IN-VITRO CONSERVATION AND GENETIC DIVERSITY STUDIES IN *GARCINIA INDICA* CHOISS. FROM MAHARASHTRA

A THESIS
SUBMITTED TO THE
UNIVERSITY OF PUNE

FOR THE DEGREE OF
**DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY**

BY
SWAPNA R. DEODHAR

**RESEARCH GUIDE
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Dedicated to My Parents

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ACKNOWLEDGEMENT

*If someone can demand that he has made me Doctor, is my **AAI- Mrs. Rekha Deodhar,** my **BABA- Mr. Ravindra Deodhar** and my Brother **Saket**. I owe my deepest gratitude and affection to them. No word would suffice to express my gratitude and, for their continuous showering of boundless affection on me and supporting me in whatever I chose or did. It is my parents' prayer, constant struggle and relentless hard work to overcome the odds of life, which has inspired me to pursue life with a greater optimism. The warmth and moral value of my parents have stood me in good stead throughout my life and I would always look up to them for strength no matter what I have to go through. This Ph. D. thesis is a result of the extraordinary wills, efforts and sacrifices of my parents. My successes are dedicated to them now and always.*

*I can't find the right words for the one person whom I like most, my husband **Rahul**. He has experienced all my ups and down. Without his patience, love and endless support this thesis wouldn't have been written.*

*Last but not the least; it is difficult to express my gratitude towards my in laws without whose encouragement and unstinted moral support, this endeavor would have never been possible. I would like to thank to my mother in-law **Mrs. Suvarnalata Sathe,** my father in-law **Mr. Shankar Sathe** and my brother in-law **Rohit,** who have been very patiently waiting for me to get this thesis done and made me indulgent from household responsibilities especially in my last crucial days of thesis writing.*

January 2010   *Swapna R. Deodhar*

CERTIFICATE

This is to certify that the work incorporated in the thesis, **"*IN-VITRO* CONSERVATION AND GENETIC DIVERSITY STUDIES IN *GARCINIA INDICA* CHOISS. FROM MAHARASHTRA"** submitted by **Ms. Swapna R. Deodhar,** 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.

Place – Pune

Date -   **Dr. (Mrs.) S.R. Thengane**

                                                                (Research Supervisor)

                                                                Dr. R.J.Thengane

                                                                (Co-Guide)

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **"*IN-VITRO* CONSERVATION AND GENETIC DIVERSITY STUDIES IN *GARCINIA INDICA* CHOISS. FROM 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.

Place – Pune

Date -   (Ms. Swapna R. Deodhar)

Key to abbreviations

°C	Degree Celsius
v/v	Volume/volume (concentration)
w/v	Weight/ volume (concentration)
ANOVA	Analysis of variance
ppm	parts per million
MS	Murashige and Skoog Medium (1962)
WPM	Woody Plant Medium
GR	Plant Growth Regulator
BAP	6-Benzyl amino purine
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
NAA	α-Naphthaleneacetic acid
IAA	Indole-3-Acetic acid
IBA	Indole-3-Butyric acid
2,4-D	2,4-di chlorophenoxy acetic acid
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)]
HCA	Hydroxy Citric Acid

ABSTRACT

Rationale of the study

Garcinia indica Choiss., family Clusiaceae, occurs in tropical rain forests of Western Ghats of India from 0 Mtrs to 1132 Mtrs above mean sea level. It is a polygamodioceous plant (Anonymous 1956).

Fruit rind of *Garcinia indica* is a rich source of (-)- Hydroxy Citric Acid (HCA) along with yellow pigmented garcinol and a dark red anthocyanin pigment. (-)- HCA is reported to possess biological activities; most important of it is its ability to inhibit lipidogenesis and thus acts as an antiobesity drug. Epidemiological data shows that the frequency of obesity in human beings is rising steeply, probably because of reduced physical activities and bad eating habits (Katsilambros N, 2002). Several synthetic antiobesity drugs like orlistat, fluoxetine etc. are available in market but side effects like heart diseases have been reported from these drugs (Linne MD *et. al.*, 2004). Though weight loss of 2 to 10 kg is reported upon use of these drugs, the weight regain after terminating drug therapy is a matter of concern (Bray GA, 1997). (-)-HCA in *G. indica* inhibits the conversion of carbohydrates to fats through an enzyme ATP – Citrate lyase and thus reduces cholesterol formation (Shara *et al.*, 2004; Preuss *et al.*, 2004; Jena *et al.*, 2002; Watson *et al.*, 1969). There are no side effects reported on use of (-)-HCA as antiobesity drug. A number of health products, which contain (-)- HCA or extract of *Garcinia* fruit rind, are in the market for the use as antiobesity drug (Vrikshamla - Himalaya Health Care, Citrimax Plus - Chrome Mate).

'Kokum butter' prepared from fruits is a specific remedy for dysentery and diarrhoea. Fruits act as demulcent and emollient (Chopra *et al.*, 1956; Nadakarni *et al.*, 1908). The fruit rind extract possesses antifungal and antioxidant properties (Salvi *et al.*, 2003). Yellow pigmented garcinol exerts anti-inflammatory effects and is neuroprotective (Liao *et al.* 2005). Fruit rind of *Garcinia indica* is traditionally used in curry preparation.

Tree is cross pollinated and of polygamodioecious nature where differentiation between male and female can be done only after flowering stage (7-8 years). Tree growth is very slow and in horticultural practices propagation is done by seeds and soft wood grafting (George et al., 2002). The seeds are recalcitrant due to high sensitivity to desiccation and freezing with shelf life of only 4 weeks (Malik et al., 2005).

Thus, it is not possible to raise seedlings throughout the years. In an orchard established with

seedlings, 50% plants are generally male.

Grafting is another method used for clonal propagation of *G. indica* (Nawala and Karmarkar, 1997; Haldankar et al., 1993). It is season dependent, space requiring. Grafted plants change in the canopy has been observed which reduces fruit yield. Thus it has limited applications. Availability of root stock is another limitation.

Garcinia indica being polygamodioceous plant, identification of female plants is a hurdle in the multiplication of this species. These difficulties can be overcome by application of *in-vitro* techniques, which may enable the production of a large number of female plants. In addition, micropropagation by somatic embryogenesis offers an alternative and efficient means for plant multiplication and moreover is a prerequisite in plant improvement programs.

There is wide a variation in size, shape, quality and yield of the fruits of *G. indica* according to different natural habitat. The average fruit yield per plant is approximately 10-15 Kg annually, but there are some plants, which yield 50 Kg per annum. This variation may be genetic and /or environmental.

In order to develop the strategies for plant improvement and preserve the unique germplasm, it is necessary to understand genetic diversity of *Garcinia indica*. Different molecular markers like ISSR / SSR can be used for study of genetic diversity.

The present research work entitled **"In-vitro conservation and genetic diversity studies in G*arcinia indica* choiss. from Maharashtra"** is designed to address some of these limitations.

Objectives of work:

a) To study the ecological distribution of *Garcinia indica* in Maharashtra.

b) Development of micropropagation protocol by clonal propagation / organogenesis / somatic embryogenesis for large scale propagation.

c) To study chemo diversity among the population of *Garcinia indica* occurring in Maharashtra with respect to -(-)HCA content using fruit rind.

d) To study Genetic Diversity among population of *Garcinia indica* occurring in Maharashtra using molecular markers.

Chapter 1. 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 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* and detailed information about species *G.indica* in particular.

Chapter 2. Materials and Methods

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

Chapter 3. Biodiversity studies

This chapter will be divided into three sections

- ### *Ecological distribution of Garcinia indica*

This chapter will give an introduction to *Garcinia indica* found in Maharashtra and their therapeutic uses. It will also contain the geographical distribution of the species in Maharashtra and the ecological parameters like latitude, longitude, MSL, soil type, Humidity of the locations. The chapter will also describe about Western Ghats of Maharashtra and   Konkan region of Maharashtra.

- ### *Genetic diversity of Garcinia indica*

The genetic diversity *G.indica* occurring in Maharashtra at the population level using ISSR markers is dealt with in this chapter along with Dendrogram and similarity matrix.

- *Analysis of (-)-HCA content in Garcinia indica*

This chapter will deal with the estimation of -(-)HCA content in the fruits of *G. indica* collected from different locations in Maharashtra and its quantitative analysis by HPLC.

Chapter 4. Repetitive Somatic Embryogenesis

This chapter will consist of *in vitro* regeneration of *Garcinia indica* by repetitive somatic embryogenesis using seed explant. For confirmation and origin of somatic Embryogenesis histological studies were carried out. Effect of different hormones like BAP, NAA on somatic embryo induction will be discussed. Effect of subculturing of primary somatic embryos on number of secondary somatic embryos will be highlighted.

Chapter 5. Clonal propagation of female plants

The chapter will deal with Clonal Propagation of female plants of *G. indica* using apical and axillary buds. Results on the optimization of sterilization protocol, influence of basal medium WPM and different hormones on bud sprouting, influence of TDZ on multiple shoot induction, shoot elongation and effect of IBA/IAA on root induction will be presented.

Chapter 6. Organogenesis

The chapter will describe the different parameters for induction of *de novo* shoot regeneration on roots of somatic embryo derived plantlets of *G. indica*. Effect of different stages of mother explant on this shoot induction will be discussed.

Chapter 7. Summary and Conclusion

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

1.1 Introduction

Throughout the ages, nature has provided humans with the sources of essential commodities for life including food, raw materials for manufacture of clothing, shelter and medicines. For centuries man has utilized the properties of plants for health and well-being.

There has been growing interest in the alternative therapies and the therapeutic use of natural products especially those derived from plants (Goldfrank, 1982; Vulto and Smet, 1988; Mentz and Schenkel, 1989). Herbal extracts and preparations still comprise the major part of primary health care for some 75–90% of the world's rural population. Plants still provide the basic raw materials for some 25% of prescription drugs (Farnsworth and Morris, 1976).

Natural products play a highly significant role in the drug discovery and development process. This was particularly evident in the areas of cancer and infectious diseases where, over 60% to 75% of these drugs were shown to be of natural origin (Cragg *et al*., 1997).

The transition from herbal preparations to defined, single chemical entity prescription medicine is well illustrated and marked by the discovery of the cardiac glycoside digitalis from the foxglove, *Digitalis purpurea*. The use of the foxglove in the British Isles and elsewhere goes back well before recorded history, to Celtic times, perhaps 2000 years or more ago and possibly derived from earlier Druidical influence.

