica*

Results and Discussion:

Due to the acidic nature of the leaves DNA isolation and storage was very difficult. A modified protocol of Lodhi et al. 1994 was used for DNA isolation. Even though 28 primers gave amplification in initial screening all the primers could not be used for the genetic diversity studies, as out of the 28 primers only 6 primers gave repeatable scorable bands for all the DNA samples. A total of 108 scorable bands were generated by the 6 primers with an average of 18 bands per primer. UBC 817 produced the lowest number of bands (4) while UBC 886 produced the highest number of bands (34). Most of the primers that gave polymorphic bands were anchored dinucleotide (GA) repeats showing that GA repeats are more common in *Garcinia indica*. The AT/TA motif has been considered as the most abundant microsatellite sequences, followed by GA/CT in plant nuclear genomes (Wang et al. 1994). Similar results have been observed in *Lycoris* (Shi et al. 2006). The 6 primers gave an average of 98.14% polymorphic bands. Except UBC 817 all the primers gave 100% polymorphic bands. The percent polymorphism showed a high level of genetic

diversity in the species. The presence and absence of bands were subjected to Dice similarity coefficient and a dendrogram was made using UPGMA from the software NTSYS. The dendrogram showed the 5 populations to be distributed into 2 clusters. Cluster 1 containing the plants from Diveargar and one plant from Amboli. Cluster 2 consisting of the plants from Amboli, Rajapur, Nevre and Guhagar. The second group was further divided into two groups. One group comprising of plants from Rajapur and Amboli and the other group comprising of plants from Nevre and Guhagar and one plant from Rajapur. The genetic diversity within the population was low compared to the genetic diversity between the population. For high genetic diversity cross pollination is required, but even though the plant is polygamodioecious propagation by vegetative means (root suckers) and apomictic seeds has been found. Moreover for cross-pollination the distance becomes a limiting factor depending upon the pollinator. So plants within a population even though undergoing crosspollination had some amount of similarity. Since there was no sharing of genetic material between populations due to the geographical distance the similarity between the populations was low. It was found that the geographical distance between the locations had a marked influence on genetic similarity between the populations, with closely located populations sharing the same genetic pool like Rajapur and Amboli; and Nevre and Guhagar.

3.B.2 Genetic diversity studies in *Garcinia talbotii*

10 plants from 4 different locations were selected. 100 ISSR primers were screened. 17 primers gave amplification. 8 primers were used to make the dendrogram

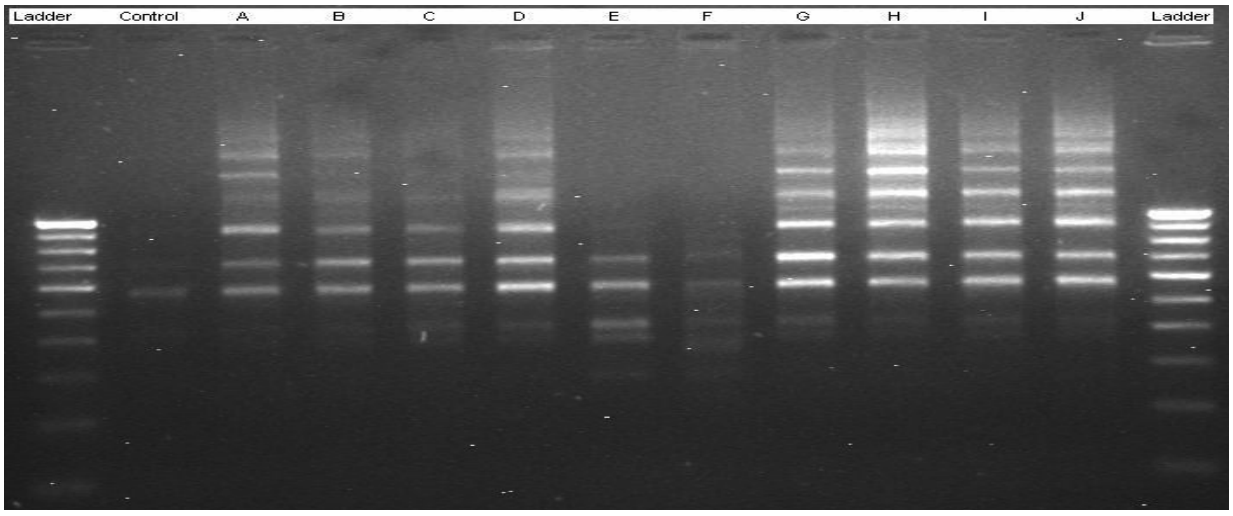
Fig 3.B.4: Map showing the locations of the plants used in genetic diversity studies

Table 3.B.3: Geographical data of the locations of *Garcinia talbotii*

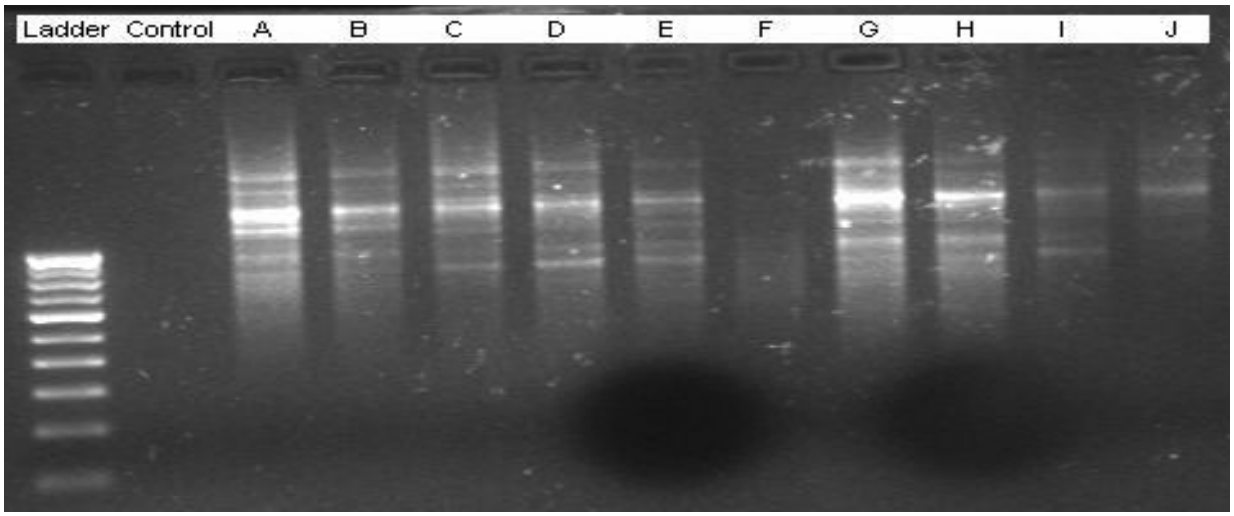
Location	District	Latitude	Longitude	Altitude(m)
Amboli	Sindhudurg	15º 57.266´ N	73º 69.913´ E	749
Dajipur	Sindhudurg	16º 22.338´ N	73º 55.342´ E	795
Mulshi	Pune	18º 28.242´ N	73º 24.929´ E	600
Ratnagiri	Ratnagiri	16° 98´N	73° 3´ E	0

Table 3.B.4: Primers used for the Genetic diversity studies in *Garcinia talbotii*

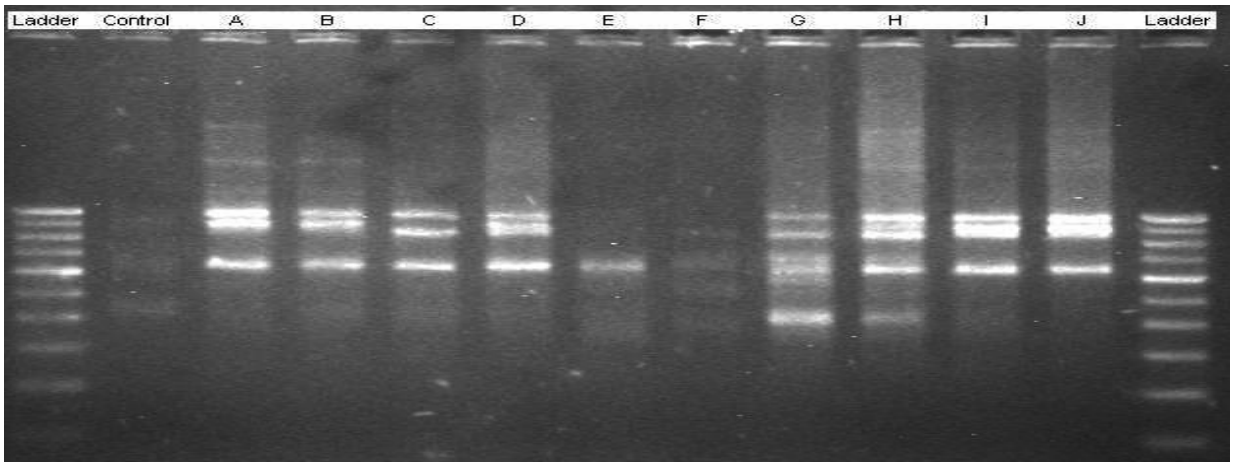
Primer no	Sequence	Annealing Temperature	No of scorable bands	No of Polymorphic bands	% Polymorphic bands
UBC 12	GAG AGA GAG AGA GAG AA	54	9	6	66.66
UBC 13	CTC TCT CTC TCT CTC TT	50	14	13	92.85
UBC 18	CAC ACA CAC ACA CAC AG	50	7	7	100
UBC 26	ACA CAC ACA CAC ACA CC	54	16	15	93.75
UBC 36	AGA GAG AGA GAG AGA GYA	50	14	12	85.71
UBC 41	GAG AGA GAG AGA GAG AYC	48	19	19	100
UBC 54	TCT CTC TCT CTC TCT CRG	54	14	14	100
UBC 85	BHB GAG AGA GAG AGA GA	50	18	15	83.33



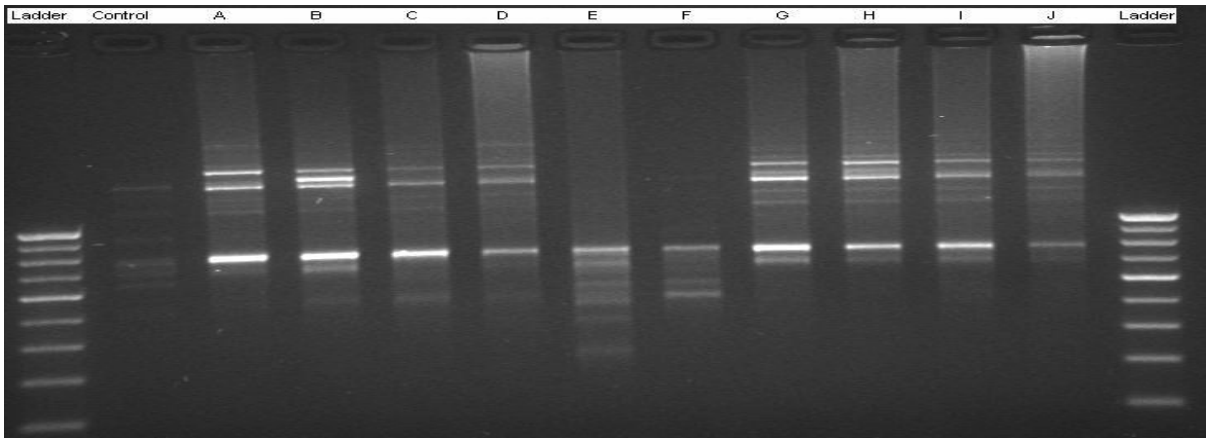
Primer: UBC 812



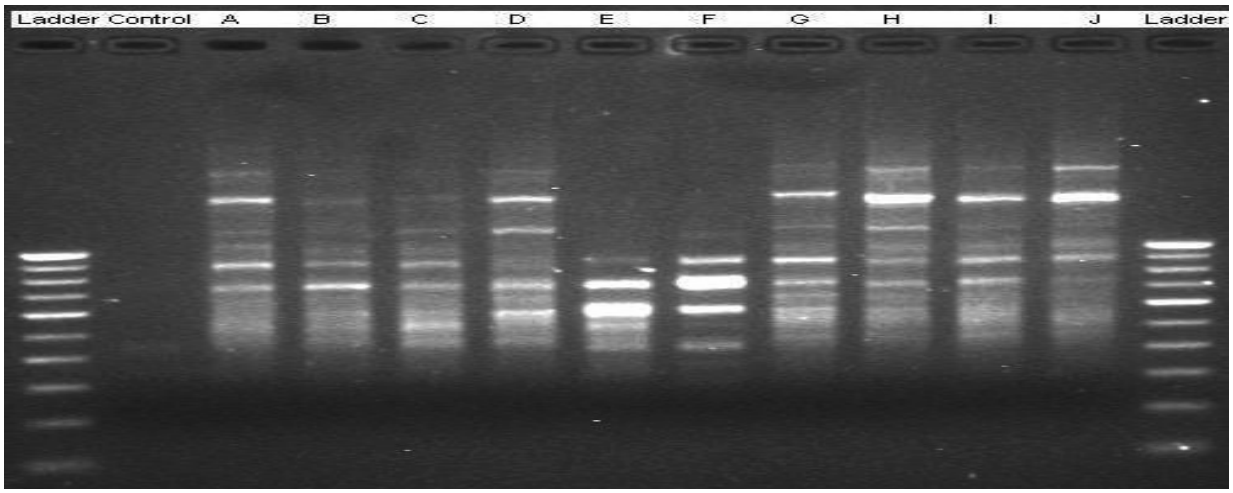
Primer: UBC 813



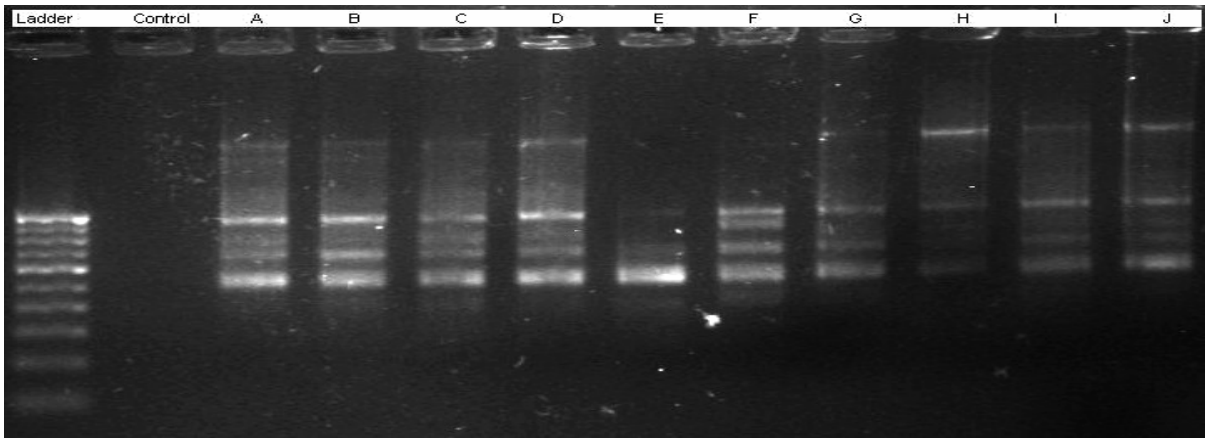
Primer: UBC 818



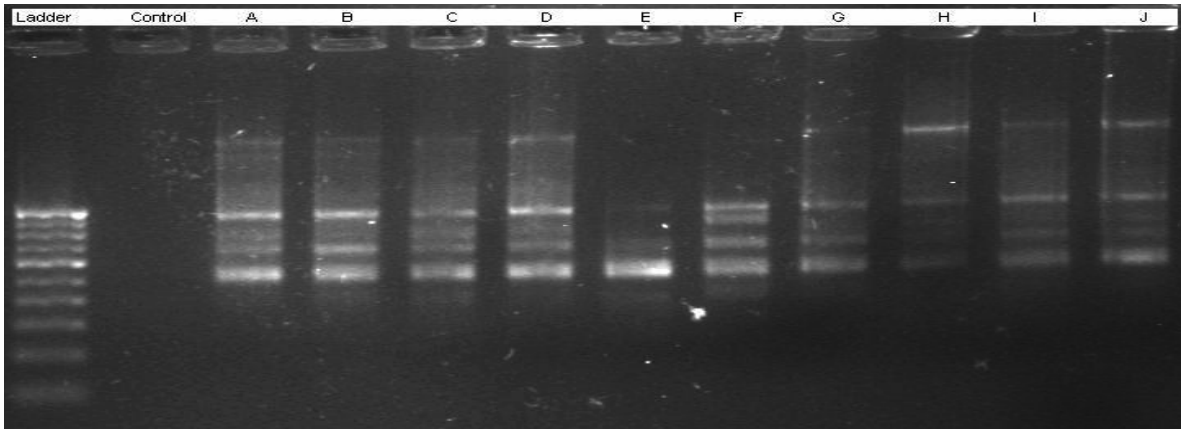
Primer: UBC 826



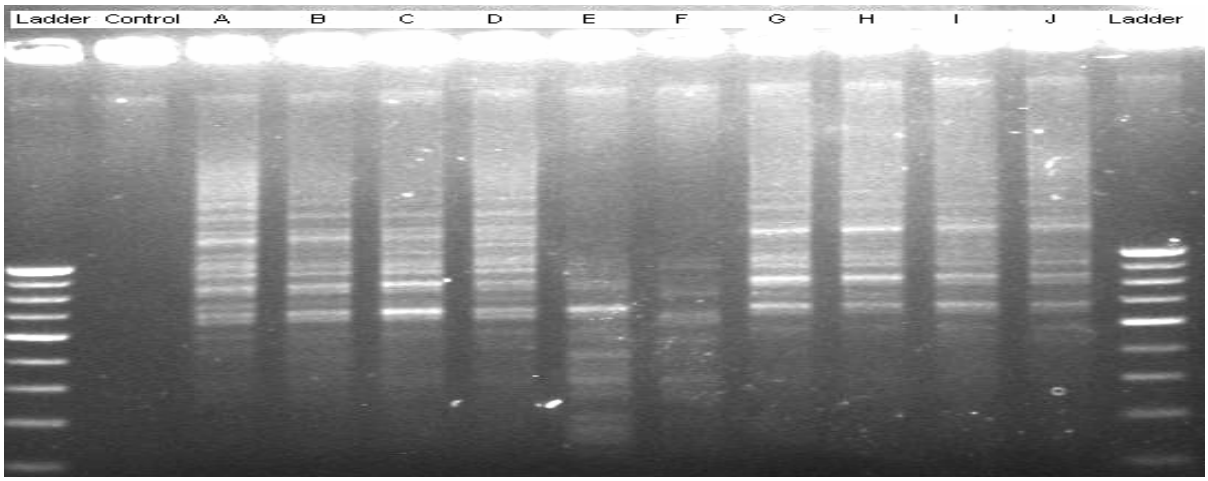
Primer: UBC 836



Primer: UBC 841



Primer: UBC 854



Primer: UBC 885

Fig 3.B.5: Banding pattern of the 10 plants of *Garcinia talbotii.*

A-B-Amboli,
C-Dajipur,
D-G-Mulshi,
H-J-Ratnagiri

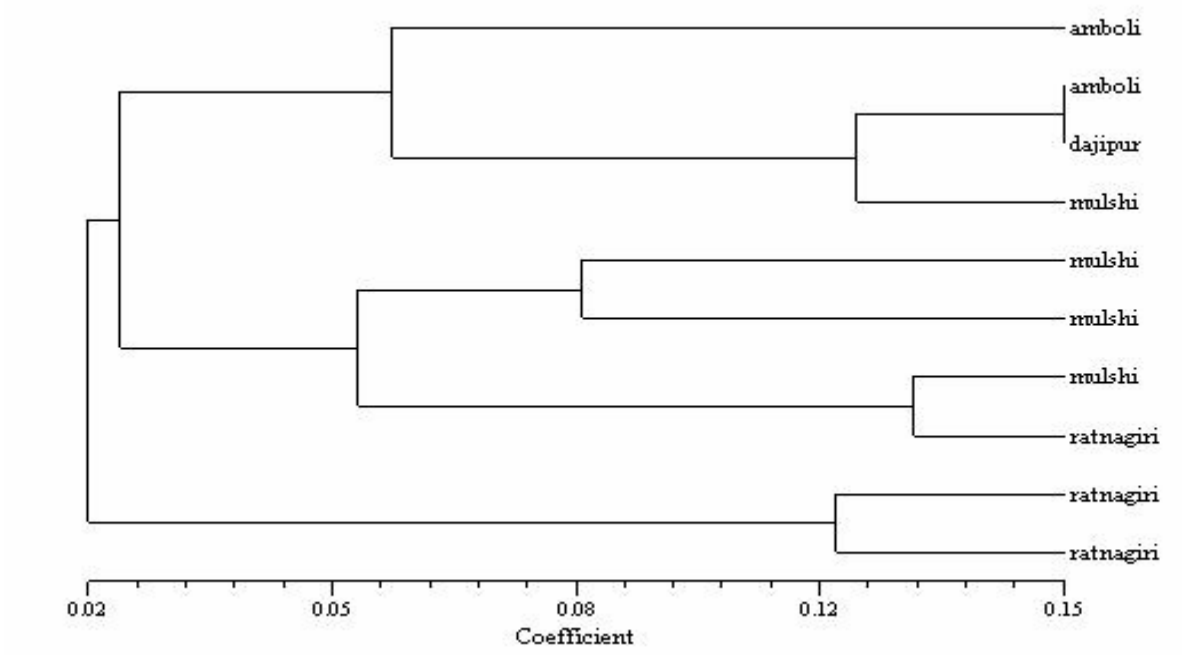


Fig 3.B.6: Dendrogram showing the genetic diversity in *Garcinia talbotii*

Results and Discussion:

ISSR markers can be used in population genetic studies of plant species as they effectively detect very low levels of genetic variation (Zietkiewicz et al 1994). Since the species is endangered not many plants could be obtained for the genetic diversity studies. A total of 10 plants from 4 different locations were sampled for the study. 8 primers were used to prepare the dendrogram. 108 bands were generated by the 8 primers with an average of 16 bands per primer. UBC 18 produced the lowest number of bands (7), while UBC 41 produced the highest number of bands (19). The primers were mostly GA/TC rich showing that these repeats are more common in this species. The primers produced an average of 96.29% polymorphic bands. A high level of genetic diversity was found in the species owing to polygamodioecious nature of the plant. Propagation by vegetative means was not observed in this species. So even though the species is endangered the genetic pool is large and the future prospects of the plant are good if appropriate measures are taken. The plants from the 4 locations could be clustered into 2 groups. 1 group comprising of 2 plants from Ratnagiri while the rest of the plants belonged to the other group. The plants from Mulshi showed strange banding pattern. While one plant was similar to plant from Dajipur, one plant showed similarity with plants from Ratnagiri the rest two formed a separate cluster. *Garcinia talbotii* did not show population level similarity but showed the altitudinal level similarity. While Ratnagiri was located in the coastal region the rest of the locations were situated at an altitude of 600-800 m. The plant is thus affected by the environmental factors.

The genetic diversity studies for *Garcinia spicata* and *Garcinia xanthochymus* could not be conducted at the population level as these plants are rare in Maharashtra and very few accessions of these plants could be collected. But these plants were included in the inter species genetic diversity studies.

3.B.3 Genetic diversity studies between the species

5 plants of *Garcinia indica* from 5 different locations, 5 plants of *Garcinia talbotii* from 4 different locations, 4 plants of *Garcinia xanthochymus* from 4 different locations and 2 plants of *Garcinia spicata* from 2 locations were used for the study. 100 ISSR

primers were screened for the study. 15 primers gave amplification. Out of these 15 primers only 8 primers were used to make the dendrogram.

Fig 3.B.7: Map showing the locations of the 4 species

Table 3.B.5a: Geographical data of the five plants of *Garcinia indica*

Location	District	Latitude	Longitude	Altitude(m)
Diveagar	Raigarh	18º 19.082´ N	72º 57.41´ E	7
Guhagar	Ratnagiri	17º 51.35´ N	79º 07.700´ E	135
Nevre	Ratnagiri	17º 06.062´ N	73º 17.080´ E	78
Rajapur	Ratnagiri	16º 29.090´ N	73º 31.025´ E	0
Amboli	Sindhudurg	15º 56.11´ N	73º 54.053´ E	39

Table 3.B.5b: Geographical data of the four plants of *Garcinia talbotii*

Location	District	Latitude	Longitude	Altitude(m)
Amboli	Sindhudurg	15º 57.266´ N	73º 69.913´ E	749
Dajipur	Sindhudurg	16º 22.338´ N	73º 55.342´ E	795
Mulshi	Pune	18º 28.242´ N	73º 24.929´ E	600
Ratnagiri	Ratnagiri	16° 98´N	73° 3´ E	0

Table 3.B.5c: Geographical data of the four plants of *Garcinia xanthochymus*

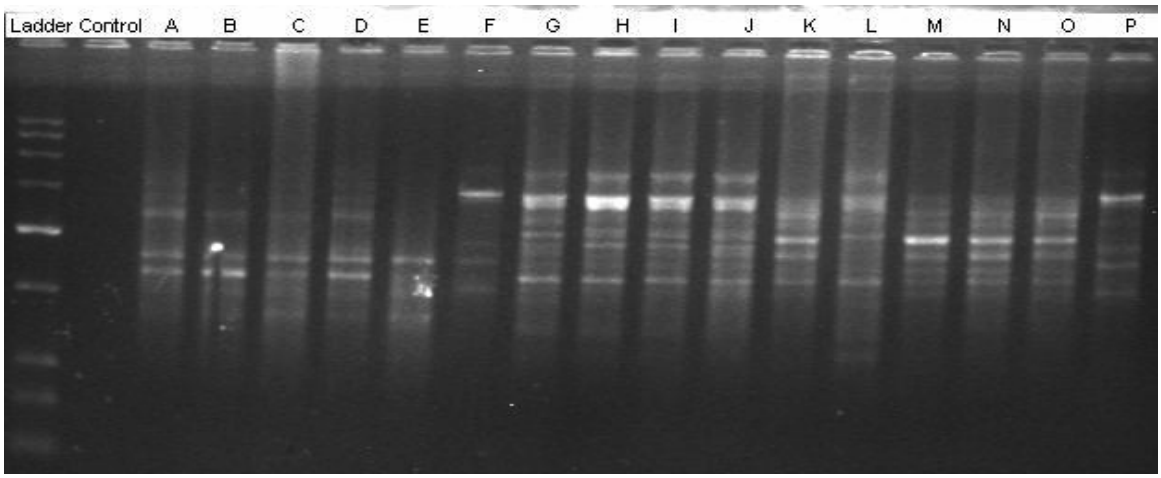
Location	District	Latitude	Longitude	Altitude(m)
Dapoli	Ratnagiri	17° 46′N	73° 10.60´ E	04
Diveagar	Raigarh	18º 19.082´ N	72º 57.41´ E	7
Ganpatipule	Ratnagiri	17º 09.737´ N	73º 16.141´ E	06
Malgund	Ratnagiri	17º 09.963´ N	73º 16.104´ E	025

Table 3.B.5d: Geographical data of the two plants of *Garcinia spicata*

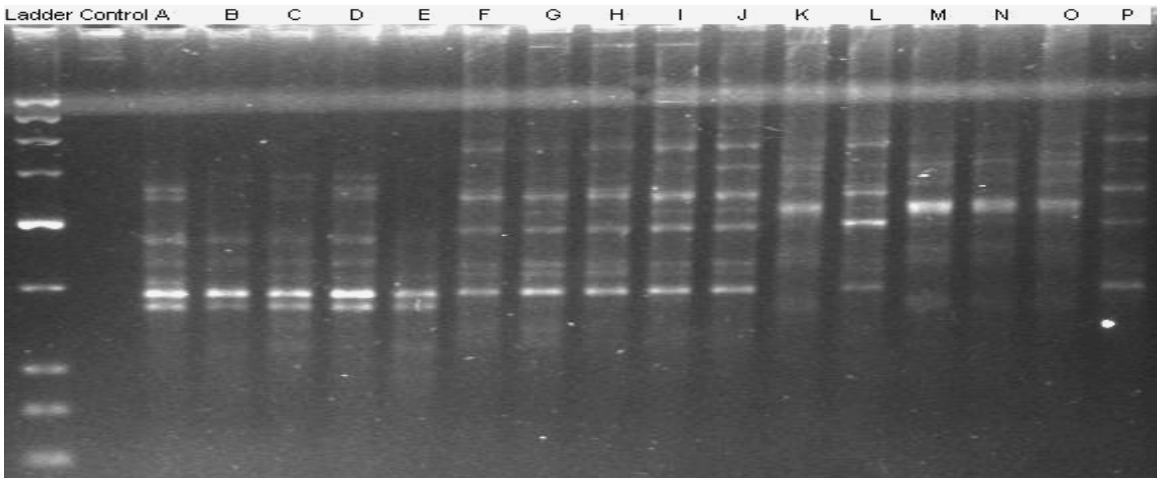
Location	District	Latitude	Longitude	Altitude(m)
Amboli	Sindhudurg	15º 57.266´ N	73º 69.913´ E	749
Mulshi	Pune	18º 28.242´ N	73º 24.929´ E	600

Table 3.B.6: Primers used for the Genetic diversity studies in *Garcinia* species

Primer no	Sequence	Annealing Temperature	No of scorable bands	No of Polymorphic bands	% Polymorphic bands
UBC 11	GAG AGA GAG AGA GAG AC	54	19	19	100
UBC 12	GAG AGA GAG AGA GAG AA	50	22	22	100
UBC 17	CAC ACA CAC ACA CAC AA	50	17	17	100
UBC 18	CAC ACA CAC ACA CAC AG	54	20	20	100
UBC 36	AGA GAG AGA GAG AGA GYA	50	20	20	100
UBC 46	CAC ACA CAC ACA CAC ART	48	25	25	100
UBC 68	GAA GAA GAA GAA GAA GAA	54	17	17	100
UBC 80	GGA GAG GAG AGG AGA	50	19	19	100



Primer: UBC 811



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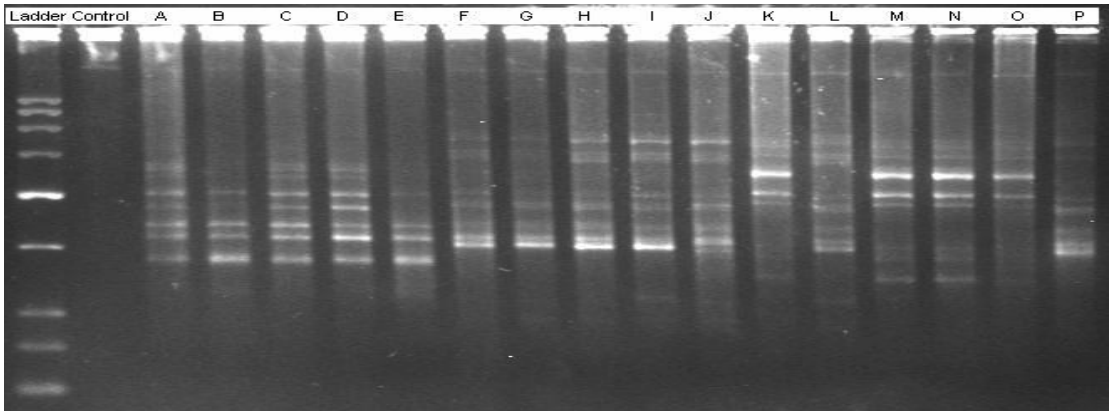

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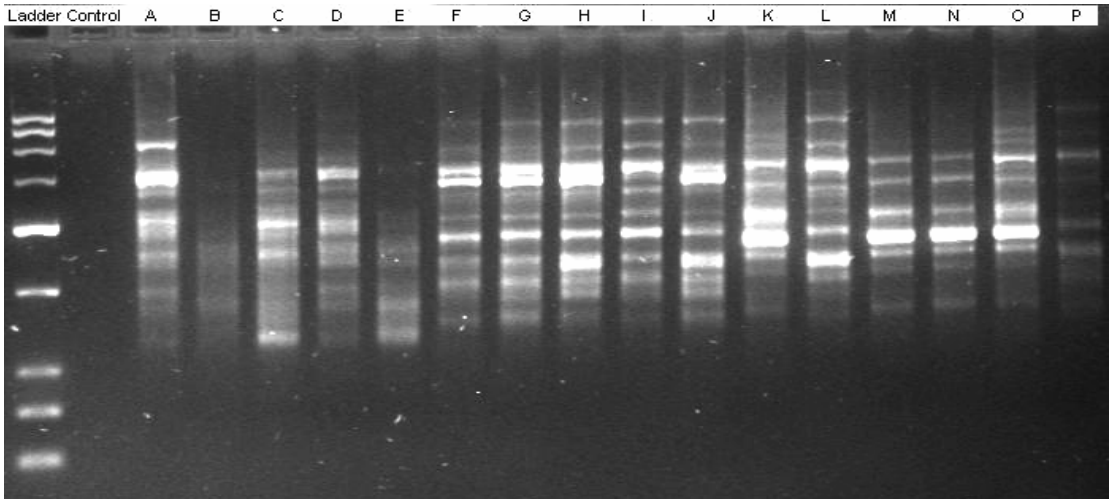

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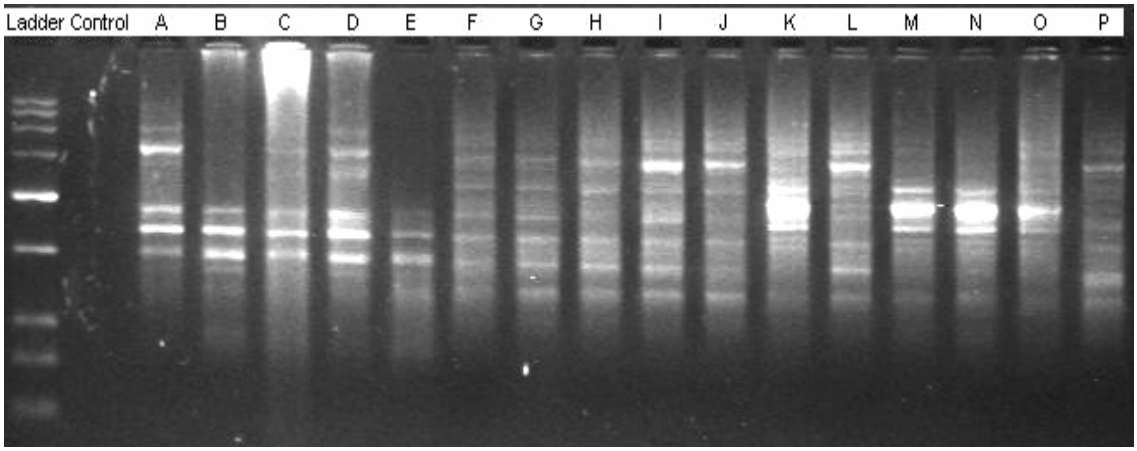

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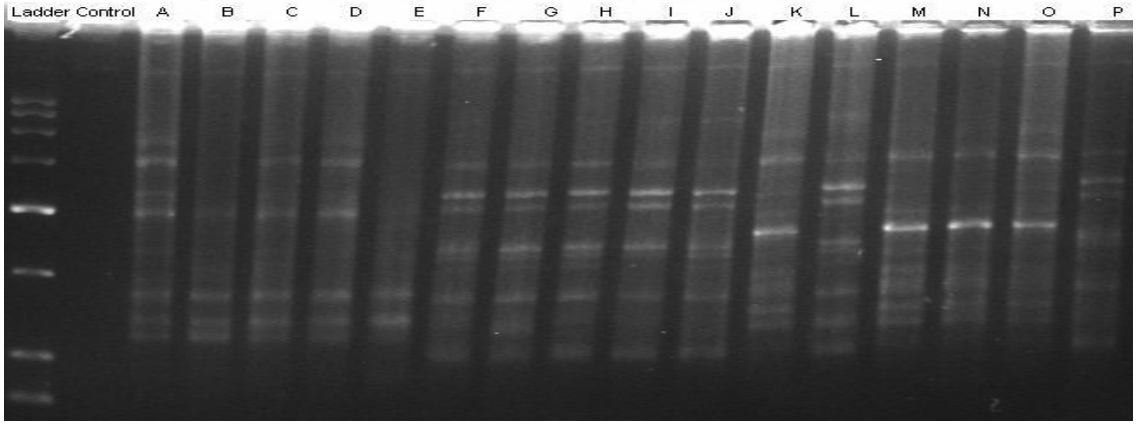

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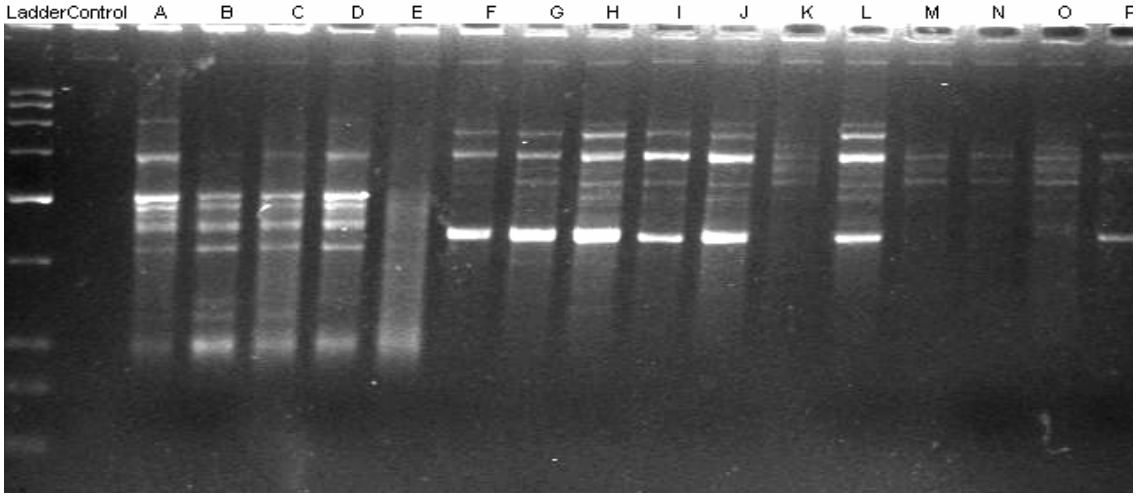

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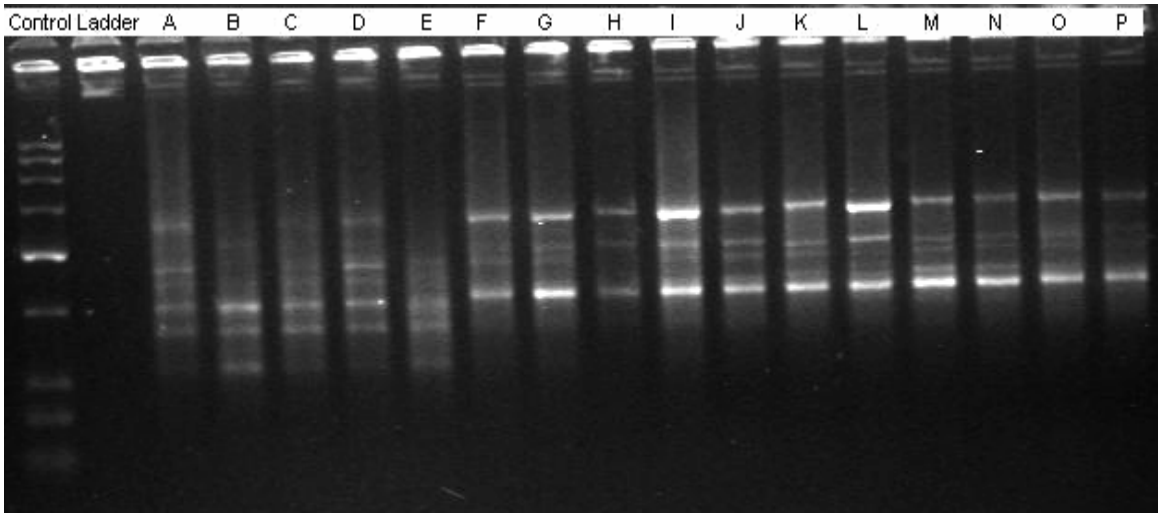


Primer: UBC 880

Fig 3.B.8: Banding pattern of the 4 species of *Garcinia.*

Garcinia indica: A-Amboli, B-Diveagar, C-Guhagar, D-Nevre, E-Rajapur

Garcinia talbotii: F-Amboli, G-Dajipur, H-Mulshi, I-J-Ratnagiri

Garcinia xanthochymus: K-Dapoli. L-Diveagar, M-Ganpatipule, N- Mulgud

Garcinia spicata: O-Amboli, P-Mulshi

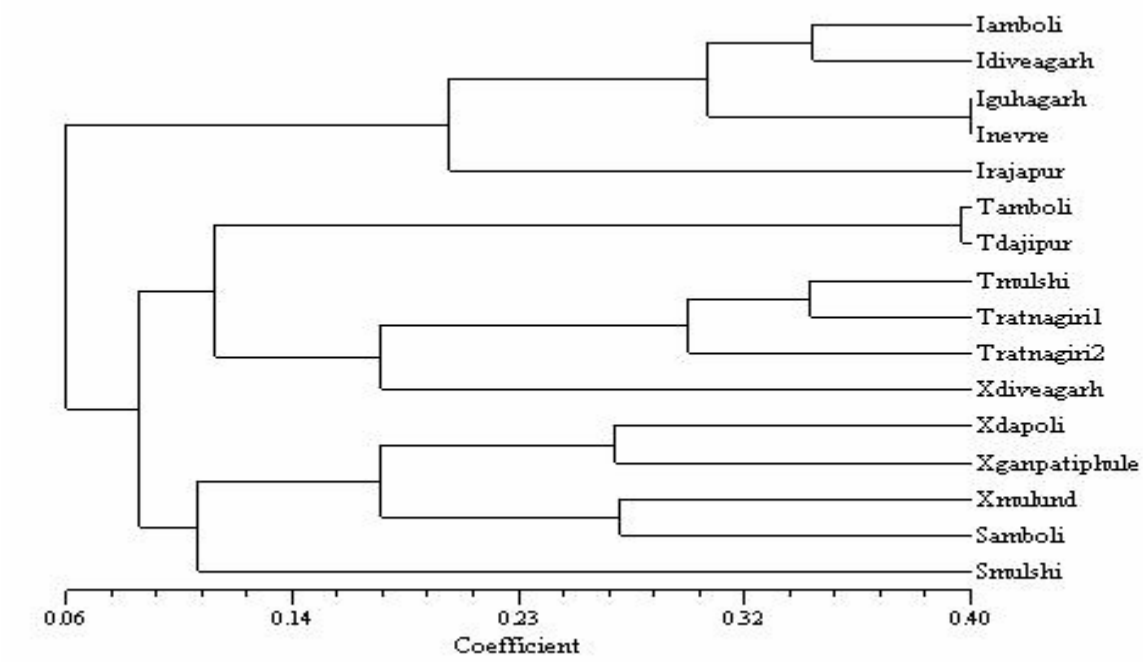


Fig 3.B.9: Dendrogram showing the genetic diversity between the 4 species of *Garcinia,* I-*Garcinia indica*, T-*Garcinia talbotii*, X-*Garcinia xanthochymus*, S-*Garcinia spicata*

Results and Discussion:

The 8 primers used for the preparation of dendrogram produced a total of 159 bands. All the bands were found to be polymorphic showing a high level of genetic diversity in the genus. The primers used were found to be AG rich which showed the genus to be rich in AG repeats. The four species could be divided into 2 groups. One group comprising of *Garcinia indica* alone and the rest of the species falling into the second group. This is in line with the classification given by Maheshwari (1964) on the basis of morphological characters. Phenotypically *Garcinia indica* is very different from the rest of the three species. The second group was again divided into 2 clusters. One cluster comprising mainly of *Garcinia talbotii* and the other group comprising of both *Garcinia xanthochymus* and *Garcinia spicata*. The dendrogram shows *Garcinia xanthochymus* to be similar to both *Garcinia spicata* and *Garcinia talbotii*, when according to the phenotypic characters *Garcinia talbotii* is similar to *Garcinia spicata* and Garcinia xanthochymus forms a separate group (Maheshwari 1964). ISSR has been used previously to clear the ambiguity raised by phenotypic characters in the species of Mulberry (Awasthi et al. 2004), *Cucurbita* (Heikal et al. 2008) and Sulla (Chennaoui-Kourda et al. 2007).

3.C Analysis of (-)-HCA content in Garcinia species

For the past several years a number of small and complex molecules have been isolated from the various species of *Garcinia*, which include xanthones and xanthone derivatives (Bennet and Lee 1989, Rama Rao et al. 1980, Minami et al. 1994), flavanoids (Koshy et al. 2001), terpenoids (Weng et al. 2004), biflavones (Gunatilaka et al. 1984, Joshi et al. 1970, Okunji and Iwu 1991, Terashima et al. 1999, Okunji et al. 2002) etc. However, the isolation of (-)-hydroxycitric acid [(-)-HCA] from the fruit rind in a few species of *Garcinia* and its biological properties have attracted the attention of biochemists and health practitioners. The physiological and biochemical effects of (-)-HCA have been studied extensively for its unique regulatory effect on fatty acid synthesis, lipogenesis, appetite and weight loss. The derivatives of (-)-HCA have been incorporated into a wide range of pharmaceutical preparations in combination with other ingredients for the claimed purpose of accelerating weight loss, cardioprotection, correcting conditions of lipid abnormalities, and endurance in exercise.

Hydroxycitric acid

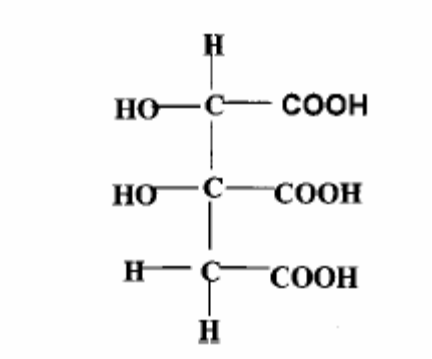


Fig 3.C.1: Hydroxy citric acid

Hydroxycitric acid has 2 assymetrical carbon atoms and so 4 different isomers are possible. Out of the 4 isomers (-)- HCA is found naturally in *Garcinia* species and (+)-HCA is found in *Hibiscus* species. Some *Streptomyces* species have been found to produce (+)-HCA (Yamada et al. 2007).

Biochemistry of (-)-HCA

ATP:citrate lyase enzyme catalyzes the extramitochondrial cleavage of citrate to oxaloacetate and acetyl-CoA. Watson et al. (1969) encountered the powerful inhibition of ATP:citrate lyase by (-)-HCA with purified enzyme from rat liver. The biological effect of (-)-HCA stems from the inhibition of extramitochondrial cleavage of citrate to oxaloacetate and acetyl-CoA. The ultimate source of all the carbon atoms of fatty acids is acetyl-CoA, formed in the mitochondria by the oxidative decarboxylation of pyruvate, the oxidative degradation of some of the amino acids, or the β-oxidation of long –chain fatty acids. The synthesis of fatty acids is maximal when carbohydrate is abundant and the level of fatty acids is low. The conversion of carbohydrate into fat involves the oxidation of pyruvate to acetyl-CoA. Fatty acids are synthesized in the cytosol, whereas acetyl-CoA is formed from pyruvate in the mitochondria. For fatty acid biosynthesis, the acetyl group of acetyl-CoA must be transferred from the mitochondria to the cytosol. Acetyl-CoA as such cannot pass out of the mitochondria into the cytosol. The barrier to acetyl-CoA is bypassed by citrate, which carries acetyl groups across the inner mitochondrial membrane. Citrate is formed in the mitochondrial matrix by the condensation of acetyl-CoA with oxaloacetate. When present at high levels, citrate is transported to cytosol, via the tricarboxylate transport system. In cytosol, acetyl-CoA is regenerated from citrate by the ATP:citrate lyase, which catalyses the following reaction: citrate + ATP + CoA $\rightarrow$ acetyl-CoA + ADP + Pi + oxaloacetate (Watson and Lowenstein 1970). ATP:citrate lyase is widely distributed in animal tissues (Srere 1959). ATP:citrate lyase has been suggested to play a physiological role in lipogenesis from carbohydrate (Spencer et al. 1964) and gluconeogenesis (D′ Adamo et al. 1965). The changes in activity of citrate cleavage enzyme correlate with changes in the rate of fatty acid synthesis and provide evidence for the involvement of the citrate cleavage reaction in fatty acid synthesis (Kornacker and Lowenstein 1965). The activity of citrate cleavage enzyme varies in accordance with the nutritional status of the animal. Thus during starvation or when fed on a high-fat diet, the enzyme levels fall drastically and on feeding a high-carbohydrate diet elevated levels of ATP:citrate lyase were detected.

It was found that (-)-HCA acts as an inhibitor of lypogenesis only if cytoplasmic acetyl-CoA is produced by citrate cleavage enzyme reaction, but it will activate fatty acid

synthesis wherever an alternative source of acetyl-CoA, for example, acetate is available. Sheehan and Yeh (1984) observed that (-)-HCA inhibited fatty acid synthesis in neonatal rat lung from glucose, pyruvate and β-hydroxybutyrate but had no effect on that from acetoacetate. Mathias et al. (1981) reported that in hepatocytes isolated from female rats, meal-fed a high-glucose diet, (-)-HCA depressed the incorporation of H_2O, alanine, leucine into fatty acids and cholesterol. Hood et al. (1985) have shown that (-)-HCA reduced the synthesis of fatty acids from lactate and glucose in bovine adipose tissue and rat adipose tissue respectively, and suggested that the conversion of lactate to fatty acids probably occurs by way of citrate. (-)-HCA equivalently reduced the biosynthesis of triglycerides, phospholipids, cholesterol, diglycerides, cholesteryl esters, and free fatty acids in isolated liver cells from normal and hyperlipidemic rats (Hamilton et al. 1977).

Other biological effects of (-)-HCA

Brunengraber et al. (1978) observed that in rat liver, the inhibition of fatty acid synthesis by (-)-HCA was associated with an increase in the tissue content of glucose 6-phosphate and fructose 6-phosphate and a diminution in glycolytic intermediates from fructosebisphosphate to phosphoenol pyruvate. Presumably the citrate content is elevated in cytoplasm in the presence of (-)-HCA. This can be expected to result in a reduced activity of phosphofructokinase because citrate is well known as an inhibitor of this enzyme. It has also been seen that AMP contents reduce in the presence of (-)-HCA. This can also be expected to reduce the activity of phosphofructokinase, because AMP is an activator of this enzyme. The inhibition of phosphofructokinase by (-)-HCA in rat hepatocytes has also been reported by McCune et al. (1989). It can be concluded that in rat liver, the inhibition of phosphofructokinase by (-)-HCA leads to the accumulation of glucose 6-phosphate and fructose 6-phosphate and the decrease of glycolytic intermediates beyond fructosebisphosphate as the reaction catalysed by phosphofructokinase in glycolysis is irreversible and controls the glycolysis.

Possible mode of action on reduction in food intake

The *in vitro* studies showed that (-)-HCA can depress acetylcholine synthesis in nerve tissues, although so far there is no evidence of reduced synthesis of acetylcholine in intact animals with the administration of (-)-HCA. Sullivan et al. (1974) suggested that if

(-)-HCA could penetrate the blood –brain barrier, then the depression of acetylcholine levels or rate of turnover in the brain resulting from a decreased pool could affect cholinergic receptor systems that might be involved in feeding behaviour.

Fatty acid synthesis regulates fatty acid oxidation by a well characterized mechanism. Malonyl-CoA levels rise during fatty acid synthesis and result in inhibition of carnitine palmitoyl transferase 1 (CPT 1) mediated uptake of fatty acids into the mitochondria. This results in elevation of cytoplasmic long-chain fatty acyl (LCFA)-CoAs and diacylglycerol molecules that may play a role in signaling, which leads to the proposal that malonyl-CoA levels act as a signal of the availability of physiological fuels. Saha et al. (1997) reported that inhibition of ATP:citrate lyase by (-)-HCA markedly diminished the malonyl-CoA level, indicating that citrate was the major substrate for the malonyl-CoA precursor, that is, cytosolic acetyl-CoA, and also there is sufficient evidence that (-)-HCA inhibits ATP:citrate lyase, limiting the pool of cytosolic acetyl-CoA, the precursor of malonyl-CoA. This type of regulation of malonyl-CoA level may affect the signaling of fuel status in hypothalamic neurons regulating feeding behavior, lending support that (-)-HCA may represent a biochemical target for the control of appetite/feeding behavior and body weight. Thus, (-)-HCA seems to act at the metabolic level and not directly at the central nervous system as classical appetite depressants do.

(-)-HCA May Promote Glycogenesis, Gluconeogenesis, and Lipid Oxidation

The inhibition of ATP:citrate lyase by (-)-HCA causes less dietary carbohydrate to be utilized for the synthesis of fatty acids, resulting in more glycogen storage in the liver and muscles. In a well-fed state, the hepatic carnitine levels are far too low to activate CPT 1. The rate-limiting enzyme for hepatic lipid oxidation, CPT 1, is activated by exogenous carnitine and inhibited by malonyl-CoA. The lipogenesis inhibitor (-)-HCA can decrease the production of malonyl-CoA in hepatocytes by potent inhibition of ATP:citrate lyase and may activate CPT 1, which can facilitate lipid oxidation. Earlier studies demonstrated that (-)-HCA can reduce body-weight and fat accumulation in growing rats, owing to the reduction in appetite. From the above views McCarty (1994) hypothesized that joint administration of (-)-HCA and carnitine should therefore promote hepatic lipid oxidation, gluconeogenesis, and satiety. In a recent study, joint administration of pyruvate, (-)-HCA,

and carnitine to obese subjects was associated with a remarkable rate of body-fat loss and thermogenesis, strongly suggestive of uncoupled fatty acid oxidation. The reduction of free fatty acids by stimulating hepatic oxidation with (-)-HCA and carnitine may ameliorate risk factors associated with abdominal obesity. (-)-HCA is available commercially in the brand names of Citrimax, SuperCitrimax etc.

(-)-HCA has been reported in the fruit rinds of *Garcinia indica*, *Garcinia cambogia*, *Garcinia artroviridis*, *Garcinia cowa* and *Garcinia pedunculata*. Due to the limited sources of (-)-HCA and the difficulty in the synthesis of the stereospecific isomer the need for finding newer sources as well as the finding of the elite plant in terms of (-)-HCA content arises. The present work was undertaken to estimate the % (-)-HCA content in the four species of *Garcinia* located in Maharashtra and to study the effect of the ecological factors on the (-)-HCA content.

(-)-HCA content in Garcinia species

Method:

Fresh fruits of *Garcinia indica*, *Garcinia talbotii*, *Garcinia spicata* and *Garcinia xanthochymus* were collected from different locations in Maharashtra. The method followed for the extraction as well as estimation of (-)-HCA was as reported by Jayaprakasha and Sakariah (1998) with some modifications. 25 g of the fruit rind from fresh fruits was autoclaved at 15 lbs/inch^2 with 100 ml of distilled water for 20 min and filtered. Autoclaving and filtration was repeated twice for complete extraction of the acid. The coloured extract was decolorized using activated charcoal and filtered. The decolorized extract was concentrated to 25 ml on rotary evaporator and was treated with 50 ml of ethanol to remove pectinaceous material and centrifuged. The supernatant was concentrated under reduced pressure to 5 ml and stored at 4 °C until further use. The solution was dissolved in 20 ml of 10 mM H_2SO_4 and centrifuged. 1 ml of this solution was filtered and used for HPLC analysis. The HPLC unit used was from Perkin Elmer, series 200 fitted with bondapack C18 column. The analysis was done with 10 mM H_2SO_4 under isocratic conditions at a flow rate of 0.7 ml/min. Detection was done by DAD (diode Array Detector) at a wavelength of 214 nm. Standard HCA with 80% purity obtained from

Cipex (Mumbai, India) was used for quantification. Three readings were taken for each sample and the standard. %HCA content was calculated from the following formula

Weight in mg of standard X (Area of sample/Area of standard) X (Volume of sample injected/Volume of standard) X 66.67 X 10^5= μg per 100 g of fruit

Results and Discussion:

The (-)-HCA extracts of *Garcinia talbotii*, *Garcinia spicata* and *Garcinia xanthochymus* were colourless while the (-)-HCA extracted from the fruits of *Garcinia indica* varied in colour from light brown to darkbrown as shown in Fig 3.C.2.

Fig 3.C.2: (-)-HCA extracts of *Garcinia indica*

The retention time of HCA was found to be 11 min (Fig 3.C.3a). The amount of HCA in *Garcinia indica* varied from 2.52-10.65 g per 100 g of fruit rind (Table 3.C.1). The plants located at the seashore (Diveargar, Guhagar and Nevre) showed higher (-)-HCA content than the plants located in the inland region (Amboli and Rajapur). The (-)-HCA content in the fruit rinds of *Garcinia indica* have been reported to be between 12.5-15.1% (Jayaprakasha and Sakariah 2002). The fruits in this case were collected from the coastal region of Karnataka. The tropical climate of Karnataka may have led to the high (-)-HCA content in the species. Moreover in the report it is not specified whether the fruits were fresh or dried. The amount of (-)-HCA in *Garcinia talbotii* was around 7.8 g per 100 g of fruit rind (Table 3.C.2), while (-)-HCA content in *Garcinia spicata* was found to be 5.04 g. *Garcinia xanthochymus* located at Dapoli showed the highest percentage of (-)-HCA of about 11.49 g per 100 g of fruit rind. Thus according to the (-)-HCA content the plants can be arranged in the descending order of *Garcinia xanthochymus* > *Garcinia indica* >

Garcinia talbotii > *Garcinia spicata.* There are no reports on the presence of (-)-HCA in the fruit rinds of *Garcinia xanthochymus, Garcinia talbotti* and *Garcinia spicata*. The (-)-HCA content in the fresh fruits of the 4 species were very high as compared to the (-)-HCA content in fresh fruits of *Garcinia peduncalata* which was reported to be 3% (Jayaprakasha et al. 2003) and in the fresh fruits of *Garcinia cowa* which was reported to be 2.3% (Jena et al. 2002). The HCA content in the dried rinds of *Garcinia peduncalata* was reported to be 20.1%, in *Garcinia cambogia* to be 16-18% (Jayaprakasha and Sakariah 1998) and in *Garcinia cowa* to be 12.7%.

Table 3.C.1: %HCA content in fruits of *Garcinia indica* collected from different locations in Maharashtra

Locations	%HCA content	Locations	%HCA content
Amboli 1	3.91±0.72	Guhagar4	10.62±2.97
Amboli 2	4.05±0.31	Guhagar5	3.36±1.19
Amboli 3	3.73±0.55	Nevre 1	7.68±0.21
Amboli 4	3.66±0.83	Nevre 2	6.72±0.33
Amboli 5	3.13±2.00	Nevre 3	6.35±0.30
Diveargar 1	6.11±0.28	Nevre 4	7.39±0.19
Diveargar 2	8.44±1.95	Nevre 5	6.24±0.33
Diveagar 3	6.42±1.14	Rajapur 1	3.54±0.51
Diveagar 4	4.86±0.48	Rajapur 2	3.30±0.57
Diveagar 5	4.42±0.63	Rajapur 3	3.69±1.39
Guhagar 1	7.31±0.28	Rajapur 4	2.52±1.03
Guhagar2	4.56±2.43	Rajapur 5	3.85±0.12
Guhagar 3	10.65±0.03*	--	--

*Maximum (-)-HCA content

Table 3.C.2: %HCA content in fruits of *Garcinia* sps collected from different locations in Maharashtra

Garcinia species+Location	%HCA content
Garcinia talbotii, Mulshi 1	7.91±0.30
Garcinia talbotii, Mulshi 2	7.69±1.12
Garcinia talbotii, Mulshi 3	8.35±0.41

Garcinia talbotii, Mulshi 4	8.80±0.25
Garcinia talbotii, Mulshi 5	6.57±0.27
Garcinia spicata, Mulshi1	5.03±0.23
Garcinia xanthochymus, Anjarle	6.89±0.22
Garcinia xanthochymus, Dapoli	11.47±3.75*

*Maximum (-)-HCA content

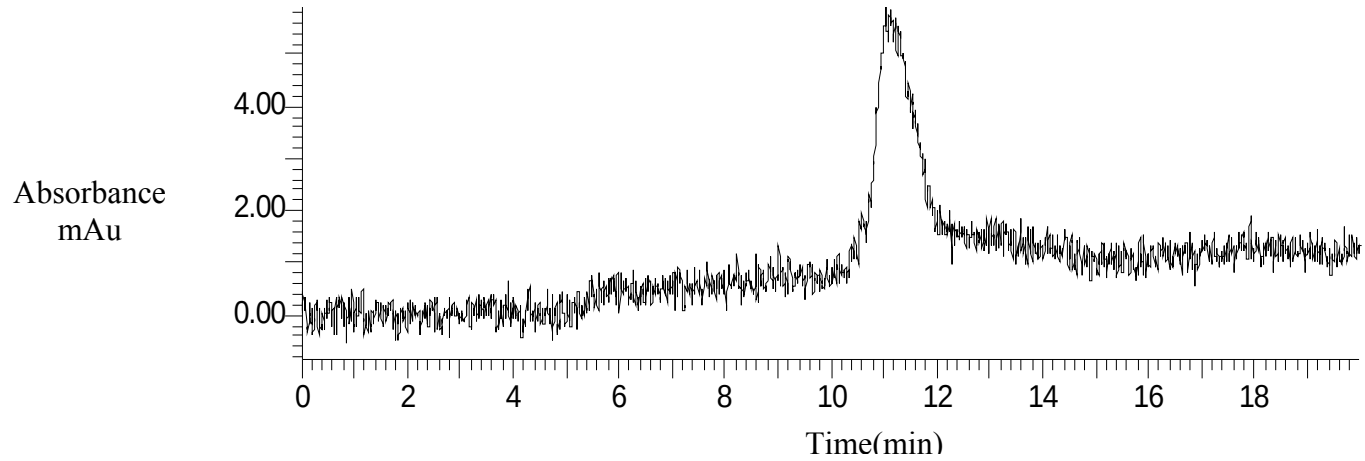


Fig 3.C.3a: Chromatogram of standard (-)-HCA

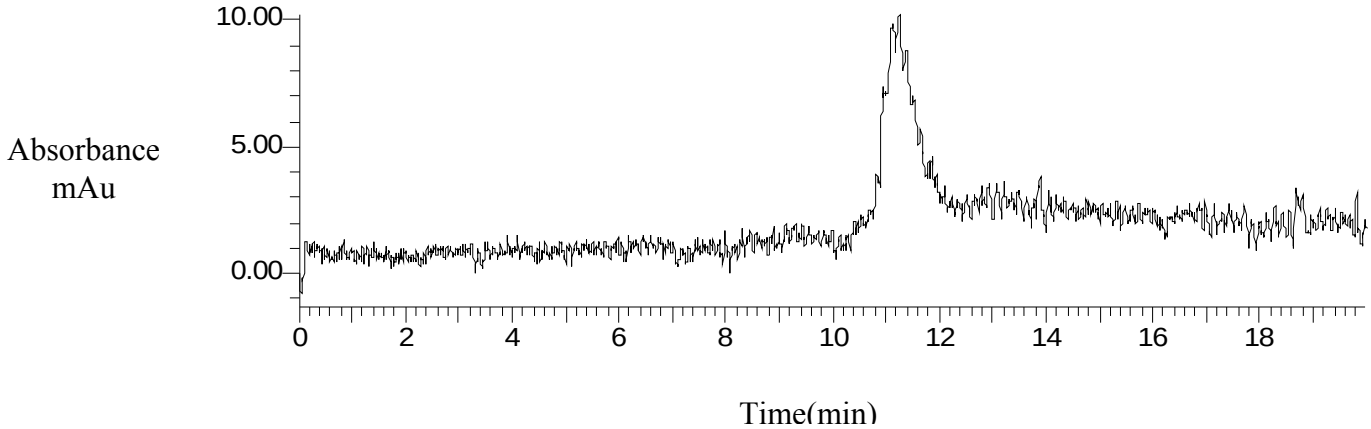


Fig 3.C.3b: Chromatogram of fruit extract of *Garcinia indica* (Diveagar)

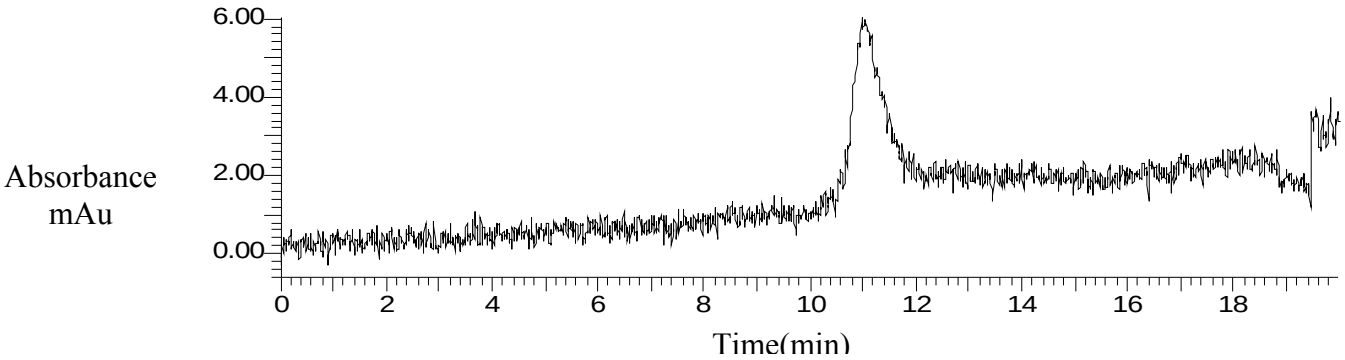


Fig 3.C.3c: Chromatogram of fruit extract of *Garcinia talbotii* (Mulshi)

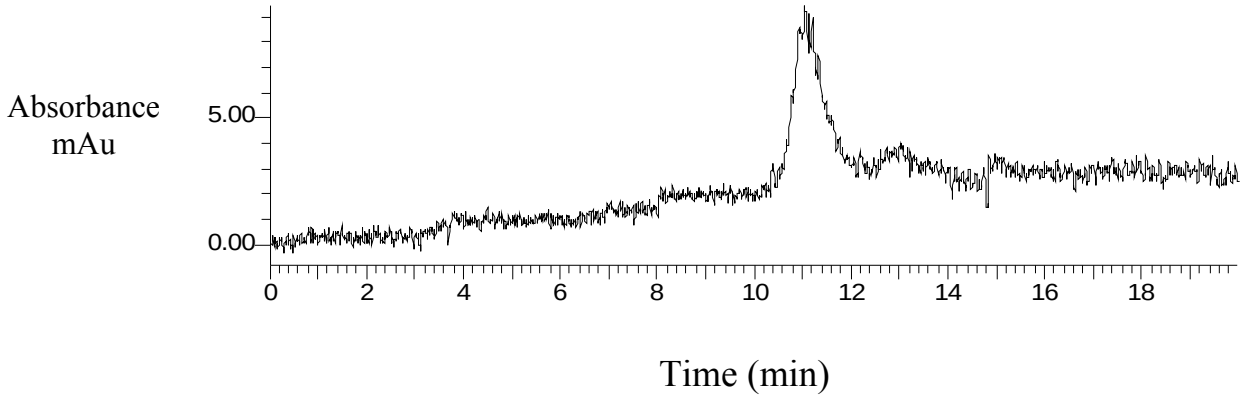


Fig 3.C.3d: Chromatogram of fruit extract of *Garcinia spicata* (Mulshi)

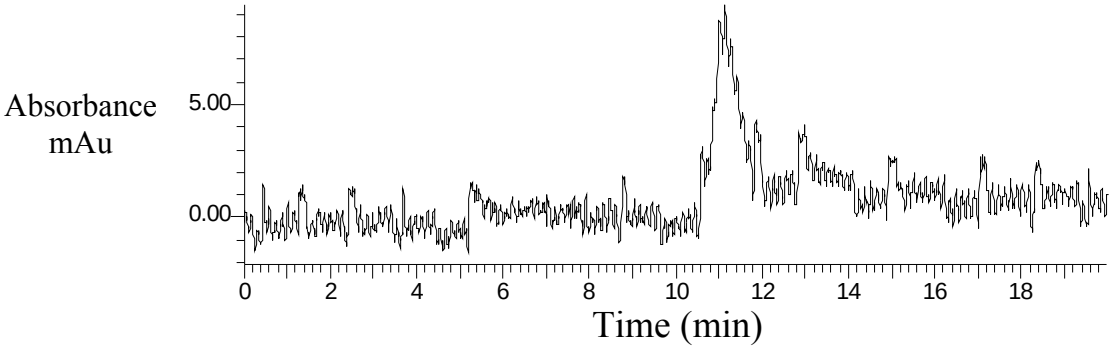


Fig 3.C.3e: Chromatogram of fruit extract of *Garcinia xanthochymus* (Anjarle)

CHAPTER 4

IN VITRO STUDIES IN *GARCINIA* SPECIES

4 Introduction

When plant cells and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plants can be subsequently regenerated. This regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of genetic potential is called 'totipotency'. In practical terms though, identifying the culture conditions and stimuli required to manifest this totipotency can be extremely difficult and it is still a largely empirical process.

The culture environment

When cultured *in vitro*, all the needs, both chemical and physical, of the plant cells have to met by the culture vessel, the growth medium and the external environment (light, temperature, etc.). The growth medium has to supply all the essential mineral ions required for growth and development. In many cases (as the biosynthetic capability of cells cultured *in vitro* may not replicate that of the parent plant), it must also supply additional organic supplements such as amino acids and vitamins. Many plant cell cultures, as they are not photosynthetic, also require the addition of a fixed carbon source in the form of a sugar (most often sucrose). One other vital component that must also be supplied is water, the principal biological solvent. Physical factors, such as temperature, pH, the gaseous environment, light (quality and duration) and osmotic pressure, also have to be maintained within acceptable limits.

The in vitro culture of plant cells and organs has several advantages

It can be used

- for the large scale propagation of elite plants in a limited time and space
- for the production of secondary metabolites by cell and suspension culture
- for the production of disease free plants
- for the production of genetically transformed plants
- for the production of hybrid plants between incompatible species by protoplast fusion.

4.A *In vitro* germination studies

Most of the plants propagate in nature by means of seed. The seed contains an embryo and stored food reserves wrapped in a seed coat. Under favorable conditions, the seeds germinate and form the seedling.

Requirements for seed germination

The germination of seeds is dependent on both internal and external conditions. The most important external factors include: temperature, water, oxygen and sometimes light or darkness.

Water - is required for germination. Mature seeds are often extremely dry and need to take in significant amounts of water relative to the seeds dry weight, before cellular metabolism and growth can resume. Most seeds respond best when there is enough water to moisten the seeds but not soak them. The uptake of water by seeds leads to the swelling and breaking of the seed coat. Water also activates the hydrolytic enzymes which breaks the food reserves in the seed. Once the seedling starts growing and the food reserves are exhausted, it requires a continuous supply of water, nutrients and light for photosynthesis, which now provides the energy needed for continued growth.

Oxygen - is required by the germinating seed for metabolism. If the soil is waterlogged or the seed is buried within the soil, it might be cut off from the necessary oxygen it needs. Oxygen is used in aerobic respiration, the main source of the seedling's energy until it has true leaves, which can photosynthesize its energy requirements. Some seeds have impermeable seed coats that prevent oxygen from entering the seeds, causing seed dormancy. Impermeable seed coats to oxygen or water are types of physical dormancy which is broken when the seed coat is worn away enough to allow gaseous exchange or water uptake between the seed and its environment.

Temperature - affects cellular metabolic reactions and eventually growth rates. Different seeds germinate over a wide range of temperatures, with many preferring temperatures slightly higher than room-temperature while others germinate just above freezing and few

others responding to alternation in temperature between warm to cool. Some seeds may require exposure to cold temperature (vernalization) to break dormancy before they can germinate. As long as the seed is in its dormant state, it will not germinate even if conditions are favorable. Seeds those are dependent on temperature to end dormancy, have a type of physiological dormancy. Some seeds will only germinate when temperatures reach hundreds of degrees, as during a forest fire. Without fire, they are unable to crack their seed coats; this is a type of physical dormancy e.g. fire ephemerals.

Light or darkness - can be a type of environmental trigger for germination in seeds and is a type of physiological dormancy. Most seeds are not affected by light or darkness, but many seeds, including species found in forest settings will not germinate until an opening in the canopy allows them to receive sufficient light for the growth of the seedling.

Scarification mimics natural processes that weaken the seed coat before germination. In nature, some seeds require particular conditions to germinate, such as the heat of a fire (e.g., many Australian native plants), or soaking in a body of water for a long period of time. Others have to be passed through an animal's digestive tract to weaken the seed coat and enable germination.

The seeds of *Garcinia* are produced once in a year. The plant is dioecious so only 50% of the population produces seeds. Moreover the seeds are known to have low seed viability and are susceptible to cold and desiccation. Freshly harvested seeds of the three *Garcinia* species viz. *Garcinia indica*, *G. cambogia* and *G. xanthochymus* are reported to retain viability for 30 days at ambient temperature. Seed longevity in all the three species could be enhanced to almost two times by storage at 15°C while chilling temperature of 5°C and freezing temperature of −20°C were unfavorable. The three *Garcinia* species were found to be highly recalcitrant (Malik et al. 2005). So in nature very few plants are produced from seeds, which explain the rarity of this genus.

In vitro germination studies were carried out to study the factors responsible for the germination of these medicinally important plants.

4.A.1 Germination studies in *Garcinia indica*

Material and Methods:

Fully mature both ripe and unripe fruits were collected in the month of May from the Konkan region of Maharashtra, India. The fruits were surface sterilized by the following method. They were washed under running tap water for one hour and then cleaned with 1% (v/v) labolene (Qualigens, India), followed by 10% (v/v) savlon (Johnson and Johnson Ltd, India) for 3-5 min. The fruits were later treated with 1% (w/v) bavistin (BASF, India) for 1 hour. After each treatment, the fruits were washed 3-4 times with single distilled water. Further treatments were carried out under sterile conditions in a laminar airflow unit. The seeds were excised from the fruits, rinsed with 70% (v/v) ethanol and washed 3-4 times with sterile distilled water followed by treatment with 0.05% (w/v) $HgCl_2$ (Qualigens, India) for 10 mins and again washed 3-4 times with sterile distilled water. The seeds after removing the seed coat were inoculated in different media combinations.

- Effect of basal salts: Three different basal media (WPM, MS, B5) and their ½ strengths (only macro half) were tried for germination. All the media contained 2% sucrose
- Effect of sucrose concentration: Four different sucrose concentrations (0.5-3%) were used to study the effect of sucrose concentration on germination. The basal medium used was ½ strength WPM (only macro half).
- Effect of different growth regulators: The effect of different concentrations of BAP (4.44-11.97 µM), Kinetin (4.65-14.15µM) and GA_3 (1.44-5.77µM) was observed. The basal medium used was full strength WPM containing 2% sucrose.

All media contained 0.7% agar. 10 seeds were used for each treatment and the experiment was repeated 3 times. The seeds were incubated in dark till the emergence of radical or plumule then grown in 16 hr photoperiod. The seeds were grown in different media combinations for 1 month then shifted to full strength WPM medium. The germination percentage was noted after 1 month and the length of the root and shoot was noted after 4 months.