In particular, higher plants have been the source of medicinal agents since earliest times and today they continue to play a role in the primary health care of about 80 % of the world's population (Famsworth *et al*., 1985). Natural products and medicinal agents derived from plants, are also an essential features in healthcare system of the remaining 20% of populations residing mainly in developed countries, with more than 50 % of all the drugs in the clinical use having natural product origin (Balandrin, 1993).

About 25% of the drugs prescribed worldwide come from plants out of which 121 active compounds are being in current use. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). In total, 119 plant derived compounds are used in western medicine, and of the worlds 25 best selling pharmaceutical agents, 12 are derived from natural products (Famsworth *et al.*, 1985; Baker *et al.*, 1995).

According to the OPS (Arias, 1999), a *medicinal plant* is -

(1) any plant used in order to relieve, prevent or cure a disease or to alter physiological

and pathological process, or

(2) any plant employed as a source of drugs or their precursors.

Recently the ability of chemists to synthesize purely artificial medicinal compounds lead to the production of many effective medicines. However, as new diseases and drug-resistant strains of existing pathogens continue to emerge, the potential of wholly synthesized compounds with simple structures and known modes of action has started diminishing. As such, attention is again being focused on natural sources of lead compounds, in which exists a wealth of more complex structures and novel modes of action.

This interest in the drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient (e.g. side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs resulting in side effects and other problems. In spite of the advances of synthetic chemistry and the contribution that it has made to the discovery of pharmaceuticals and the development during the later part of the 20th century, the use of herbal remedies and plant-derived drugs remains substantial. According to recent trade statistics, the apparently growing Pharmaceutical industry once again beginning to look at the plant kingdom as a source of chemical scaffolds for drug synthesis, often coupled with highly innovative molecular approaches.

Higher plants, many of which are threatened with extinction, are used as sources of pharmaceuticals as ingredients of traditional medicines and are of value in new drug discovery. Plant biotechnology offers the possibility of improved production methods of cultivated medicinal plants as well as alternative approaches to the production of natural products for the preparation of pharmaceuticals.

However, the potential use of higher plants as a source of new drugs is still poorly explored. Of the estimated 250,000–500,000 plant species, only 2% have been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties; in most cases, only pharmacological screening or preliminary studies have been carried out. It is estimated that 5000 species have been studied for medical use (Payne *et al*., 1991).

India is a treasure chest of biodiversity which hosts a large variety of plants and has been identified as one of the eight important 'Vavilorian' centers of origin of crop's diversity. Although its total land area is only 2.4% of the total geographical area of the world, the country accounts for 8% of the total global biodiversity with an estimated 49,000 species of plants of which 4,900 are endemic (Kumar and Asija 2000). The ecosystems of the Himalayas, the Khasi and Mizo hills of northeastern India, the Vindhya and Satpura ranges of northern peninsular India, and the Western Ghats contain nearly 90% of the country's higher plant species and are therefore of special importance to traditional medicine. Currently more than 75% of the herbal requirement is met through wild collections. While the demand for medicinal plants is increasing, their survival in their natural habitat is under growing threat. Species like *Rauwolfia serpentina, Terminalia chebula, Sapindus laurifolius, Jatropha curcas* are becoming uncommon in the Western Ghat forests (Anonymous, 2001). Collection of herbs from the wild by destructive harvesting followed by unscientific handling has resulted in poor quality products as well as extinction of many species.

A phytopharmaceutical preparation or herbal medicine is any manufactured medicine obtained exclusively from plants (aerial and non-aerial parts, juices, resins and oil), either in the crude state or as a pharmaceutical formulation.

1.2 Conservation

The nature is a potential source of useful drugs and its importance has been recognized since ancient time. Large numbers of medicinal plants are used to treat various diseases and some drugs in today's medicine are based on their traditional use (Verpoorte, 1998). Clearly, plants hold a prominent position in the availability of resources of natural bioactive molecules. The sessile character of plants required the development of large array of molecules to effectively respond to biotic and abiotic stimuli *in situ*. The chemical diversity of plants has made them the source of choice for the isolation of pharmacologically important metabolites. Metabolites derived from plants can be used in several different ways in the development of drugs.

Biodiversity, "the diversity of living forms" has attracted the great interest and concern because of its ability to provide large array of molecules for the development of drugs. Decline in the biodiversity is largely the result of human activities such as drastic transformation of natural landscape and deforestation. The consequence of reduction in biodiversity through loss of species constitutes the serious threat to human survival. The loss of plant species has reduced the availability of natural products used for futuristic drug developments. In medical science, loss of species could reduce the opportunity for treatment of disease through the loss of medical model and a new medicine.

The biodiversity found on Earth today consists of many millions of distinct biological species, which is the product of nearly 3.5 billion years of evolution. Rainforests are the examples of biodiversity on the planet, and typically possess a great deal of species biodiversity.

A tropical rainforest is an ecosystem usually found around the equator. They are common in Asia, Australia, Africa, South America, Central America, Mexico and on many of the Pacific Islands. Rainforests are home to half of all the living animal and plant species on the planet. Tropical rain forests are also called as the "world's largest pharmacy" because, over one-quarter of modern medicines originate from its plants. The rainforests are a perfect home to more species or populations than all other biomes

combined. Eighty two percent of the world's biodiversity could be found in tropical rainforests. Tropical rain forests have been subjected to heavy logging and agricultural clearance throughout the 20th century, because of which the area covered by rainforests around the world is rapidly shrinking. There are 25 biodiversity hotspots in the world which represent tropical rain forest.

In India, Western Ghats and North-Eastern Himalayas represent the biodiversity hotspots. Western Ghats of India, also known as the Sahyadri Mountains, is a mountain range along the western side of India. It 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. 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 meters. The area is one of the world's ten "Hottest biodiversity hotspots" and has over 5000 species of flowering plants, 139 mammal 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 as 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.
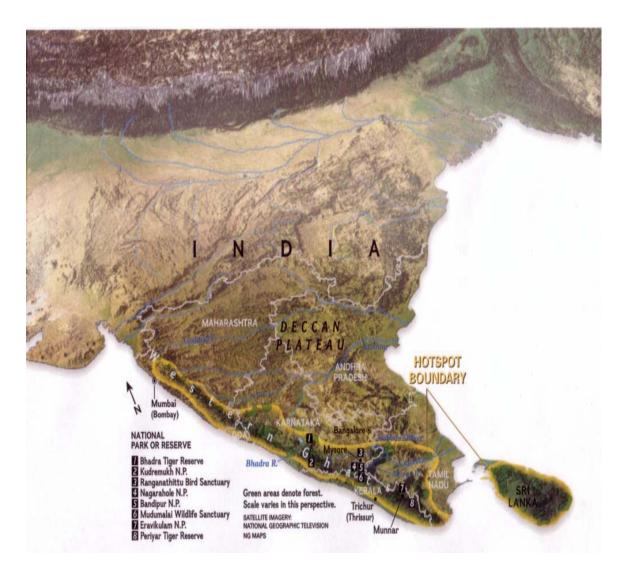
Figure 1.1 Map showing the Western Ghats of India

Conservation can be defined as is the science of protection and management of biodiversity. Sustainable conservation of forest resources is a major concern today. As modern medicine is getting more and more interest in medicinal plant therapies, the consequences of expanding population is over exploitation of important plant species. Conserving and recycling of plant genetic diversity is vital to meet future developmental needs. 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). It is necessary to preserve wild population of different plant species with their inherent intra specific diversity for further evolution.

Indian herbal Industry is at a blooming stage; however cultivation of medicinal plants is not so easy. It is a challenging task because less is known about their seed biology. Much effort has not been made to search elite specimens and their propagation. There is a need for a scientific approach for propagation of medicinal plants and to collect relevant information regarding agrotechnology, genuine planting material, economics of field cultivation, high yielding varieties etc. One has to explore wild medicinal plant species and to bring them under cultivation. Sometimes plants selected from the wild population may be suitable for the cultivation and there is no immediate necessity for any improvement programmes in it. Scientific cropping of industrially important medicinal plant is an integrated approach with agriculture and forestry with optimum land and water resources utilization. In India, various medicinal plants are cultivated and domesticated; therefore, International trade is looking to procure medicinal plant materials from India for the production of pharmaceutical, nutraceutical and cosmeticeutical preparations. India is also making efforts for cultivation, collection, characterization, planned hybridization programs for improvement and conservation of medicinal plants restoring their genetic resources for commercial cultivation. The breeders of medicinal plants play key role in the improvement of medicinal plants because they care for stable produce, uniform plant growth and maturity, economics to grow and good quality. Indian farmers are facing various problems in cultivation of medicinal plants because of lack of proper agro-technology, high fees for packages developed by various organizations, lack of reliable and standardized technology package, lack of planting material, market potential and system, cultivated vs wild plants, organic farming techniques, etc. In addition, Knowledge of post-harvest processing technology of plants for the extraction of chemicals and preparations of active formulation is lacking. For this purpose one has to take help of research organizations, private institutes, farmers, NGO's public sector institutes etc. for better technical know-how of cultivation and post-harvest techniques of medicinal plants. Role of women in

particular in harvesting most of this forest produce and collective fuel wood is of paramount importance. This has generated income for them and in turn improved quality of their life (Purohit and Vyas , 2007).

Conservation of genetic diversity of tropical recalcitrant seed species is presently being undertaken on a limited scale by maintaining trees in gene banks. *In vitro* conservation and cryopreservation offers the only safe and cost-effective option for long term conservation of genetic resources of recalcitrant species (Engelmann, 2000).

In situ conservation-

In-situ conservation means "on-site conservation". It is the process of protecting an endangered plant or animal species in its natural habitat, either by protecting or cleaning up the habitat itself, or by defending the species from predators. This term refers also to the conservation of genetic resources in natural populations of plant or animal species and is increasingly being applied to conservation of agricultural biodiversity in agroecosystems by farmers, especially those using unconventional farming practices.

One benefit of *in-situ* conservation is that it maintains recovering populations in the surrounding where they have developed their distinctive properties. Another is that this strategy helps to ensure the ongoing processes of evolution and adaptation within their environments.

For *in situ* conservation public awareness is required so that overexploitation of 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 –

Ex situ conservation is simply defined as the management (or conservation) of genetic resources or species outside their natural habitat. It is an off-site conservation

where endangered species of plant can be protected by removing part of the population from a threatened habitat and placing it in a new location.

Ex situ conservation methods include gene banks or cold storage facilities and other methods of cryobiological preservation of plant material like seed bank and collection of individual resource stocks maintained in botanical gardens, plant introduction and propagation facilities.