Results and Discussion:

Ex vitro germination of the seeds with or without seed coat on moist filter paper was not successful. The field experiments done for germination of the seeds in the green house was also not successful. In the *in vitro* studies when the seeds were inoculated without removing the seed coat on ½ strength plain WPM medium, contamination was observed and the seeds took 20 days to germinate. Even the germination percent was low. Only 10% of the seeds germinated. When the seeds were inoculated after removing the seed coat 42% of the seeds germinated in 3 days. Removing the seed coat also reduced the contamination in the cultures. Removal of the seed coat has reduced contamination in cherimoya seeds (Padilla and Encina 2003). Removal of the seed coat did not improve germination in cherimoya (Padilla and Encina 2003, Jubes et al. 1975), in *Annona diversifolia* (Gonzalez-Esquinca et al. 1997), in *Annona squamosa* and atemoya (Ferreira et al. 1997). The germination percentage of the decoated seeds was around 30% in distilled water but the seeds took 16 days to germinate. Nutrient media was thus found beneficial for germination. Among the different basal media tested ½ WPM gave the best response with 42% of the seeds germinating in 3 days and the germination percentage increased to 76.18% in 1 month, but the growth of the seedlings was retarded and so the seeds were shifted to full strength WPM media after 1 month. Full strength WPM induced germination in 48% of the seeds in 3 days, but the germination percentage never increased beyond 53.15%. The high salt content in MS, B5 and WPM reduced the germination percentage of *Garcinia indica* seeds. High salt content has been shown to inhibit germination in a number of species like *Annona cherimola* (Padilla and Encina 2003) and *Annona muricata* (Lemos and Blake 1996). But the decoated seeds of *A. muricata* germinated in ½ MS medium (Bejoy and Hariharan 1992) and the seeds of *A. squamosa* without the seed coat germinated on White´s basal medium (Nair et al. 1986). Thus it can be concluded that non-dormant seeds require small amounts of nutrients for germination.

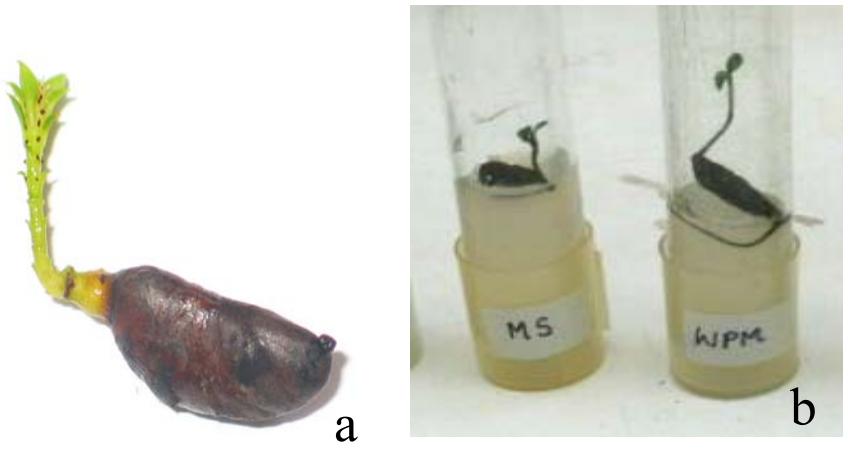


Fig 4.A.1: Effect of different basal media on *Garcinia indica* seed germination,

a-effect of B5 medium,

b-effect of MS and WPM medium

Table 4.A.1: Effect of different basal media on *Garcinia indica* seed germination

Medium	% Germination (mean ± s.d.)*after 1 month	Average shoot length (cms) after 4 months	Average root length (cms) after 4 months
WPM	53.15 ± 8.09^{ab}	2	6
1/2WPM	76.18 ± 29.74^{a}	1	4
MS	22.91 ± 3.6^{bc}	1.25	4
B5	32.0 ± 1^{abc}	0.1	0
1/2B5	0^{c}	0	0

*values followed by the same letter do not vary significantly at $p<0.01$

A carbon source was found essential for the germination of *Garcinia indica* seeds. 2% sucrose was better as compared to the other sucrose levels (0.5-1.5%). At 2% sucrose 76.18% of the seeds showed germination in 1 month. Since the basal medium used was ½ WPM the germinated seeds were shifted to full strength WPM for proper growth of the seedlings. The root and shoot length noted after 3 months on full strength WPM was 4 cm and 1 cm respectively. While at lower concentrations of sucrose (0.5-1.5%) the germination percentage was (0-63.66%), there was no root formation and the shoots never elongated beyond 0.2cm. The positive effect of sugar concentration may be because of its role on increasing the osmotic pressure of the medium, which stimulates mitochondrial activity and presumably production of energy required for shoot initiation (Bonga and

Aderkas 1992). At higher sucrose concentration (3%) many cultures were lost to bacterial contamination and the germination frequency was also reduced drastically.

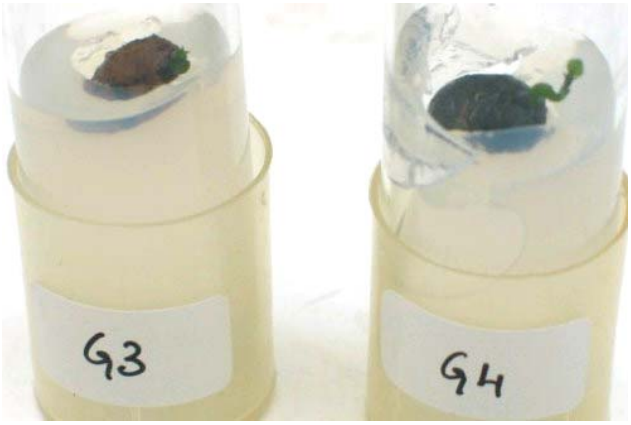


Fig 4.A.2: Effect of sucrose concentration on *Garcinia indica* seed germination,

G3-1.5%, G4- 2%

Table 4.A.2: Effect of different sucrose concentration

Sucrose (%)	% Germination (mean ± s.d.)*	Average shoot length (cms) after 4 months	Average root length (cms) after 4 months
0.5	0^{b}	0	0
1	50.0 ± 0^{ab}	0.2	0
1.5	63.66 ± 28.86^{ab}	0.2	0
2	76.18 ± 29.74^{a}	1	4
3	8.33 ± 2.88^{b}	0.2	0

*values followed by the same letter do not vary significantly at $p<0.01$

Growth regulators did not have a significant effect on the germination percentage of *Garcinia indica* seeds. In fact the growth regulators negatively affected the germination percentage of *Garcinia indica* seeds. While germination percentage was recorded as 53.15% for plain WPM medium, the germination percentage never increased beyond 50% in PGR supplemented medium. The occurrence of germination on PGR free media may be explained by the presence of large quantities of stored carbon, minerals and hormones in the cotyledons that support the growth and development of the seedlings (Hopkins and Hüner 2004). Similar results have been reported in *Labisia pumila* (Hartinie and Jualang

2007), *Kniphofia uvaria* (Bringmann et al. 2002) and *Swainsona salsula* (Yang et al. 2001). But it was found that even though the germination percentage was not much improved the growth of the shoots was enhanced by the use of PGRs. BAP 11.97 µM gave 6 cm shoot and GA_3 (1.44-5.77 µM) gave around 4 cm shoot. Among the growth regulators tested GA_3 5.77 µM gave the highest percentage (50%) of germination. Low concentration of BAP (4.44 µM) gave normal germination while at high concentration (11.97 µM) multiple shoot formation with long shoots and no root was observed. Development on BAP has been reported to induce multiple shoot formation in *Labisia pumila* (Hartinie and Jualang 2007) and *Lotus corniculatus* (Nikolić et al. 2006). Kinetin was deleterious to seed germination. The shoots formed were thin and weak and there was no root formation. Mackay et al. (1995) noted the poor growth of seedlings of *Cercis canadensis* in kinetin. After a month all the seeds were shifted to plain WPM basal medium. From the experiments conducted it can be concluded that ½ WPM containing 2% sucrose is the best medium to induce germination. Later the seedlings should be shifted to full strength WPM medium containing BAP 11.97 µM or GA3 (1.44-5.77 µM) for shoot elongation. Under *in vitro* conditions the germination percentage of *Garcinia indica* was increased considerably. *In vitro* studies have led to the increase in germination percentage in other species as well e.g. cherimoya seeds (Padilla and Encina 2003)

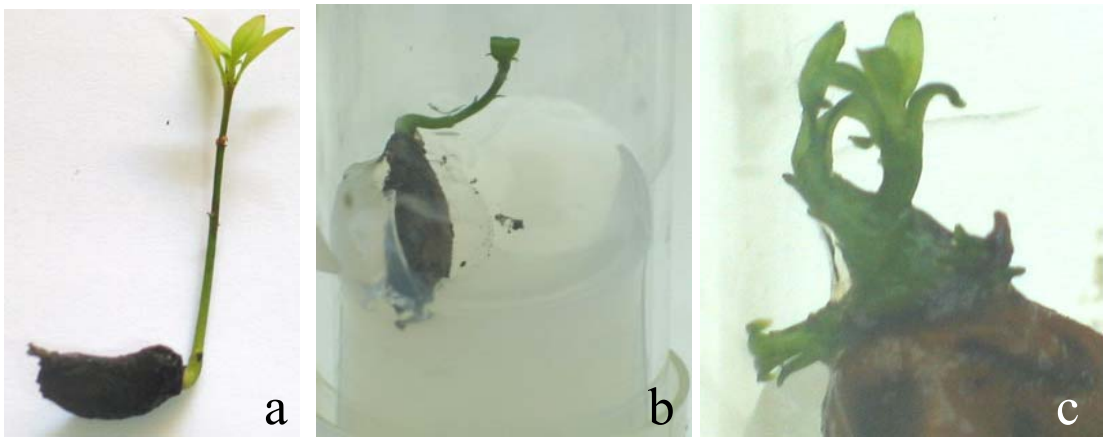


Fig 4.A.3: Effect of different growth hormones on *Garcinia indica* seed germination,

a-effect of GA_3(5.77 µM),

b-effect of kinetin (6.66 µM),

c-effect of BAP (11.97µM)

Table 4.A.3: Effect of different concentrations of growth regulators on seed germination of *G. indica*

Hormones(μM)	% Germination*	Average shoot length (cms) after 4 months	Average root length (cms) after 4 months
BAP 4.44	8.33 ± 2.88	1	4.25
BAP 11.97	13.80 ± 6.27	6	0
Kinetin 4.65	35.89 ± 4.44	1	0
Kinetin 14.15	6.66 ± 5.77	0.2	0
GA3 1.44	22.00 ± 19.05	4	0
GA3 5.77	50.0 ± 43.30	4.5	0

*nonsignificant at $p<0.05$

4.A.2 Germination studies in *Garcinia talbotii*

Materials and Methods:

Fruits were collected in the month of April from the semievergreen forests of Mulshi (18°28'N, 73°27'E, 610m) in Maharashtra, India. The method of surface sterilization was similar to that of *Garcinia indica*. The seeds after removing the seed coat were inoculated in different media combinations.

- Effect of different basal media:

Three different basal media- WPM, MS, B5 and their half strengths (except vitamins everything half) were used. All the media contained 2% sucrose.

- Effect of different carbon sources:

Glucose, sucrose, maltose and fructose at varied concentrations (2, 4, 6 and 8%) were incorporated in full strength WPM media and their effect on germination was studied. Full strength WPM without any carbon source was used as control.

- Effect of different growth regulators:

For the effect of different growth regulators, different concentrations of BAP ((4.44-22.19 μM), Kinetin (4.65-13.95 μM), TDZ (0.062-6.24 μM) and GA_3 (0.29-5.77 μM) were studied. The basal medium used was full strength WPM containing 2% sucrose.

All media contained 0.7% agar. Five seeds were used for each treatment and there were ten replicates for each treatment. The seeds were incubated in the experimental media for two months then shifted to plain WPM media. The seeds were cultured in 16hr

photoperiod. Germination percentage was noted every month and after three months the length of shoot, root, no. of nodes and no. of opened leaves were also noted down.

Results and Discussion:

- Effect of different basal media:

Three different basal media-WPM, MS and B5 and their half strength were tested. It was found that half strength WPM medium gave the best results with 66.66% germination in 30 days with an average shoot length of 2.85 cms and root length of 5.0 cms after 3 months. The high salt content of MS and B5 inhibited the germination as well as the root and shoot growth of *Garcinia talbotii* seeds as is found in the germination studies of *Annona cherimola* (Padilla and Encina 2003). A seed is supposed to contain food reserves to sustain it through the process of germination till the formation of new leaves. Moreover, mineral demand during the process of germination depends upon the species and is related to the amount of reserves in the seed. *Annona muricata* could be germinated on moist filter paper (Lemos and Blake 1996) and *Annona cherimola* could be germinated in distilled water (Padilla and Encina 2003). When decoated, *A. muricata* seeds were germinated aseptically on half strength MS medium (Bejoy and Hariharan 1992) and decoated *A. squamosa* seeds on White´s basal medium (Nair et al. 1986). In general, non-dormant seeds are germinated *in vitro* in media with low salt content. The same has been observed in *Garcinia talbotii*.

Table 4.A.4: Effect of different basal media on *Garcinia talbotii* seed germination. (value±s.d.)

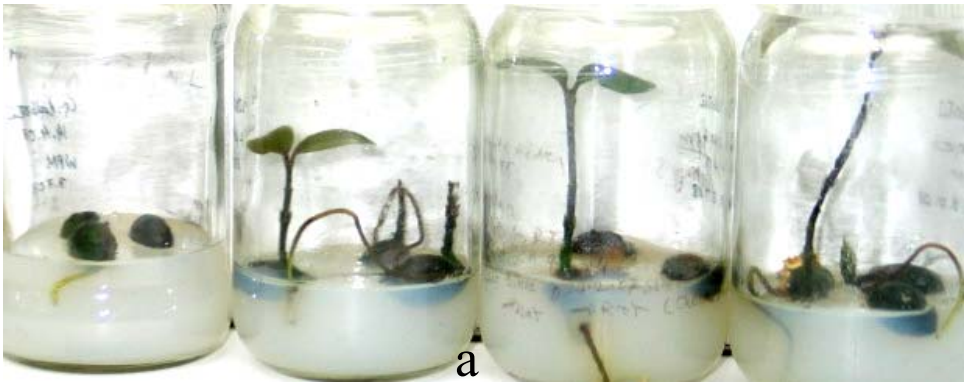
Medium	Germination %* after 30 days	Germination%* after 60 days	Germination%* after 90 days	Average shoot length (cms)	Average root length (cms)	Average no of nodes	Average no of opened leaves
WPM	22.22±19.24^{bc}	55.55±19.24^{ab}	55.55±19.24^{ab}	0.54±0.35	0.8±1.09	2±1.41	0
½ WPM	66.66±0^{a}	66.66±0^{a}	66.66±0^{a}	2.85±3.74	5±7.07	4±4.24	1 pair±1.4
MS	33.33±0^{b}	33.33±0^{bc}	33.33±0^{b}	0.1±0	0	0	0
½ MS	0^{c}	0^{e}	66.66±0^{a}	0.1±0	0	0	0
B5	0^{c}	33.33±0^{bcd}	33.33±0^{b}	0.23±0.23	0.16±0,28	0.66±1.15	0
½ B5	22.22±19.24^{bc}	22.22±19.24^{cde}	22.22±19.24^{b}	3.5±0	1±0	7±0	1pair±0

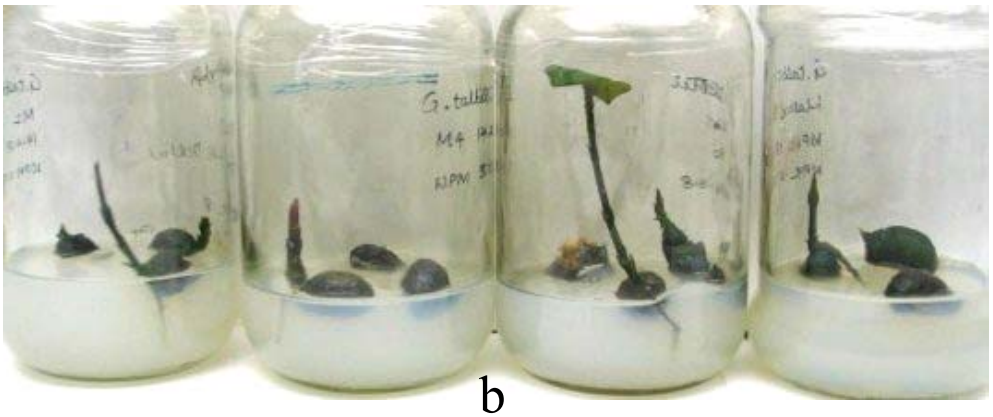
*values followed by the same letter do not vary significantly at $p<0.05$

- Effect of different carbon sources:

Glucose, sucrose, fructose and maltose at varied concentrations (2%, 4%, 6% and 8%) were incorporated in full strength WPM medium and their effect on germination was studied. Plain WPM media without any carbohydrate was used as control. The seeds were cultured in the different media for 2 months then shifted to plain WPM medium. The different carbon sources and their varied concentrations did not show a significant difference on the germination percentage of *Garcinia talboti* but affected the growth of the seedlings. The results are in line with those found in olive (Garcia et al. 2002). A carbon source is required in the later stages of seedling growth as found in olive (Garcia et al. 2002), or for other developmental purposes as in *Labisia pumila* (Hartinie and Jualang 2007), *Quercus suber* (Romano et al. 1995), *Amygdalus communis* (Gürel and Gülsen 1998) and *Chrysanthemum morifolium* (Karim et al. 2003). Sucrose was the best carbon source followed by maltose, fructose and glucose. Sucrose at 4% was found optimum giving 77.77% germination in 30 days. The maximum average shoot and root length, 4.92 and 3.42 cms respectively were recorded at this concentration after 3 months. Sucrose at 8% concentration gave 77% germination in 60 days and 100% germination in 90 days with average shoot length of 1.95 cms and a root length of 4.05 cms. At 6% sucrose concentration even though the germination percentage was not very high (33% in 30 days) it was found that this concentration gave the highest number of opened leaves. The results are in line with those found in *Labisia pumila, Quercus suber, Amygdalus communis* and *Chrysanthemum morifolium*. Sucrose has been the sugar of choice for most of the studies because it is easily translocatable and resistant to enzymatic degradation due to its non-reducing nature. Maltose gave 49% germination at 4% concentration in 30 days which later increased to 83% in 90 days. The shoot and root length, 0.58 and 0.36 cms respectively at this

concentration were very low. While maltose at 6% concentration even though gave only 44% germination in 30 days the shoot (1.8cms) and root lengths (0.5cms) were higher than those induced at 4% maltose. Multiple shoot formation was also observed at 4 and 6% maltose concentration. 6% fructose was found effective for inducing germination in 66% (almost equal to control) of the seeds in 60 days. The root and shoot length recorded at this concentration after 3 months was 1.17 and 2.12 cms respectively. Glucose was not found good for germination. At 2 and 4% glucose level no germination was observed in 30 days. At 6% concentration the germination percentage recorded was 49.99% (less than control) in 60 days. The shoot and root length at this concentration was 0.73 and 1.3cms respectively. But in all cases we found that a carbon source was required for the growth of the root and shoot of the germinated seedling. Among all the carbon sources sucrose at 4% concentration was found best for germination in *Garcinia talbotii* seeds.





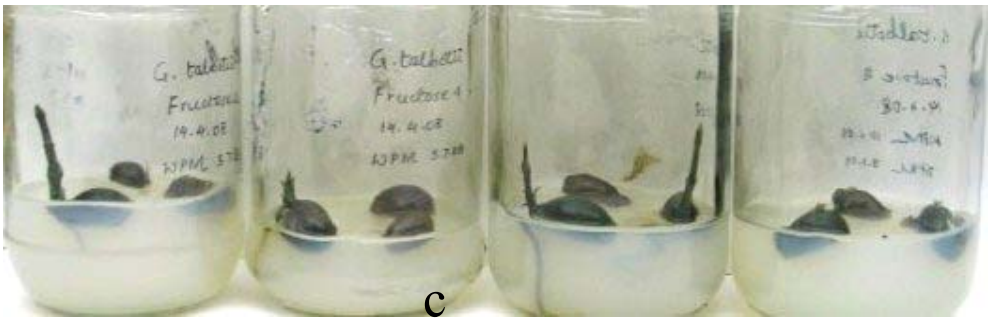


Fig 4.A.4: Effect of different carbon sources on *Garcinia talbotii* seed germination,

a-effect of different sucrose concentrations (2, 4, 6, 8)%,

b-effect of different concentrations of maltose (2, 4, 6, 8)%,

c-effect of different concentrations of fructose (2, 4, 6, 8)%

Table 4.A.5: Effect of different carbon sources on *G. talbotti* germination. (value±s.d.)

Medium	Germination %* after 30 days	Germination % *after 60 days	Germination %* after 90 days	Average shoot length (cms)	Average root length (cms)	Average no of nodes	Average no of opened leaves
control	33.33±0	66.66±47.14	100±0	0.2±0.24	0	0.5± 1.22	0
Sucrose 2%	22.22±19.24	55.55±19.24	55.55±19.24	0.54±0.35	0.8±1.09	2± 1.41	0
Sucrose 4%	77.77±38.49	77.77±38.50	77.77±38.50	1.32±1.33	4.92±5.58	3.42± 3.25	0.14 pairs± 0. 37
Sucrose 6%	33.33±47.09	33.33±47.09	49.95±23.54	1.73±2.83	3±5.16	1.6± 2.88	0.33 pairs± 0.57
Sucrose 8%	55.55±38.49	77.73±19.28	100±0	1.95±2.13	4.05±3.82	4.11± 2.89	0.11 pairs± 0.33
Maltose 2	44.44±50.92	55.53±38.50	66.66±33.35	0.91±1.33	0.33±0.81	2± 2.4	0
Maltose 4%	49.95±23.54	49.95±23.54	83.3±23.61	0.58±1.07	0.36±0.8	1.6± 3.5	0
Maltose 6%	44.44±50.92	55.55±38.49	44.44±19.24	1.8±2.5	0.5±1	2.75± 3.4	0.25 pairs± 0.5
Maltose 8%	0	33.33±0	49.95±23.54	0.86±1	3±3	2±2	0±
Fructose 2%	16.66±23.56	49.95±23.54	49.95±23.54	1.2±1.5	0.5±0.86	2.3± 2.51	0
Fructose 4%	33.33±0	66.66±0	33.33±0	1±0	2±0	5±0	0
Fructose 6%	33.33±47.13	66.66±0	66.66±0	1.17±1.25	2.12±2.46	3± 3.46	0
Fructose 8%	0	33.33±0	100±0	0.1±0	0	0	0
Glucose 2%	0	11.11±19.24	11.11±19.24	0.1±0	0	0	0
Glucose 4%	0	22.22±19.24	22.22±19.24	0.25±0.21	0	1± 1.41	0
Glucose 6%	22.22±19.24	49.99±23.56	49.99±23.56	0.73±1.09	1.3±2.30	1.3± 2.30	0
Glucose 8%	33.33±0	33.33±0	33.33±0	0.1±0	0	0	0

*values were found non significant at $p<0.05$

- Effect of different growth regulators

The effect of different growth regulators *viz*. BAP (4.44-22.19 µM), Kinetin (4.65-13.95 µM), Thidiazuron (0.062-6.24 µM) and GA_3 (0.29-5.77 µM) on the germination of *Garcinia talbotii* seeds was studied. BAP was found to be the most effective growth regulator for germination as well as multiple shoot formation. With increase in BAP concentration there was an increase in germination percentage along with the induction of multiple shoots. BAP 4.44 µM and 8.87 µM gave both shoots and roots. At BAP 13.32 µM 100% germination was observed in 60 days with 26 ± 24.24 shoots per seed. But there was no root formation. At BAP 22.19 µM the germination percentage observed was 77.77% in 30 days but the number of shoots induced per seed was low (7.57±7.36). This finding was in agreement with the results obtained in *Labisia pumila* where multiple shoots were obtained from seeds at 1-3µM BAP (Hartinie and Juglang 2007). BAP was reported to be the most effective cytokinin for shoot formation in *Polygonatum odoratum* seedling explants as well (Yoon and Choi 2002).

Fig 4.A.5: Effect of different concentrations of BAP (2.22-22.19) µM on *G. talbotii* Seed germination

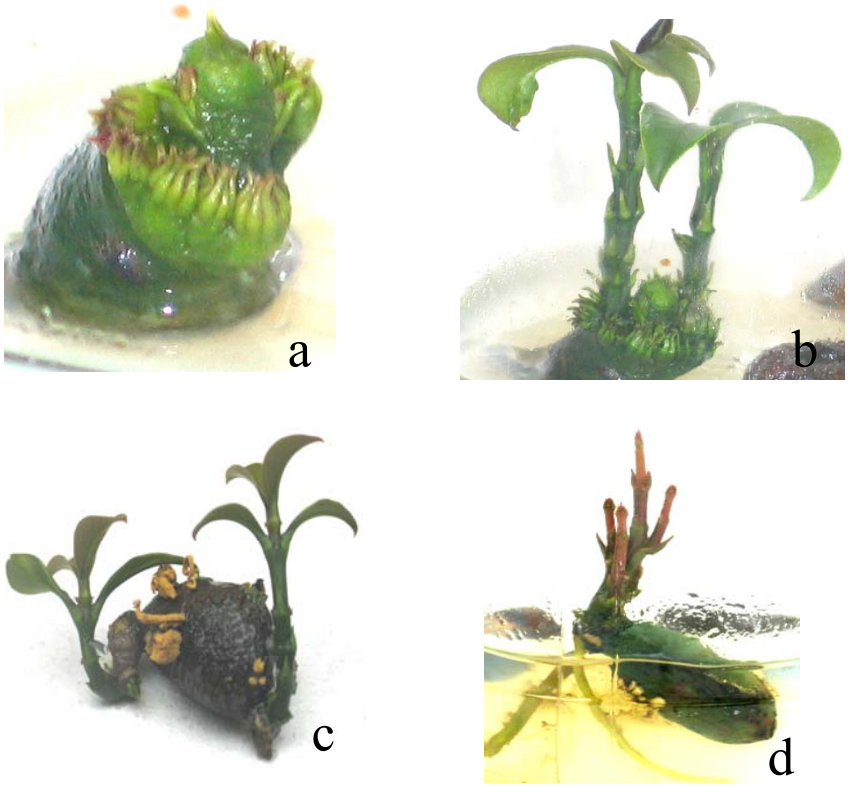


Fig 4.A.6: Multiple shoot formation from seeds of *G. talbotii*

a&b-multiple shoot induction and growth on BAP 13.34 µM,

c-shoot formation from both shoot and root pole on BAP 22.19 µM,

d- effect of 8.87 µM BAP on seed germination

Kinetin was not much useful for germination. At 4.65µM concentration it gave only 33.33% germination in 30 days which was much less than that obtained in plain ½ WPM. With the increase in kinetin concentration (9.29 µM) there was an increase in germination percentage (66.66%) but there was no root formation. Above 9.29 µM of kinetin the germination percentage decreased as well as the shoots were stunted. In other plant species such as *Myrica esculenta*, kinetin showed the best performance with regards to shoot proliferation (Bhatt and Dhar 2004). TDZ at 0.04 µM induced 66.66% germination in 30 days, but the shoots did not survive and the germination percentage was reduced to 33.33% at the end with no shoot elongation. TDZ at 0.45 µM gave 44.44% germination in 30 days

and 100% germination at the end of 90 days and the shoots formed showed elongation. Above 0.45 µM TDZ was found inhibitory for germination, the seeds bulged out at several places and became distorted. At 4.54 µM although 66.66% germination was observed the seedlings did not survive. Moreover, in all concentrations of TDZ no root development was observed. GA_3 was not found very effective for germination. At all concentrations (0.29-5.77 µM) the germination percentage was not more than that of plain WPM media (55.55%) and there was no rooting. GA_3 is known to induce germination in seeds. In the present study GA_3 was not found very effective may be because the concentration used in the present study was not appropriate.

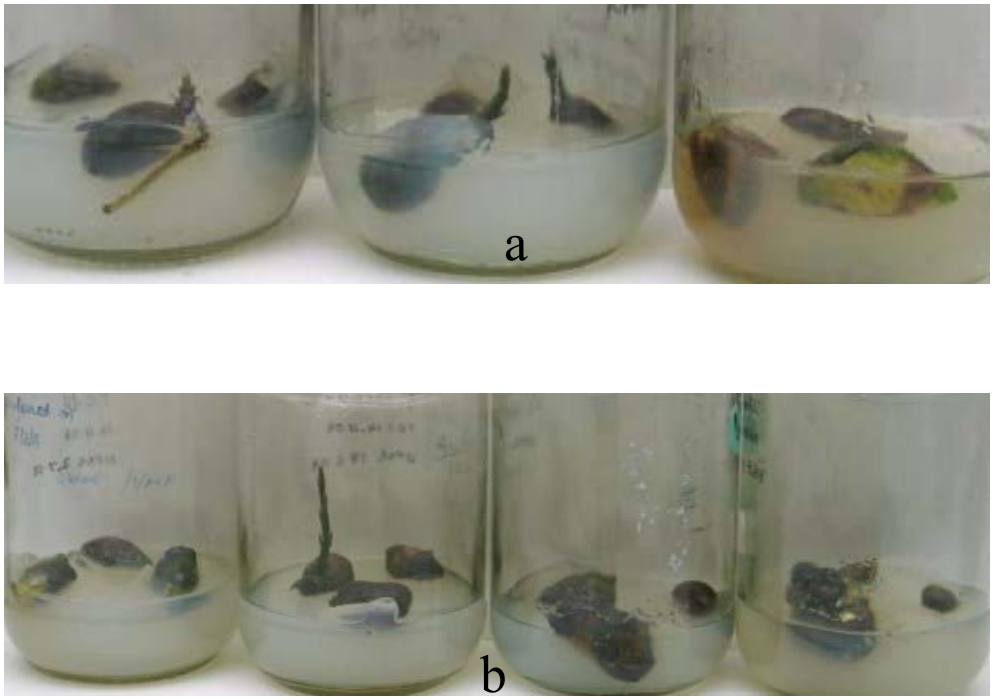


Fig 4.A.7: Effect of growth hormones on *G.talbotii* seed germination

a-Effect of different concentrations of kinetin (4.65-13.94 µM),

b-Effect of different concentrations of TDZ(0.04-4.54 µM)

Table 4.A.6: Effect of different growth hormones on *G. talbotii* seed germination. (value±s.d.)

WPM+ Hormones (μM)	Germination % *after 30 days	Germination% *after 60 days	Germination%* after 90 days	Average no of shoots from a single seed	Average no of roots from a single seed	Average shoot length (cms)	Average root length (cms)	Average no of nodes	Average no of opened leaves (in pairs)
KN 4.65	33.33 ± 0^{abcd}	66.66 ± 0^{abc}	66.66 ± 0^{abc}	1±0	0.5±0.7	0.3±0.28	1±1.41	2±2.82	0
KN 9.29	66.66 ± 0^{ab}	66.66 ± 0^{abc}	66.66 ± 0^{abc}	1±0	0	1.5±0.7	0	3.5±2.12	0
KN 13.94	33.33 ± 0^{abcd}	66.66 ± 0^{abc}	66.66 ± 0^{abc}	1±0	0	0.1±0	0	0	0
BAP 4.44	55.55 ± 19.24^{abc}	66.66 ± 0^{abc}	66.66 ± 0^{abc}	1.66± 1.63	0.16±0.4	1.28±2.6	0.5±1.22	1.6±2.65	0.66±1.21
BAP 8.87	33.33 ± 0^{abcd}	33.33 ± 0^{bcd}	33.33 ± 0^{bcde}	12.5± 7.7	1±1.411	0.42±0.79	4±3.60	0.64±	0
BAP13.31	33.33 ± 0^{abcd}	100 ± 0^{a}	100 ± 0^{a}	26± 24.24	0	0.2±0.4	0	0.26±1.2	0.01±0.11
BAP22.19	77.77 ± 19.24^{a}	77.77 ± 19.24^{ab}	77.77 ± 19.24^{ab}	7.57± 7.36	0.01±0.37	0.23±0.53	0.21±0.56	0.24±0.82	0
TDZ 0.04	66.66 ± 0^{ab}	33.33 ± 0^{bcd}	33.33 ± 0^{bcde}	1±0	0	0.1±0	0	0	0
TDZ 0.45	44.44 ± 38.48^{abcd}	44.44 ± 38.48^{bcd}	100 ± 0^{a}	1±0	0	0.66±1.38	0	1.3±3.26	0
TDZ 2.27	22.22 ± 19.24^{bcd}	22.22 ± 19.24^{cd}	33.33 ± 47.13^{bcde}	1±0	0	0.1±0	0	0	0
TDZ 4.54	66.66 ± 0^{ab}	66.66 ± 0^{abc}	0^{e}	0	0	0	0	0	0
GA_3 0.29	0^{d}	55.55 ± 38.49^{abcd}	55.55 ± 38.49^{abcd}	1±0	0	0.1±0	0	0	0
GA_3 1.44	11.11 ± 19.24^{cd}	33.33 ± 0^{bcd}	33.33 ± 0^{bcde}	1±0	0	0.1±0	0	0	0
GA_3 2.89	11.11 ± 19.24^{cd}	22.22 ± 19.24^{cd}	22.22 ± 19.24^{cde}	1±0	0	0.4±0.42	0	1.5±2.12	0

GA_3 5.77	11.11±19.24^{cd}	11.11±19.24^{d}	22.22±38.48^{de}	1±0	0	0.1±0	0	0	0

*values followed by the same letter do not vary significantly at $p<0.05$

4.A.3 Germination studies in *Garcinia xanthochymus*

Materials and Methods

Fruits were collected in the month of February from the semievergreen forests of Dapoli ($17^0$45'N, $73^0$10'E, 162m) in Maharashtra, India. The method of surface sterilization was similar to that of *Garcinia indica*. The seeds after removing the seed coat were inoculated in different media combinations.

- Effect of basal media

WPM and MS media and their half strengths were tried for germination studies

- Effect of different hormones BAP(4.44-13.22 µM), TDZ(0.04-0.45 µM) and GA_3(1.44-5.77 µM) was studied.

All media contained 2% sucrose and 0.7% agar. There were 10 replicates for each experiment and the experiment was repeated three times. The seeds were incubated in 16 hr photoperiod.

Results and Discussion

- Effect of different basal media

No germination was observed on plain WPM and MS media and their half strengths even after 3 months of incubation in the medium.

- Effect of different hormones

While no germination was observed on GA_3, TDZ and BAP induced germination in *Garcinia xanthochymus.* BAP 4.44 µM induced the best response. The seeds germinated in 4 weeks and the shoots elongated to 5cm in 3 months. But there was no root formation. TDZ at all concentrations (0.04-0.45 µM) induced germination. At low TDZ 0.04 µM, the roots (2cm) were longer than the shoots (0.2cm), but at high TDZ 0.45 µM both the root and shoot were 2cm in length. Cytokinins are plant hormones implicated in the regulation of various processes of growth and development. The effects of cytokinins on seed germination stand apart from their role in shoot morphogenesis. Modern analytical methods have shown their active metabolism in all phases of germination, from imbibition to radicle emergence and the start of seedling establishment (Stirk et al. 2005, Chiwocha et al. 2005). On the other hand, exogenous cytokinins have various effects on

seed germination in different species. Their promotive effects are mostly related to the alleviation of stress factors. In seeds of several large-seeded grain legumes, cytokinins did not affect seed germination (Malik and Saxena 1992). BAP and TDZ have been reported to reduce the time required for germination in *Lotus corniculatus* (Nikolic´ et al. 2006). TDZ was also found effective in the germination of *Pyrus serotina* (Linn et al. 1994) which otherwise required chilling for germination.

Table 4.A.7: *In vitro* germination studies in *Garcinia xanthochymus*

Hormones (μM)	Response	% Germination after 3 months	Shoot length (cm) after 3 months	Root length (cm) after 3 months
BAP 2.22	No germination	0	0	0
BAP 4.44	germination	65	5	0
BAP 13.22	No germination	0	0	0
TDZ 0.04	germination	65	0.2	2
TDZ 0.22	germination	70	1	0
TDZ 0.45	germination	60	2	2

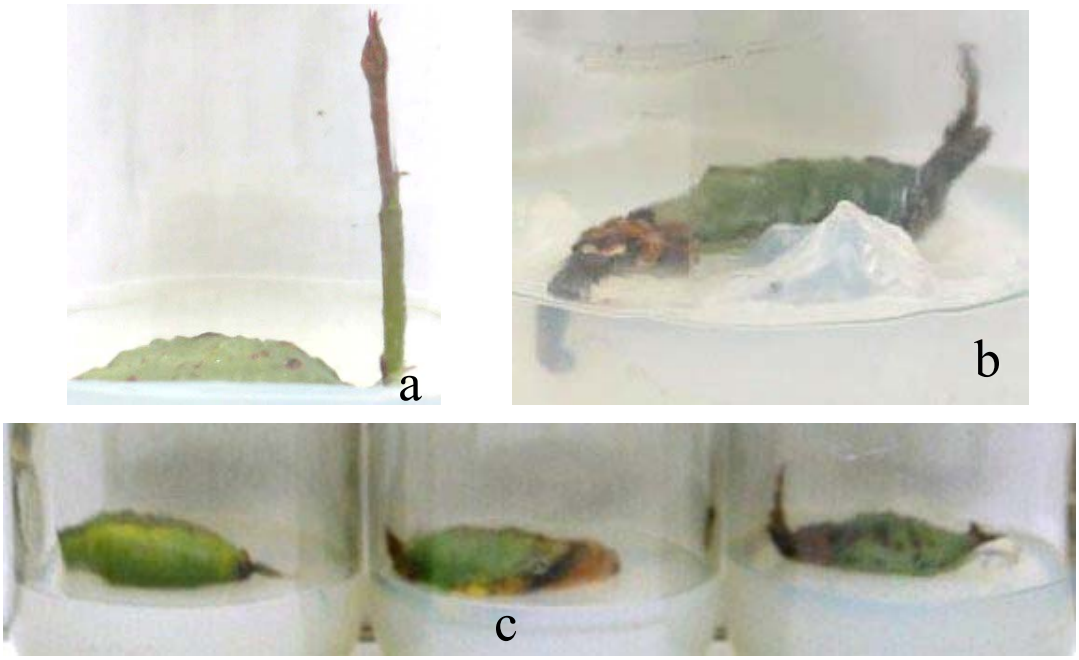


Fig 4.A.8: *In vitro* germination of *Garcinia xanthochymus* seeds,

a-germination on BAP 4.44 µM,

b-germination on TDZ 0.45 µM,

c-germination on TDZ(0.04, 0.22, 0.45 µM)

4.B *In vitro* regeneration by direct organogenesis in *Garcinia* species

In nature, stem, leaf, and root pieces of several taxa are able to differentiate shoots and roots leading to the establishment of new individuals (Dore 1965). Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. Direct organogenesis can either be from pre existing meristem like apical bud, axillary bud or embryo or it can be from fully differentiated tissue like cotyledons, hypocotyls, epicotyl, root or leaf. Indirect organogenesis involves callus formation. Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium. In particular, it is the auxin to cytokinin ratio of the medium that determines which developmental pathway the regenerating tissue will take. It is usual to induce shoot formation by increasing the cytokinin to auxin ratio of the culture medium. These shoots can then be rooted relatively simply. The earliest report on the formation of shoot buds in tissue culture was reported by White in 1939 on tobacco explant.

Organogenesis is not only an important tool for some plant application fields (Banerjee and de Langhe 1985, Levin et al. 1988, Brown and Thorpe 1995, Jain 2001, Chaturvedi et al. 2004), such as plant modification and improvement, clonal propagation and germ plasm storage, but also provides a useful experimental system for studying regulatory mechanisms of plant development (Guan and Zhang 2006).

Since the idea of culturing plant cell was first proposed by Gottlieb Haberlandt at the beginning of the 20th century (Haberlandt 1902), various types of organs, including vegetative organs and reproductive organs, have been successfully regenerated from hundreds of plant species (Tran Thanh Van 1973, Hicks and McHughen 1974, Lu et al. 1988, 1999, 2000, Li and Zhang 1999, Li et al. 2002, Lu 2002, 2003, An et al. 2004, Xu et al. 2004, Guan et al. 2006). In plant tissue culture, the balance of exogenous auxin and cytokinin in the medium is essential for *de novo* organogenesis (Skoog and Miller 1957). Based on the temporal requirement for a specific balance of exogenous hormones, three phases can be conceptually characterized during plant organogenesis (Christianson and Warnick 1983, 1984, 1985, Sugiyama 1999, Zhang and Lemaux 2004). First, explant cells respond to phytohormones and acquire organogenic competence. Then, quiescent cells re-enter the cell cycle and cell fate is determined to form specific organ primordia, which is

the key step during *de novo* organogenesis. In the final stage, the morphogenesis of *in vitro* organs occurs.

Role of phytohormones during *in vitro* plant organogenesis

Earlier evidence for the regulatory effects of auxin and cytokinin on shoot organogenesis *in vitro* was provided by Skoog and Miller in the mid-20th century (Skoog and Miller 1957). The balance of auxin and cytokinin in the medium plays a role in the determination of the morphological fate of callus (Skoog and Miller 1957, Christianson and Warnick 1985). Different organ types are achieved by regulating the ratio between auxin and cytokinin. A relatively high ratio between auxin and cytokinin promotes the regeneration of root. Whereas, the reverse leads to the formation of shoot. The explant cells proliferate to form callus when the same level of auxin and cytokinin are added into the medium. Thus, it is indicated that the ratio of auxin to cytokinin is the critical factor in triggering developmental events *in vitro*. Other studies indicate that the type of *de novo* organs formed is influenced by the concentration of exogenous phytohormones too. In the *in vitro* floral organogenesis of *Hyacinthus orientalis*, higher levels of cytokinin and auxin trigger tepal formation from the explants (tepals). After being transferred to medium containing low levels of hormones, ovaries and ovules can be induced from regenerated floral buds (Lu et al. 1988, 1999, 2000). Taken together, both the concentration and the ratio of exogenous hormones are important to determine cell fate during *de novo* organogenesis.

Organogenesis at the molecular level

Thorpe and Murashige (1970) by histochemical analysis examined the changing status of nucleic acid, protein and carbohydrate in differentiating and non differentiating calli of tobacco. The two tissues did not vary much in the level of DNA/cell but RNA and proteins were higher in the shoot forming callus. The starch content was also higher in the shoot forming callus. It has been suggested that starch together with the free sugars in the medium serve as energy supply for the developing shoot. Gibberellin which inhibits shoot formation, prevents starch accumulation from reaching a threshold level required for shoot bud differentiation by decreasing starch synthesis and increasing starch degradation (Thorpe and Meier, 1975)

Molecular analysis of *de novo* organogenesis shows that inductive expression of organ identity genes is essential for organ regeneration under cultured conditions (Li et al. 2002, An et al. 2004, Xu et al. 2004, Guan et al. 2006, Gordon et al. 2007). In *Hyacinthus*, induced expression of HAG1 by high level phytohormones promotes the regeneration of floral buds, whereas low level phytohormones could activate HoMADS1 that is responsible for formation of *in vitro* ovule (Li et al. 2002, Xu et al. 2004). The induction of TFL1 is critical to *Arabidopsis* inflorescence regeneration (Guan et al. 2006). Promoter analysis shows that there are three auxin response elements, suggesting that auxin may activate TFL1 expression (Woodward and Bartel 2005, Guan et al. 2006). Although the direct link between phytohormones and organ identity genes has not been elucidated, the organ identity genes might be activated by exogenous phytohormones, and then their expression regulates the cell fate of progenitor cells. The study of a versatile floral induction system provides further evidence for this conclusion. For example, ectopic overexpression of LEAFY in the callus directly induces formation of flowers from root explants bypassing normal vegetative development in *Arabidopsis* (Wagner et al. 2004), indicating that the genes involved in organ identity play an important role during the *in vitro* organogenesis. However, the pathway by which the organ identity genes are activated by phytohormones remains unclear.

Until recently, standardization of successful methods for regeneration has proceeded empirically. The literature abound with methods have taken years to develop because of the lack of the basic understanding of the regulatory processes of morphogenesis. Furthermore, knowledge gained from successful manipulation of one species or cultivar is often not applicable to other species or cultivars even if the plants are genetically closely related.

4.B.1 Organogenesis in *Garcinia indica*

Material and Methods:

The fully mature ripe and unripe fruits of *Garcinia indica* were collected in the month of May from the Konkan region of Maharashtra. The fruits were stored in the cold room until they were inoculated. All fruits were inoculated within 2-3 days. The fruits

were surface sterilized by the following method. The fruits were washed under running tap water for 1 hr. They were then cleaned with few drops of detergent and treated with 10% (v/v) savlon for 3-5 min. Kept in 1% (w/v) bavistin for 1 hr with occasional stirring. After each treatment the fruits were washed with single distilled water 4-5 times. Further treatments were carried out in the laminar air flow cabinet. The seeds were excised from the fruits rinsed with 70% (v/v) alcohol and washed with sterile distilled water 2-3 times to remove traces of alcohol. The seeds were then treated with 0.05% (w/v) $HgCl_2$ for 10 min and washed several times with sterile distilled water. The seed coat was removed and the seeds were germinated in half strength WPM medium (only macro elements halved) under dark conditions. On germination the seeds were shifted to light conditions with 16 hr photoperiod. After 1 month on ½ WPM the germinated seeds were shifted to full strength WPM for proper growth of the seedlings.

When the seedlings were around 2 cm in height the shoots were decapitated and the seedling along with the attached seed was grown in WPM basal medium containing different concentrations of the growth regulators viz. BAP (2.22-26.64 μM), TDZ (0.45-2.27 μM). The induced shoot buds were elongated on WPM basal medium containing 0.2% activated charcoal. All media contained 2% sucrose and was gelled with 0.7% agar. When the shoots were 2-3 cm in length they were rooted in WPM basal liquid medium containing IBA (4.9-14.7 μM) singly or in combination with NAA 5.37 μM. Well developed plants having both root and shoot were subjected to hardening in plastic cups containing sterile soil: sand in 1:1 ratio. The plants were watered, covered with plastic bags to maintain humidity and kept in 24 hr light in culture room. The plants were watered at regular intervals. After 1 month of hardening the plants were shifted to green house.

Ex vitro rooting was also tried. Shoots about 2-3 cm in length were dipped in commercial rooting mixture and planted in plastic cups containing sterile soil: sand mixture in the ratio 1:1. The plants were watered and covered with plastic bags to maintain humidity. These plants were then kept in the culture room under 24hr light condition. Well developed plants having both root and shoot were transferred to green house. 10 seedlings were used for each treatment and the experiment was repeated 3 times. The data was subjected to statistical analysis.

For histological studies the shoot buds induced on TDZ were used because they remained in the bud state for a long time, where as the shoot buds on BAP media showed induction with elongation and thus were not found suitable for histological studies. Moreover, in TDZ the buds were all crowded together and thus better results could be expected from this explant. The explants showing organogenesis in TDZ media after 1 month culture in the elongation media were fixed for histological studies. At this stage the shoot buds could be counted. Histological studies were done to confirm organogenesis.

Results and Discussion:

The seeds germinated within 3 days in dark. The sterilization method developed gave almost 91% sterile cultures. The position of the embryo varied in the seeds as shown in fig (4.B.1c).

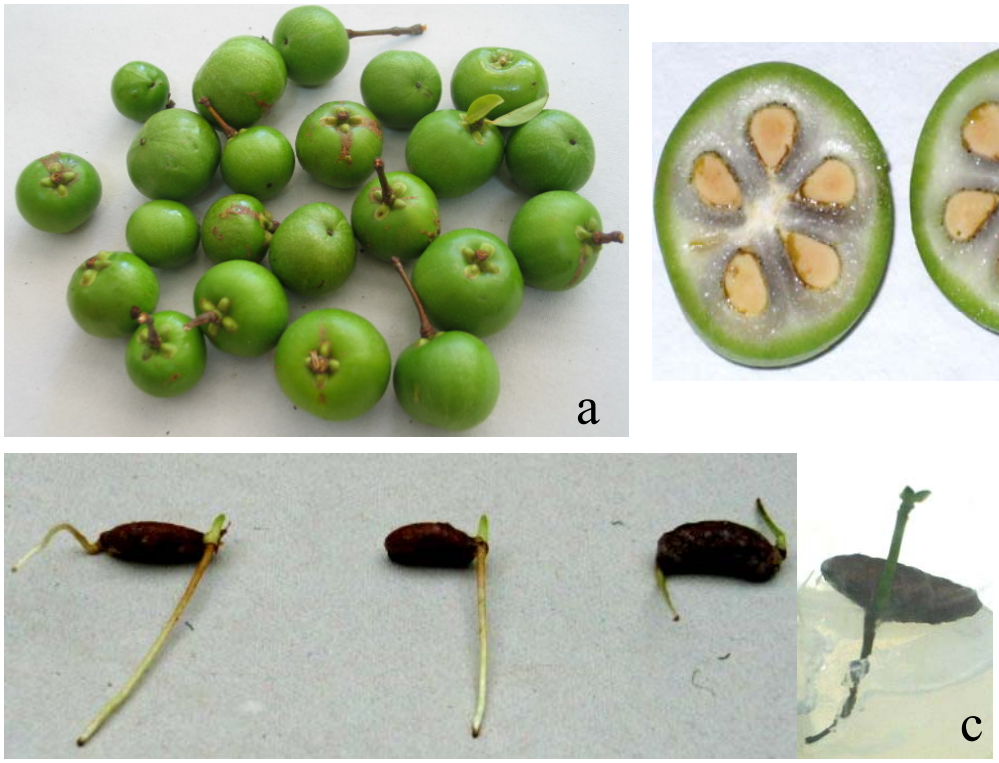


Fig 4.B.1: a-fruits of *Garcinia indica,* b- T.S. of fruit showing 5 seeds, c-different positions of embryo showing germination

As soon as the plumule appeared the seeds were shifted to light conditions under 16 hr photoperiod. After a month in ½ WPM the germinated seeds were shifted to full strength WPM for proper growth of the seedlings. *Garcinia indica* being a tree species the growth of the seedlings was slow. In two months the shoots were about 2 cm in length. The seedlings were then decapitated and the seedlings still attached to the seeds were inoculated in different media compositions. Within 3 weeks (25 days) multiple shoots were formed from the cotyledonary node region of the seedling as well as from the roots of the seedlings on BAP and TDZ containing media (Fig 4.B.2).

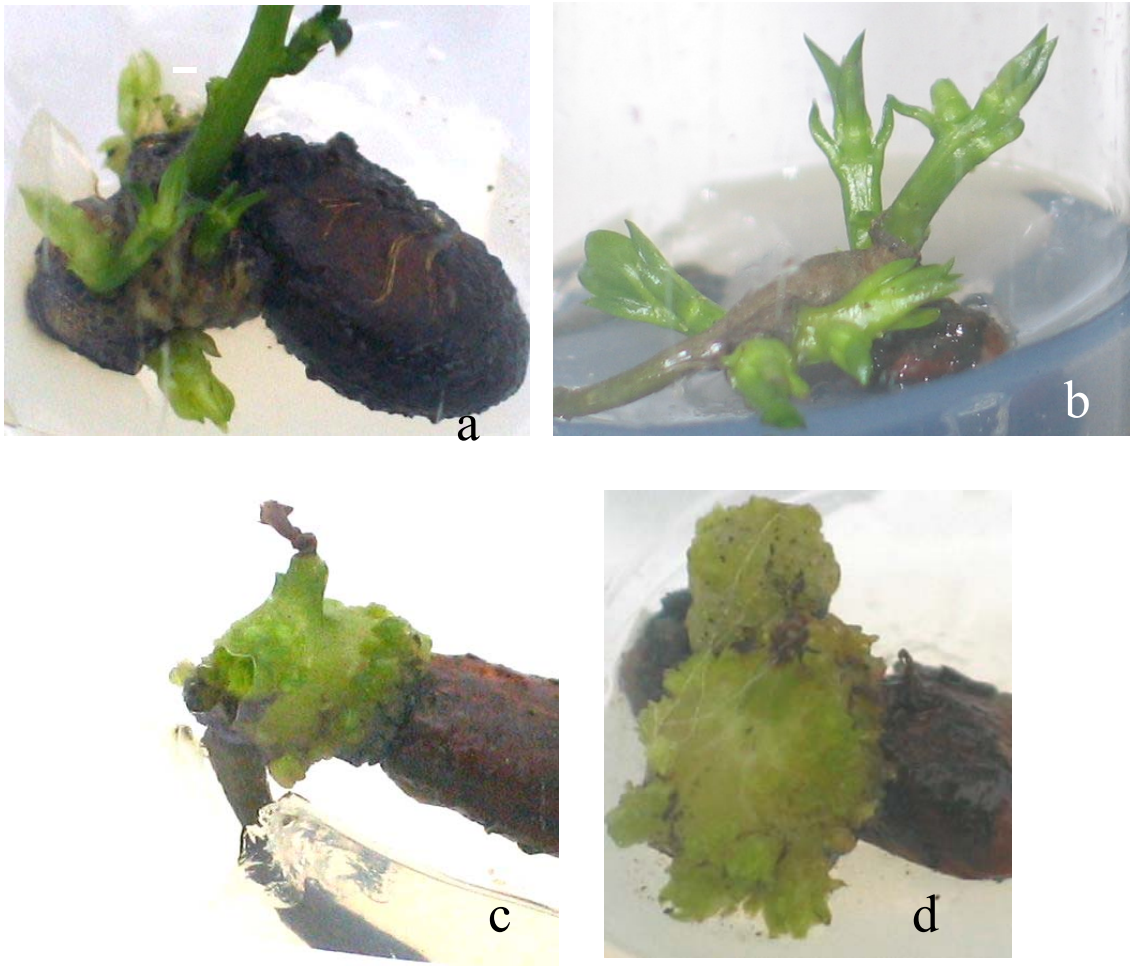


Fig 4.B.2: Induction of organogenesis in *Garcinia indica*

a- multiple shoot formation from cotyledonary node of decapitated seedling on BAP medium

b- multiple shoot formation from root,

c&d- organogenesis in TDZ medium from cotyledonary node region

Multiple shoot induction:

The concentration of cytokinin in the culture medium is known to be critical for shoot organogenesis. At higher concentrations of BAP (17.76 µM) multiple shoot formation was observed in 90% of the explants in 3 weeks but at lower concentrations of BAP (2.22 µM) multiple shoot formation was observed in only 20% of the explants with very few multiple shoots (Table 4.B.1). With the increase in BAP concentration from 2.22 to 17.76 µM there was an increase in the number of organogenic buds formed, with further increase in BAP to 26.67 µM the number of organogenic buds decreased drastically. BAP at 17.76 µM was found optimum giving an average of 10.20 shoots per seedling. Some amount of callus formation was observed at the cotyledonary node region on BAP concentrations above 8.87 µM (Fig 4.B.2a).

Table 4.B.1: Effect of different growth regulators on organogenesis in *Garcinia indica*

Hormones(µM)	% response	Type of Response	Average number of shoots*
Control	0	No response	0
BAP 2.22	20	Shoots from cot. node and root of seedling	1.55 ± 0.99^{c}
BAP 4.44	60	Shoots from cot. node and root of seedling	4.33 ± 2.28^{bc}
BAP 8.87	80	Shoots from cot. node and root of seedling	4.35 ± 3.53^{bc}
BAP 17.76	90	Shoots from cot. node and root of seedling. Callus at cot. node	10.20 ± 9.58^{b}
BAP 26.64	50	Shoots from cot. node and root of seedling. Callus at cot. node	5.02 ± 1.0^{bc}
TDZ 0.45	70	Green compact callus with shoots on both root and cot. node	17.0 ± 8.09^{a}
TDZ 0.91	30	Green compact callus with shoots on both root and cot. node	17.3 ± 4.23^{a}
TDZ 2.27	10	Green compact callus with shoots on both root and cot. node	10.0 ± 2.50^{b}

*Values followed by the same letter do not vary significantly at $p<0.01$

All concentrations of TDZ (0.45-2.27 µM) induced organogenesis along with callus formation. 0.91 µM TDZ although gave maximum no of organogenic buds, the number of explants showing response was very low (only 30%). TDZ at 0.45 µM induced 17 buds

(which was not significantly different from the number induced on TDZ 0.91 μM) per explant and the number of explants showing response was also high (70%). At higher concentrations of TDZ (2.27 μM) the number of organogenic buds as well as the percent response was reduced. Even though TDZ induced the highest number of organogenic shoots it took 3 months to elongate. TDZ facilitates efficient micropropagation of many recalcitrant woody species (Huettman and Preece 1993), however, the same was not observed in the present study. In the present study, BAP was found to be more potent as compared to the other cytokinin used. Superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue (Zaerr and Mapes 1982). The promotory effect of BAP in inducing multiple shoots has been previously reported in *G. mangostana* from leaf (Goh et al. 1990), in *Garcinia indica* from root explant (Deodhar et al. 2008) and seed explant (Malik et al. 2005) and in shoot tip cultures of seedlings of *Acacia seyal* (Al-Wasel 2000).

Histological Studies:

The seeds were hard so 10μm sections were not found suitable for histological studies. Instead 7μm sections were taken. Histological examinations of the regeneration process revealed areas of active cell division in the subepidermal region of the explant. These developed into meristematic pockets that appeared on the surface of explants (Fig 4.B.3a). Leaf primordia started developing (Fig 4.B.3b) and formed recognizable shoot meristems by the third week of culture (Fig 4.B.3c). The sections showed vascular connection of the shoots with the mother explant

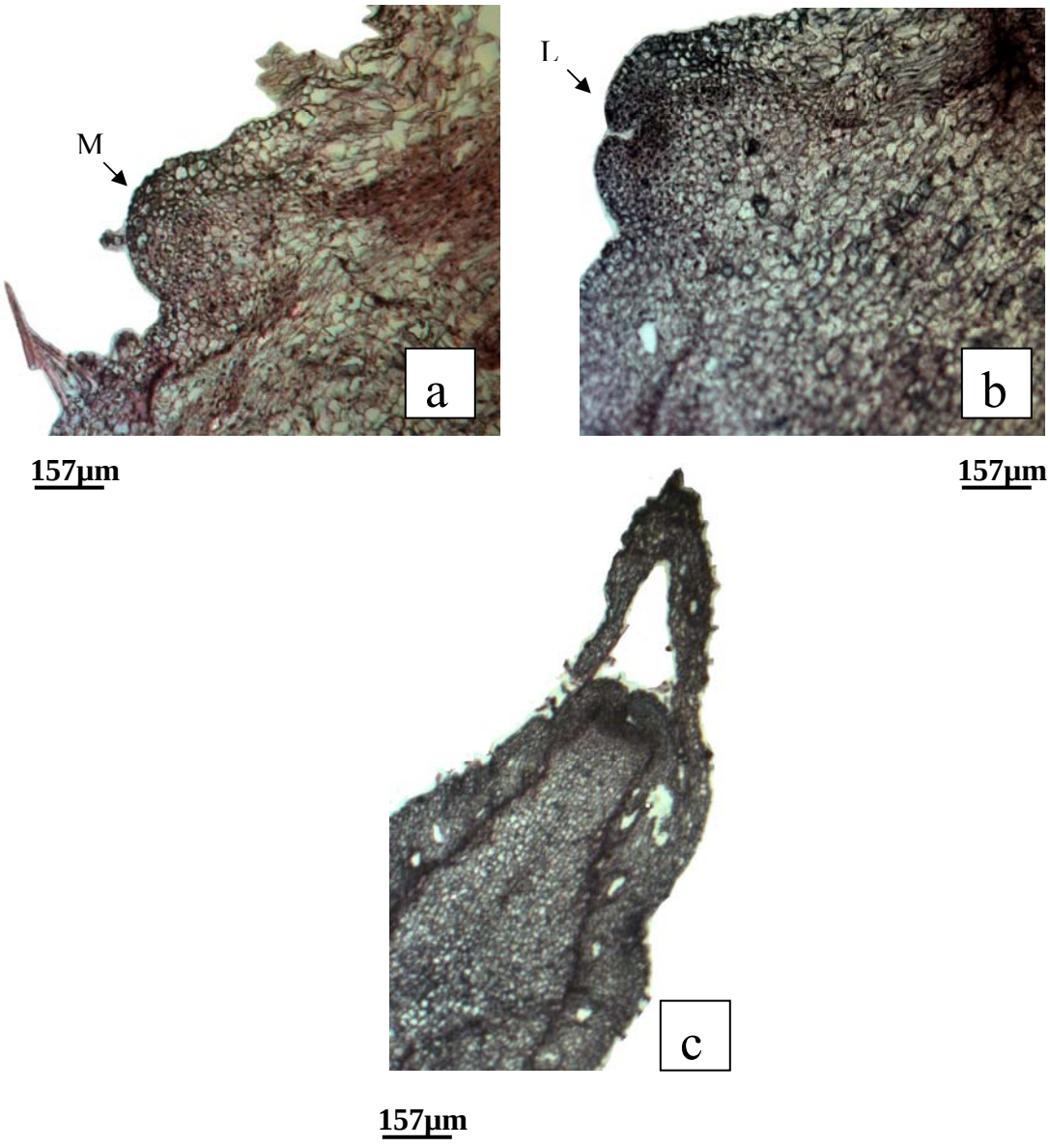


Fig 4.B.3: Histology of organogenesis in *Garcinia indica*

a-Meristematic region protruding out of the explant, M-meristematic region

b-Initiation of leaf primordia, L-leaf primordia

c-L.S. of shoot bud showing young leaves

Elongation of multiple shoots:

The organogenic buds were shifted to plain WPM medium or plain WPM medium containing 0.2% charcoal. It was found that plain WPM medium was not as effective as charcoal containing medium for elongation. The shoots induced on lower concentrations of BAP (2.22-8.87 µM) showed maximum elongation in 1 month. Similar results have been reported in previous studies on *Garcinia indica* (Malik et al. 2005) and in *Pinus roxburghii* (Kalia et al. 2001). While the shoots induced on TDZ had to be repeatedly subcultured 2-3 times at 1 month interval to get 2 cm shoots.

Fig 4.B.4: Elongation on charcoal medium

***In vitro*/ *Ex vitro* Rooting:**

Liquid media was chosen for the rooting experiments as the nutrients are more readily available in liquid media and there is no physical resistance to the developing roots. IBA (4.90-14.7 µM) alone or in combination with NAA 5.37 µM were used for *in vitro* root induction. Rooting was observed in all the media tried with varying frequencies in 25 days. The plants were kept in the same medium for 1 month for elongation of the roots. IBA at 14.7 µM gave roots in 80% of the explants (Table 4.B.2) with an average of 2 roots per shoot but the roots formed were short and thick and did not look normal (Fig 4.B.5b). 9.8 µM IBA induced normal long roots and was considered the optimum concentration for root induction (Fig 4.B.5a). NAA was not found suitable for rooting. The roots formed were short and thick with callus formation at the root shoot junction (Fig 4.B.5c). Similar results have been reported in the earlier studies of this species (Deodhar and Kulkarni 2002). IBA is considered as the most

effective auxin in root induction in tropical fruit tree species (Litz and Jaiswal 1990). *Ex vitro* rooting helps to reduce cost in tissue culture by combining the rooting and hardening steps into one. In e*x vitro* rooting experiments 50% success was observed, and was a good alternative to *in vitro* rooting in *Garcinia indica* (Table 4.B.3). The roots were induced in 25 days and the roots were normal. No phenotypic difference was found in the *in vitro* and the *ex vitro* rooted plants. On an average 10 plants per seed can be produced by this method in 6-7 months.

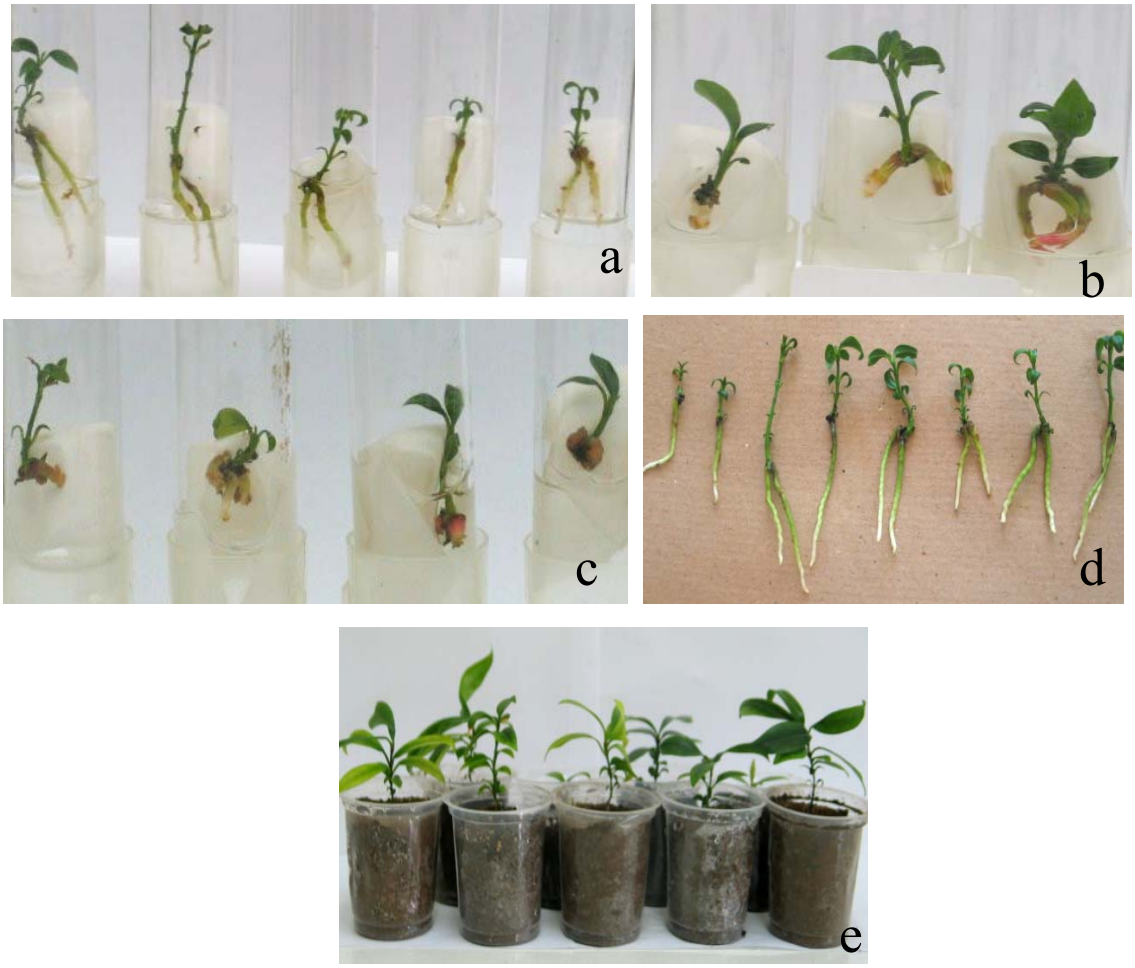


Fig 4.B.5: a-rooting of *Garcinia indica* shoots on IBA 9.80µM, b-rooting on IBA 14.8 µM, c-rooting on IBA 9.80 µM +NAA 5.37 µM, d-complete plantlets, e-2 months old hardened plants in soil mixture

Table 4.B.2: *In vitro* rooting in *Garcinia indica*

Medium		Rooting %	Average no. of roots	Average root length(cms) after 45 days of inoculation
IBA(μM)	NAA(μM)			
4.90	--	18.18	1.0	3.0
9.80	--	77.17	1.7	2.0
14.7	--	80.0	2.0	1.5
4.90	5.37	60.0	1.6	1.0
9.80	5.37	80.0	1.0	0.5

Table 4.B.3: *Ex vitro* rooting in *Garcinia indica*

Chemical used	Rooting %
Commercial rooting mixture	50.0

4.B.2 Organogenesis in *Garcinia talbotii*

Materials and methods:

Fully mature unripe green coloured fruits were collected in the month of April from the semievergreen forests of Mulshi (18°28' N, 73°27' E, 610 m) in Maharashtra, India. The fruits were stored in the cold room till inoculation. All fruits were inoculated within 2-3 days. The fruits were surface sterilized by the method followed for the fruits of *Garcinia indica*. The seeds after removing the seed coat were cut into three parts and inoculated in WPM media containing TDZ (0.045-9.08 μM)/ BAP (2.22-44.38 μM)/ BAP (2.22-44.38 μM) in combination with NAA 2.69 μM or BAP (2.22-44.38 μM) in combination with kinetin 4.65 μM. The explants were cultured in the TDZ media for 1 month and then shifted to low BAP (0.44 μM). The explants were grown in BAP, BAP in combination with NAA or BAP in combination with kinetin for 2 months with 1 subculture to the same medium after 1 month and then shifted to low BAP (0.44 μM). The shoots were rooted on ½ WPM+0.44 μM BAP. All media contained 2% sucrose and were gelled with 0.2% (w/v) phytagel. The explants at different stages of morphogenic response were fixed for histological studies. 12 seeds were used for each treatment and the experiment was

repeated 3 times. All cultures were grown at 25±2 ºC under 16 hr photoperiod provided by cool white fluorescent tubes with a photon flux density of 30 μmol m^{-2} s^{-1} at the culture level.

Fig 4.B.6.: a-fruits of *Garcinia talbotii*, b-fruit cut into halves to show the seeds, c-decoated seeds of *Garcinia talbotii*

Results:

Effect of BAP: The seeds were cut into 3 parts and inoculated in different concentrations of BAP. No response was observed in the first 6 days. On the 7th day initiation of germination was observed from the micropylar end of the seeds in some of the plates. Germination was observed in 50% of the explants on 4.44 μM BAP while BAP 44.38 μM showed germination in 75% of the seeds in 7 days (Table 4.B.4). But at the end of 45 days the germination percentage increased to 50% at BAP 2.22 μM and reached 100% at BAP 22.19 μM and above. Thus it was observed that high concentrations of BAP induce germination in less time. While low concentrations of the hormone can be equally effective if used for a longer duration. While low concentrations of BAP (2.22-4.44 μM) induced normal germination with both root and shoot formation (Fig 4.B.7a), high concentrations of BAP (8.87-44.38 μM) induced multiple shoot formation with no root formation (Fig 4.B.7b). At low concentrations of BAP the germinating shoots were normal but above BAP 22.19 μM the shoots formed were stunted.

Table 4.B.4: Effect of BAP concentration on seed germination

BAP (μM)	% Germination in 7 days	% Germination in 45 days	No. of shoots from germination point after 68 days	No.of roots from germination point after 68 days
2.22	0	50	1	1
4.44	50	50	1	1
8.87	50	80	3	0
22.19	50	100	5	0
35.52	50	100	7	0
44.38	75	100	10	0

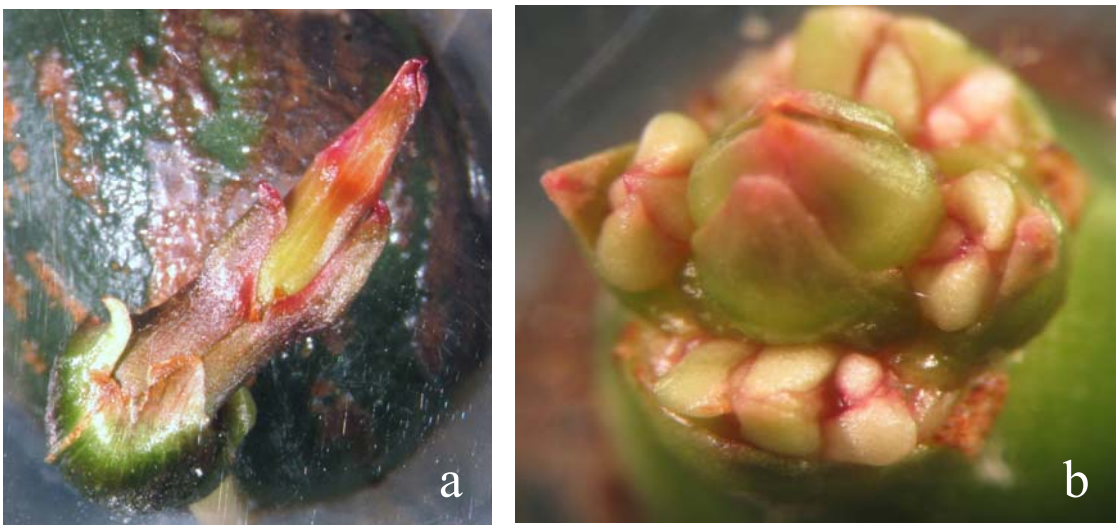


Fig 4.B.7: a-germination of *G. talbotii* seed on low BAP, b-multiple shoot formation on BAP 44.38μM

Since only germination was observed in 30 days, the explants were subcultured to the same medium. After 45 days globular and heart shaped embryos could be observed on BAP 4.44 μM while organogenic buds were observed on BAP 8.87-44.38 μM. The % response of the explants for organogenesis varied from 0-66% with BAP 35.52 μM showing the highest response of 66% and an average of 11 shoots per explant (Fig 4.B.8b, Table 4.B.5). With further increase in BAP there was a decline in % response and no of shoots formed. The

organogenesis observed was direct with no callus formation. No relation was found between the part of the seed and the response. On the middle explant some green round morphogenetic structures were formed in a ring as shown in fig 4.B.8d. on all concentrations of BAP. The explants were kept in the medium for 2 months then shifted to BAP 0.44 μM.