Although it is typically a simple task to remove some wild germplasm resources (or genetic resources) from their native environments and place them in an appropriate *ex situ* environments, it is impractical, if not impossible, to preserve the entire gene pool of a population, by *ex situ* means. By carefully and systematic sampling of the germplasm resources within a gene pool of a wild species, a significant proportion of the genetic diversity available can be adequately sampled for *ex situ* conservation (Oldfield, 1989). From the definition of *ex situ* conservation, botanical gardens have a significant role in the conservation of plant biodiversity.

Biotechnological approaches for conservation of medicinal plants-

Medicinal plants are of great interest in biotechnology which includes 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 made 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).

1.3 Micropropagation

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

Tissue culture is an important component of an integrated approach towards conservation. It offers great potential for rapid multiplication of superior individuals. The powerful technique of Plant cell and tissue culture, recombinant DNA and bioprocess technologies coupled with sophisticated analytical tools such as NMR, HPLC, GC-MS, LC-MS etc. have offered man kind a great opportunity to exploit the totipotent, biosynthetic and biotransformation capabilities of plant cells under *in vitro* conditions.

The application of micropropagation technique for medicinal plants gives many benefits to the breeder as it enables-

- ➢ Increase in propagation rate of plants
- ➢ Rapid multiplication of plants which in particular climate do not produce seeds or whose seeds have no germination capacity.
- ➢ Availability of plants throughout years i.e. in all the seasons
- ➢ Uniform plants of elite chemotype
- ➢ Conservation of genetic resources of species and threatened plants
- ➢ 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 regeneration by micropropagation can be achieved in three different ways-

1. regeneration from existing meristem

2. regeneration from adventitious meristem

3. regeneration by Somatic embryogenesis

Regeneration from existing meristem by Axillary bud proliferation

The principal advantage of axillary shoot methods for multiplication is that all propagules are derived from preformed buds, thereby enhancing the likelihood that propagules will be true to type. In this method, development and multiplication of the pre-existing meristem is achieved. From pharmaceutical point of view the most useful way of propagating medicinal plants is from existing meristem as by this method identical plants with desired traits are obtained. The plants regenerated from meristems are genetically identical with the donor plants (Hu and Wang, 1983).

Regeneration from adventitious meristem by Organogenesis

Organogenesis is *de novo* production of plant organ (bud, shoot, leaf, hypocotyls, seed and root) from organized tissue or callus. Organogenesis proceeds via *de novo* organization of meristem. It involves the induction of localized meristematic activity by treatment with phytohormones. It leads to formation of primordium and eventually the formation of shoot. Recently, genes which play critical roles in each phase of organogenesis *in vitro* are being identified largely through genetic analysis and some of them have been already isolated (Phillips, 2004) (Table 1.1).

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.

Table 1.1 Genes involved in shoot organogenesis

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

Regeneration by Somatic embryogenesis

It is the *de novo* production of structures resembling zygotic embryos, either from organized tissue or from callus. Structure classified as somatic embryos are bipolar and have no vasular connection to the source tissue. Somatic embryogenesis has been cited by many authors as the *in vitro* regeneration system of choice for economial prodution of clonal populations of forest trees (Gupta et al., 1991). A major advantage is the potential for very high frequency regeneration, as well as liquid cultures allow the production and

handling of thousands of embryos at one time (bioreactors). The similarity of somatic embryo with zygotic embryo, led to the idea of somatic embryo encapsulation to produce synthetic seeds which would facilitate mechanical handling and automated planting of somatic embryos. In a situation where a large number of plants are to be regenerated in a short time, somatic embryogenesis is promising as somatic embryos can be produced in astounding number per gram of callus (Sluis and Walker, 1985). For the commercial purposes they can be also used for the production of artificial seeds (Redenbough *et al.*, 1986). Propagation through somatic embryogenesis, have advantages over organogenesis, particularly direct somatic embryogenesis because it can potentially scale up propagation using bioreactors and produce synthetic seeds (Rani and Raina, 2000). Direct somatic embryogenesis has a lower probability of genetic variation than other propagation methods (Markle, 1997).   The general aspects about the specific genes involved in somatic embryogenesis are reviewed by Phillips *et al.*, 2004. The discovered genes were listed in the Table 1.2.

Table 1.2 Genes involved in somatic embryogenesis

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

Artificial seed technology involves the production of Tissue culture derived somatic embryos encased in protective coating. Artificial seed are also referred as Synthetic Seeds. Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that retain this potential also after storage.

Based on technology established so far, two types of synthetic seeds are known: *desiccated* and *hydrated*. The desiccated synthetic seeds are produced from somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyoxr) followed by their desiccation. Desiccation can be achieved either slowly over a period of one or two weeks sequentially using chambers of decreasing relative humidity, or rapidly by unsealing the petri dishes and leaving them on the bench overnight to dry. Such types of synthetic seeds are produced only in plant species whose somatic embryos are desiccationtolerant. On the contrary, hydrated synthetic seeds are produced in those plant species where the somatic embryos are recalcitrant and sensitive to desiccation. Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogel capsules.

Synthetic seed technology using encapsulation of vegetative propagules of woody plant species has become a potentially cost effective clonal propagation system. Encapsulation of vegetative propagules could be used for mass clonal propagation at a reasonable cost. In addition, these propagules may be used in germplasm conservation of an elite plant species and exchange of axenic plant materials between laboratories (Fabre and Dereuddre, 1990; Maruyama, 1997). Synthetic seeds, consisting of *in vitro* regenerated vegetative propagules encapsulated in a protective coating, have many advantages over organogenesis for propagation including ease of handling, potential long-term storage, high scale-up production and low cost of production.

In vitro regeneration studies in medicinal plants

The genetic diversity of medicinal plants in the world is getting endangered at an alarming rate because of ruinous harvesting practices and over-harvesting for production of medicines, with little or no regard to the future. Also, extensive destruction of the

plant-rich habitat as a result of forest degradation, agricultural encroachment, urbanization, etc. are other factors. Hence there is a strong need for proactive understanding in the conservation, cultivation, and sustainable usage of important medicinal plant species for future use.

An efficient and most suited alternative solution to the problems faced by the phytopharmaceutical industry is development of *in vitro* systems for the production of medicinal plants and their extracts. The *in vitro*-propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for biochemical characterization and identification of active constituents (Wakhlu and Bajwa, 1986; Miura *et al*., 1987). In addition, compounds from tissue cultures may be more easily purified because of simple extraction procedures and absence of significant amounts of pigments, thus possibly reducing the production and processing costs (Chang *et al*., 1992, 1994).

Large-scale plant tissue culture is found to be an attractive alternative approach to the traditional methods of plantations, as it offers a controlled supply of biochemicals independent of plant availability and more consistent product quality (Sajc et al., 2000). Tissue culture techniques can play an important role in the clonal propagation of elite clones and germplasm. The technique have been increasingly applied to many medicinal plants in particular for mass propagation, *In vitro* regeneration studies have been carried out in many medicinal plants by somatic embryogenesis or organogenesis (Table 1.3).

Table 1.3 *In vitro* regeneration studies in medicinal plants

Plant	Cultured explant	Response	Reference
Abrus picatorius	Epicotyl segments	Plantlets	Narula *et al*., 2001
Ammi majus	Nodal segments	Multiple shoots	Pande *et al*., 2002
Artemisia annua	Cotyledonary leaves	Multiple shoots	Gulati *et al*., 1996
Arnica montana	Nodal segments	Plantlets	Butiuc-keul *at al*.,2001
Azadirecta indica	Nodal segment	Plantlets	Joshi and Thengane, 1996
Bacopa monniera	Nodal segments	Callus, multiple shoots, plantlets	Ali *et al*., 1996
Cineraria maritima	Nodal segment	Plantlets	Banerjee *et al*., 2004
Cinnamomum camphora	Nodal segment	Plantlets	Babu *et al*., 2003
Clerodendrum inerme	Leaf segment	Callus, multiple shoot, plantlet	Baburaj *et al*., 2000
Datura innoxia	Anthers	Multiple shoots	Srivastava *et al*., 1993
Garcinia indica	Mature seed	Direct organogenesis	Malik *et al*., 2005
Garcinia mangostana	Leaf from seedlings and mature trees	Direct organogenesis	Goh *et al*., 1990
Garcinia mangostana	Leaf from seedlings	Direct organogenesis	Goh *et al*., 1994
Garcinia mangostana	Leaf from seedlings	Indirect organogenesis	Te-chato and Lin, 2000
Garcinia mangostana	Seed	Direct organogenesis	Normah *et al*., 1992
Nothapodytes foetida	Leaf segment	Plantlets	Thengane *et al*., 2001

Peganum harmala	Cotyledonary node with shoot tip	Multiple shoots	Saini and jaiswal, 2000
Phyllanthus caroliniensis	Nodal segment	Multiple shoots	Catapan *et al*., 2000
Rauwolfia serpentine	Shoot apices	Multiple shoots	Sehrawat *et al*., 2002
Rotula aquatica	Nodal segment	Plantlets	Martin, 2003
Sterculia foetida	Cotyledonary nodes	Multiple shoots, Plantlets	Anitha and Pullaiah, 2001
Stevia rebaudiana	Nodal segment	Multiple shoots	Sivaram *et al*., 2003
Tylophora indica	Leaf segment Callus Somatic embryos	Callus Somatic embryos Plantlets	Jayanthi and Mandal, 2001
Typhonium flagelliforme	Shoot buds (from rhizomes)	Plantlets	Sai *et al*., 2000
Valeriana jatamansi	Shoot buds	Multiple shoots Plantlets	Kaur *et al*., 1999
Vitex negundo	Nodal segment	Plantlets	Thiruvengadam and Jayabalan, 2000
Withania somnifera	Leaf segment	Multiple shoots Plantlets	Kulkarni *et al*.,1996

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 plants 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 1.4 *In vitro* productions of bioactive secondary metabolites

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	Tetrahydroanthr acene 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
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 glabra	Flavanoids	MS+IAA(1mg/l), Kinetin(0.1mg/l)	Callus	Ayabe *et al.,* 1990
Hyoscyamus niger	Tropane alkaloids	LS+NAA(10-5M), BA(5×10-6M)	Callus	Yamada and Hashimoto, 1982
Lithospermum erythrorhizon	Shikonin derivatives	LS+IAA(10-6M), Kinetin(10-5M)	Suspension	Fujita *et al.*, 1981
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

In order to obtain high yields suitable for commercial production, efforts have been focused on exploiting the biosynthetic capacity of cultured cells, achieved by optimizing the cultured conditions, selecting high producing strains, 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). 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 valuable molecule in a target plant. 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 1.5 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–	Zdravkovic-Korac *et al*.,

				plants	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

1.4 Genetic diversity

The development of molecular techniques for genetic analysis has led to a great increase in our knowledge. Understanding the structure and behavior of genomes will be useful for the improvement of tree. These molecular techniques, in particular the DNA fingerprinting has been used in monitoring DNA sequence variation in and among the species their by increasing their chances of finding a marker that segregates with the specific phenotype of interest. DNA markers have the advantage that they do not change in response to environmental factors or the developmental stage of a particular plant tissue.

Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based classification. DNA markers offer several advantages over traditional phenotypic markers, as they

provide data that can be analyzed objectively. Genetic markers can give insight not only into genetic processes in populations (e.g. mating system, gene flow), but also into past demographic events (e.g. bottlenecks, founder events), both kinds of information being necessary to understand population history to propose conservation measures.

Knowledge of genetic population structure of wild species related to cultivated types would be very useful in developing an efficient selection strategy. The exploitation of this genetic resource accompanied with identification of suitable accessions characterized by high genetic diversity may contribute to conservation practices.

As the tree species show variation, it is important to study the genetic composition of the germplasm of existing species (germplasm chacterization) and related species. This will not only provide information on their relationship but will also indicate a chance of finding new and useful genes, as the accessions with most distinct DNA profiles are likely to contain a greater number of novel alleles. DNA markers will provide information on the genetic composition of the germplasm, amount of genetic variation that exists among them and an access to the novel alleles.

DNA markers have also been used for testing the genetic fidelity during micropropagation/ *ex situ* conservation on the one hand and for characterization of plant genetic resources (PGRs) on the other. Molecular markers have particularly been suggested to be useful for confirmation of genetic fidelity in micropropagated tree species, where life span is quite long and performance of micropropagated plant could only be ascertained after their long juvenile stage in field conditions (Gupta and Varshney, 1999). A large number of RFLPs were recorded in some tree species like populous, eucalyptus etc., for studying the variation and genetic fidelity during micropropagation.

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

1. They are unlimited in number because the RFLP and PCR techniques are able to detect differences at the DNA level in both translatable and non translatable DNA regions.

2. They are phenotypically and environmentally neutral because sequence polymorphisms are detected directly at the DNA level and not by assaying gene products which may be influenced by alterations in gene coding sequence or by changes in the level of gene expression.

3. DNA markers mediated selection in breeding programs can be carried out in a non destructive process to the original plant because only a small amount of tissue is required.

4. They enable selection of specific recessive genotypes in a heterozygous form where the recessive genotype cannot express because a dominant allele masks a recessive allele. This advantage can be achieved with markers which are co-dominant in nature.

5. They do not show either dominant recessive interaction or epistasis and are devoid of pleotropic effect.

6. DNA markers in the genome can be considered as entry point to the genes. Thus, they facilitate the chromosome walking techniques for gene cloning.

7. DNA markers are not tissue specific and can be used at any stage of the plant growth.

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

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

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

Restriction Fragment Length Polymorphism (RFLPs), Random amplified polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphism (AFLPs), Simple Sequence Repeats (SSRs), Single Nuleotide Polymorphism (SNPs) etc., are some of the DNA markers in use. The relative advantages and disadvantages of some of these techniques are summarised in Table 1.6.

Inter simple sequence repeats (ISSR), a promising DNA marker, combines most of the benefits of AFLP and microsatellite analysis with the universatality of RAPD. The details regarding the nature and utility of this marker are dealt in chapter 2.

Table 1.6- Features of different DNA markers (Korzun, 1999)

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

1.5 Genus - *Garcinia*

The genus *Garcinia*, belonging to the family Clusiaceae (formerly known as Guttiferae) includes some 400 species found in the Old World tropics, especially Asia and Africa. Out of the 35 species found in India. 31 are native of India and others are introduced. Fourteen are endemic to the Western Ghats region (lying along western coastal India), thirteen in the Andaman and Nicobar Islands and four in the North-Eastern region of India.

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 *Garcinia* appears as symmetrical, medium-sized, straight-stemmed trees with horizontal branches. They produce new leaves in flushes at intervals. The *Garcinia* 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; close grained, yellowish white, red or grey. Exudates are often yellow and resinous. Branches are usually opposite, horizontal or pendulous. Flowers are polygamodioecious or

pseudohermaphrodite, the female or pseudohermaphrodite always in lesser number, axillary or terminal, solitary or in cymes, small or medium sized and hypogynous. Sepals are 4, decussate or 5, imbricate, rarely 2. Petals are 4, alternate with the sepals, imbricate, seldom 5. In Male flower: stamens are indefinite, rarely few, free or united surrounding a rudimentary pistil. Female flowers have minute, various free or united staminodes. Ovary in superior, bi to multilocular with solitary ovule. Fruit is a berry enclosing several large seeds. Embryo is a solid homogeneous mass (tigellus); cotyledons are either 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 and gama mangostin have shown inhibitory effect against topoisomerase I and topoisomerase II. 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*. Of these *Garcinia talbotii* and *Garcinia indica* are listed as endangered.

Garcinia indica or **kokum** is a fruit tree, of culinary, pharmaceutical, and industrial uses. It is a potential underexploited fruit and oil seed crop, currently getting

much commercial and medicinal importance. The tree is also ornamental, with a dense canopy of green leaves and red-tinged tender emerging leaves. It is indigenous to the Western Ghats region of India, along the western coast. It is found in forest lands, riversides, and wasteland, and also gets cultivated on a small scale. The crop prefers a warm and moderately humid tropical climate with a total rainfall range of 2500-5000 mm and grows under a mean annual temperature of 20-30^{0}c. The crop requires 60-80% humidity and grows at altitude of 800 meters.

Trees are 10-12 m tall with drooping branches. Young branches subterete irregularly striate, about 2.5 mm thick. Bark of the tree is generally light brown, shining, very thin, smooth. Wood is of grayish brown colored and hard. Leaves 6.5-11 x 1.5-4 cm, lanceolate or obvate-oblong, acute or acuminate, base contracted in petiole, membranous, shining, dark green, midrib prominent below. Petiole 5-10 mm long, slender.

Male flower – small, 4-8 in axillary and terminal fascicles, pedicels 6 mm long, sepals 4 yellowish –orange / pinkish orange, cariaceous, base narrow, petals thick

Female flowers – solitary, terminal, pedicels 3 mm long, ovary 4-8 locular, subglobose, stigma 4-8 rayed.

Fruit – Tree bears mostly dark purple to black or rarely white or cremish yellow fruit. Rind of kokum is sticky with curled edges when dry. It is smaller and thinner than most of the species in *Garcinia.*Fruit size 2.5–3 cm in diameter and encases 5-8 seeds. Freshly harvested fruits are redish green and turn to full red purple in a day or two.

Normal shelf life of fruits is about 5 days. Common method prescribed for preservation is sun drying. For this fresh fruits are cut into halves and fleshy portion with seeds is removed. Fruit rind or skin is then repeatedly soaked in juice or pulp during drying. Product obtained after sun drying is called amasul, ratamba or kokum.

Figure 1.1 – a: Habitat of *Garcinia indica*, b: Flowering twig, c: Fruits of *G. indica*

The process of manufacture of kokum syrup is simple and standardized. Fresh and ripe kokam fruits are cleaned and cut into 2 pieces and seeds are removed. Then these pieces along with sugar are kept in transparent glass jars and jars are kept under sunlight for about 8-10 days. With melting of sugar, juice is naturally extracted from fruits and over a period of time syrup is formed. This syrup is filtered and some preservatives are added into it before packing it in 1 ltr. capacity plastic jars. Seeds and shells of fruit are sold to oil extractors and other processors who make products for curry making. From 100 kgs. of kokam fruits, around 35 ltrs. of syrup is made whereas by-products like shell and seeds account for 15 kgs. Rests of the ingredients are waste or process loss.

Fruits have agreeable flavor and sweetish acid taste valued for its nutritional and medicinal properties. Kokum has been traditionally used as an acidulatant. Dried fruit rind is excellent substitute for tamarind and lime. Fruit of *G. indica* is anthelmintic, cardiotonic and useful in treatment of piles, dysentery, tumors, pains and heart complaints. Syrup from fruit juice is given in bilious affections. Root is astringent. Seeds yield a valuable edible fat known as kokum butter. It is extracted mostly in the cottage industry by crushing kernels and boiling the pulp by solvent extraction method. Yield of fatty oil is about 25%. Kokum butter is also used as substitute for cocoa butter due to its uniform triglyceride composition. Kokum butter is more quickly absorbed in skin without greasy feeling. Kokum butter is composed of beneficial compounds that help to regenerate skin cells. It is commonly used in skin heeling lotions, emollient and astringent. Various parts of trees like root, bark, fruit, seed are used for treating piles and abdominal disorders. Kokum rind contains 2 to 3 % anthocyanine pigment. It is a promising source of natural colorant for acidic foods. Processing conditions for this are standardized by CFTRI, Mysore.

Fruit rind of *G. indica* contains hydroxycitric acid –(-) HCA and garcinic acid. (-) HCA is a well known antiobesity drug due to powerful inhibitory action of citrate cleavage enzyme (Shara *et al.*, 2004; Preuss *et al.*, 2004; Jena *et al.*, 2002; Watson *et al.*, 1969). It inhibits the body's ability to store fat, possibly causing more fat from foods to pass through the body without being stored. It is suggested that, HCA may cause the

body to use existing body fat for energy during prolong exercise. Because of this property now a days *G. indica* is the most potent antiobesity herbal drug in the market. The derivatives of (-)-HCA have been incorporated into a wide range of pharmaceutical preparations in combination with other ingredients for the purpose of facilitating weight loss, cardioprotection, correcting conditions of lipid abnormalities and endurance of exercise. Many ayurvedic antiobesity products in market use fruit extract of kokum (eg. Himalaya drug for antiobesity-Citrimax).