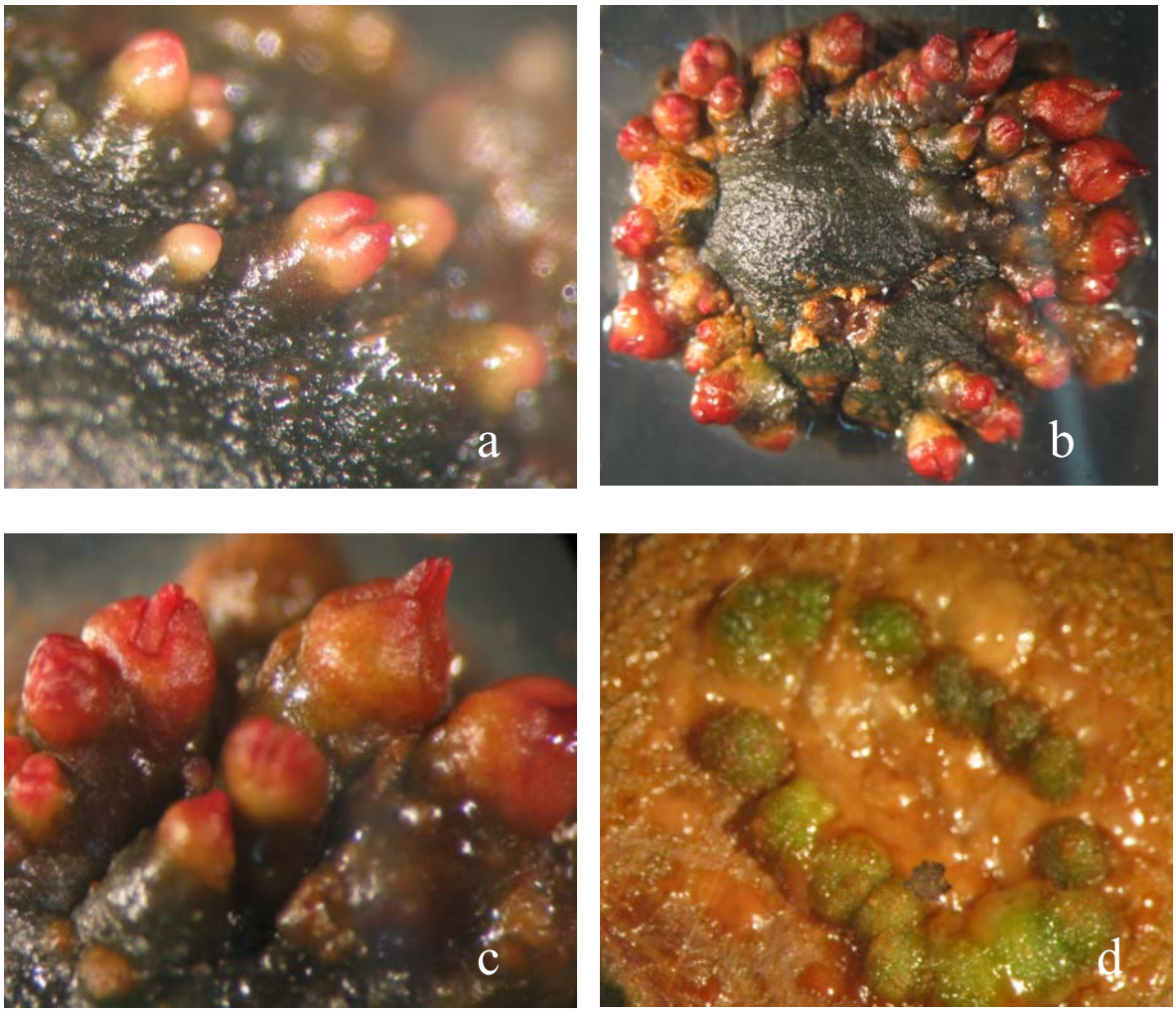


Fig 4.B.8: Organogenesis in *G. talbotii*

a-initiation of organogenic primordia on BAP 22.19 μM,

b-organogenesis on BAP 32.52 μM,

c-magnified view of organogenic buds,

d-morphogenetic structures in a ring on middle explant

Effect of BAP+kinetin: Germination of the seeds was observed within 7 days of inoculation on all BAP+kinetin combinations. It was observed that BAP+kinetin combination was more effective for germination. After 45 days in the induction medium direct organogenesis was observed on the explants. Some amount of compact green callus was also observed on most of the explants. Organogenesis was observed on all BAP+kinetin combinations till BAP 35.52 μM+kinetin 4.65 μM. The % response on BAP+kinetin varied from 0-100% and even the number of shoots formed was higher than on BAP alone (Table 4.B.5). Very high BAP (44.38 μM) +kinetin 4.65 μM was inhibitory and no organogenic buds were observed at this combination. Instead compact green callus was observed on BAP 44.38 μM+kinetin 4.65 μM. The organogenic buds observed on BAP+kinetin medium were green in colour (Fig 4.B.9a) while that on BAP alone were red in colour (Fig 4.B.8a). In some of the explants the buds broke into white friable callus (Fig 4.B.9b). BAP 8.87 μM +kinetin 4.67 μM gave the best response with about 47 shoots per explant. No relation was found between the part of the seed and the response. In some of the cultures green morphogenic structures were observed on the middle explant as in fig(4.B.8d).The cultures were grown on BAP+kinetin medium for 2 months with 1 subculture to fresh medium after 1 month then shifted to 0.44 μM BAP.

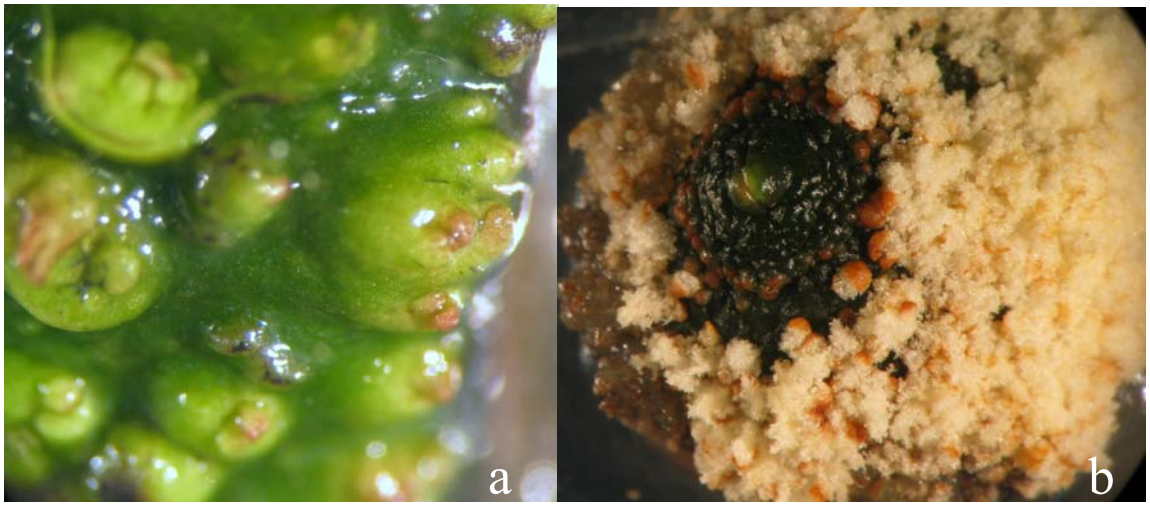


Fig 4.B.9: a-green coloured organogenic buds on BAP+Kinetin, b-buds breaking into white friable callus on BAP 4.44 μM+kinetin 4.67 μM

Table 4.B.5: Effect of cytokinins alone or in combination on organogenesis in *G. talbotii*

Medium			%response	Average no of buds after 68 days
BAP(μM)	Kinetin (μM)	NAA (μM)		
2.22	--		0	0
4.44	--		0	0
8.87	--		33.33	3.0±0
22.19	--		33.33	7.0±1.50
35.52	--		66.66	11.0 ±3.0
44.38	--		33.33	9.0 ±0
2.22	4.65		33.33	7.0 ±0
4.44	4.65		66.66	16.66 ±1.15
8.87	4.65		100	47.0 ±42.03
22.19	4.65		33.33	4.0 ±0.50
35.52	4.65		33.33	4.50 ±0.50
44.38	4.65		0	0
35.52	--	2.69	25.0	4.0±0.50
44.38	--	2.69	33.33	4.2±0.25

Effect of BAP+NAA: After 45 days germination of the seeds was observed on BAP+NAA combination. On BAP 2.22 μM+NAA 2.69 μM 60% germination was observed which increased to 100% on BAP 4.44 μM+NAA 2.69 μM and BAP 8.87 μM+NAA 2.69 μM. On further increase in BAP there was a decrease in germination %. At 22.19 μM BAP+NAA 2.69 μM the germination percentage observed was 25%. There was callus formation in all combinations of BAP with NAA. The amount of callus formation was dependent upon the BAP:NAA ratio. The callus formation increased from 73% at BAP 2.22 μM+NAA 2.69 μM to 100% at BAP 8.87 μM+ NAA 2.69 μM. On further increase in BAP there was reduction in callus formation. Some amount of organogenic buds were formed at BAP 32.52 μM+ NAA 2.69 μM and BAP 44.38 μM+ NAA 2.69 μM but the frequency and number of buds formed were very low (Fig 4.B.10). The organogenic buds were accompanied with brown callus and root formation. The green morphogenetic structures in a ring on the middle explant observed on BAP and BAP+kinetin media was observed only on BAP 44.44 μM +NAA 2.69 μM.

Fig 4.B.10: organogenesis on BAP+NAA medium

Effect of TDZ: There was no organogenic response in any of the TDZ concentrations.

Histological studies:

The seeds were hard so 10µm thick sections were not possible. 7µm sections were used for the histological studies. Meristematic activity was observed in the subepidermal region of the seed in the 4th week of culture on BAP and BAP+Kinetin medium (Fig 4.B.11a). The meristematic region formed small buds on the surface of the explant in the 5th week of culture (Fig 4.B.11a). Leaf primordia were also seen developing from both sides of the shoot bud (Fig 4.B.11b-d). Complete shoots were formed in the 7th week (Fig 4.B.11e-f). The shoots formed showed vascular connection with the mother explant (Fig 4.B.11f).

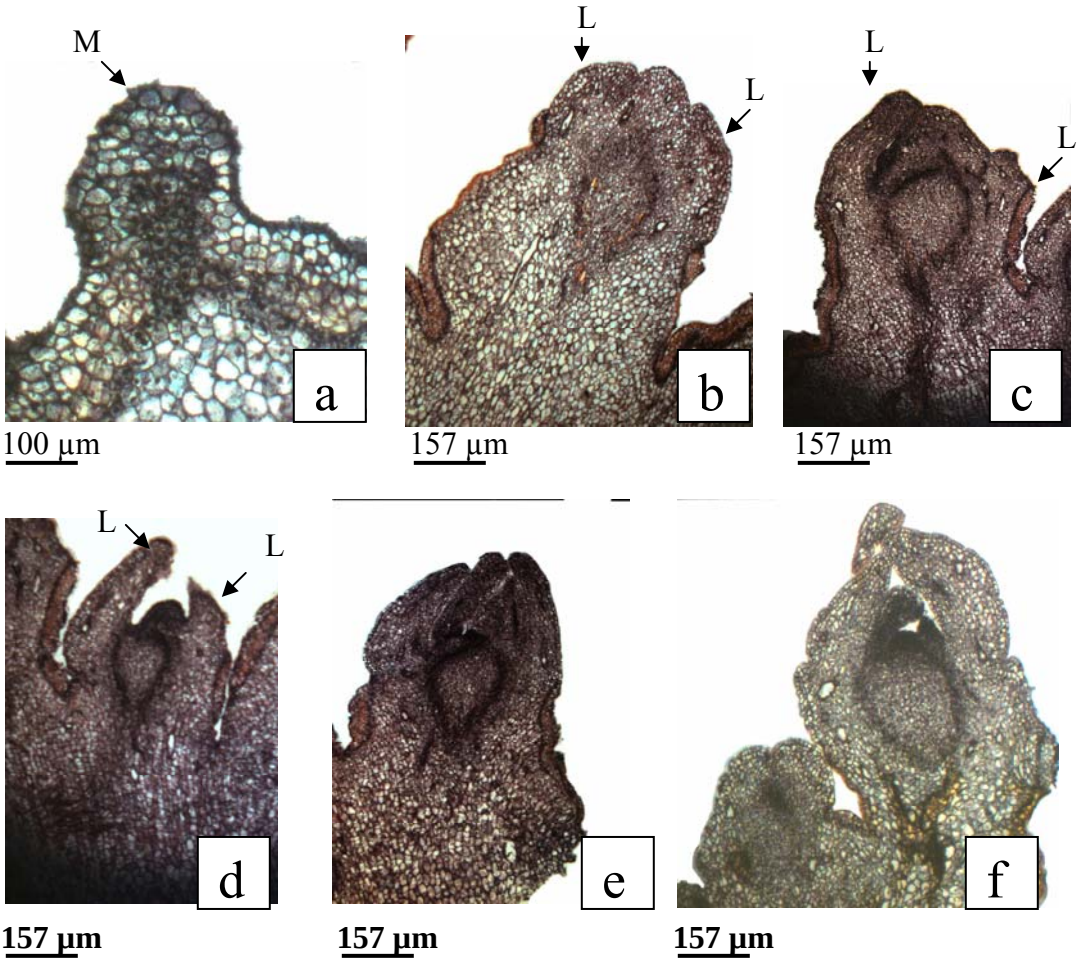


Fig 4.B.11: Histology of organogenesis in *Garcinia talbotii*

a-initiation of organogenic bud, (M-meristematic region)

b-d-formation of leaf primordia, (L-leaf primordia)

e&f-shoot formation,

g-shoots showing vascular connection with mother explant.

Elongation and rooting:

For elongation the shoot buds were shifted to BAP 0.44 µM. The shoot buds were cultured in this medium for two months with one subculture to fresh medium after one month. Further incubation on the same media resulted in browning of the shoots so they were shifted to ½ WPM+4.44 µM BAP where the shoots grew properly and formed roots in 1 month. The plantlets were washed in running water to remove phytagel and then shifted to soil. The plantlets were hardened in plastic cups containing soil:sand (1:1), watered and covered with transparent plastic bags. The plants were then kept in 24 hr light

culture room for one month. Later the plastic bags were removed and the plants were shifted to green house where the plants grew with 98% survival. Thus an average of 140 plants per seed can be produced in 6 months.

Fig 4.B.12: a-elongation of the shoots on BAP 0.44 μM, b-elongation with rooting on ½ WPM+BAP 0.44 μM, c-complete plantlets, d-hardened plants in sand:soil (1:1)

Discussion: A marked response to the different phytohormones as well as to the different concentrations of phytohormones was observed in *Garcinia talbotii*. While BAP and BAP with kinetin gave organogenesis, BAP with NAA gave callus formation with adventitious roots and a few organogenic buds, TDZ gave embryogenesis. We also observed the differential effect of BAP concentrations on the type of morphogenetic response in

Garcinia talbotii. While a low concentration of BAP led to embryo formation a higher concentration led to organogenesis. Generally, in dicots, for the same explant, auxins with or without low levels of cytokinins have been reported to induce somatic embryogenesis. Elevated cytokinin levels or cytokinin alone are usually required for the induction of shoot organogenesis (Matsuoka and Hinata 1979, Tabei et al. 1991, Tang et al. 2000, Nikolic´ et al. 2006). In the present study we found that while BAP at low concentrations led to embryogenesis at high concentrations it led to organogenesis. BAP has been used earlier to induce organogenesis in *Garcinia indica* from root explant (Deodhar et al. 2008), from seed explant (Malik et al. 2005) and in *Garcinia mangostana* from leaf explant (Goh et al. 1990). Also a high cytokinin level reduced the number (Table 4.B.5) and length of shoots, which is in accordance with the results in *Dendrocalamus asper* (Arya et al. 1999) and *Albizia chinensis* (Sinha et al. 2000) and could be attributed to the decrease in apical dominance by the cytokinin (Gaspar et al. 1996). We also found that a combination of BAP+kinetin just doubled the number of shoots produced per explant. Similar results have been reported in Guava (Singh et al. 2002).

4.B.3 Organogenesis in *Garcinia spicata*

Materials and methods: Fully mature unripe green fruits of *Garcinia spicata* were collected in the month of April from the semievergreen forest of Mulshi (18°28' N, 73°27' E, 610 m) in Maharashtra, India. The fruits were surface sterilized by the method followed for the fruits of *G. indica* and *G. talbotii* and the seeds after removing the seed coat were cut into three parts and inoculated in WPM media containing TDZ (0.045-9.08 μM)/ BAP (2.22-44.38 μM)/ BAP (2.22-44.38 μM) in combination with NAA 2.69 μM or BAP (2.22-44.38 μM) in combination with kinetin 4.65 μM. The explants growing on BAP, BAP in combination with Kinetin and BAP in combination with NAA were cultured in the media for 8 weeks with one subculture in the same media after 4 weeks. The explants were then shifted to three different media for the elongation of the shoot buds- (1) plain WPM medium with 0.2% charcoal, (2) BAP 0.44 μM, (3) BAP 0.44 μM+IBA 0.49 μM. The elongated shoots were rooted on ½ WPM+BAP 0.44 μM. The explants growing on TDZ were shifted after 4 weeks to 2 different media combinations- BAP 0.44 μM and 0.88 μM.

All media contained 2% sucrose and were gelled with 0.2% (w/v) phytagel. Histological studies were done to confirm the organogenetic nature of the shoots. 12 seeds were used for each treatment and the experiment was repeated 3 times. All cultures were grown at 25±2 ºC under 16 h photoperiod provided by cool white fluorescent tubes with a photon flux density of 30 µmol m^{-2} s^{-1} at the culture level.

Results:

Effect of BAP alone and BAP with kinetin: No organogenic buds were observed when the explants were grown in BAP alone or in BAP with kinetin. Germination was observed in BAP alone and BAP with kinetin. The length of the seedling increased with the increase in BAP and at BAP 44.38 µM a well-developed root and shoot was observed. There was no root formation at lower concentrations of BAP.

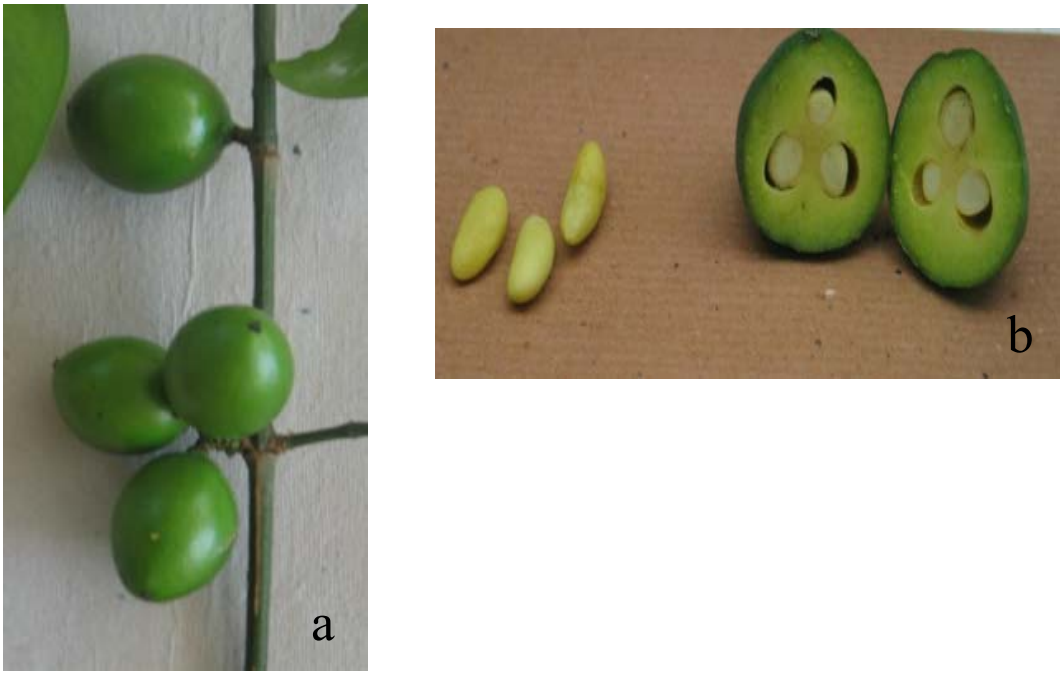


Fig 4.B.13: a-fruits of *Garcinia spicata*, b-T.S. of fruit showing the seeds and separated seeds

Effect of BAP+NAA: Since the seeds belonged to a recalcitrant tree species the number of seeds showing response was very low. Germination of the seeds was observed in 7 days in

all BAP (4.44-44.4 μM) + NAA 2.69 μM combinations with varying frequencies. The explants were cultured in BAP+NAA medium for 8 weeks with one subculture to the same medium after 4 weeks where initiation of shoot buds was observed. These cultures were then shifted to 3 different media- (1) plain WPM medium with 0.2% charcoal, (2) BAP 0.44 μM, (3) BAP 0.44 μM+IBA 0.49 μM. BAP (0.44-13.32 μM) with 2.69 μM NAA in the induction medium led to root and callus formation along with few organogenic buds after 8 weeks. BAP (22.19-44.38 μM) +NAA 2.69 μM induced direct organogenesis immediately when the explants were shifted to BAP 0.44 μM media after 4 weeks, but continuous culture in the same BAP+NAA medium gave more number of organogenic buds. Table 4.B.6. shows the organogenic response of *Garcinia spicata* seeds cultured in BAP+NAA medium. It was found that with the increase in BAP (4.44-44.38 μM) concentration there was an increase in the percent response (0-100%) as well as the number of organogenic buds formed. BAP 44.38 μM+NAA 2.69 μM gave the highest number of organogenic buds (19.33 buds per explant or approximately 60 buds per seed). It was also noticed that higher concentration of BAP in induction medium decreased the length of the root and shoot. No relation was found between the part of the explant and the response.

Table 4.B.6: Effect of BAP concentration on the organogenic response of *Garcinia spicata*

Medium		No of explants showing response(%)	Average no of organogenic buds after 3months*	Average length of shoot (cms) after 6 months	Average length of root (cms) after 6 months
BAP(μM)	NAA(μM)				
2.22	2.69	0	0	0	0
4.44	2.69	0	1.00±0	2.00	3.00±0
8.87	2.69	66.66	4.33±2.88	1.65±2.28	10.00±0
13.32	2.69	88.88	4.00±2.64	1.55±1.50	6.00±2.30
22.19	2.69	100	6.00±3.46	2.07±2.50	5.20±4.80
17.76	2.69	100	9.66±8.96	1.97±1.30	2.50±0.50
44.38	2.69	100	19.33±18.58	1.85±0.85	0.40±0

*Values were found to be non significant at $P<0.05$

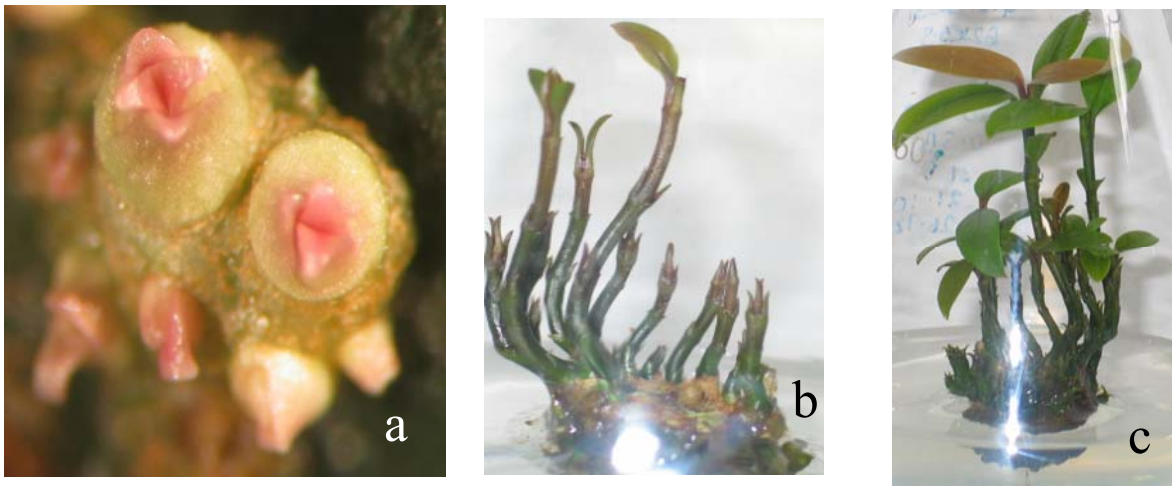


Fig 4.B.14: a-Initiation of organogenic buds, b&c-elongated shoots on BAP 0.44 μM

Effect of TDZ: No organogenetic response on any of the TDZ concentrations

Histological studies:

Histological examinations of the regeneration process revealed areas of active cell division in the subepidermal region of explant after 4 weeks of culturing (Fig. 4.B.15a) on BAP 44.38 μM+NAA 2.69 μM. These developed into meristematic regions that appeared on the surface of explants and formed recognizable shoot meristems by the 5th week of culture (Fig 4.B.15b). Leaf primordia were seen forming from the organogenic buds (Fig 4.B.15b-c) and complete shoots were formed by the end of 6th week (Fig 4.B.15d). The regenerated shoots showed vascular connection with the mother explant (Fig 4.B.15d).

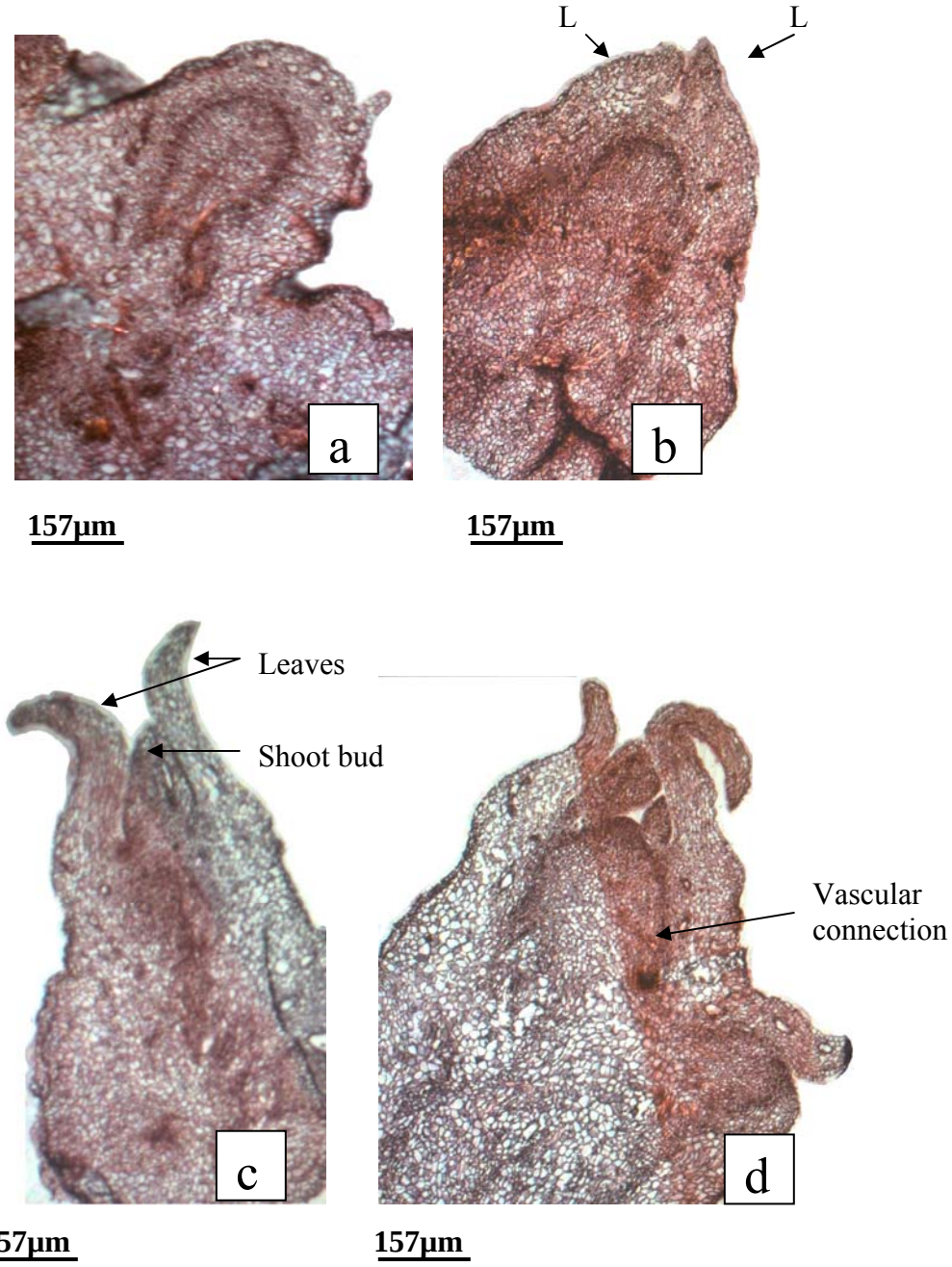


Fig 4.B.15: Histology of organogenesis in *Garcinia spicata*

a-initiation of organogenic bud,

b-initiation of leaf primordia, (L-leaf primordia)

c-fully formed shoot bud,

d-shoots showing vascular connection with mother explant

Elongation and Rooting:

The organogenic buds induced on BAP+NAA medium were shifted to 3 different media combinations for elongation- plain WPM medium with 0.2% charcoal, WPM+BAP 0.44 µM or WPM+BAP 0.44 µM+IBA 0.49 µM. Charcoal media led to callus formation. Combination of BAP 0.44 µM+IBA 0.49 µM showed elongation in the shoots which were induced only on BAP 22.19 µM + NAA 2.69 µM and above. For buds induced on BAP (2.22-13.32 µM) +NAA 2.69 µM shifting to BAP 0.44 µM+IBA 0.49 µM led to callus formation. 0.44 µM BAP was the only medium that led to elongation of the shoots induced on all the BAP concentrations. The shoots were cultured in this medium for 2 months then shifted to ½ strength WPM medium with 0.44 µM BAP. The shoots elongated and formed roots by themselves probably because of the NAA in the induction medium (Fig 4.B.16a,b) The plantlets formed were subjected to hardening in plastic cups containing 1:1 soil, sand ratio (Fig 4.B.16c). The plants were covered with plastic bags and kept at 24 hr light conditions. The plants were watered at regular intervals. One month old hardened plants were shifted to green house where the plants grew further with 98% survival.

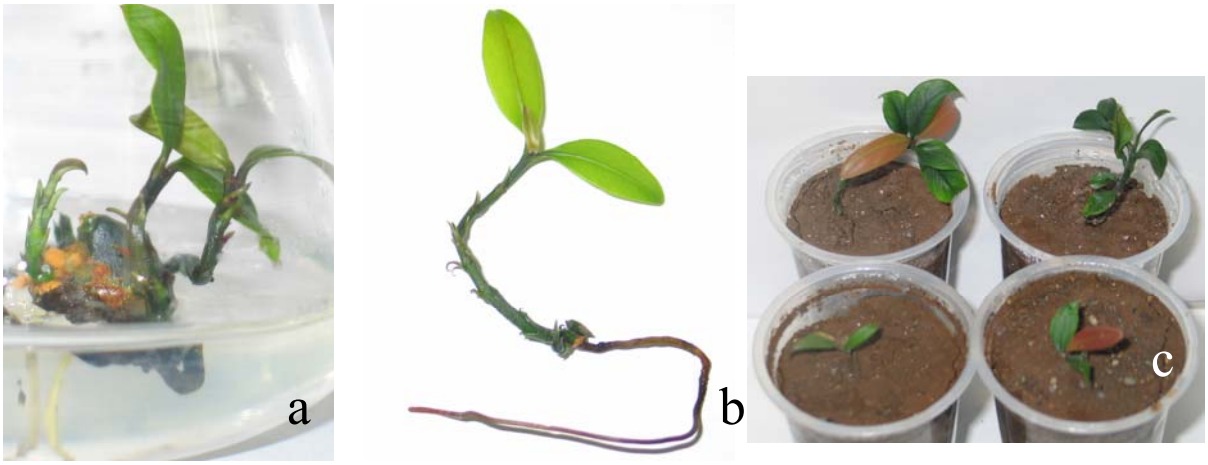


Fig 4.B.16: a-rooting on ½ WPM+0.44 µM BAP, b-complete plant, c-plants transferred to soil

Discussion: A marked response to the phytohormones was seen in *Garcinia spicata.* While BAP or Kinetin led to germination, a balance of auxin and cytokinin was required for organogenesis. BAP at low concentration (2.22-4.44 µM) with NAA led to callus formation or rhizogenesis. BAP at higher concentrations with NAA led to multiple shoot

formation. An average of 60 plants was obtained from a single seed in a period of 6 months. BAP+NAA has been used to produce multiple shoot formation in *Garcinia indica* from explants derived from seedling (Kulkarni and Deodhar 2002), Banana (Rahman et al. 2004), *Dianthus chinensis* (Kantia and Kothari 2002) and *Gladiolus* (Torabi-Giglou and Hajeghrara 2008). Elongation and rooting of the shoots was also not a problem. Most of them elongated and developed roots in the induction medium only. The shoot buds formed in higher BAP concentrations rooted when shifted to elongation medium.

4.B.4 Organogenesis in *Garcinia xanthochymus*

Materials and Methods: Fully mature green fruits were collected in the month of February from Dapoli (17°45.222′ N, 73°10.932′ E, 162 m) in Maharashtra. The fruits were surface sterilized by the following method. They were washed under running tap water for one hour and then cleaned with 1% (v/v) labolene (Qualigens, India), followed by 10% (v/v) savlon (Johnson and Johnson Ltd, India) for 2-3 min. The seeds were later treated with 1% (w/v) bavistin (BASF, India) for 1 hr. After each treatment, the fruits were washed 3-4 times with single distilled water. Further treatments were carried out under sterile conditions in a laminar airflow unit. The seeds were excised from the fruits, rinsed with 70% (v/v) ethanol and washed 3-4 times with sterile distilled water followed by treatment with 0.05% (w/v) $HgCl_2$ (Qualigens, India) for 10 mins and again washed 3-4 times with sterile distilled water. The seed coat was removed and the seeds were cut into 7-8 pieces and inoculated in WPM media containing TDZ (0.045-5.54 μM)/ BAP (2.22-44.38 μM) or BAP (2.22-44.38 μM) in combination with NAA 2.69 μM. The explants were grown in TDZ media for one month then shifted to either the same media, to BAP 2.22 μM/ BAP 4.44 μM or to TDZ 0.04 μM. The explants were grown in BAP containing media for 2 months then shifted to BAP 2.22 μM. The explants that were grown in BAP+NAA medium for 1 month were shifted to BAP medium without NAA. For elongation and rooting the explants showing organogenic response was shifted to BAP 0.44 μM. All media contained 2% sucrose and were gelled with 0.2% (w/v) phytagel. Histological studies were done to confirm the morphogenic nature of regeneration. 12 seeds were used for each treatment and the experiment was repeated 3 times.

Results:

Effect of BAP: All the explants grown in various concentrations of BAP for 2 months were shifted to BAP 2.22 µM. BAP at low concentrations (2.22-8.87 µM) gave friable callus in all the explants. BAP at 13.32 µM gave 2 shoot formation in 1 explant and adventitious root formation in all the explants. Brown friable callus was also common at this BAP concentration. Above BAP 13.32 µM induction of organogenic buds was observed. The shoot buds formed were direct and did not involve an intermediary callus phase. These explants were shifted to low BAP 0.44 µM for further growth of the shoots where they grew and formed roots. Table 4.B.7 shows the effect of BAP concentration on the organogenic reponse. The percent response was in general low. Only at BAP 22.19 µM all the explants showed response and the average number of shoots formed was 23.66.

Fig 4.B.17: a-fruits of *Garcinia xanthochymus*, b-seeds of *Garcinia xanthochymus*, c-initiation of organogenesis, d-organogenic bud formation

Effect of BAP+NAA: The explants were inoculated in BAP (2.22-44.38 µM)+ NAA 2.69 µM medium. After 1 month adventitious roots were observed growing from the explants on all the BAP+NAA media. The explants were then shifted to BAP medium without NAA. After 1 month BAP (2.22-4.44 µM) showed adventitious roots, while multiple shoots with adventitious roots were observed above BAP 4.44 µM. The shoots and the roots formed were direct and did not involve any callus formation. The number of explants showing response in all BAP+NAA combinations was low (20-25%). BAP 35.52 µM+NAA 2.69 µM showed the highest number of organogenic buds (63.33).

Table 4.B.7: Effect of BAP and BAP in combination with NAA on organogenic response in *Garcinia xanthochymus*

Medium(mM)		%response	Average no. of buds*
BAP	NAA		
2.22	--	0	0^{d}
4.44	--	0	0^{d}
8.87	--	0	0^{d}
13.34	--	20	1.66 ± 0.57^{c}
22.19	--	100	23.66 ± 15.27^{b}
35.52	--	50	24.33 ± 0.57^{b}
44.38	--	25	23.66 ± 3.21^{b}
2.22	2.69	0	0^{d}
4.44	2.69	0	0^{d}
8.87	2.69	20	20.00 ± 2.22^{c}
13.34	2.69	20	24.00 ± 0^{b}
22.19	2.69	22	24.50 ± 0^{b}
35.52	2.69	25	63.33 ± 1.52^{a}
44.38	2.69	25	5.00 ± 1.0^{c}

*Values followed by the same letter do not vary significantly at $p<0.01$

Effect of TDZ: No organogenic response observed in any of the TDZ concentrations

Histology:

Explants at different stages of morphogenic response were fixed for histological studies. 7µm sections were cut to study the regeneration process. Meristematic activity was

observed in the subepidermal region of the explant. The meristematic region formed small buds on the surface of the explant in the 8th week of culture (Fig 4.B.18). These buds were very slow growing so proper leaf primordia were not observed.

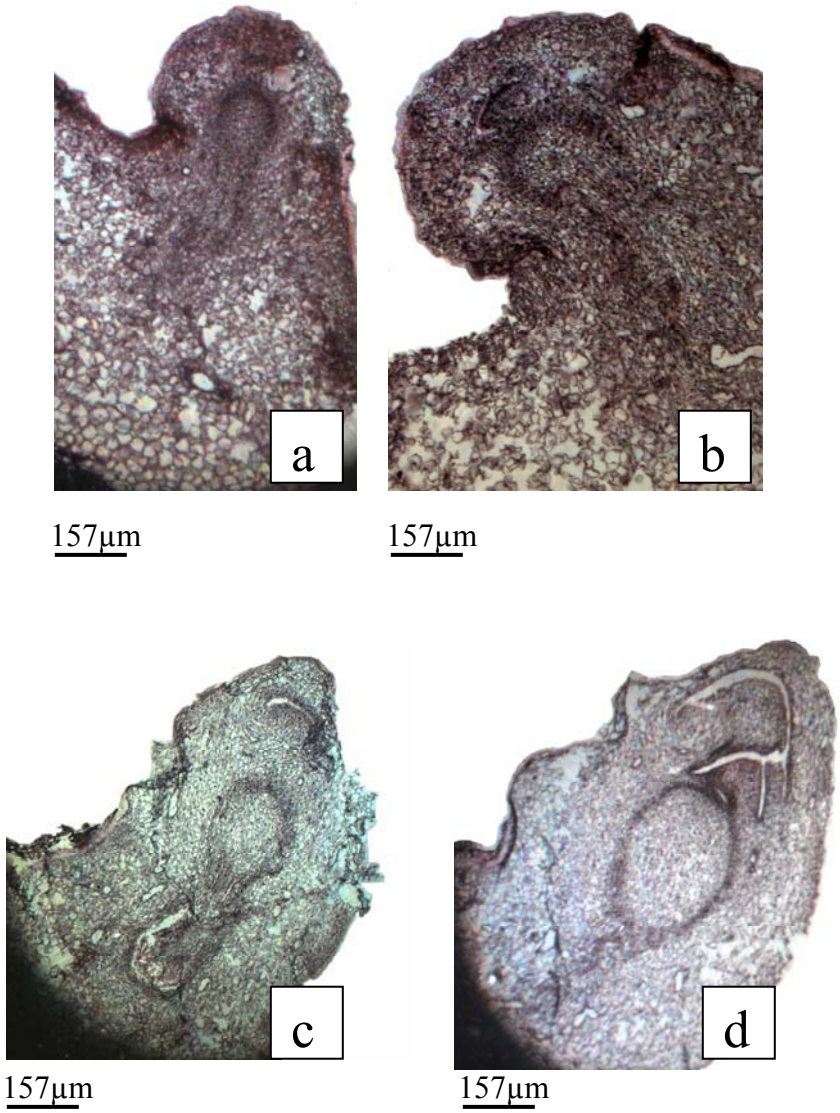


Fig 4.B.18: Histology of regeneration in *G. xanthochymus*

a-induction of organogenic bud

b-initiation of leaf primordia

c-fully formed shoot bud

d-shoot showing large number of unopened leaves.

Elongation and rooting:

For elongation all explants were shifted to low concentration of BAP (0.44 µM) where elongation of buds with rooting was observed. Further shifting to the same media resulted in an increase in root length so the shoots were further shifted to 4.44 µM BAP. Shoots about 2 cm in length were transferred to soil for hardening. After one month hardened plants were shifted to green house where the plants grew with 80% survival. The plants were very slow growing. No leaf opening was observed even after transfer to soil.

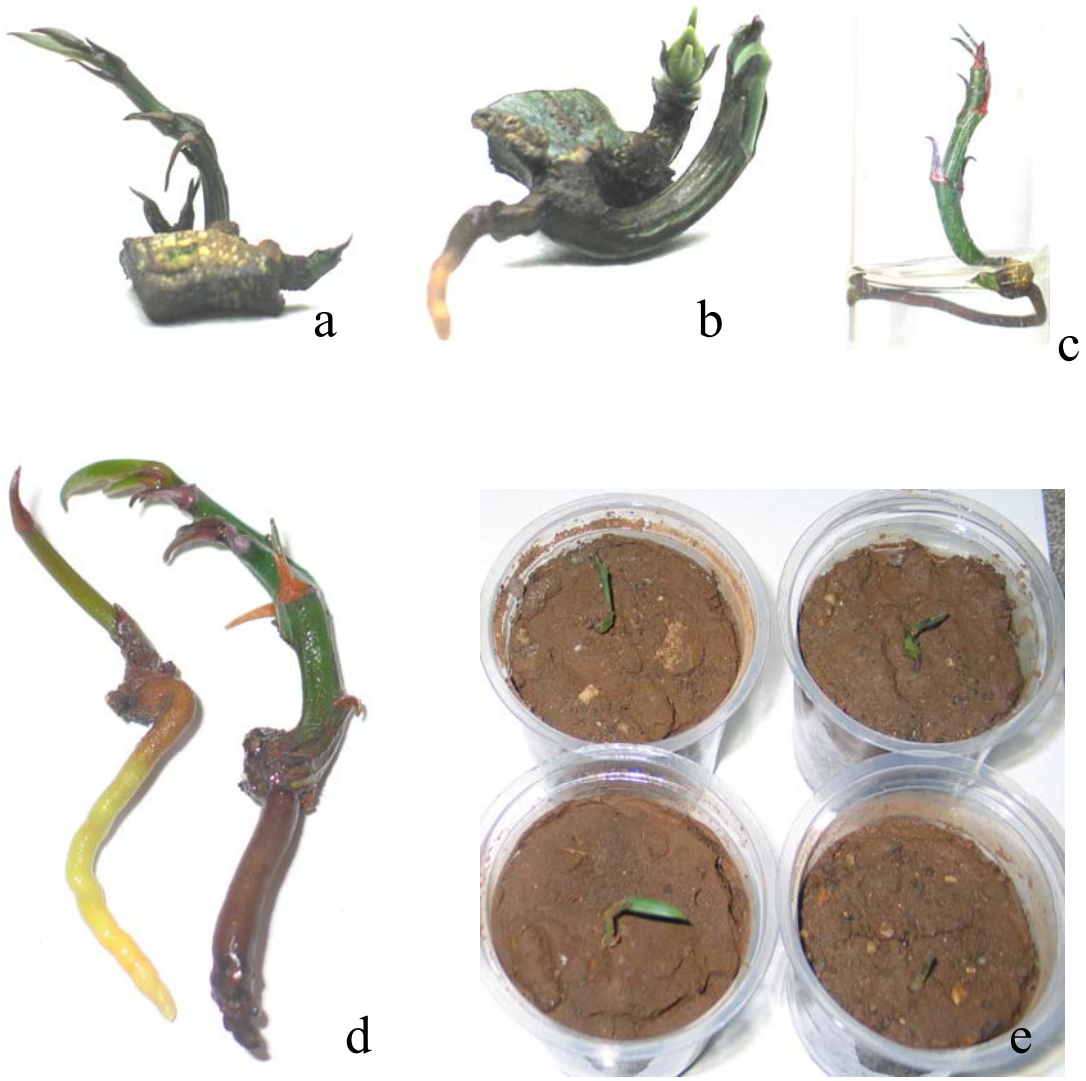


Fig 4.B.19: a-elongated shoots still attached to seed, b-rooting on BAP 0.44 µM, c&d-complete plantlets without leaf opening, e-hardened plants

Discussion: BAP and BAP+NAA led to direct organogenesis. Even though BAP was more responsive to organogenesis, the number of buds formed was higher in BAP+NAA. Similar results have been reported in *Garcinia indica* (Kulkarni and Deodhar. 2002) and Gladiolus (Torabi-Giglou and Hajieghrari 2008; De Bruyn and Ferriera 1992). BAP+NAA has been shown to produce multiple shoot formation in a number of plants, e.g. banana (Rahman et al 2004), *Dianthus chinensis* (Kantia and Kothari, 2002) and *Saussurea involucrata* (Guo et al. 2007).

4.B.5 Micropropagation

Micropropagation is the *in vitro* multiplication of selected genotypes using tissue culture techniques under aseptic and controlled environmental conditions.

It can broadly be divided into four stages:

Stage 0 – Mother plant selection and preparation

The mother plant, which is typical of the variety and disease free, is selected. Preparation of the mother plant is important to avoid contamination of the cultures. Initially this stage was introduced to overcome the huge problem of contamination. Raising stock plants in green house under more hygienic condition can considerably reduce the risk of contamination. It will not only reduce the number of microorganisms growing on the plant surface but it will also produce quality plants for stage 1 due to the regular application of fertilizers, fungicides and insecticides. The physiological state of the stock plant can also be changed in the green house by altering the light, temperature or by the application of growth regulators.

Stage I - Establishment of culture:

The initial step in the micropropagation process is to obtain an aseptic culture of the selected plant material. Firstly for the success at this stage the explants should be safely transferred to the culture environment and secondly that there should be an appropriate response (e.g. growth of a shoot tip or formation of callus on a stem piece). Usually a batch of explants is transferred to culture media at the same time. Stage 1 should be regarded as satisfactorily completed if an adequate number of explants had survived without contamination and are showing growth. This stage is dependent on many factors like

- Explant- Any part of the plant (vegetative or reproductive) can be used as explant. The age of the stock plant, the physiological and developmental state as well as the size of the explant determines the success rate in stage 1.
- Sterilization- treatment of the plant by biocides to remove microorganisms is important to obtain axenic cultures.
- Culture conditions- like medium, light and temperature

Stage II - Proliferation of shoots in culture:

In this stage, shoots are multiplied in culture, which then serves as the source of shoots for subsequent propagation as well as the material that is required to maintain the stock.

Multiplication of shoots is achieved by sub culturing the shoot cultures at regular intervals in appropriate medium. Variant plants may arise from the cultures maintained *in vitro* for a long period. For the purpose of micropropagation, usage of shoots from the cultures maintained for several passages and having higher subculture numbers is avoided to ensure genetic fidelity.

Stage III – Rooting:

In this stage the shoots obtained from multiplication media are separated and shifted to conditions that favor root initiation and shoot elongation. Rooting of shoots can be induced either *in vitro* or *extra vitrum*. For rooting *in vitro*, shoots are cultured either in growth regulator free medium, in auxin containing medium or subjected to pulse treatment of auxin prior to transferring to an auxin free medium for root induction. For *ex vitro* rooting, shoots are excised as small cuttings (micro cuttings), treated with commercial rooting mixture and planted in soil. These are then placed in a high humidity chamber for rooting. Rooting *ex vitro* has several advantages (Debergh and Read 1991):

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

Stage IV – Hardening of tissue culture raised plants:

In tissue culture, plants are produced in high humidity and low light intensity. This results in there being less epicuticular wax than on plants grown in the green house or in field. Tissue cultured plants therefore lose water rapidly when moved to external conditions. Under culture conditions the plants are supplied with sucrose and other

nutrients and kept in low light conditions, they are not dependent on their own photosynthesis and are heterotrophic. Hence, it is necessary to acclimatize plantlets gradually until they develop new leaves that are more adapted to the ambient conditions under which plants are normally grown to ensure survival. In practice, plantlets are removed from stage 3 containers, washed to remove agar, transplanted in sterile rooting mixture (such as sand:soil mixture) and kept for several days under high humidity and low light intensity in culture rooms. Intermittent water misting may be applied or plants placed inside a clear plastic enclosure. The plants after hardening are shifted to green house.

Propagation from mature trees

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

- Cloning of mature trees is generally preferred over cloning of embryos or young seedlings because often it is not possible to determine if these embryos or seedlings have the genetic potential to develop the desired qualities later in their life cycle.
- Considering the genetic heterozygosity of most of the tree species, cloning of mature trees is the only method that permits immediate and total capturing of genetic gains (Franclet et al. 1987) and to obtain true-to-type large-scale clonal propagules.
- Preferential cloning of mature trees is desired when establishing plantations of dioecious species. For example, in some dioecious species only the female trees have economic value. Since the gender of these trees is not known before maturity, they are cloned preferentially from sexually mature trees. Examples of such preferential cloning are *Carica candamarensis* (Jordan et al. 1983), *Phoenix dactilifera* (Reynolds 1982) and *Simmondsia chinensis* (Rost and Hinchee 1980).

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

A. Selection of juvenile tissues within mature tree:

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

B. Rejuvenation by special treatments:

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

Successful clonal propagation of tree species is no more a difficult phenomenon. Several woody species such as Poplars, Wild cherry, Eucalyptus, Redwood, Radiata pine, Teak, Sandalwood, Birch, Loblolly pine, Shorea and Dalbergia are being standardized for mass multiplication and are commercially micropropagated (Rodriguez and Vendrame 2003).

Garcinias are dioecious in nature with the female plant being more sought after by the local people for its fruits. The plant is usually propagated by seeds and softwood grafting (George et al. 2002). The seeds of *Garcinia indica*, *Garcinia xanthochymus* and *Garcinia cambogia* are recalcitrant with a shelf life of only 4 weeks which further decreases due to desiccation and freezing (Malik et al. 2005). Thus, it is not possible to raise seedlings throughout the year. The sex of the plant is known only after 7-8 years when the plant comes into bearing. In an orchard of *Garcinia* produced from seeds 50% of the population is male. So a fool-proof method giving only female plants needs to be developed. Softwood grafting, another method used for clonal propagation of *G. indica*

(Nawala and Karmarkar 1997, Haldankar et al. 1993) is season dependent, space requiring and cumbersome in nature thus finding limited application. Another bottleneck is the limited availability of rootstocks for grafting. The grafted trees are phenotypically modified and the yield of the fruits is reduced. *In vitro* clonal propagation of female mature trees is the only way to obtain large no of female trees in limited time and space. Most of the studies on *in vitro* culture of *Garcinia* has been conducted in *G. mangostana* using seed (Normah et al. 1995) and leaf explants (Goh et al. 1990, 1994, Te-Chato and Lim 2000). The seed raised plants of *Garcinia mangostana* bear fruits in 8 yrs while the tissue culture raised plants bear fruits in 5 yrs (Te-chato and Lim 2004). Limited attention has been given to *G. indica*, which has only recently been recognized as an important horticultural and medicinal tree species. Kulkarni and Deodhar (2002) used immature seeds, young leaves, apical and axillary buds of *in vitro* raised seedlings for micropropagation of *G. indica*. Deshpande et al. (1999) and Mathew et al. (2001) conducted preliminary studies on *in vitro* establishment of kokum using apical buds. Malik et al. (2005) regenerated the plants by *de novo* organogenesis from seed explants. Deodhar et al. 2008 regenerated *Garcinia indica* plants by *de novo* organogenesis from root explant. Regeneration of *Garcinia indica* by somatic embryogenesis has also been reported (Thengane et al. 2006). But in all these studies the sex of the plant is not known due to the heterozygous nature of the seeds. The present experiments were conducted to obtain large number of female plants in *Garcinia talbotii*. There are no reports on the micropropagation of *Garcinia talbotii*.

Microprpagation of *Garcinia talbotii*

Materials and Methods:

Shoots from mature trees of *Garcinia talbotii* were collected in different seasons from Mulshi. 2-3 cm shoot pieces containing apical or axilllary buds were used as explant. The leaves were removed from the explant keeping the petiole intact. The explants were subjected to different sterilization procedures (Table 4.B.8) to obtain axenic cultures. The effect of season and different sterilants (like bavistin, benovit, cefotaxim and PPM) was studied. Different growth regulators BAP (2.22-22.19 µM) alone or in combination with kinetin (2.32-4.65 µM) were used for sprouting. The effect of IBA (4.90 µM) in combination with BAP (2.22-4.44 µM) and kinetin (2.32 µM) was also tried to obtain

sprouting. The effect of charcoal (0.2%) on sprouting was also tested. WPM basal media containing 2% sucrose and 0.8% agar was used for all the experiments. Cultures were incubated in 16 hr photoperiod. After a month the number of explants showing sprouting was noted and the frequency of sprouting was recorded in percentage. The cultures were shifted to the same media to obtain multiple shoots. Multiple shoots were then elongated on low BAP (0.44-0.88 µM)+GA3 (0.58-1.15 µM) media. After one month in the elongation media the shoots 3-4 cm in height were subjected to rooting experiments.

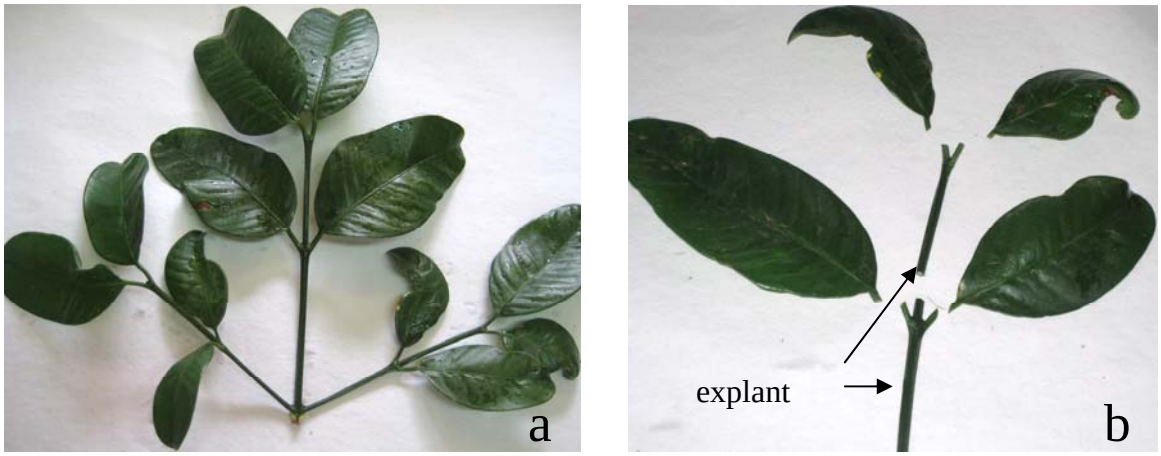


Fig 4.B.20: a-twig of *Garcinia talbotii*, b-explants used for micropropagation

Results and Discussion:

The explants were obtained from field grown mature trees so standardization of the sterilization procedure was the first step towards the development of a regeneration protocol. The work could be done only from Jan-May when around 32.5-53.0% established cultures could be obtained. The sterilization procedure had to be varied for different times of the year. The same method did not give the above % of established cultures. In January although the different sterilization methods did not give any significant difference in the number of sterile cultures, the percent established cultures was affected. 0.5% benovit for ½ an hour gave 32.5% established cultures. In May treating the plants with 1% bavistin +50 mg/l cefotaxim for 1 hr gave 53.42% established cultures. Although the other treatments gave more sterile cultures but the explants did not respond and so could not be used for further studies. The sterilization procedure could not be improved by adding

bavistin, cefotaxim or PPM to the medium. All these sterilants delayed and drastically reduced the sprouting percentage of the explants. Sprouting is seen in 7-8 days on explants inoculated in media without any biocides, while sprouting occurs after 30 days in media containing bavistin, PPM or cefotaxim. Heavy contamination was observed in the rainy season (June-September). Selection of explants, season and age of the plant are important factors to get shoot proliferation (Purohit et al. 1998). Success in establishment of aseptic cultures of field grown trees is influenced by the season during which explants are taken (Hu and Wang 1983). *In vitro* seasonal effect on bud growth was reported in many tree species such as: *Tectona grandis* (Gupta et al. 1980), sweetgum (Sutter and Barker 1985), gauva (Amin and Jaiswal 1987), *Tecomella undulata* (Rathore et al. 1991).

Table 4.B.8: Effect of different sterilization methods on *Garcinia talbotii*

Sterilization method	January		May	
	%sterile cultures	%sterile cultures showing response	%sterile cultures	%sterile cultures showing response
1% bavistin for ½ an hour	37.77±1.52	18.33±0.57^{b}	77.5±1.52^{a}	42.5±0.5^{b}
1% bavistin for 1 hour	38.56±1.56	20.14±0.56^{b}	65.62±1.52^{b}	12.5±1.15^{c}
1% bavistin for 1hour+50mg/l cefotaxim	40.20±1.0	22.36±1.52^{b}	58.9±1.0^{c}	53.42±2.51^{a}
1% bavistin for 1hour15min+50mg/l cefotaxim	37.62±1.0	20.54±1.1^{b}	13.93±2.08^{d}	6.46±2.08^{d}
1.5% bavistin for ½ an hour+50ppm bavistin in media	45.30±2.51	17.56±1.2^{b}	69.0±2.08^{b}	42.0±1.0^{b}
1.5% bavistin for 1hour	60.71±25.64	24.88±19.85^{b}	10.20±0.57^{d}	4.25±1.15^{d}
1.5% bavistin for 1hour+2 ml PPM in medium	35.4±1.0	1±1.0^{b}	10.20±1.52^{d}	2.00±1.0^{d}
0.5% benovit for ½ an hour	41.25±1.0	32.5±1.25^{a}	65.32±1.0^{b}	12.5±1.52^{c}
ANOVA	NS	S1%	S1%	S1%

*values followed by the same letter do not vary significantly at $p<0.01$

Different growth regulators were tried for sprouting. Sprouting started after 1 week of inoculation. It was found that BAP 13.32 µM+kinetin 2.32 µM gave the highest percentage of sprouted explants (91.66%) followed by BAP 8.87 µM+kinetin 2.32 µM which induced sprouting in 80.53% of the explants. When continued to grow in BAP or BAP+kinetin media multiple shoots were formed in 45 days. The number of shoots formed per explant ranged from 1.2-4.5 with BAP 8.87 µM+kinetin 2.32 µM giving the best response of 4.5 shoots per explant. Charcoal reduced the sprouting frequeny in *Garcinia talbotii*. It was found that axillary buds were more responsive than apical buds for multiple shoot induction. This differential morphogenetic response could be due to differences between the physiological states of the buds on different regions of a stem (Vieitez et al. 1985) Similar results were also reported in *Agel marmelos* (Ajithkumar and Seeni 1998), *Syzygium cuminii* (Yadav et al. 1990) and *Morus australis* (Pattnaik et al. 1996).

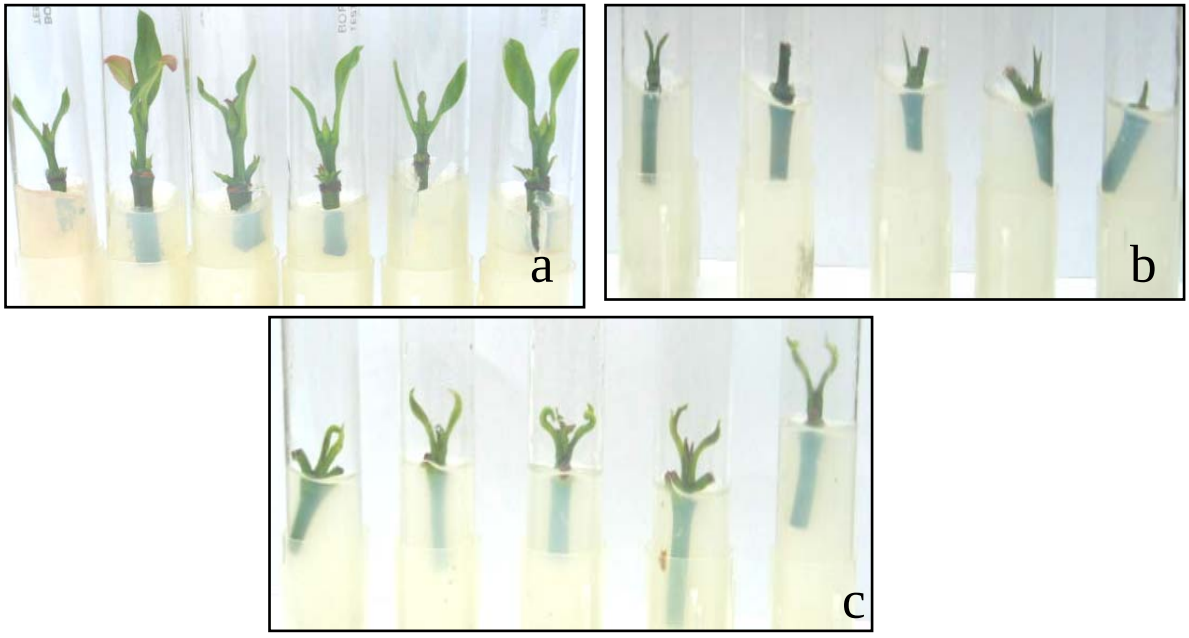


Fig 4.B.21: a-sprouting on BAP 2.22 µM, b-sprouting on BAP 8.87 µM, c-sprouting on BAP 4.44 µM+kinetin 4.65 µM

Table 4.B.9: Effect of BAP, Kinetin and IBA on sprouting of shoot buds in *Garcinia talbotii*

Medium WPM			sprouting after 30 days	Sprouting after 30 days in media with charcoal
BAP	Kinetin	IBA		
2.22	-	-	65.55±15.03^{abc}	0
4.44	-	-	66.66±28.88^{abc}	0
8.87	-	-	56.81±23.33^{abc}	0
13.32	-	-	82.1±3.65^{ab}	33.33
22.19	-	-	65.53±10.04^{abc}	16.66
2.22	2.32	-	55.53±9.58^{abc}	22.22
4.44	2.32	-	64.16±32.24^{abc}	0
8.87	2.32	-	80.53±17.37^{ab}	50
13.32	2.32	-	91.66±7.63^{a}	14.28
22.19	2.32	-	54.83±4.53^{abc}	11.11
2.22	4.65	-	50.0±2.0^{abc}	--
4.44	4.65	-	45.13±0.80^{abc}	--
8.87	4.65	-	43.0±1.0^{abc}	--
13.32	4.65	-	61.5±4.09^{abc}	--
22.19	4.65	-	27.5±2.17^{c}	--
2.22	2.32	4.90	37.66±1.52^{bc}	--
4.44	2.32	4.90	38.0±1.0^{bc}	--

*****the means followed by the same letter do not vary significantly at $p<0.01$

Table 4.B.10: Effect of different combinations of BAP+Kinetin on multiple shoot formation in *Garcinia talbotii*

Medium		Average no. of buds after 50 days
BAP(µM)	Kinetin(µM)	
2.22	--	1.2 ± 0.75^{c}
4.44	--	2.0 ± 0.94^{b}
8.87	--	2.9 ± 0.73^{ab}
13.32	--	2.9 ± 0.73^{ab}
22.19	--	3.4 ± 0.51^{ab}
2.22	2.32	3.0 ± 0^{ab}
4.44	2.32	3.1 ± 0.31^{ab}
8.87	2.32	4.5 ± 1.08^{a}
13.32	2.32	4.0 ± 2.62^{a}

*****the means followed by the same letter do not vary significantly at $p<0.01$

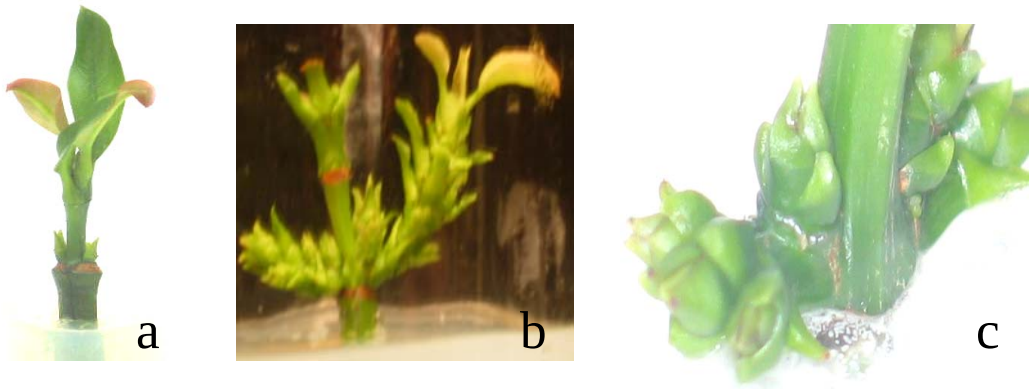


Fig 4.B.22: a-multiple shoot formation on BAP 2.22 µM, b- multiple shoot formation on BAP 8.87 µM + Kinetin 2.32 µM, c-multiple shoot formation on BAP 13.32 µM +Kinetin 2.32 µM

Effect of TDZ on multiple shoot formation:

TDZ was not found suitable for multiple shoot induction. Most of the shoots died and those, which survived, produced fasciated shoots and buds. Formation of stunted shoots or the fasciation of shoots on TDZ containing medium was reported in other species (Chalupa 1988, Pradhan et al. 1998). The average no of shoots formed were also less.

Elongation:

The shoots were elongated in WPM media containing 0.44 µM BAP or BAP 0.88 µM and GA_3 0.58 µM. The multiple shoots formed in BAP 2.22 µM elongated readily while those induced in higher concentrations of cytokinin elongated slowly. The elongated shoots were then subjected to rooting.

Fig 4.B.23: elongation on BAP 0.88 µM + GA_3 0.58 µM

Rooting and Hardening:

Liquid media was used for the rooting experiments. Different combinations of IBA (9.80 - 39.2 µM) alone and in combination with NAA 10.74 µM were tried but was not successful. Pulse treatment with high concentrations of IBA (245, 490, 735 and 960 µM) for 72hrs was tried. After pulse treatment the cultures were shifted to ½ strength WPM (only macro ½) containing 1% sucrose and 0.7% agar. Rooting was observed with very low frequency (10%) on 490 µM IBA after 2 months. The rooted plants were washed to remove agar. They were then subjected to hardening in plastic cups containing soil: sand (1:1) ratio. The plants were then covered with plastic bags and kept under 24 hr photoperiod in culture room. The plants were watered regularly and after a month the

plastic bags were removed and the plants were shifted to green house where the plants grew with 95% survival. Through this method an average of 4.5 shoots can be produced per explant.

Fig 4.B.24: a-rooted plant b-hardened plants of *Garcinia talbotii*

CHAPTER 5

IN VITRO REGENERATION BY SOMATIC EMBRYOGENESIS IN *GARCINIA* SPECIES

5 Introduction

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

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

Applications of SE may be summarized as follows: -

1. Clonal propagation : Somatic embryos offer potential advantages over conventional micropropagation system which includes:

- high proliferation rate: As many as 1.35 million embryos per litre suspension culture (Janick 1993).
- singulation: Each embryo being separate package can be handled without the physical separation required in organogenesis or axillary branching systems.
- bipolarity: Well-developed embryo contains root and shoot meristems. Thus conversion to seedling can be obtained in single step.
- promise of automations : via synthetic seed technology.

2. Crop improvement: Somatic embryogenesis offers a number of options for crop improvement.

- Somatic embryogenesis can be utilized as a regeneration technique for cell selection of spontaneous or induced mutations. Selection for salt tolerance and disease resistance has proven efficient in embryogenically competent callus tissues of citrus (Litz 1985). Hammerschlag (1990) and Hammerschlag and Ognjanov (1990) reported *in vitro* selection of embryogenic cultures of peach (*Prunus persica*) for resistance to the toxins produced by *Xanthomonas campestris* pv *pruni*, the causative agent of bacterial leaf spot, and *Pseudomonas syringae* pv *syringae*, the cause of bacterial canker.
- Chimerism is one of the major hurdles to be overcome in the course of the development in particularly woody plant mutant. Somatic embryos obtained from single irradiated cells help avoiding chimeras during selection stages. After the mutagenic treatment, well developed somatic embryos can be germinated into whole plants. It ensures that shoot as well as root system is of the mutated type. Once mutant embryogenic cell lines are obtained, mass production of plants can be envisaged all year round. This steady supply can be of economical importance for newly released cultivars.
- Plants regenerated from somatic embryos display high genetic uniformity. Thus, large populations can be obtained from highly heterozygous and outbreeding species. This uniform material is an ideal population for mutagenesis treatment. Especially, if certain

experiments have to be repeated, the constant supply of even-aged embryos or plants is advantageous (Heinze and Schmidt 1995).

- Immature embryos from incompatible interspecific crosses (Merkle et al. 1990) may be rescued by culturing them for secondary somatic embryogenesis and simultaneously the plant is multiplied. This has been exploited as means of obtaining plants from intergeneric hybrid embryos (McGrahanhan et al. 1986, Ozias-Akins et al. 1992). It is a widely used technique to rescue embryos that normally abort in wide crosses and is an established technique to obtain viable plants from incompatible interspecific crosses.
- Breeding of haploids reduces the time needed to produce homozygous plants which express the recessive genes. Haploid plants can be obtained *in vivo* through gynogenesis, androgenesis, genome elimination, semigamie and by chemical and physical treatments such as heat shock, X-rays and UV light (Pierik 1987). The frequency of these events is however, low.

  Theoretically, all steps necessary in a haploid breeding scheme can be conducted by using somatic embryogenesis (Raemakers et al. 1995). In numerous species large number of haploid embryos can be obtained by culture of microspores or anthers (Von Aderkas and Dawkins 1993, Aslam et al. 1990, Loh and Ingram 1983). The haploid embryogenic lines can be multiplied and maintained by secondary somatic embryogenesis. Embryos of the selected lines can be used to double the ploidy level. In *Brassica napus* (Loh and Ingram 1983), *B. campestris* (Aslam et al. 1990) and citrus (Hikado et al. 1982) this was accomplished by culturing somatic embryos for secondary embryogenesis in the presence of colchicine. In *Asparagus officinalis* (Feng and Wolyn 1991) and *Camellia japonica* (Kato 1989) the ploidy level of embryos doubled spontaneously in a low frequency.
- Because of the absence of vascular connections between the nucellus and other maternal tissues, polyembryogenic species are generally free of infections that might have affected the parent plant. Similarly, plants derived via embryogenesis from nucellus or nucellus callus would also be free of pathogens including viruses (Janick 1993).

3. Production of metabolites: Seeds are storehouses of many important products such as starch, fats, oils, proteins. Therefore, cell/organ cultures have been suggested as a means to

synthesize desirable metabolites (Al-Abta et al. 1979). Lipid synthesis has been followed in embryo cultures in number of species including cocoa, jojoba, borage, rape seed, *Brassica napus* (Nehlin et al. 1996) and carrot (Janick 1991, Weber et al. 1992)

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

5. Transformation: Somatic embryogenesis is the most often used tissue culture system in woody plants for genetic transformation (Handley 1995). In woody plants *Agrobacterium* mediated transformation has been used to regenerate transformants of mango (Mathews et al. 1992), *Carica papaya* (Fitch et al. 1993), *Juglans* (McGranahan et al. 1988), *Picea mariana* (Mill.) BSP, *Pinus strobilus* L and *Populus spp*. (Tian et al. 1999) etc. Direct DNA-mediated transfer methods such as biolistics have been used on embryogenic cultures of *Picea glauca* (Ellis et al. 1993), *Liriodendron tulipifera* (Wilde et al. 1992) and *Carica papaya* (Fitch et al. 1990), *Picea mariana* (Mill.) BSP, *Pinus strobilus* L and *Populus spp*. (Tian et al. 1999). Each of these transformation methods has been used in SE tissue culture system. The advantages in using such system are, more number of regenerants can be obtained originating from few or single cells which increase the likelihood of achieving transformed cells.

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

The process of embryo initiation and development is called embryogenesis. It is distinct from organogenesis in that the product formed is autonomous and is not connected with any other structure *via* vessels. In practice all the processes in which shoot and root

poles develop more or less synchronously are included in this term (Thorpe 1983). Several types may further be distinguished based on the origin of the material (Table 5.1)

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

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

The first report of SE appeared when Reinert (1958) demonstrated the phenomenon using carrot callus. Embryogenesis that occurs directly from cells of the explant without a callus phase is identified as direct embryogenesis. Indirect embryogenesis involves callus proliferation from explants prior to differentiation of embryos. It would be convenient to distinguish a third type viz. repetitive embryogenesis, where embryos arise directly from primary somatic embryos (Thorpe 1983). Somatic embryos formed either directly on explants or indirectly through a callus phase often show a tendency to undergo secondary embryogenesis to give rise to new embryos. This phenomenon has been referred in literature as repetitive or accessory embryogenesis (Ammirato 1987), recurrent embryogenesis (Lupotto 1983) and proliferative embryogenesis (Stamp and Meridith 1988).

Once embryogenic determination has occurred the commitment of a group of cells to integrated development as an embryo depends on the maintenance of coordinated embryogenic behavior and the suppression of any tendency of individual cells to act independently (Williams and Maheswaran 1986). Secondary embryogenesis then can be explained as an expression of cells, which have broken away from the integrated control. Secondary embryogenesis is considered as one of the major causes for absence of synchrony in populations of somatic embryos (Ammirato 1987). In manipulating embryogenesis, two media components in particular play crucial roles. These are auxin and nitrogen (Kohlenbach 1978). Halperin and Witherell (1964) first recognized the

importance of auxin. Further studies showed that the process of SE normally takes place in two stages. First the induction of cells with embryogenic competence (referred to as embryogenic masses or clumps, proembryos, proembryogenic tissue) in the presence of high concentrations of auxin. Second, development of the embryogenic masses into embryos in absence or in presence of a lowered concentration of auxin. 2,4-Dichlorophenoxyacetic acid is the most commonly used auxin. Reduced nitrogen in the form of NH4 is also required (Kohlenbach 1978, Wetherell 1979). There are few recent reports on induction of somatic embryogenesis in presence of cytokinins like Thidiazuron (Qureshi and Saxena 1992, Saxena et al. 1992) and BAP (Malik and Saxena 1992) etc.

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

All somatic cells within a plant contain the entire set of genetic information necessary to create a complete and functional plant. Temporal and spatial expression of genes is tightly regulated to permit the differentiation of various organ systems as the plant develops (Goldberg 1986). The induction of SE must then consist of the termination of the existing gene expression pattern in the explant tissue, and its replacement with an embryonic gene expression program in those cells of the explant tissue which will give rise to somatic embryos. This concept was first embodied by Sharp et al. (1982) and Evans et al. (1981), who used the term “IEDC”, for “induced embryogenic determined cell” to

describe an embryonic cell that had originated from a nonembryonic cell. Cells from plant embryos, which already express an embryonic gene expression program, were termed "PEDC", for "preembryogenic determined cells" (Sharp et al. 1982). For the purpose of regeneration, once obtained, both IEDCs and PEDCs are functionally equivalent, and both may be referred as "EDCs" for "embryogenic determined cells" (William and Maheswaran 1986) or simply, "ECs" for "embryogenic cells" (Carman 1990, Merkle et al. 1990). The later term is preferred, because the formation of SE is not inevitable fate for an EC, indicating an existing plasticity in the cells not adequately conveyed by the term "determined" (Carman 1990). Treatments to obtain SE thus depend on whether the explant tissue consists of PEDCs or non ECs. In the first case, a stimulus for cell division may be sufficient for the formation of a somatic embryo on a tissue explant, in a process referred to as direct embryogenesis, as the somatic embryos appear to rise directly from the explant tissue. In contrast, non EC tissue must undergo several mitotic divisions in the presence of an auxin during the induction of the EC state. Cells resulting from these mitotic divisions are manifested as a callus, and the term indirect regeneration is used to indicate that a callus phase intervenes between the original explant and the appearance of somatic embryos. Any cells, which can develop into somatic embryos, are said to possess embryogenic competence. Whether these cells are target cells which respond to special signals or whether most cells have this capability is not yet known. The selection of specific developmental stages of explant material, conditioning media, sequential transfers and appropriate environmental conditions are generally necessary for successful embryogenesis. The primary events required for cells to enter the developmental program of embryogenesis are unknown, but the techniques are available to begin to address this question (Tulecke 1987).