Kokum fruit rind contains 2-3% Garcinol, a polyisoprenylated benzophenone derivative a yellow pigment. It has some antibiotic properties and has been found to be a potent inhibitor of histone acetyltransferase and is considered as a potential anti-cancer agent. Yamaguchi *et.al.*, 2000 reported antioxidative activity, chelating activity, free radical scavenging activity, and anti-glycation activity. They have suggested that garcinol might be beneficial as a potent antioxidant and a glycation inhibitor under specified conditions. Garcinol has also shown *in vivo* cancer chemopreventive activity against colonic aberrant crypt foci in an animal model. Therefore, the Japanese research group regards, benzophenone derivatives as useful candidates for drug development as anti cancer agents. They confirmed that garcinol has potent free radical scavenging activity in three kinds of free radical generating systems. Hydroxyl radical is regarded as the most dangerous ROS, and, therefore, garcinol is expected to be useful for preventing diseases caused by that radical, such as stress-induced gastric ulcer and nonsteroidal anti-inflammatory drug-induced gastric ulcer. These results suggest that garcinol, a free radical scavenger, may have potential as an antiulcer drug. Although the mechanism of its antiulcer activity is not yet understood, garcinol may scavenge reactive oxygen species on the surface of gastric mucosa, thus protecting cells from injury.

Some useful non traditional properties:-

1. Organogelation:- from Indian Institute of science, Bangalore, used Kokum fat to solidify organic liquids in 12 hours at room temperature and in just 1 hour at 4ºC. Further experiments showed that any saturated fatty acid that has between 10 and 31 carbon atoms can perform the same trick, although the gelling efficiency was more efficient with

smaller molecules. Moreover, saturated wax esters—a certain type of derivative of fatty acids used to make cosmetics--were just as efficient.

2. Kokum wine- The red Kokum juice has about 4 percent sugars and can be fermented to produce wine. Kokum wine is prepared in Goa using the traditional method with commercial bakers yeast. An improved method is also been developed using natural wild *Saccharomyces* sp.

3. Kokum honey - Honey is concentrated floral nectar. So far no efforts are reported to establish apiculture units in a kokum plantations but if this is done then 'Kokum honey' can be obtained with excellent medicinal qualities.

4. Kokum based sunscreens - The acidic juice contains pigments which strongly absorb rays in the DNA damaging Ultraviolet range. This property is useful in producing sunscreens in cosmetics industry.

5. Kokum pigment based pH indicators and biosensors - It has been recently reported that increasing alkaline conditions affect the colour of the fresh juice and this property can be utilized to develop pH indicators and pH sensitive biosensors for a pH range of 3-13.

United States Patent database –

List of Patents granted from 1976 on *Garcinia indica*

PAT. NO.Title

1. 6,818,234 Dietary food supplement containing natural cyclooxygenase inhibitors and

   methods for inhibiting pain and inflammation

2. 6,780,440 Herbal compositions and methods for diabetes and weight loss management

3. 6,770,782 Process for the production of potassium hydroxy citric acid, and

compositions containing the potassium hydroxy citric acid

4. 6,706,899 Acyclic chiral compound from garcinia acid and process for preparing the

   same

5. 6,489,492 Chiral derivatives of Garcinia acid bearing lactone ring moiety and process

   for preparing the same

6. 6,395,296 Soluble double metal salt of group IA and IIA of hydroxycitric acid, process

   of preparing the same and its use in beverages and other food products without

   effecting their flavor and properties

7. 6,391,928 Process for preparing a novel synergistic solid/semi-solid organic

   composition

8. 6,294,190 Antiobestic agent containing procyanidin as the active ingredient

9. 6,221,901 Magnesium (-)hydroxycitrate, method of preparation, applications, and

   compositions in particular pharmaceutical containing same

10. 6,217,898 Pharmaceutical composition comprising carnitine or alkanoyl Lcarnitine,

    for the prevention and treatment of diseases brought about by lipid metabolism

    disorders

11. 6,160,172 Soluble double metal salt of group IA and IIA of (-) hydroxycitric acid,

    process of preparing the same and its use in beverages and other food products

    without effecting their flavor and properties

12. 6,147,228 Convenient method for the large scale isolation of garcinia acid

13. 6,126,950 Composition useful for healing and protecting skin

14. 6,113,949 Weight control product and method of treating hyperlipidemia and increasing vigor with said product

15. 5,972,357 Healthy foods and cosmetics

16. 5,783,603 Potassium hydroxycitrate for the suppression of appetite and induction of weight loss

17. 5,656,314 Hydroxycitric acid concentrate and food products prepared therefrom

18. 5,536,516 Hydroxycitric acid concentrate and food products prepared therefrom

19. 4,594,194 Fat fractionation

20. 4,348,423 Method of preparing a cocoa butter substitute

21. 4,157,405 Cocoa butter substitutes and their preparation

22. 6,822,141 Diacylglycerol acyl transferase proteins

23. 6,713,117 Vegetable oil having elevated stearic acid content

24. 6,649,781 Recovery of minor components and refining of vegetable oils and fats

25. 6,517,883 Satiety product

26. 6,444,876 Acyl CoA: cholesterol acyltransferase related nucleic acid sequences

27. 6,103,292 Food products containing structured triglycerides

28. 5,679,393 Method of preparing fat fractions of vegetable origin enriched with

unsaponifiable materials and use of said fractions for preparing cosmetic and/or

pharmaceutical compositions, in particular dermatological compositions

29. 5,135,769 Fat and oil composition

30. 5,111,860 Compression log debarking apparatus

31. 5,023,101 Shortening for hard butter product and process for producing hard butter

product

32. 5,022,446 Compression log debarking apparatus

33. 4,956,287 Process for producing oleaginous composition

34. 4,948,618 Process for tempering edible plastic fat products

35. 4,940,845 Esterification process of fats and oils and enzymatic preparation to use

therein

36. 4,910,037 Tempering accelerator and use thereof in chocolate

37. 4,880,658 Obtaining a fat fraction with azeotropic solvents

38. 4,877,636 Chocolate and chocolate additive

39. 4,875,511 Apparatus for removing bark from logs

40. 4,874,699 Reaction method for transesterifying fats and oils

41. 4,844,940 Hard butter

42. 4,839,192 Hard butter composition containing triglycerides, and process for

production thereof

43. 4,839,191 Fat fractions and mixtures thereof

44. 4,735,900 Enzyme preparation for interesterification

45. 4,613,514 Cacao butter substitutes and chocolates containing the same

46. 4,601,857 Process for fat fractionation with azeotropic solvents

47. 4,420,560 Method for modification of fats and oils

48. 4,308,350 Method for producing cacao butter substitute

49. 4,276,322 Chocolate having defined hard fat

50. 4,219,584 Hard butter composition

51. 4,032,405 Method for producing cacao butter substitute

Composition of Fresh kokum rind

Moisture - 80%	Crude Fiber – 14.28%	Tannin – 2.85%
HCA - 9 -11%	Ascorbic acid – 0.06%	Starch – 1%
Protein – 1.92%	Total Ash - 2.57%	Crude fat – 10%
Pigment - 2.4%	Carbohydrates – 35%	

Propagation method

Tree is cross pollinated and of polygamodioecious in nature where differentiation between male and female can be done only after flowering stage (7-8 years). Tree growth is very slow and in horticultural practices propagation is done by seeds and soft wood grafting (George *et al.*, 2002). The seeds are recalcitrant due to high sensitivity to desiccation and freezing and have shelf life of only 4 weeks (Malik *et al.*, 2005). Thus, it is not possible to raise seedlings throughout the year. Provided in an orchard established

with seedlings, 50% plants are generally male. One of the possibilities of reducing number of male trees is gradual removal of non productive trees. Few female trees observed were more than 100 years old and still fruiting profusely. When propagated through seeds, male and female trees and very rare trees with both the sexes are obtained. Absence of differentiated embryo, endosperm and embryonic axis indicated that so called seed is not true seed. Germination characteristics of seed showed clear-cut presence of polarity. Regeneration of multiple seedlings from whole seed and seed pieces further indicated apomictic nature of seed. Based on seed characteristics and field examination of natural and raised populations, facultative agamospermy of *G. indica* is reported (Malik *et al*., 2005).

Grafting is another method used for clonal propagation of *G. indica* (Nawala and Karmarkar, 1997; Haldankar *et al*., 1993). It was found that inarch grafting is successful on 10 to 18 month old seedlings of Kokum, when done in the month of December-January. For grafting purpose about 3-4 month old up-right growing shoots from scion plant should be selected. If horizontal growing scion shoot is used, the graft will have straggling habit with top heaviness on one side. About 90% success is observed in inarch grafting method.

Recently soft wood grafting has been found to be successful and easier than inarching. For soft wood grafting 9-12 month old seedlings and 3 to 6 month old scions are suitable. The period from April-May is suitable. Graft union occurs within 2 months. About 80% success is observed in this method. However, the method is season dependent and space requiring. Grafted plants have been shown to change the canopy which reduces fruit yield. Thus it has limited applications. Availability of root stock is another limitation.

Area under cultivation of kokum is 1200 ha. in South Maharashtra and total production of 10200 tones with ripe fruits yield 8.5 tones/hact. Total export of kokum during 2001-2002 as dried pericarp was 2.56 tones valuing Rs. 216 million. Kokan krishi vidyapeeth, Dapoli, Maharashtra in 1997, has released early maturing variety with yield

of 138 kg/tree and average fresh fruit weight of 35 gms. Data on world area production and productivity are not available as it is native and endemic to India, but it is reported to be introduced to France, Bourbon and Mauritius as a crop.

Taking into consideration the above mentioned importance of the species the work was carried out with the following aim:

a) To Study the ecological distribution of *Garcinia indica* in Maharashtra.

b) To develop micropropagation protocol by clonal propagation / organogenesis /

   embryogenesis for large scale propagation.

c) To study chemo diversity among the population of *Garcinia indica* occurring in

   Maharashtra with respect to HCA content using fruit rind.

d) To study Genetic Diversity among population of *Garcinia indica* occurring in   \

   Maharashtra using ISSR markers

2.1 Introduction

This chapter describes the materials and general techniques routinely practiced in-
(A) Plant tissue culture, (B) Isolation and amplification of DNA and (C) Chemical analysis of -(-)HCA. The materials and methods, specific to particular experiment, are dealt in details in respective chapters.

2.2 Plant Tissue Culture

2.2.1 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 (25x150mm), conical flasks (250ml capacity), bottles with caps (200 ml capacity), pipettes (0.1, 0.2,1,2,5,10 ml capacity) and measuring cylinders (25ml, 100ml, 1000ml capacity) of Borosil, India were used for culturing the tissues and for preparation of media. Autoclavable screw cap bottles (100, 250 & 500ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (60ml capacity), slides (Blue Star, India) and cover slips (Micro-Aid, India). Plasticwares including sterile disposable plastic petriplates of 55 & 85 mm diameter were procured from Tarson, India.