The induction of SE in woody perennials is restricted due to the characters that make them more intractable for studies. These are:

(1) Short seasonal period for the availability of any particular tissue or a developmental stage for culture, (2) long period required for regeneration, (3) frequent production of phenolic compounds, etc. (Tulecke 1987). Inspite of these limitations SE has been achieved in a number of angiosperms, both dicotyledons and monocotyledons. Still most woody plants, appear to be recalcitrant and routine regeneration of plants be it either

organogenesis or SE is rare. Problems and potentials of tissue culture in forest tree improvement have been discussed extensively (Haissig et al. 1987). Although axillary shoot culture is the most promising short-term (2-5 years) possibility for commercial propagation, it is presumed that in the long-term (10-25 years), SE is likely to replace shoot culture

5.1 Somatic Embryogenesis in *Garcinia talbotii*

Material and methods:

Fully mature unripe fruits of *Garcinia talbotii* were collected in the month of April from the semievergreen forest of Mulshi (18°28'N, 73°27'E, 610m) in Maharashtra, India. The fruits were surface sterilized by the following method. They were washed in running tap water for 1 hr. They were cleaned with a few drops of detergent. Treated with 10% savlon for 3-5 min and treated with 1% bavistin for 1 hr. After each treatment the fruits were washed 2-3 times with single distilled water. Further treatments were carried out in the laminar air flow unit. The seeds were excised from the fruits, rinsed with 70% (v/v) ethanol and washed 3-4 times with sterile distilled water followed by treatment with 0.05% (w/v) $HgCl_2$ for 10 mins and again washed 3-4 times with sterile distilled water. The seeds after removing the seed coat were cut into three parts and inoculated in WPM media containing TDZ (0.045-9.08 μM), BAP (2.22-44.38 μM), BAP (2.22-44.38 μM) in combination with NAA 2.69 μM or BAP (2.22-44.38 μM) in combination with 4.65 μM kinetin. The explants that were cultured in the TDZ media were shifted to BAP 0.44 μM or BAP 0.88 μM after 1 month of incubation in TDZ medium. Different media combinations were tried for the conversion of embryo- ½ WPM, ½ WPM+BAP (0.88-22.19 μM), full strength WPM medium with or without 4.44 μM BAP. The explants that were grown in BAP, BAP in combination with NAA or BAP in combination with kinetin were cultured in the same medium for 2 months with one subculture after 30 days, then shifted to low BAP (0.44 μM). All media contained 2% (w/v) sucrose and were gelled with 0.2% (w/v) phytagel. 12 seeds were used for each treatment and the experiment was repeated 3 times. All cultures were grown in 16 hr photoperiod. Explants at different stages of morphogenic

response were fixed for histological studies.

Results:

Effect of BAP: After 45 days in the induction medium globular and heart shaped embryos could be observed on BAP 4.44 μM (Fig 5.1) while organogenic buds were observed on BAP 22.19-44.38 μM. The embryos were kept in the same medium for a further period of 15 days then shifted to low BAP (0.44 μM). The growth of the embryos was retarded on BAP 0.44 μM so after 1 month the embryos were again shifted to BAP 4.44 μM where complete plantlets were obtained.

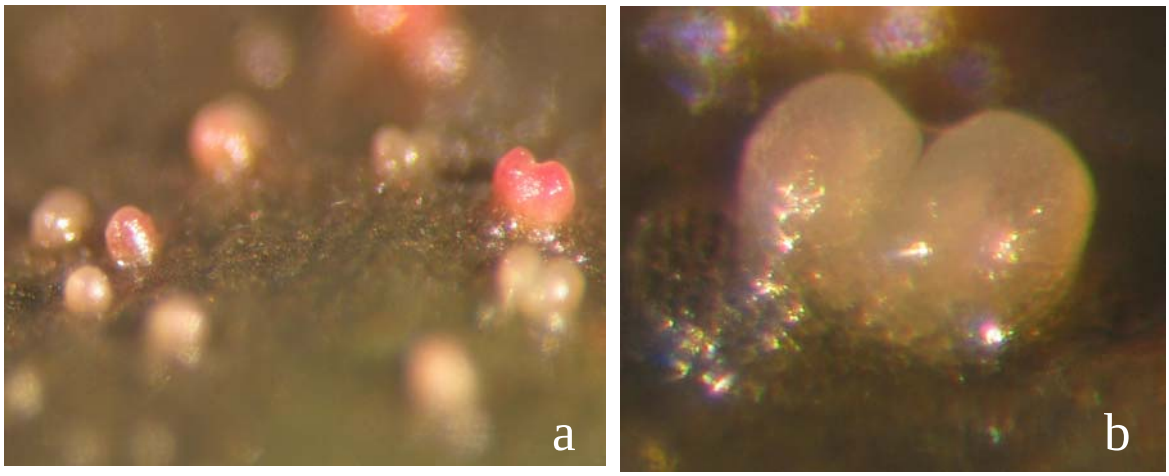


Fig 5.1: Induction of somatic embryogenesis in *G. talbotii* on BAP 4.44 μM

a-globular and heart shaped somatic embryos,

b-heart shaped somatic embryo

Effect of BAP in combination with kinetin: BAP in combination with Kinetin induced organogenesis.

Effect of BAP in combination with NAA: BAP + NAA led to callus formation with few shoots and adventitious roots at higher BAP:NAA ratios.

Effect of TDZ: The explants were cultured in TDZ medium for 1 month. No response was observed during this time. The explants were then shifted to BAP 0.44 μM or BAP 0.88 μM. The zygotic embryos present in the explants germinated after 15 days of shifting to BAP medium. The shoots formed were stunted. TDZ 0.04 μM induced white compact callus formation (Fig 5.2a), while TDZ (0.22-0.45 μM) induced direct somatic embryogenesis after 15 days of shifting to BAP medium. TDZ 0.22 μM induced large number of crescent shaped pale yellow coloured somatic embryos from the surface the explant (Fig 5.2b). TDZ 0.45 μM induced secondary somatic embryogenesis (Fig 5.2c&d). 1-2 secondary somatic embryos were seen growing from the base of each primary somatic embryo. The explants inoculated on TDZ (0.45-9.08 μM) had to be subcultured to BAP 0.44 μM one more time to get somatic embryos. Thus after 90 days all TDZ concentrations gave direct somatic embryogenesis (Table 5.2). The embryos formed were synchronous. BAP 4.44 μM was the best media to induce somatic embryogenesis. It gave 100% response with an average of 27.66 embryos per explant. Moreover the embryos induced on BAP 4.44 μM showed 100% conversion. TDZ 0.22 μM even though produced significantly large number of somatic embryos (44 embryos per explant) the percent response was very low (44.44%) and even the conversion percentage was not very high (33%). It was observed that high TDZ in the induction medium adversely affected the conversion percentage of the somatic embryos.

Table 5.2: Effect of BAP and TDZ on embryogenesis

Hormones(μM)	%response	Average no of embryos/explant after 90 days*	Percentage of germinated embryos
BAP 4.44	100	27.66 ± 23.02^{ab}	100
TDZ 0.04	33.33	10.00 ± 0^{ab}	10.00
TDZ 0.22	44.44	44.00 ± 17.00^{a}	33.00
TDZ 0.45	100	34.66 ± 23.18^{ab}	42.85
TDZ 4.54	50	5.33 ± 2.30^{b}	5.00
TDZ 6.81	100	15.00 ± 3.46^{ab}	3.20
TDZ 9.08	33.33	24.00 ± 0^{ab}	2.00

*Values followed by the same letter do not vary significantly at $p<0.01$

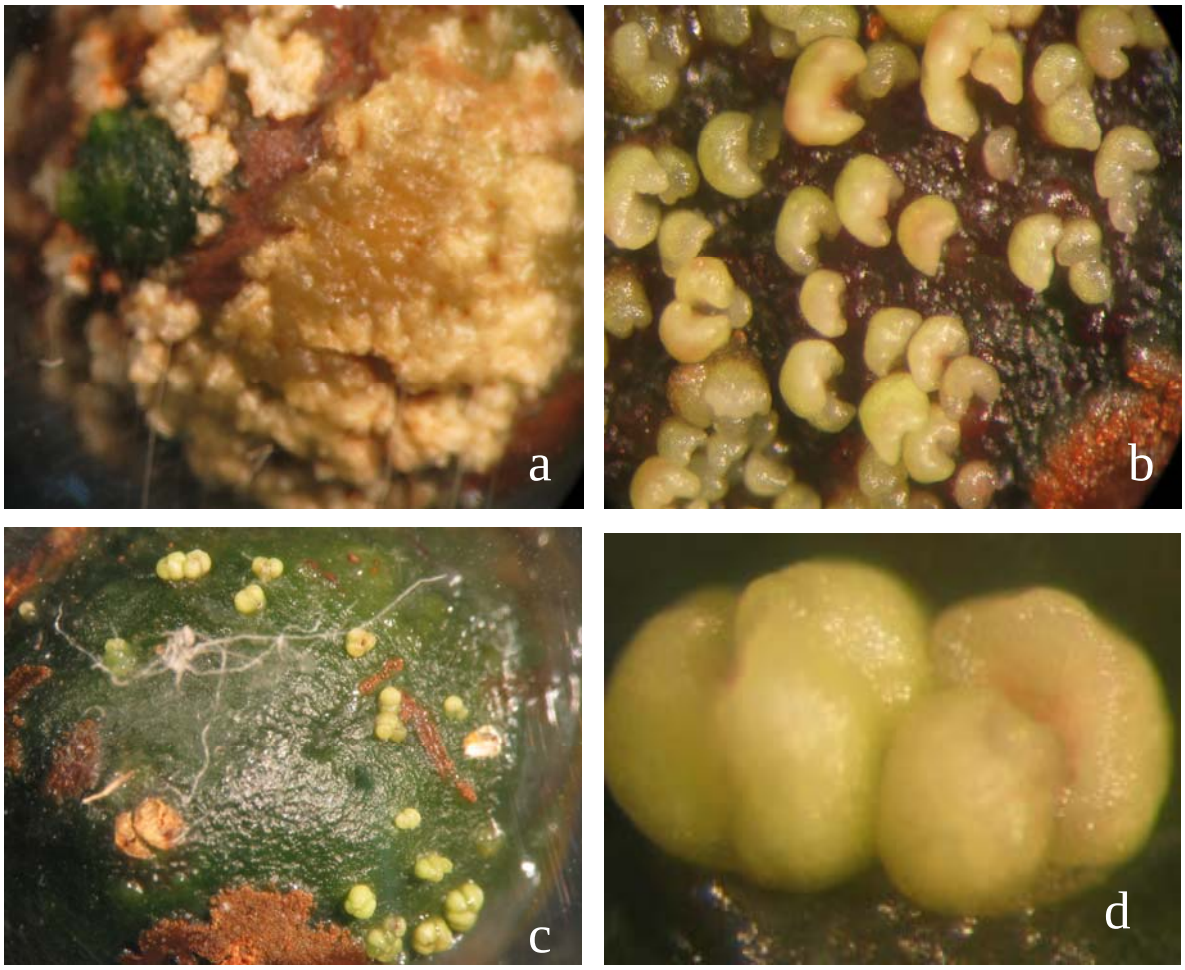


Fig 5.2: Effect of different concentrations of TDZ on *G. talbotii* seed explant

a-callus formation on TDZ 0.04 μM,

b-induction of somatic embryogenesis on TDZ 0.22 μM,

c&d-secondary somatic embryogenesis on TDZ 0.45 μM

Maturation:

The maturation medium had an influence on the growth of the somatic embryos. While well defined somatic embryos were observed on BAP 0.44 μM (Fig 5.3b), the embryos formed on BAP 0.88 μM were not well formed (Fig 5.3a). The embryos developed through the stages of globular, heart, torpedo (Fig 5.3c) and cotyledonary stages (Fig 5.3d) on being subcultured to the same medium after 1 month. But further subculturing to the same medium was leading to necrosis of the somatic embryos. So the embryos were shifted to conversion medium.

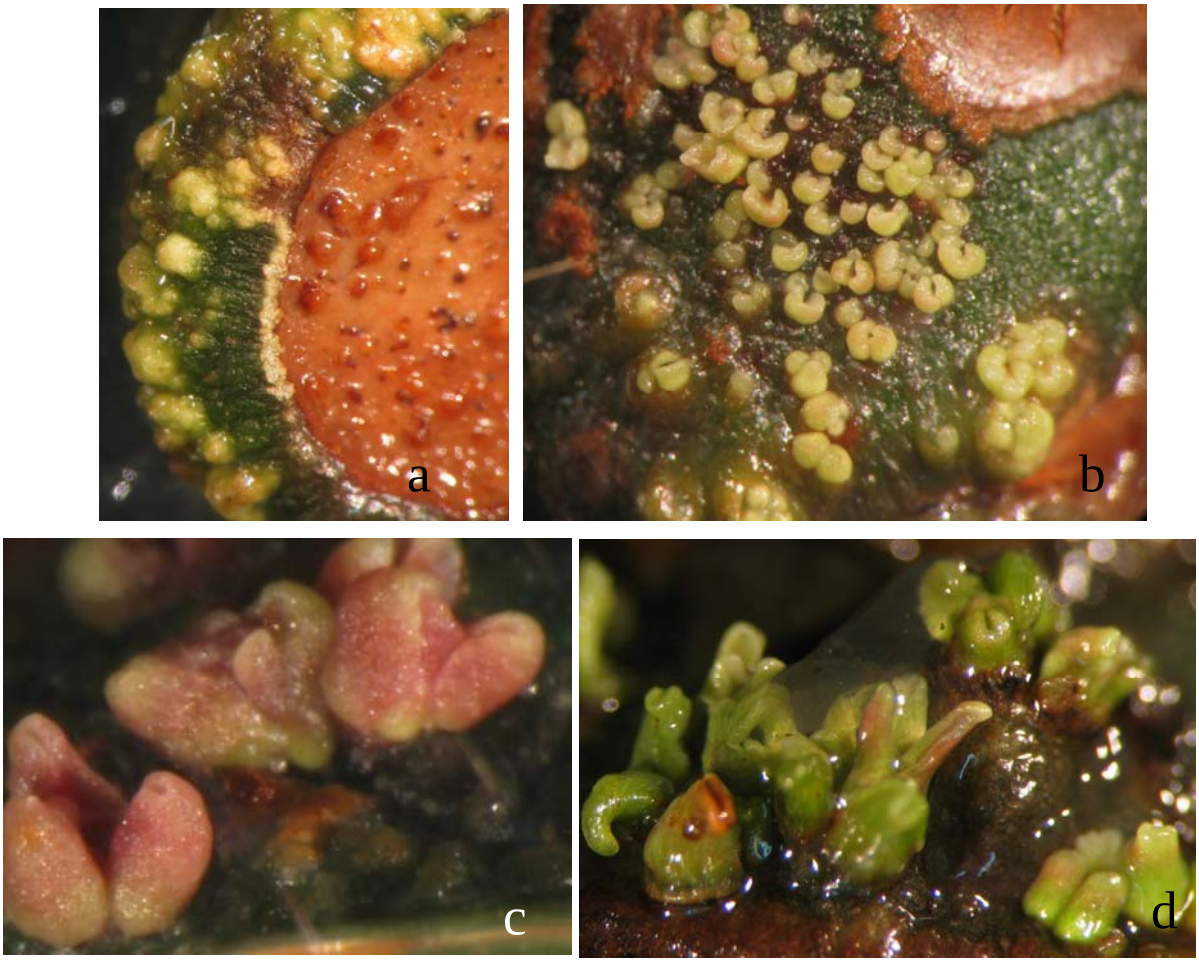


Fig 5.3: Maturation of *G. talbotii* somatic embryos

a-Somatic embryos on BAP 0.88 μM,

b-somatic embryos on BAP 0.44μM

c-Somatic embryos in torpedo shape,

d-somatic embryos in cotyledonary stage

Histology:

Histological studies revealed the direct induction of somatic embryos without callus formation. The somatic embryos originated from the subepidermal layer of the seed. The embryos showed multicellular origin. The embryos developed through the stages of globular, heart (Fig 5.4a), torpedo (Fig 5.4b) and cotyledonary stages. The secondary somatic embryos formed at higher concentrations of TDZ originated from the base of primary embryos (Fig 5.4c). The somatic embryos did not show vascular connection with

the mother explant.

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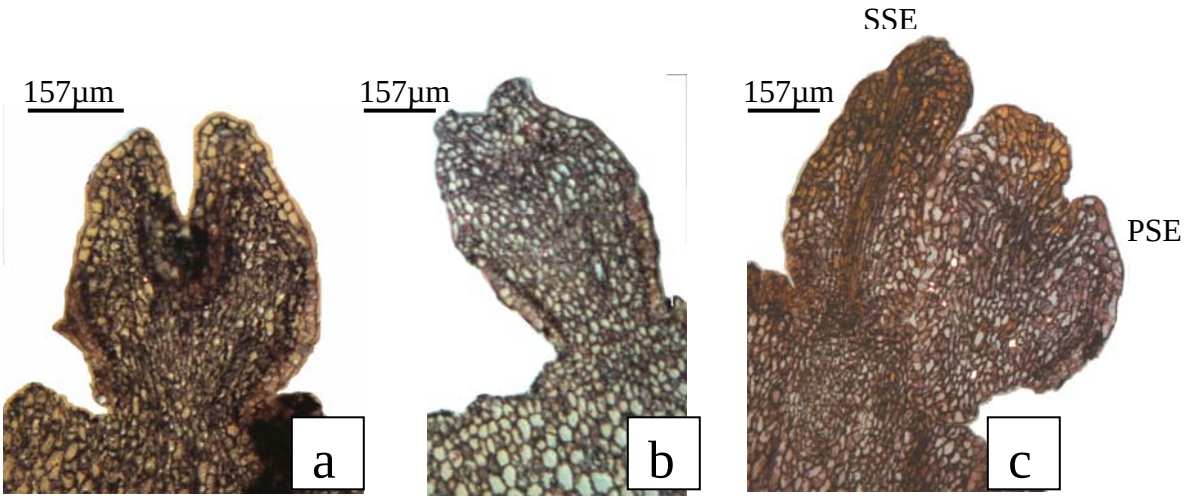

Fig 5.4: Histological sections of somatic embryogenesis in *G. talbotii*

a-heart shaped somatic embryo,

b-torpedo shaped somatic embryo,

c-histology showing swcondary somatic embryogenesis.

Conversion of Somatic embryos:

The embryos induced on BAP 4.44µM matured on BAP 0.44 µM and germinated on BAP 4.44 µM. The embryos showed 100% conversion. For the embryos induced on TDZ, different media combinations were tried. ½ WPM + BAP 4.44 µM proved to be the best media giving 40% conversion of the embryos.

Table 5.3: %conversion of somatic embryos in different media

Medium	% conversion
½ WPM	Necrosis of embryos
½ WPM+0.88 µM BAP	Necrosis of embryos
½ WPM+4.44 µM BAP	40
½ WPM+22.19 µM BAP	10
WPM	20
WPM+4.44 µM BAP	12

The embryos induced on TDZ took 7 months to form complete plants while those induced on BAP took 5 months to form complete plants. Even the conversion percent was lower than that compared with embryos induced on BAP. Fully formed plants were subjected to hardening in plastic cups containing soil:sand (1:1). The plants were watered and covered with plastic bags to maintain humidity. They were kept in 24hr light in the culture room. After a month in the culture room the plants were shifted to green house where the plants grew with 98% survival.

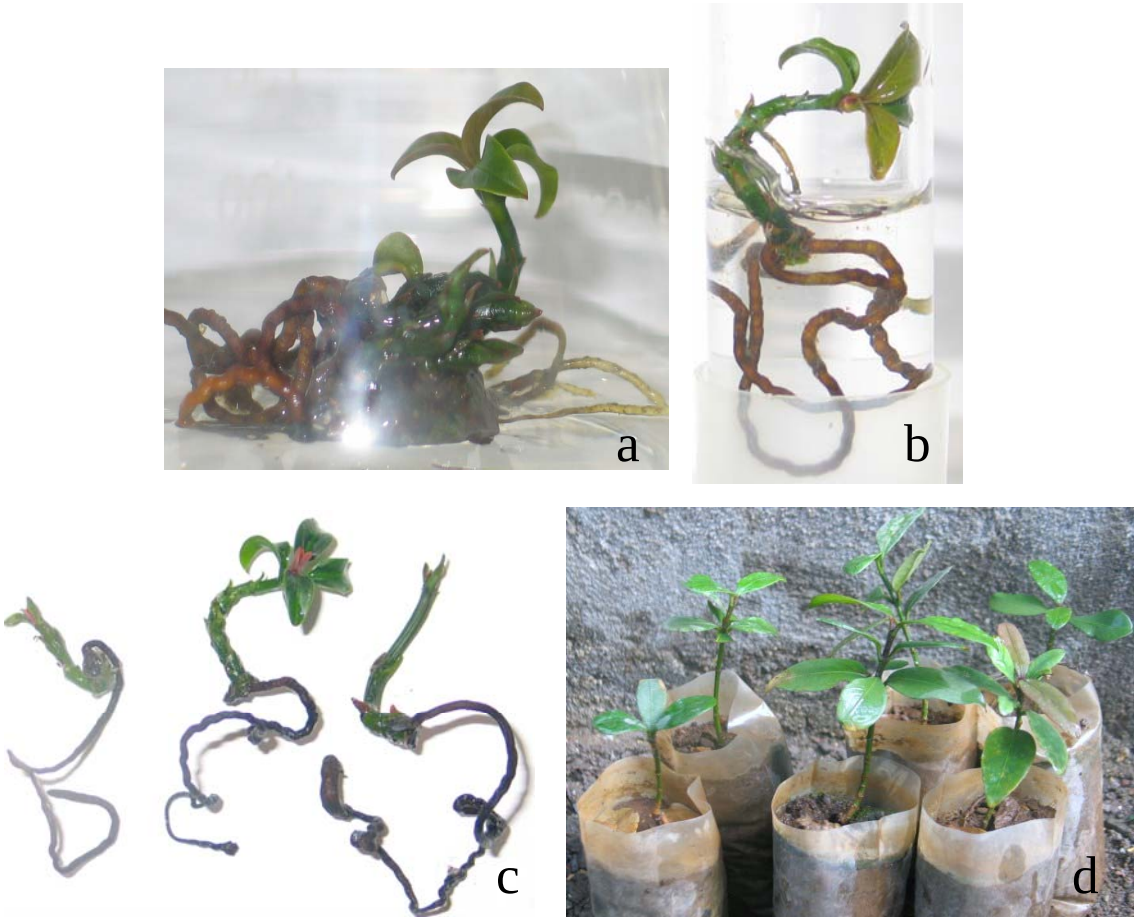

Fig 5.5: a-conversion of somatic embryos on WPM+4.44 μM BAP, b&c-complete plantlet, c-hardened plants

Discussion: In the present study we found that while BAP at low concentration led to embryogenesis at higher concentration it led to organogenesis. TDZ at all concentrations used in the present study induced direct somatic embryogenesis. TDZ is known as a potent

cytokinin, and in the present study TDZ had the same effect as low concentration of BAP. Thus TDZ in this species induced somatic embryogenesis by altering the ratio of auxin to cytokinin (Visser et al. 1992). At high concentrations the embryogenesis was delayed and there was induction of secondary somatic embryogenesis. The embryos induced on BAP formed complete plants in 5 months while embryos induced on TDZ formed complete plants in 6-7 months. This is because of the persistent nature of TDZ, which affects the conversion of the embryos.

5.2 Embryogenesis in *Garcinia spicata*

Materials and methods:

Fully mature unripe green fruits of *Garcinia spicata* were collected in the month of April from the semievergreen forests of Mulshi (18°28'N, 73°27'E, 610m) in Maharashtra, India. The fruits were surface sterilized by the method followed for the fruits of *Garcinia talbotii* and the seeds after removing the seed coat were cut into three parts and inoculated in WPM media containing TDZ(0.045-9.08 μM), BAP (2.22-44.38 μM), BAP (2.22-44.38 μM) in combination with NAA 2.69 μM or BAP (2.22-44.38 μM) in combination with kinetin 4.65 μM. The explants growing on TDZ were shifted after 4 weeks to 2 different media combinations- BAP 0.44 μM and BAP 0.88 μM. ½ WPM+0.44 μM media was used for conversion of the somatic embryos. The explants growing on BAP, BAP in combination with Kinetin and BAP in combination with NAA were cultured for 8 weeks with one subculture in the same media after 4 weeks. The explants were then shifted to three different media - (1) plain WPM medium with 0.2% charcoal, (2) BAP 0.44 μM, (3) BAP 0.44 μM+IBA 0.49 μM. All media contained 2% (w/v) sucrose and were gelled with 0.2% (w/v) phytagel. 12 seeds were used for each treatment and the experiment was repeated 3 times. All cultures were incubated at 16hr photoperiod. The explants showing morphogenic response were fixed for histological studies.

Results:

Induction of Somatic embryogenesis:

BAP with NAA gave direct organogenesis. BAP alone or in combination with kinetin did not show regeneration but led to normal germination of the seeds. TDZ induced direct somatic embryogenesis. The explants were grown in TDZ media for 4 weeks then shifted to low concentrations of BAP 0.44 μM or 0.88 μM. Direct embryogenesis was induced by TDZ 0.45 μM after 2 weeks in low BAP (Fig 5.6a). Lower concentrations of TDZ (0.04-0.22 μM) did not gave any response, while green compact callus was observed at higher concentrations of TDZ (4.54-9.08 μM) after 2 weeks in BAP medium (Fig 5.6b).

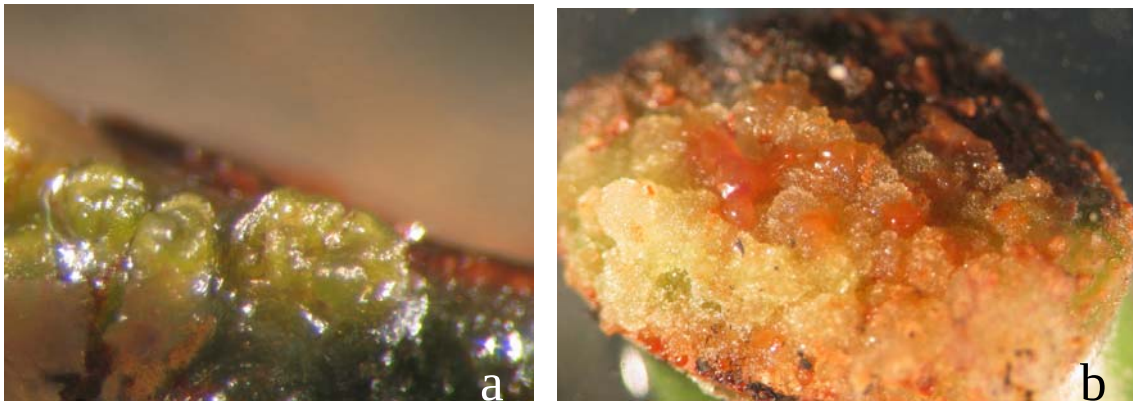

Fig 5.6: Effect of different concentrations of TDZ on *G. spicata* seeds

a-initiation of somatic embryos induced by TDZ 0.04 μM,

b-callus induced by TDZ 6.81 μM

Since the explants cultured on TDZ (4.54-9.08 μM) induced green compact callus, three different media combinations were tried at this stage. The explants were shifted to plain WPM medium containing 0.2% charcoal, BAP 0.44 μM or BAP 0.44 μM+ IBA 0.49 μM. The results obtained were highly dependent on the TDZ concentration of the induction medium. While TDZ 4.54 μM induced direct somatic embryogenesis on BAP 0.44 μM, callus formation was observed on explants shifted to BAP 0.44 μM+ IBA 0.49 μM and there was no response in the charcoal medium. TDZ 6.81 μM induced direct somatic embryos in some of the explants when shifted to charcoal medium, while the rest had to be

continuously shifted to IBA 0.49 μM for two subcultures to obtain direct somatic embryos (Fig 5.7a). It was observed that the explants inoculated on TDZ 9.08 μM induced callus when shifted to BAP 0.44 μM, but gave direct somatic embryogenesis when shifted to BAP 0.44 μM+ IBA 0.49 μM. Secondary somatic embryogenesis was also observed on TDZ (6.81-9.08 μM) (Fig 5.7b,c).

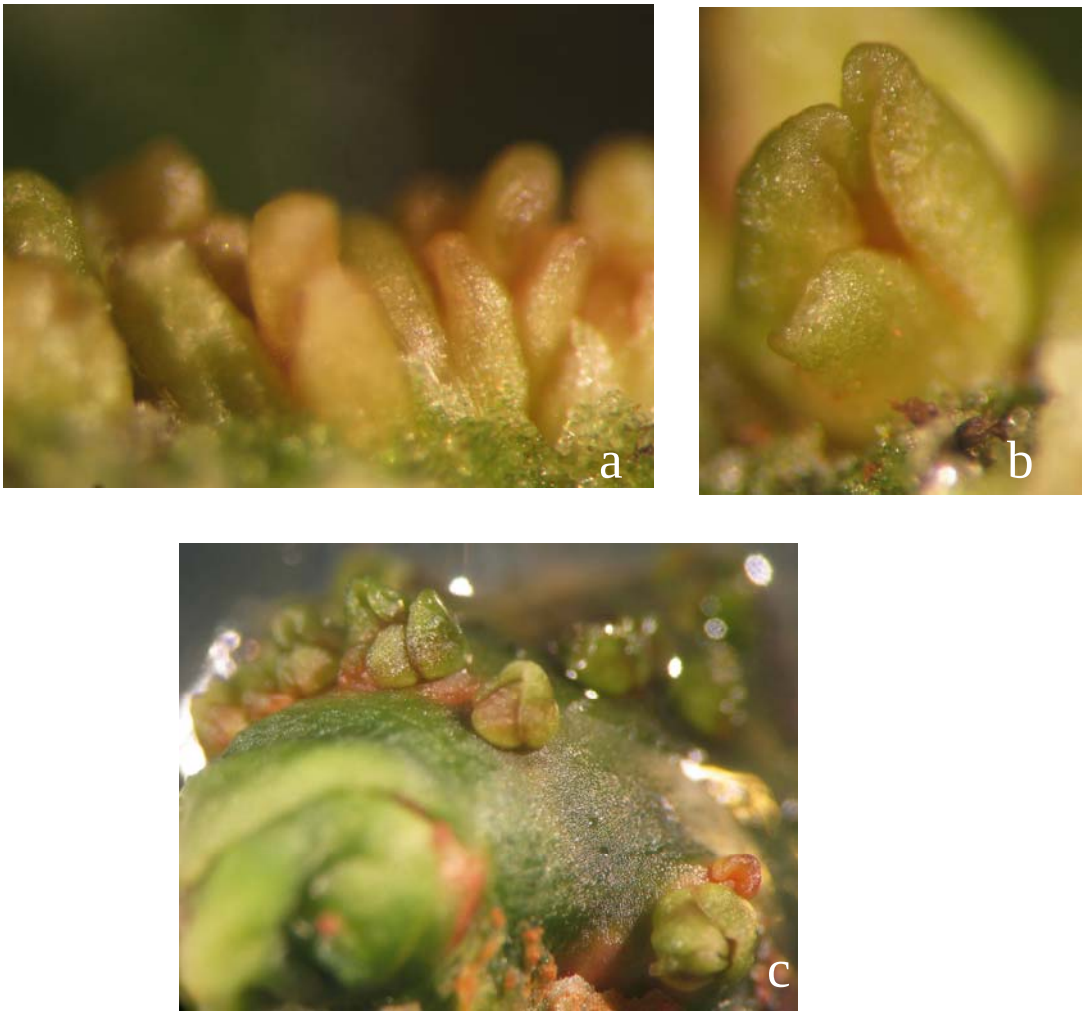

Fig 5.7: Somatic embryogenesis in *G. spicata*

a-somatic embryogenesis induced on TDZ 6.81 μM,

b-secondary somatic embryo induced on TDZ 6.81 μM,

c-secondary somatic embryos induced on TDZ 9.08 μM

Table 5.4: Effect of TDZ concentration on the embryogenic response in *Garcinia spicata*

Medium +TDZ(μM)	Percent response	Average no of embryos after 8 weeks*	Average no of embryos after 16 weeks*	Percent germination after 16 weeks
0.04	0	0^{b}	0^{b}	0
0.22	0	0^{b}	0^{b}	0
0.45	88.88	9.90 ± 3.25^{ab}	18.75 ± 3.58^{ab}	30
4.54	100.00	18.00 ± 2.00^{a}	28.00 ± 0^{a}	12.28
6.81	66.66	12.00 ± 0^{a}	30.33 ± 16.80^{a}	0
9.08	33.33	8.66 ± 9.81^{ab}	11.50 ± 8.50^{ab}	0

*values followed by the same letter do not vary significantly at $p<0.01$.

Histological studies:

Histological studies showed that the regeneration was by means of direct somatic embryogenesis and there was no intermediary callus formation involved. The somatic embryos formed were synchronous and had multicellular origin. The embryos formed from the sub epidermal region of the seed (Fig 5.8.a). The embryo developed through the stages of globular (Fig 5.8c), heart (Fig 5.8d), torpedo (Fig 5.8e) and cotyledonary stages. Secondary somatic embryos could also be seen originating from the suspensor of the primary embryo (Fig. 5.8f)

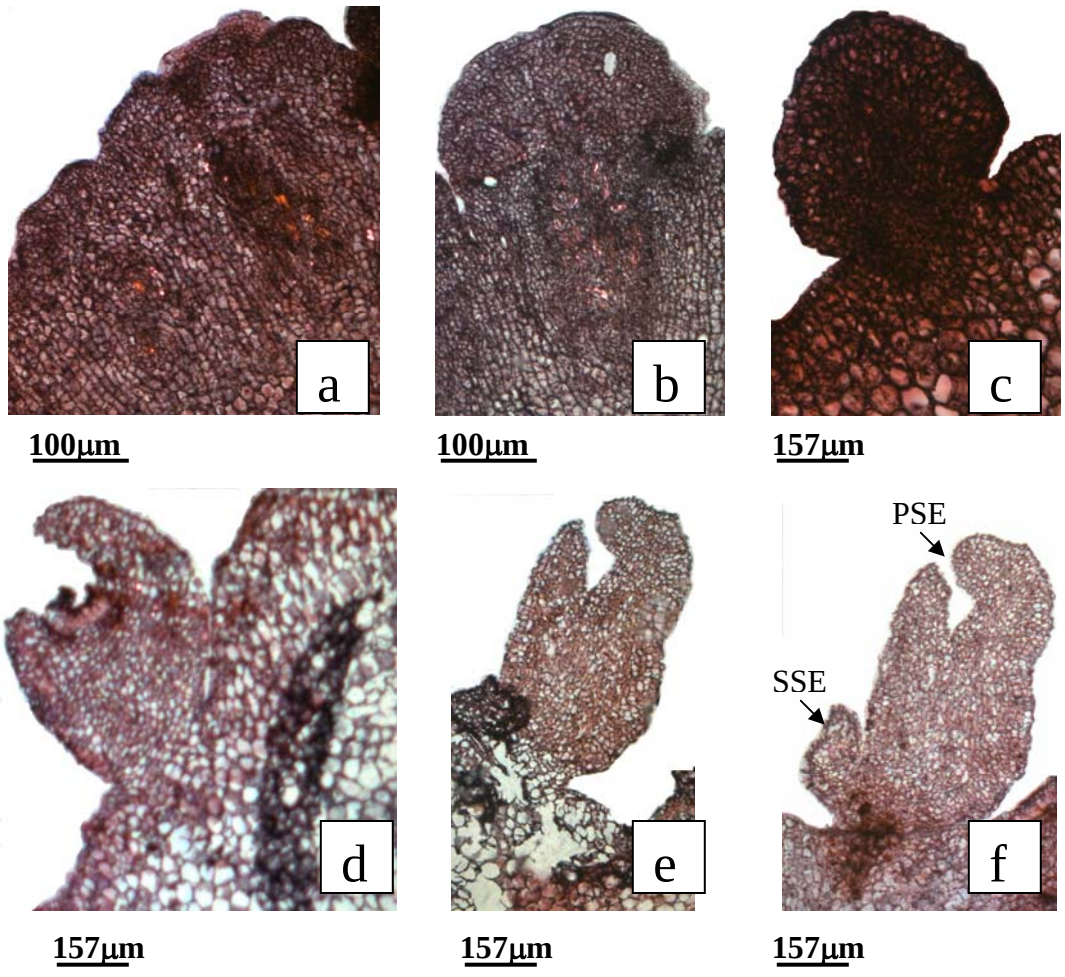

Fig 5.8: Histology of somatic embryos of *G. spicata*

a-meristematic activity in the sub epidermal layer of the seed.

b-protusion of the actively dividing cells from the seed surface, distinct root pole and shoot pole can be seen.

c-globular shaped somatic embryo.

d-heart shaped somatic embryo.

e-torpedo shaped somatic embryo.

f-secondary somatic embryogenesis.

Maturation of somatic embryos:

BAP 0.44 µM was the best medium for the maturation of the embryos. Continuous subculturing on BAP 0.44 µM led to germination of the embryos. Half strength WPM

medium with 0.44 μM BAP was found very effective for elongation and root formation. The frequency of germinating embryos was very low. TDZ 4.54 μM was the best medium to induce embryogenesis. While it induced 28 somatic embryos per explant which was not significantly different from that produced by TDZ 6.81 μM (30.33 embryos per explant), it was also not found inhibitory to the conversion of the somatic embryos. High concentrations of TDZ (6.81-9.08 μM) in the induction medium inhibited the conversion of somatic embryos.