Chemicals:

Chemicals used for surface sterilization procedures were Bavistin® (BASF, India), Savlon (Johnson and Johnson Limited, USA) and Mercuric chloride (Qualigens Fine Chemicals, India). A media additive sterilant, Plant Preservative Mixture (PPM) was procured from Sameer Science Laboratory, India. An antibiotic, Cefotaxime (Alkem, India) was often used for controlling the bacterial growth in plant tissue culture medium. Inorganic salts and vitamins used for preparation of culture media and for other experiments were of Analytical grade (BDH, Hi-Media and Qualigens Fine Chemicals, India). Sucrose was purchased from Hi-Media (India) and Qualigens Fine Chemicals (India). Agar agar (bacteriological grade), used as gelling agent in the semisolid culture

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

Growth regulators including Kinetin (KN), 6-benzyl adenine (BA), N-phenyl-N'-1, 2, 3-thidiazol-5-ylurea (Thidiazuron, TDZ), α-naphthalene acetic acid (NAA) Indole-3-Acetic Acid (IAA), Indole -3-Butyric Acid (IBA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were obtained from Sigma or Aldrich (U.S.A.).

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

Miscellaneous items-

> Distilled Water,
> Ordinary grade filter paper, Whatman filter paper, Brown Paper,
> Non Absorbent Cotton (Safe surgical industries, Beawar, India)
> Klin Wraps
> Forcep, Scalpel, Blade Holder, Surgical Blade (No. 11 and 24), Needles
> Autoclavable Bags, Zip lock bags.
> micropipette of different precision measurements (1000μl, 200μl, 100μl, 20μl, 10μl & 2μl) (Gilson, USA)

Equipments:

The major equipments used include:

pH meter: pH is the negative logarithm of hydrogen ion concentration. The measurement of pH in pH meter (Thermo Orion, USA) 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) used for quick weighing and for analytical purposes. This is a single pan balance capacity 100-200gm, sensitivity 0.1mg operating on 230 V 50 H2 AC mains. Precision of ± 0.005g, weighing range 0-1, 200g, digital read out was used for making stock solutions of growth regulators and for other fine weighing.

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

Laminar airflow ultra clean unit: All aseptic manipulations were carried out on this unit. In laminar (Klenzaids/ Microfilt, India), with the help of air pump air is passed through HEPA filters of pore size, 0.22 micron. Due to positive pressure, the entry of any contaminant is restricted from the open side of the bench. The instrument is fitted with UV tubes in addition to the fluorescent tubes.

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

Source of Explants:

The plant material was collected along the Western Ghats of Maharashtra. Fruits were collected in the month of March. Collected fruits were stored in zip lock polythene bags and stored at 4°c till further use. With the help of GPS (Geographical Positioning System, Magellan Sportrak, India) altitude, latitude and MSL of particular location were noted down. pH of the soil of particular location were taken by pen pH meter (Merck, India). Seeds were excised from these fruits aseptically. These seeds were used as explant for somatic embryogenesis and organogenesis.

For clonal propagation actively growing shoots were collected in different seasons of the year. Apical and axillary buds were excised from these shoots in laboratory. These buds served as explant for clonal propagation. Eight months old *in vitro* grown somatic embryo derived plantlets were used as explant for root organogenesis.

2.2.2 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 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. In case of tubes for liquid media, paper boat of Whatman Filter paper was prepared and inserted in tube before autoclaving. 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 alternately 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 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 *et al*., 1968), SH (Schenk and Hildebrandt, 1972), WPM (Lloyd and McCown, 1980) etc. are now available. In the present studies WPM and MS basal media 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	WPM medium
KNO_3	1900	400
NH_4NO_3	1650	-
$CaCl_2.2H_2O$	440	96
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
K_2SO_4	-	990
$Ca(NO_3)_2.4H_2O$	-	556
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0.83	-
$CuSO_4.5H_2O$	0.025	0.25
$Na_2MoO_4.2H_2O$	0.25	-
$CoCl_2.6H_2O$	0.025	-
$FeSO_4.7H_2O$	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.3
Myo-inositol	100	100
Thiamine-HCl	0.1	0.1
Nicotinic acid	0.5	0.5
Pyridoxine-HCl	0.5	0.5
Glycine	2.0	2.0

Stock solutions of growth regulators (GR) were prepared by adding few drops of appropriate solvent in 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

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 molten autoclaved media by micropipette at 60^{0}c and the media were allowed to solidify.

Media containing Plant Preservative Mixture (PPM): 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) was weighed and added in required quantity (1%, 2%) 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 before autoclaving. The media were then heated in water bath or steamer for the agar to melt. Molten medium was later 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 few drops of detergent (Labolene, Qualigens, India) and washed with distilled water. They were then 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 10 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. In case of petriplates, 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 followed by washing and stored in 70% ethanol till further use. Tissues were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (MP 58-60°C). Serial sections of 10 μm were cut using a rotary microtome (Reichert-Jung 2050, Germany). Sections were double stained with 1% Haematoxylin-1% eosin and mounted with DPX (Qualigens, India) for studies under microscope.

Preparation of Haematoxylene: 1g 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, filtered and stored.

Statistical Analysis:

Statistical analysis was used for comparison of treatment means during optimization of the parameters for micropropagation using both immature and mature explants, and for morphogenic response. For statistical analysis, analysis of variance (ANOVA) and Duncan's multiple range test were done by completely randomized block design (CRBD) using Agrobase 99 software and the angular transformation values derived according to Snedecor & Cochran, 1967.

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 Isolation and Amplification of DNA:

2.3.1 Materials

Glasswares and Plasticwares:

Micropipette of different precision measurements (1000, 200, 100, 20, 10 & 2μl) was procured from Gilson medical Electronics, France. Micro tips, eppendorff 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, (India). Storage vials (Laxbro, India) were used for storage of crushed leaf sample at -70^{0}C.

Reagents and Chemicals:

- Liquid Nitrogen
- Tris-HCl pH 8.0 (1 M); EDTA pH 8.0 (0.5 M); NaCl (5 M); CTAB (20%); NaCH$_3$COO (3 M); 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, 1.4 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)

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

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, India): 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 CA): It is used for maintaining the constant temperature, in which, temperature setting, temperature indicator, and cooling effect are also available.

SpinWin (Tarson, India): 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, India): Used 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, 1982).

PCR Robocyler (Stratagene, USA): It is microprocessor which utilizes 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.

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

Miscellaneous

➢ Mortal and pestle
➢ Distilled water
➢ Hand gloves
➢ Filter papers
➢ Spatula

Source of plant material:

Juvenile leaves of *Garcinia indica* were collected from 11 different locations in Maharashtra. The locations were usually 50 km apart from each other. Five plants from each location were sampled. Leaves of total 55 plants were collected. These leaves were kept in zip lock polythene bags and these bags were sealed. The bags were labeled with the location and date of collection. Bags were stored in thermacol box containing Stay Cool Packs to maintain the cold temperature for 4-5 days to avoid DNA damage. In

laboratory, leaves were taken out from polythene bags, washed with distilled water, dried on filter paper and then used for crushing with liquid nitrogen. Crushed leaf samples were kept in storage vials and stored in -70^0C refrigerator till further use.

2.3.3 Methods

DNA isolation protocol:

DNA was isolated using modified CTAB method (Khanuja *et al.*, 1999). The protocol is as follows:

➢ Collected crushed young leaves were stored in -70°C until used. 2.0 g of leaves were grind using mortar and pestle in the presence of liquid nitrogen.

➢ Added preheated (at 65^0 C for 40 min) 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 for 10 min.

➢ 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 3M $NaCH_3COO$ 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 TE 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 *et al*., (1986) was followed and carried out in a total volume of 25 μl containing following components: The optimized PCR reaction for *G. indica* is as follows.

- 1X Taq Buffer (Banglore GeNei, India, Mg^{++} free)
- 2.5 mM $MgCl_2$ (Banglore GeNei, India)
- 0.2 mM dNTPs (Banglore GeNei, India)
- 0.6 U Taq polymerase enzyme (Banglore GeNei, India)
- 0.25 μM primer (Table 2.3)
- 50 ng DNA template.

The cycle programme is initial 10 min denaturation at $94\ ^0$ C followed by 40 cycles of 60 sec at $94\ ^0$ C, 60 sec at 52^0 C, 90 sec at 72^0 C and final extension of 10 min at 72^0C.

- PCR amplifications were performed on a Stratagene RoboCycler under the following conditions (Figure 2.1).
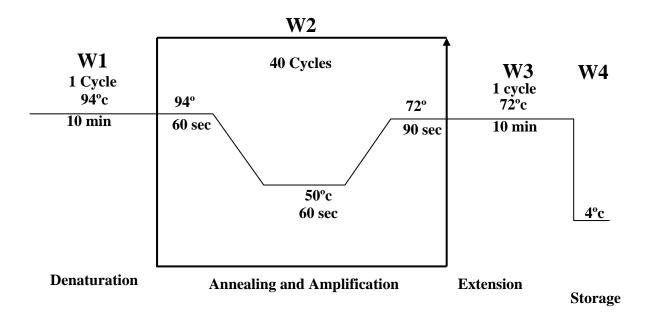
Figure 2.1 Schematic diagram of PCR reaction

- ➢ 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.

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 UPGMA. Cluster analysis was performed in TAB format using Win Boot software package.

2.4 Chemical analysis

2.4.1 Materials

Glasswares:

Autoclavable bottles (250 ml) with screwable 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 (Analytical Grade, Merck, India)
- Ethanol (HPLC Grade, Qualigens, India)
- 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 C_{18} (phenomenex) with diameter 3.90 X 300 mm and length 27 cm.

Miscellaneous:

- Chromafil Einmal filter - 0.45µm pore size (SGR Enterprises, Pune, India)
- Muslin cloth
- Spin Win

2.4.2 Methods

Preparation of Standard (-)-HCA:

The standard (-)-HCA used for quantification was procured from Cipex, Mumbai, India. Mg/ml stock of 80% standard (-)-HCA was prepared. This was centrifuged on spinwin to remove debris. 1ml supernatant was filtered and used as standard (-)-HCA.

Preparation of Samples:

Fruits of *Garcinia indica* were collected from 11 locations along the Western Ghats of Maharashtra. The locations were about 50 km apart. From each location effort was made to collect fruits from 5 plants. For the extraction of (-)-HCA from the fruits of *Garcinia indica* the method reported by Jayaprakasha and Sakariah, 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 filtration 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 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.

5 ml of this solution was dissolved in 20 ml of 10 mM H_2SO_4 and centrifuged. From this 1 ml solution was filtered and used for HPLC analysis.

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

> Mobile phase used was 10 mM H_2SO_4

> Column used was $C_{18.}$

> Flow rate 0.7 ml/min

> Chromatogram recorded at 214 nm at UV visible light.

> Retention time – 11.1 min

3.1 Introduction

"Terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; 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 organism 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 human, the earth supported more biodiversity than any other period in geological history. However, since the dominance of human, biodiversity has begun a rapid decline, with one species after another suffering extinction.

India has been divided into 15 resource development zones of which 14 fall in the main land and remaining one in the islands of Bay of Bengal and the Arabian sea. Maharashtra falls in the 9th zone known as the Western Plateau and hills region. The aim is regionalization of the Indian agricultural economy and to bring integration of plans of the agro climatic regions with the State and National plans and also faster agricultural development on regional basis.

Maharashtra is a state located on the western coast of India. It is the 3rd largest state of India located between 16 N to 22 N latitudes and 72 E to 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 is from July to September. There are large variations in the quantity of rainfall within different parts of the state. 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 ghat with an average of 600 to 700 mm. The rainfall variations from 500 to 5000 mm have been recorded with an average of 1000 mm distributed over 60-70 days.

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

1. South Kokan Coastal Zone
2. North Kokan 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

The Sahyadri Range is the physical backbone of Maharashtra. Rising on an average elevation of 1000m. It falls in steep cliffs, to the Konkan on the west. Eastwards, the hill country falls in steps through a transitional area known as Mawal to the plateau level. The 500 km long coastline between Mumbai and Goa on west coast is known as the Konkan region.

The Konkan, lying between the Arabian Sea and the Sahyadri Range is narrow coastal lowland, barely 50 km wide. Though mostly below 200 m elevation, it is far from being a plain country. Highly dissected and broken, the Konkan alternates between narrow, steep-sided valleys and low laterite plateaus. The Sahyadri Mountain range ("Western Ghats") forms the eastern boundary of the Konkan and the Arabian Sea marks the western boundary. The southern boundary is the Gangavali River. The Mayura River forms the northern boundary.

The region is placed in the northern section of the Western Ghats Biodiversity hotspot and forms part of the Sahyadri - Konkan corridor, one of the five major landscapes in the Western Ghats. Only 0.6% of the forests in Ratnagiri district within Konkan are currently protected in statutory forest reserves.

Figure 3.1: Map of India and Maharashtra showing districts

The soils in Konkan are mainly of alluvial and residual types. The residual soil occurs on the hilltops or slopes while alluvial is seen along the river valleys. Lateritic soil is formed in the hilly upland where the rainfall is more than 200 cm, which is rich in Fe, Al, Ti. Nevertheless, it is devoid of lime and poor in organic matter. Soil is lateritic in the south (Sindhudurg and Ratnagiri districts) while it is red, loamy and alluvial towards the north Thane district. There is wide variation in the physico-chemical properties of coastal salt affected soils in the Konkan. The region can broadly be classified in two types - Very high Rainfall Non-lateritic (VRN) soil zone of north Konkan and Very high Rainfall Lateritic (VRL) soil zone in the southern coastal parts. The soils from the VRN zone shows varying content and pattern of distribution of organic carbon throughout the soil depth while in the VRL zone it is in the range of low to very low in the profile. The available phosphorus is more in the soils from VRN zone than VRL zone, while the soils from both the zones are well supplied with available K. Both VRN and VRL soils contain adequate available Mn and Cu but are deficient in the micronutrients like B, Fe and Zn, which is a major problem in rice cultivation in these soils. The variation in the soil salinity according to the season is a common feature of coastal saline soils.

In general, Konkan belt experiences tropical warm, humid or marine type climate throughout the year with regular rainfall of an average 2286-2540 mm during monsoon and the temperature difference (between minimum and maximum) hardly exceed 10°C. Atmospheric temperature varies from 25-35°C with the average humidity ranging between 60-90%.

Biodiversity study of *Garcinia indica* species was carried out to evaluate the current distribution pattern of this species, to find out the agroclimatic conditions suitable to the plant and to locate the ecologically threatened locations of the plant.

3.2 Ecological distribution

3.2.1 Materials and Methods

For ecological mapping frequent trips were made to the Western Ghats of Maharashtra and datasheets were prepared. As the aim was to collect the data of female plants only, collection trips were made in the month of March as it is the fruiting season of *Garcinia indica.* Accessions were taken only from the wild plants of kokum.

The plants were identified and their geographical locations were noted down by means of GPS (Global Positioning System) which include latitude, longitude and elevation above mean sea level. The name of the village and district in which the plant occurred was noted. The soil conditions like pH and type of soil were also recorded. The observations on population structure, whether the plant exists singly or in population were also made. Each population studies were 50 kms apart from each other.

3.2.2 Results

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 800m. 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 these regions. Thus although the plant is listed endangered; the coastal region can be seen densely populated with this species. The soil found in these regions is red laterite / non laterite having pH of about 6.18. The soil is acidic having poor fertility. The rainfall in this region varies from 72-750 mm.
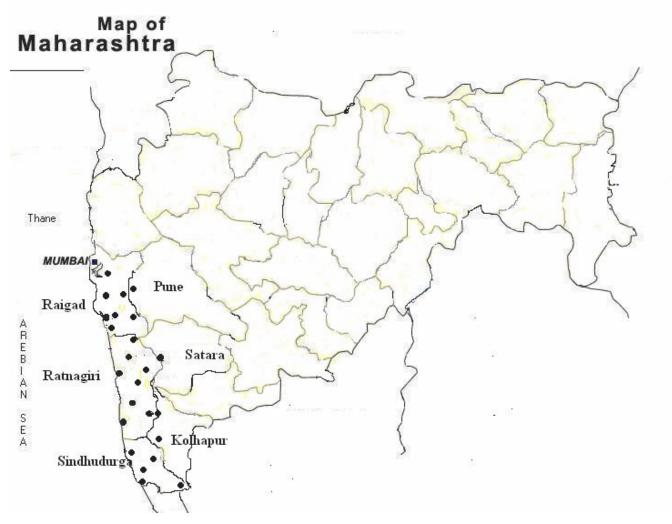
Figure 3.2 - Distribution map with locations of
Garcinia indica **in Maharashtra**

Table 3.1 - Ecological data of the different locations of *Garcinia indica* in Maharashtra

Sr. No.	Location	District	Latitude (N)	Longitude (E)	Altitude (m)	Single/ population	Summer temp.range	Winter temp.range	Rainfall (cm)
1	Amboli	**Kolhapur**	16º04.769´	74º06.027´	679	population	22- 40	10- 30.	750
2	,,	,,	16º01.174´	74º03.200´	682	population	,,	,,	,,
3	,,	,,	15º56.026´	73º58.541´	818	population	,,	,,	,,
4	,,	,,	15º56.11´	73º54.053´	39	Population	,,	,,	,,
5	Patgaon	,,	16º08.683´	73º57.699´	595	single	24-35	12-32	750
6	,,	,,	16º07.616´	73º54.228´	642	single	,,	,,	,,
7*	Shivdav	,,	16°14.36´	73°57.560´	--	single	--	--	--
8	Fonda Ghat	,,	16º22.402´	73º51.608´	532	population	22-40	12-32	378
9	,,	,,	16º21.141´	73º44.956´	110	Population	,,	,,	,,
10	,,	,,	16º21.302´	73º50.319´	110	population	,,	,,	,,
11	Radhanagri	,,	16º24.601´	73º59.975´	602	single	24-36	10-32	381
12	,,	,,	15º56.110´	73º54.530´	38	single	,,	,,	,,
13	Gaganbavda	,,	16º33.120´	73º50.747´	588	single	24-35	12-32	378
14	,,	,,	16º33.408´	73º51.474´	559	single	,,	,,	,,
15	,,	,,	16º33.296´	73º51.938´	566	single	,,	,,	,,
16	,,	,,	16º33.550´	73º52.030´	582	single	,,	,,	,,

17	Savantavadi	**Sindhudurg**	15º53.310´	73º49.579´	104	population	24-32	10-32	248.2
18	Bhedshi	,,	15º43.404´	74º01.787´	041	single	24-32	10-32	248.2
19	Danoli	,,	15º55.981´	73º54.728´	060	single	22- 40	10- 30.	235
20	,,	,,	15º55.981´	73º52.382´	046	single	,,	,,	,,
21	Achara	,,	16º13.888´	73º28.016´	002	single	22-40	12-33	227.5
22	Kankauli	,,	16º29.436´	73º22.707´	084	population	24-30	10-32	230
23	,,	,,	16º19.26´	73º43.021´	70	population	,,	,,	,,
24	,,	,,	17034.015´	73017.080´	64	population	,,	,,	
25	Deogad	,,	16º29.696´	73º22.707´	00	Single	25-32	20-32	224.7
26	,,	,,	16º01.397´	73º38.498´	020	Single	,,	,,	,,
27	,,	,,	16º01.397´	73º38.498´	020	Single	,,	,,	,,
28	Malvan	,,	15º53.280´	73º34.955´	160	single	22-40	12-33	227.5
29	Khanoli	,,	16º28.014´	73º34.955´	160	single	22-35	12-33	235
30	Vengurla	,,	15º51.459´	73º39.143´	014	Population	24-33	18-32	238
31	Ratnagiri	**Ratnagiri**	17º01.452´	73º17.557´	02	Population	25-32	19-32	378.7
32	,,	,,	16º59.057´	13º17.033´	03	Population	,,	,,	,,
33	,,	,,	16º59.057´	73º17.033´	40	Population	,,	,,	,,
34	Nevre	,,	17º06.062´	73º17.080´	78	Population	25-38	14-32	318.8
35	Malgund	,,	17009.963´	73016.104´	025	Single	25-38	14-32	318.8