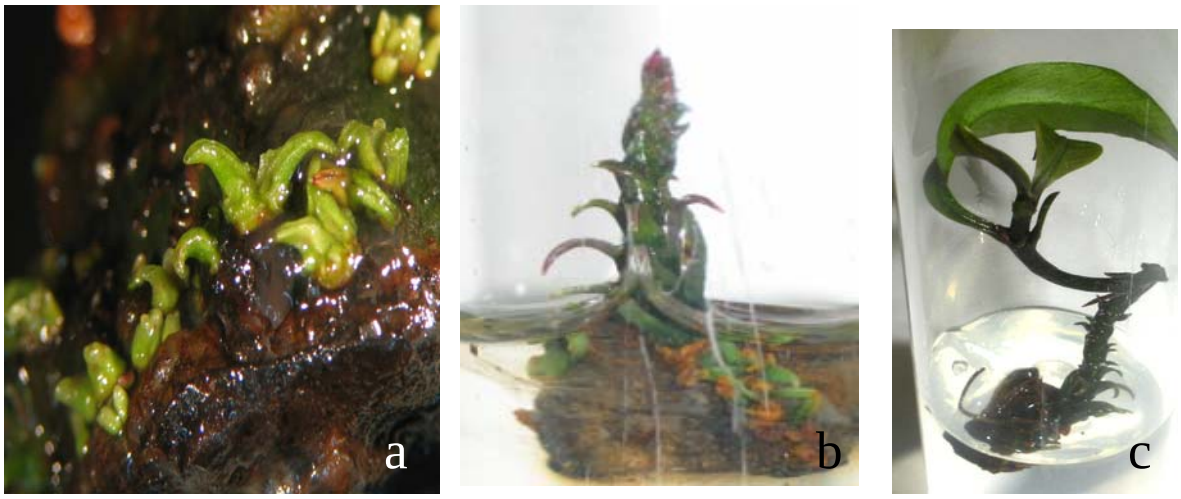

Fig 5.9: a-maturation of somatic embryos on BAP 0.44 μM, b-germinating somatic embryos, c-root formation,

Hardening:

Fully formed complete plants were transferred for hardening in plastic cups containing soil:sand (1:1). The plants were watered and covered with plastic bags to maintain humidity. The plants were kept in culture rooms in 24 hr light conditions and after a month were shifted to green house where they grew with 99% survival.

Fig 5.10: a-complete plant, b-hardened plants of *Garcinia spicata*

Discussion:

For embryogenesis in *Garcinia spicata* TDZ was found the best suitable hormone. TDZ is an effective synthetic growth regulator for the production of somatic embryogenesis in a number of plants ranging from herbaceous species to woody perennials (Gill and Saxena 1992, Visser et al. 1992). With the increase in the TDZ concentration there was an increase in embryo number with TDZ 6.81μM giving almost 90 embryos per seed. But the germination of the embryos was a major problem. While the embryos induced on TDZ 0.45-4.54μM germinated without much effort the embryos induced on higher TDZ (6.81-9.08 μM) did not germinate. High TDZ concentration (6.81-9.08 μM) also induced secondary somatic embryogenesis.

Somatic Embryogenesis in *Garcinia xanthochymus*:

Material and Methods:

Fully mature unripe green fruits of *Garcinia xanthochymus* were collected in the month of February from Dapoli (17°45.222'N, 73°10.932'E, 162m) in Maharashtra, India. The fruits were surface sterilized by the method followed for the fruits of *Garcinia talbotii* and the seeds after removing the seed coat were cut into 7-8 pieces and inoculated in WPM media containing TDZ (0.045-4.54 μM), BAP (2.22-44.38 μM) or BAP(2.22-44.38 μM) in combination with NAA 2.69 μM. The explants were grown in TDZ (0.04-4.54 μM) for one month then shifted to either the same media, or to BAP 2.22 μM/ BAP 4.44 μM or to TDZ

0.04 µM. 6 different media combinations were tried for the conversion of the embryos-plain WPM medium with BAP 0.44 µM, ½ WPM, ½ WPM+BAP 4.44 µM, ½ WPM+BAP 22.19 µM, ½ WPM+BAP 4.44 µM+IBA 0.98 µM, ½ WPM+BAP 22.19 µM+IBA 0.98 µM. The explants that were grown in BAP media were shifted after 2 months to BAP 2.22 µM. The explants that were grown in BAP+NAA medium were shifted after 1 month to BAP medium without NAA. All media were fortified with 2% (w/v) sucrose and gelled with 0.2% (w/v) phytagel. 12 seeds were used for each treatment and the experiment was repeated 3 times. The explants were cultured in 16 hr photoperiod. Explants at different stages of morphogenic response were fixed for histological studies.

Results:

BAP alone and BAP in combination with NAA did not induce embryogenesis while all concentrations of TDZ induced direct somatic embryogenesis. The explants were grown in TDZ (0.04-4.54 µM) for one month and then shifted to either the same media or to BAP 2.22 µM or BAP 4.44 µM or to low TDZ (0.04 µM). TDZ (0.22-0.45 µM) induced direct somatic embryogenesis after 15 days on BAP or low TDZ medium. While TDZ 0.04 µM in the induction medium did not induce any response in the explants, TDZ 4.54 µM induced callus formation when shifted to low BAP medium.

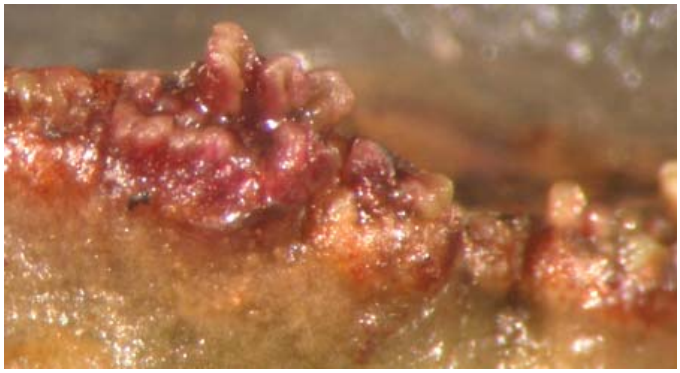

Fig 5.11: induction of somatic embryogenesis by TDZ in *G. xanthochymus*

Table 5.5 shows the number of embryos induced by different concentrations of TDZ. It was observed that with the increase in TDZ (0.04-4.54µM) the percent response increased (0-100%). TDZ 0.45 µM induced the highest number of somatic embryos (54.6 embryos per explant)

Table 5.5: Effect of TDZ on direct somatic embryogenesis in *Garcinia xanthochymus*

Medium+Hormones(µM)	% Response	Average no of embryos after 90 days*	Average no of embryos after 120 days*	% Germination of somatic embryos on BAP 0.44 µM
TDZ 0.04	0	0^{b}	0^{b}	0
TDZ 0.22	80	18 ± 1.58^{ab}	28.0 ± 15.31^{ab}	6.80
TDZ 0.45	85.71	54.6 ± 59.25^{a}	54.4 ± 46.57^{a}	5.0
TDZ 2.27	100	10.4 ± 4.27^{ab}	16.6 ± 6.30^{ab}	3.20
TDZ 4.54	100	2.6 ± 0.54^{b}	8.4 ± 5.12^{b}	0

*Values followed by the same letter do not vary significantly at $p<0.05$

Maturation of Somatic Embryos: Shifting the cultures to the same TDZ media, or to lower TDZ concentration increased the number of embryo but did not help in the maturation of the embryos. It also led to abnormal somatic embryo formation.

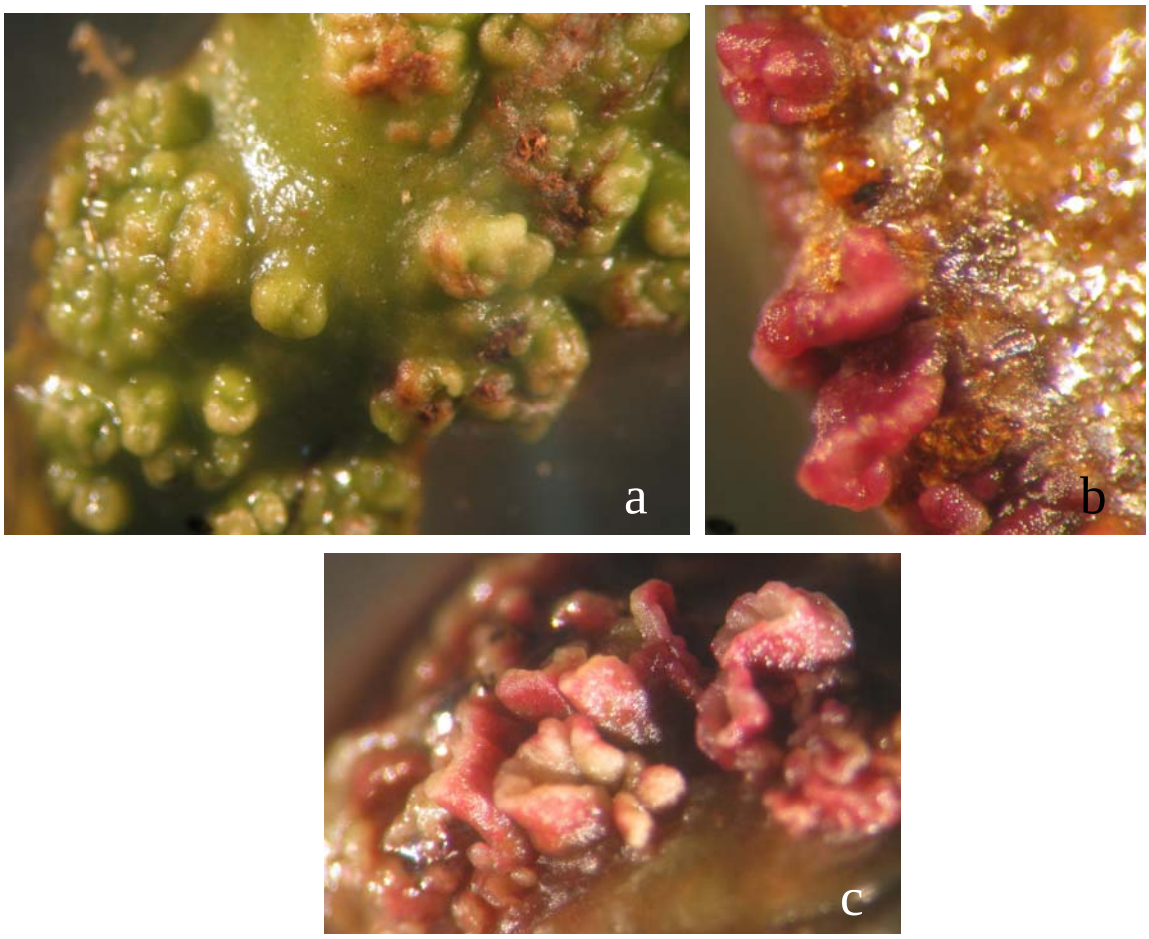

Fig 5.12: Maturation of somatic embryos on TDZ medium

a-embryos induced on TDZ 0.45 µM shifted to maturation medium (TDZ 0.45 µM),

b-embryos induced on TDZ 0.45 µM shifted to maturation medium (TDZ 0.04 µM),

c-embryos induced on TDZ 0.22 µM shifted to maturation medium (TDZ 0.04 µM)

BAP 2.22 µM was found better than the other media tried for maturation of the embryos. On this concentration the embryos developed through the stages of heart, torpedo and cotyledonary stages (Fig 5.13a,b,c,d).

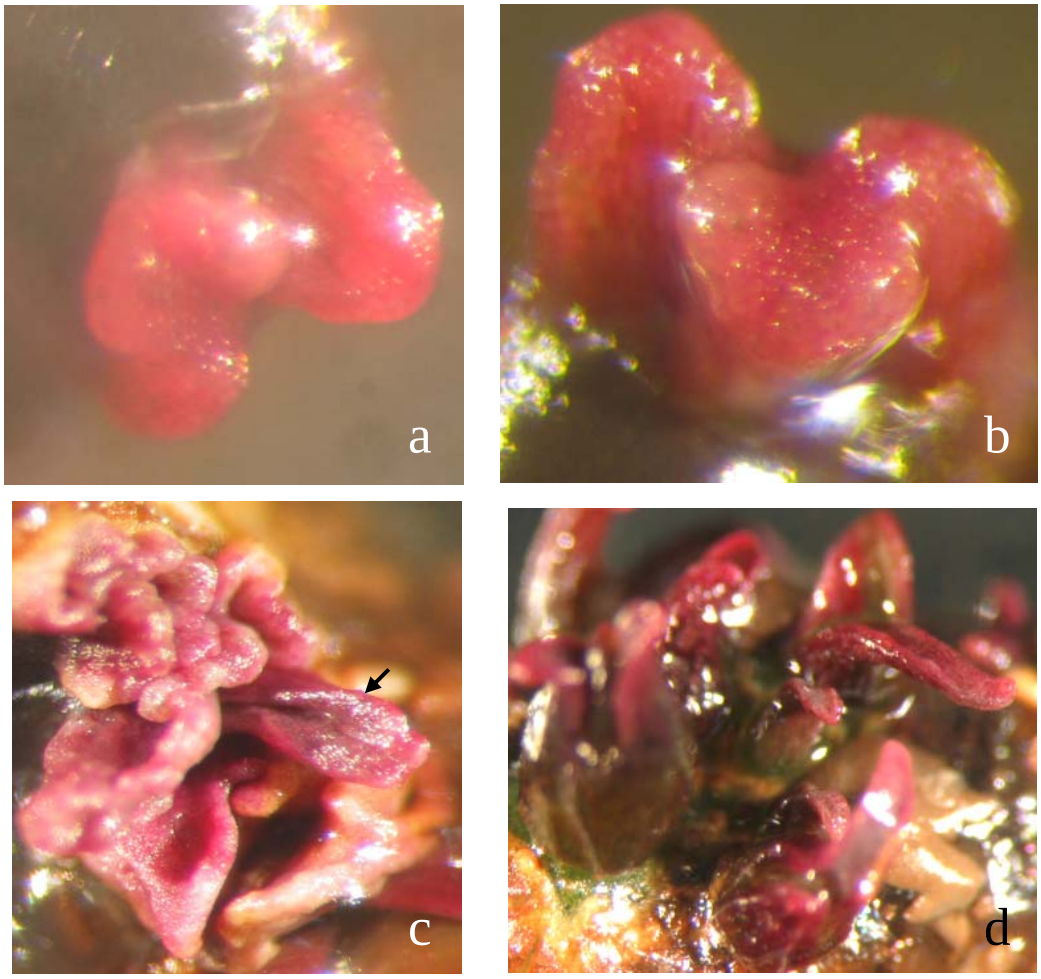

Fig 5.13: Maturation of the somatic embryos on BAP 2.22 µM

a-heart shaped somatic embryos,

b-torpedo shaped somatic embryos,

c-late torpedo shaped somatic embryos,

d-cotyledonary shaped somatic embryos

Histological studies:

7 µm sections were cut for the histological studies. The sections showed subepidermal origin of the embryos without callus formation (Fig 5.14a). Heart shaped embryos were observed after 6 weeks of culture (Fig 5.14b). The embryos showed a well developed suspensor (Fig 5.14c). The torpedo shaped embryos showed a well developed

root pole and shoot pole. The embryo formed a separate structure at this stage lined by epidermal cells and could be separated from the mother explant (Fig 5.14d).

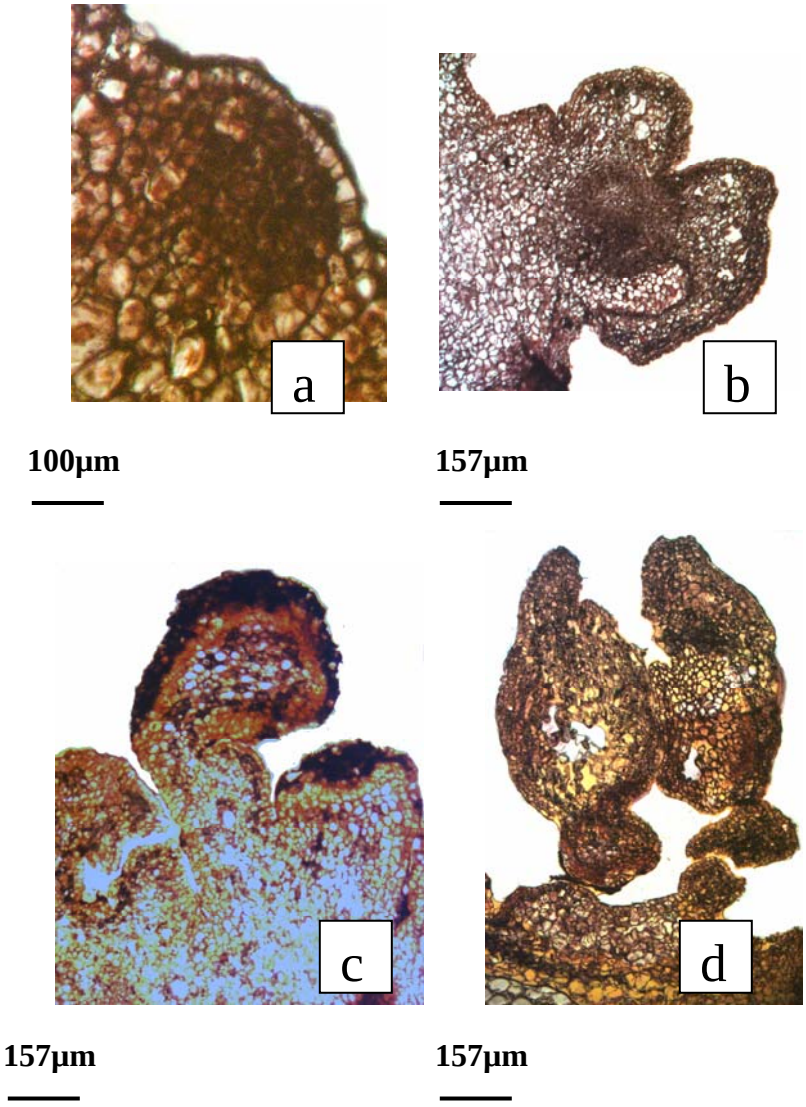

Fig 5.14. Histological sections of somatic embryogenesis in *Garcinia xanthochymus*

a-meristematic activity in the subepidermal region of explant,

b-heart shaped somatic embryo

c-torpedo shaped somatic embryo showing suspensor

f-somatic embryo with distinct root and shoot pole

Conversion of somatic embryos:

After 1 month on the maturation media (BAP 2.22 µM) the explants were shifted to BAP 0.88 µM for the further growth of the embryos. Later when the embryos were shifted to BAP 0.44 µM they started germinating on this media. But the germination frequency was very low- almost equal to I embryo/explant. At this stage the explants that were inoculated in the induction medium TDZ 0.04µM showed callus and root formation. Adventitious roots were also observed in some of the explants inoculated on the induction medium TDZ 0.45 µM while the explants that were inoculated on the induction medium TDZ 4.54 µM showed embryo formation. Further shifting the explants to BAP 0.44 µM lead to browning of the explants (Fig. 5.15).

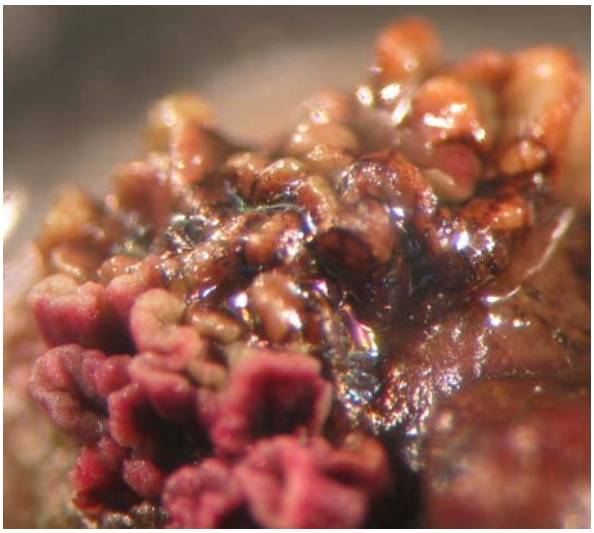

Fig 5.15: continuous subculturing on BAP 0.44 µM led to browning of the embryos

6 different media combinations were tried for the conversion of the embryos. The graph shows the effect of the 6 different media combinations used. It was observed that ½ strength WPM with 22.19 µM BAP+0.98 µM IBA was the best media for the conversion of somatic embryos where 56.2% conversion of the somatic embryos was noted.

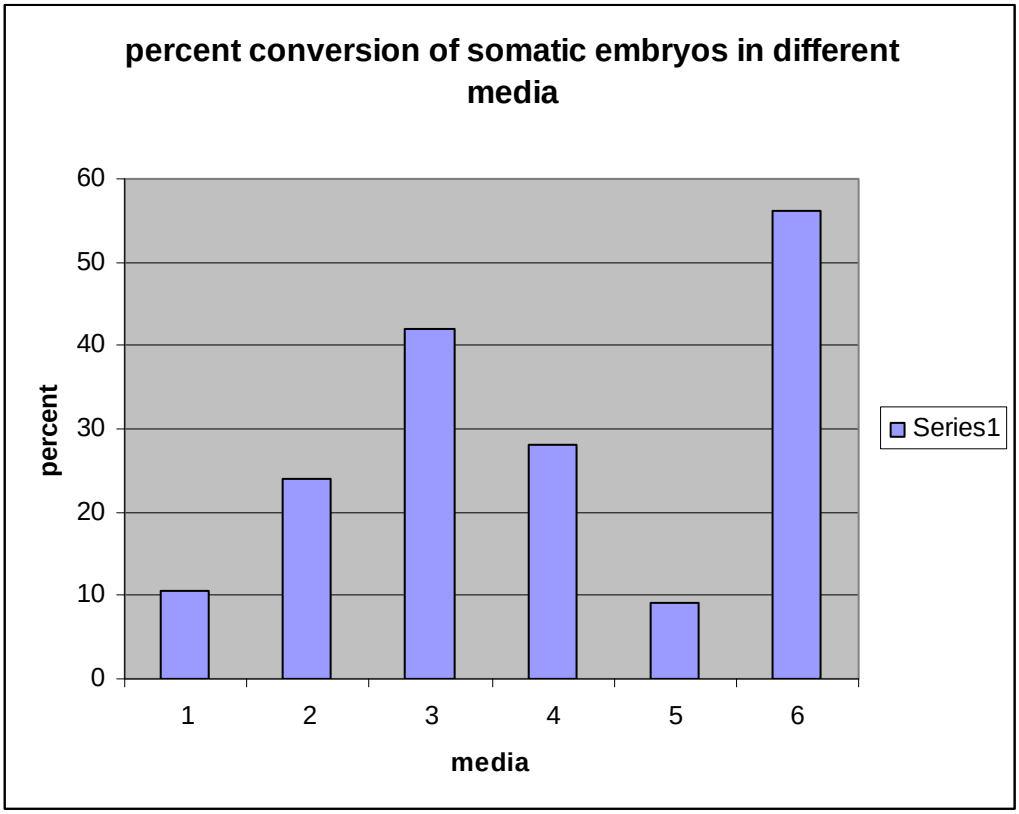

Fig.5.16: Bar graph showing percent conversion of somatic embryos in different media

1. WPM+BAP 0.44 µM
2. ½ WPM
3. ½ WPM+BAP 4.44 µM
4. ½ WPM+BAP 22.19 µM
5. ½ WPM+BAP 4.44 µM+IBA 0.98 µM
6. ½ WPM + BAP 22.19 µM+IBA 0.98 µM

Fig 5.17: Conversion of somatic embryos of *Garcinia xanthochymus*

a-c-shoot formation

d-rooted plant

Hardening:

The embryos were further grown for 1 month on the conversion medium then taken out from the culture washed to remove phytagel and subjected to hardening in plastic cups containing 1:1 soil and sand. The shoots were covered with plastic bags to maintain humidity. After 1 month the plants were shifted to green house where the plants survived with 98% survival. The growth of the plants was very slow and even after the hardening stage the most of the plants did not show leaf opening.

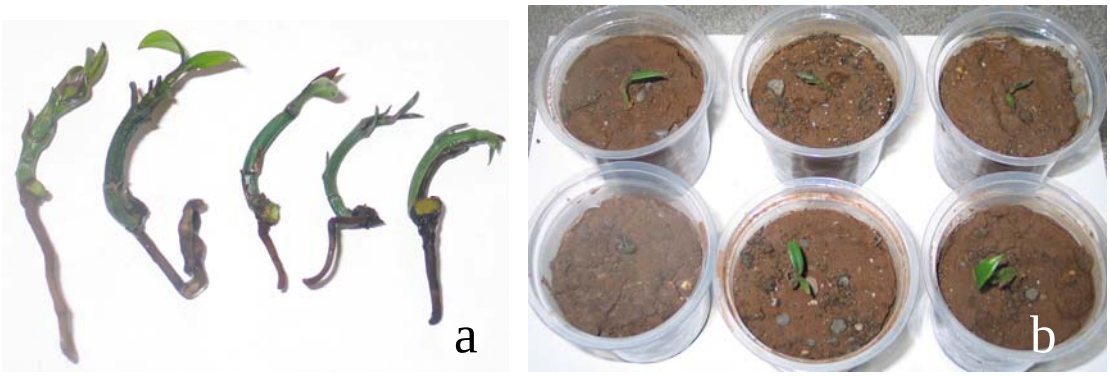

Fig 5.18: a-complete plantlets, b- hardened plants of *Garcinia xanthochymus*

Discussion:

TDZ induced direct somatic embryogenesis in *Garcinia xanthochymus*. The role of TDZ in the induction of somatic embryogenesis has also been reported in bamboo (Linn et al. 2004), cherry (Pesce and Rugini 2004) and *Sesbania drummondi* (Cheepala et al.2004). TDZ has been suggested to either increase the level of nucleosides (Laloue and Pethe 1982), increase the accumulation or synthesis of purine cytokinins or to promote the conversion of adenine to adenosine (Capelle et al. 1983). Low concentrations of TDZ (0.2–2.5 mg l^{-1}) have been reported to be more efficient in inducing organogenesis or somatic embryogenesis than other cytokinins, particularly in recalcitrant woody species (Huetteman and Preece 1993). The reasons for the high activity of TDZ in woody species have not been investigated at the physiological or molecular level. A carbon isotope study has shown that TDZ is very stable in culture media and persistent in plant tissue (Mok and Mok 1985). Saxena et al. (1992) suggested that TDZ helps to establish the optimal internal balance of cytokinin and auxin required for the induction and expression of somatic embryogenesis. Somatic embryogenesis also forms due to stress and TDZ has been shown to increase the stress in the culture medium. The maturation and germination of the somatic embryos occurred on low concentrations of BAP. While most of the reports on somatic embryos induced on TDZ either grow on the same media as in bamboo (Lin *et al.* 2004), cherry (Pesce and Rugini 2004), *Sesbania drummondii* (Cheepala *et al*. 2004) and Capsicum (Khan et al 2006) or on hormone free media - *Cajanus cajan* (Singh et al 2003), *Dendrobium* (Chung et al 2007) and neem (Murthy and Saxena 1998). This may be related

to the concentration of TDZ used in the induction media. While a low dose of TDZ can induce, mature and convert the embryos to plants, somatic embryos induced on very high levels of TDZ require growth regulator free media to mature and germinate. In the present study the TDZ level was not very high nor very low so a low amount of BAP was required for the embryos to mature and grow into plantlets

SUMMARY AND CONCLUSION

Summary and Conclusion

Garcinia, belonging to the family Clusiaceae is a genus of medicinal importance. Fourteen species of *Garcinia* are endemic to the Western Ghats of India. In Maharashtra it is represented by four species - *Garcinia indica, Garcinia talbotii, Garcinia spicata* and *Garcinia xanthochymus*. Two out of these four species i.e. *Garcinia indica* and *Garcinia talbotii* are listed as endangered while *Garcinia spicata* is rare and *Garcinia xanthochymus* is an introduced species. The fruits of *Garcinia* are rich in (-)-Hydroxycitric acid which prevents lypogenesis in humans and is commercially available in the market as anti-obesity drugs. The plant is also rich in various xanthones, depsidones, flavonoids and terpenoids having important medicinal properties. The study entitled "In vitro conservation, ecological mapping with chemical and molecular characterization of Garcinia species occurring in Maharashtra." was undertaken with the following objectives:

1. It is proposed to do detailed ecological diversity studies in these species and their mapping along the Western Ghats of India.

2. Use of *in vitro* techniques for propagation via organogenesis /embryogenesis.

3. Evaluation of %HCA among the species

4. Genetic diversity studies using ISSR/RAPD primers

In the present study it was observed that although *Garcinia indica* is listed as endangered, it grows luxuriantly in Maharashtra and found all along the Konkan region of Maharashtra. It is cultivated on a small scale and found naturally growing in 68 locations covering 6 districts of the state. *Garcinia talbotii* was found rare in the state, occurring mainly in the Western Ghats region at high altitutes (600-800m). The plant is usually found in the dense forests and it seems the destruction of the forest is the main cause of its rare occurrence. *Garcinia spicata* was found extremely rare in the state. It was found singly in only two accessions -Suleran and Mulshi in the field studies. This plant is mainly found in the Eastern Ghats region of India and it seems the climate of the Western Ghat region is not suitable for the growth of this species. *Garcinia xanthochymus* does not occur naturally in Maharashtra. It is an introduced species usually planted by people in the backyards of their homes for its fruits. This plant occurs naturally in Karnataka.

The genetic diversity studies between and among the four species of *Garcinia* was done. At the population level studies in *Garcinia indica,* it was observed that this species has a high genetic diversity. The plants within a population were more similar than plants between the populations. And geographically closely located populations were more similar than distantly located populations. This shows the cross pollinating nature of this species whose genetic diversity is dependent on the distance traveled by the pollinator. The genetic diversity studies in *Garcinia talbotii* revealed the environmental effect on the genetic make up of this species. It was observed that plants located in the coastal region were different from plants located in the high altitudes. It showed how the plants adapt to changing environmental conditions. The genetic diversity studies in *Garcinia talbotii* showed high genetic diversity in this species. So even though the plant is endangered the plant has high adaptability and conservation efforts must be taken to protect this species. Since very few accessions of *Garcinia spicata* and *Garcinia xanthochymus* were sampled population level genetic diversity studies could not be done in these two species. However, these species were included in the species level genetic diversity studies. At the species level it was observed that *Garcinia indica* is genetically different from the other 3 species. While the plants of *Garcinia indica* formed 1 cluster the other three species formed another cluster. And it was observed that *Garcinia spicata* is genetically similar to *Garcinia xanthochymus*. Morphologically however *Garcinia spicata* is similar to *Garcinia talbotii*. The genetic diversity studies thus help us to see differences beyond what eyes can see.

An analysis of the %(-)-HCA content in the four species was done. It was observed that the fruits of *Garcinia xanthochymus* located at Dapoli contained the highest percentage of (-)-HCA (11.49%), followed by the fruits of *Garcinia indica* collected from Guhagar 3 (10.65%). The (-)-HCA content in the four species varied in the range of (6.89-11.47%) in *Garcinia xanthochymus,* (2.52-10.65%) in *Garcinia indica,* (6.67-8.80%) in *Garcinia talbotii* and (5.03%) in *Garcinia spicata*. This is the first work on the (-)-HCA content in the fruits of *Garcinia talbotii*, *Garcinia spicata* and *Garcinia xanthochymus*.

Garcinias are important medicinal plants. But owing to the destruction of forests there are very few populations of this genus left. The plants are polygamodioecious and propagate by means of seeds. The seeds are highly recalcitrant and have low viability. *In*

vitro studies for the regeneration of the four species were carried out. The *in vitro* conditions not only improved the germination percentage of *Garcinia indica* seeds but also reduced the time required for the seeds to germinate. When decoated seeds of *Garcinia indica* were germinated on ½ WPM containing 2% (w/v) sucrose, the germination percentage observed was 76.18% in 30days. While addition of BAP 11.97µM to plain WPM medium containing 2% sucrose even produced multiple shoot formation. In the *in vitro* germination studies in *Garcinia talbotii* when the decoated seeds were inoculated in different basal medium, it was observed that ½ WPM was the best basal media giving 66.66% germination in 30 days. Among the different carbon sources tested it was observed that sucrose was the best carbon source followed by maltose, fructose and glucose. Sucrose at 4% gave 77.77% germination in 30 days. At BAP 13.30 µM, 100% germination was observed in 60 days with 26 ± 24.24 shoots per seed. But there was no root formation. The *in vitro* germination studies in *Garcinia xanthochymus* was very different from that of *Garcinia talbotii* and *Garcinia indica*. The decoated seeds of *Garcinia xanthochymus* did not germinate on plain basal medium. Growth hormones BAP or TDZ was required for the germination of *Garcinia xanthochymus* seeds. The growth of *Garcinia xanthochymus* seedlings was very slow. No leaf opening was observed even after 3 months of culture.

When decapitated seedlings of *Garcinia indica* were inoculated in BAP and TDZ medium multiple shoot formation was observed from the roots as well as the cotyledonary node of the seedlings in 30 days. BAP 8.87 µM was the optimum medium giving response in almost 90% of the explants and produced 10 shoots per explant. The shoots elongated on plain WPM medium containing 0.2% charcoal and rooted on IBA 9.8 µM. 10 plants per seedling were obtained in a period of 3 months.

For the *in vitro* regeneration studies in *Garcinia talbotii* the seeds after removing the seed coat was cut into 3 parts and inoculated in WPM medium containing TDZ (0.045-9.08 µM), BAP (2.22-44.38 µM), BAP (2.22-44.38 µM) in combination with NAA 2.69 µM or BAP (2.22-44.38 µM) in combination with kinetin 4.65 µM. TDZ led to direct embryogenesis, and the embryos matured on BAP 0.44 µM and germinated on BAP 2.22 µM. TDZ 0.44 µM induced 132 embryos per seed but only 33% of the embryos converted into complete plants and survived in green house conditions. Secondary somatic

embryogenesis was also observed. Complete plants were obtained in 7 months. BAP at lower (4.44 μM) concentrations gave direct somatic embryogenesis. An average of 81 embryos per seed was produced on this media. All the embryos formed on BAP converted to plants in 5 months. BAP at higher concentrations and in combination with Kinetin gave organogenesis. BAP 8.87 μM+kinetin 4.67 μM gave the best response with an average of 140 shoots per seed. Complete plants were formed in 6 months. BAP in combination with NAA gave callus formation. While the protocol for somatic embryogenesis and organogenesis can be used for mass propagation as well as genetic transformation of the species, but for propagation of the elite plant in terms of (-)-HCA content propagation from mature explant is necessary. Apical and axillary buds from mature female trees were used as explant. The explants when cultured on BAP 8.87 μM+kinetin 2.32 μM produced 4.5 shoots per explant, the shoots were elongated on media containing 0.44 μM BAP or BAP 0.88 μM and GA_3 0.58 μM. The explants were then rooted and complete plants were obtained.

In the *in vitro* regeneration studies in *Garcinia spicata* the seeds after removing the seed coat were cut into three parts and inoculated on WPM medium containing TDZ (0.045-9.08 μM), BAP (2.22-44.38 μM), BAP (2.22-44.38 μM) in combination with NAA 2.69 μM or BAP (2.22-44.38 μM) in combination with kinetin 4.65 μM. TDZ led to direct embryogenesis, and the embryos matured and germinated on BAP 0.44 μM. TDZ 0.45 μM induced an average of 54 embryos per seed. Although all the embryos germinated only 30% converted into complete plants and survived in green house conditions. Thus only 16 plants per seed were obtained by embryogenesis in 7 months. Secondary somatic embryogenesis was also observed. BAP with NAA gave direct organogenesis. The buds elongated and formed roots on low BAP (0.44 μM). 60 plants per seed could be obtained in 6 months. The part of the seed had no effect on the regeneration capacity. BAP alone or in combination with kinetin did not show regeneration but led to normal germination of the seeds.

In the *in vitro* regeneration studies in *Garcinia xanthochymus* the seeds after removing the seed coat were cut into 7-8 pieces and inoculated on WPM medium containing BAP (2.22-44.38 μM), BAP(2.22-44.38 μM)+NAA(2.69 μM) or TDZ(0.04-

4.54 µM). Direct organogenesis was obtained on BAP and BAP+NAA. BAP at 22.19µM gave 100% response with an average of 115 shoots per seed. Complete plants were obtained in 6 months. Direct somatic embryogenesis was obtained on TDZ. An average of 321 embryos were formed in 90 days on TDZ 0.45 µM. But out of these embryos only 5 % embryos germinated and survived in green house conditions. Complete plants were obtained in 7 months.

The *in vitro* regeneration studies in *Garcinia* showed that the plants are recalcitrant in nature and being a tree species are extremely slow growing. The development of complete plants by the *in vitro* methods took on an average 6-7 months. However, the *in vitro* protocols developed could be used for the conservation of the rare and endangered species of this genus. *Garcinia indica* is listed as endangered but is a good source of (-)-HCA. The plant is grown by the local people for its fruits. The traditional methods used by the people for propagation of this plant -by seed germination by cuttings or by soft wood grafting are cumbersome and less successful. The *in vitro* techniques developed for germination or multiplication by organogenesis can be used for large scale propagation of this species. *Garcinia talbotii* is also listed as endangered. For the conservation of *Garcinia talbotii* although organogenesis produced an average of 140 shoots per explant, complete plants were formed in 6 months. Embryogenesis on the other hand generated 81 plants per seed in a period of 5 months. So propagation of this plant by embryogenesis would be a better option. *Garcinia spicata* is found extremely rare in Maharashtra. However, this plant can be introduced in this state. The *in vitro* regeneration protocols formed 16 plants per seed in 7 months by embryogenesis and 60 plants per seed in 6 months by organogenesis in 6 months. Thus this plant can be propagated by organogenesis. *Garcinia xanthochymus* is an introduced species and is found rare in Maharashtra but this plant can be introduced for large scale cultivation as an alternative to *G. indica*. This species can be propagated by organogenesis where 115 plants per seed can be produced in 6 months. Since all the four species could be regenerated by organogenesis/ embryogenesis, improvement of these plants by genetic transformation can also be done. For immediate capturing of elite characters or for selective propagation of female plants the protocol developed for the regeneration from mature trees has high potential.

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