36	Pirandavane	,,	17º04.381´	73º11.793´	30	Single	25-38	14-32	318.8
37	Pavas	,,	16º51.844´	73º19.775´	00	Population	25-32	19-32	378.7
38	,,	,,	16º51.844´	73º19.775´	00	Population	,,	,,	,,
39	,,	,,	16º52.023´	73º19.035´	00	population	,,	,,	,,
40	Savarde	,,	17º23.032´	73º31.067´	105	Population	24-39	14-32	318.8
41	Lanja	,,	16º54.054´	73º31.094´	128	Single	24-39	14-32	287.4
42	Sukondi	,,	17º51.35´	79º07.700´	135	Population	24-39	14-32	287.4
43	Guhagar	,,	17º29.043´	73º13.077´	10	Population	25-34	18-32	280.0
44	Rajapur	,,	16º29.090´	73º31.025´	54	Population	25-32	19-32	378.7
45	Mhasala	**Raigad**	18º15.018´	73º04.583´	98	Population	22-38	12-33	350
46	Shrivardhan	,,	18º25.915´	73º00.844´	05	Population	22-40	12-33	249.2
47	,,	,,	18º03.06´	73º00.78´	05	Population	,,	,,	,,
48	Diveagar	,,	18º10.238´	72º59.377´	05	Population	22-40	12-33	300
49	,,	,,	18º19.082´	72º57.41´	07	population	,,	,,	,,
50	Dabhol	,,	17º34.155´	73º11.395´	24	single	22-40	12-33	250
51	Alibag	,,	18º36.866´	72º54.259´	02	single	22-40	12-33	249.2
52	Murud	,,	16º51.844´	73º19.775´	00	population	22-40	12-33	217.7
53	,,	,,	18º19.085´	73º57.38´	09	population	22-40	12-33	217.7
54	Panvel	,,	18051.568´	73006.017´	33	single	22-40	12-33	250

55	Kelshi	,,	17º46.095´	73º11.93´	09	population	22-38	12-33	399.6
56	Chaul	,,	18º36.866´	72º54.259´	02	population	22-40	12-33	249.2
57*	Khandala	,,	18°3'	74°1'	666	population	22-36	12-30	450
58*	Shivthar	,,	18º09.715´	73º36.836´	115	--	22-40	12-33	250
59	Mulshi	**Pune**	18º28.079´	73º27.023´	610	Population	20-38	6-28	72.2
60	,,	,,	18º27.825´	73º26.114´	616	Population	,,	,,	,,
61	,,	,,	18º28.410´	73º27.023´	610	Population	,,	,,	,,
62	,,	,,	18º28.247´	73º24.929´	600	Population	,,	,,	,,
63	,,	,,	18º25.482´	73º24.161´	541	Population	,,	,,	,,
64	,,	,,	18º28.235´	73º24.888´	580	population	,,	,,	,,
65	Varandha Ghat	,,	18º06.387´	73º37.494´	811	population	22-36	10-28	450
66	Pratapgad	**Satara**	17º57.216´	73º34.646´	1132	population	16-29	5-24	500
67	Ghodbunder	**Mumbai**	18º16´	72º55´	11	--	25-35	15-25	220
68	Victoria gardens	,,	18º55´	72º54´	11	--	,,	,,	,,
69	Matheran	,,	18°58.60´	73° 16'	677	--	20-30	12-25	524

Lot of difference was seen in shape and maturity of fruits. Following photographs shows the difference in shape of fruit according to different locations collected in the same season.
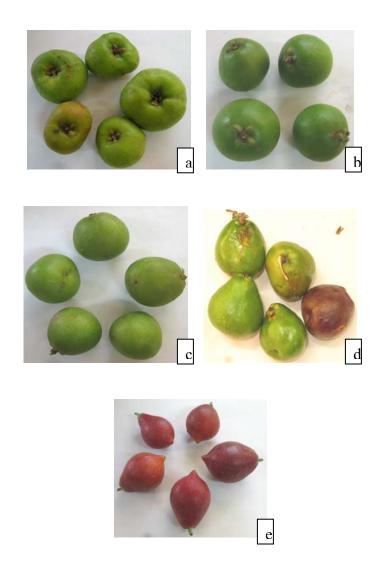
Figure 3.3 - Fruits of *G. indica* collected from different locations
a.Amboli, b.Guhagar, c. Pratapgad, d.Diveagar, e.Pavas

Fruits from Diveagar and Pavas (Figure 3.3 –d,e) show difference in shape of fruits, these are spindle shaped, while the fruits of Amboli, Guhagar and Pratapgad (Figure 3.3 – a,b,c) were round in shape. The reason behind this may be the sex of the plant (bisexual or female). Information regarding relationship between shape of fruit and sex of the plant is mentioned further in the chapter.

Along with difference in shape, maturity of the plant also varies according to different location. Plant growing at 0 MSL (Pavas) (Figure 3.3–e) show early maturation while plants growing at higher altitude i.e. 1132 MSL (Pratapgad) (Figure 3.3–c) show immature fruits in the same season.

Figure 3.4 - Fruit bearing capacity of plant at Diveagar

Plants growing at diveagar show high fruit bearing capacity than plants growing at other locations (Figure 3.4). These plants can be considered as elite plants for fruit yield.

Figure 3.5- Difference in shape of leaves a. Diveagar, b.Pratapgad

Along with difference in shape and maturity of fruits, leaves also showed difference in shape. Plant growing at Pratapgad had elongated leaves than normal leaf shape of *G. indica* (Diveagar) (Figure 3.5).

According to variation in altitude, change in temperature and rain fall of the particular location was also reported. This may be the reason behind change in leaf, fruit shape and maturity of fruits. Similar effect of altitude on variation in leaf and fruit is noted by Hovenden and Vander, 2006 in *Nothofagus cunninghamii,* Mehmet *et al.,* 2005 in Walnuts, George *et al.,* 2007 in *Clinopodium vulgare,* Krishnan *et al.,* 2000 in *Acalypha indica.*

Karnik (1978) has carried out detailed study on the types of flowers found in *Garcinia indica.*

According to his observations different flower types found are-

Type I - This is a typical flower of male tree with long pedicel and numerous stamens. All the stamens are fertile and the rudimentary pistil is absent.

Type II- This flower is found on male trees. It has long pedicel with numerous stamens as type I. All the stamens are fertile. The flower has no functional pistil, although rudimentary structure is present in the place of pistil. The structure is conical in shape, measuring about 0.5mm in length. Cross section of the pistil shows absence of ovary cells.

Type III – This flower is found on male and bisexual trees. It has long pedicel. The stamens are numerous, ranging from 30 to 40 and all the stamens are fertile. The stamens near rudimentary pistil are normally distributed, but those at the periphery appear to cluster at 4 corners. This indicates the tendency of stamens to separate into 4 tufts as noticed in female and bisexual flowers. The rudimentary pistil exhibits further increase in size, measuring about 1 mm in length. It is enlarged at the base and pointed at the apex. The stigma portion is absent. The cross section of the pistil shows absence of ovary cells. The enlargement of pistil at the base indicates tendency of the pistil to become globose or round as noticed in female or bisexual flower.

Type IV – This flower type is found on male and bisexual trees. It has long pedicel. All the stamens are fertile. The number of stamens and their tendency to fall into 4 tufts is similar to that observed in type III. The rudimentary pistil becomes more enlarged than in type III. It is 1.5 to 2 mm in length and moderately tapering towards the apex. The cross section of the pistil fails to show any presence of ovary cells, indicating strictly

rudimentary condition. The stigmatic surface makes its first appearance. It is asymmetrical, non-peltate, non-tuberceled and measuring about 0.6 mm in cross section.

Type V- This flower is observed on male and bisexual trees. It has long pedicel. All the stamens are fertile. The number of stamens ranges from 25 to 40. This decrease in the number of stamens indicated its decline from the maleness. The tendency of the stamens fall into 4 tufts is still noticeable. The stigmatic surface is enlarged; it is peltate having 3 to 4 lobes. The individual lobes are irregular with the absence of tubercles. The pistil becomes more enlarged than in type IV, measuring about 2 mm in length. Pistil is rudimentary in condition.

Type VI – This flower is found on male and bisexual flowers. Stamens are fertile with long pedicel. Pistil is reduced in length. Cross section of the pistil shows the presence of overy cells. The stigma surface shows further development. Stigma surface is peltate, having 3-4 lobes. The tendency of pistil to become globose in shape as noticed in female flower and the stigmatal development indicates the stronger tendency of this flower towards attainment of female characters.

Type VII – The flower is observed on bisexual trees only. It has long pedicel. The number of stamens ranges from 20 to 30, arranged in a whorl in radial fashion. The individual stamens are placed slightly apart from each other. All the stamens are fertile. The pistil is about 2 mm in length, tapering moderately towards both the apices. The shape resembles the spindle. The cross section of the pistil shows ovary cells ranging from 3 to 5 and irregular in shape. Stigma surface is peltate having 3 to 5 lobes with prominent tubercles on each lobe. The lobes may be symmetrical or asymmetrical. The decrease in the nmber of stamens with tendency to fall apart and well developed pistil indicates the further advancement towords femality. This flower sometimes bears spindle shaped fruit containing 0 to 2 seeds.

Type VIII – This flower is observed on bisexual trees only. The flower may have long or short pedicel. The number of stamens ranges from 12 to 25. All the stamens are fertile. The cross section of the pistil exhibits 4 to 6 ovary cells. Stigma surface is symmetrical. The fruit born by this type are spindle shaped containing 2 to 4 seeds. This is normal bisexual flower.

Type IX – This flower is found on bisexual trees only. The flower may have long or short pedicel. All the stamens are fertile. Stigma surface is symmetrical. The fruit born

by this type of flower is round to globose in shape, containing 2 to 6 seeds. This is normal bisexual flower.

Type X – This flower is observed on female trees only. It has short pedicel. The anthers are empty and contain no pollen. The staminodes are arranged in 2 tufts opposite to each other. Stigma surface is symmetrical. The fruit may be round to globose in shape, containing 2-6 seeds. This is a female flower.

Type XI – This flower is found on female trees only. It has short pedicel. The anthers are empty and contain no pollen. The staminodes are arranged in 4 tufts in decussate fashion. Stigma surface is symmetrical. The fruit may be round to globose in shape, containing 2-7 seeds. This is a female flower.

Sex types observed by Karnik are –

Type I – Male tree

Trees bear flowers which are characteristically of Type I and rarely Type II and III. They are incapable of producing any fruits, but serve as pollinators.

Type II – Hermaphrodite or bisexual

Tree bears predominantly IV, V, VI, VII, VIII and IX type of flowers. Fruits produced by this type are generally irregular in shape and varied in size containing 0 to 6 under developed seeds.

Type III – Female Tree

This tree bears flowers which are characteristically of Type XI and in few cases Type X. The adult tree bears heavy crop. The fruits are globose to round and dark red in color and contain 1 to 7 well developed seeds.

It can be seen from the present study that sex variation on kokum is on the basis of increasing functionality of pistil as noticed by gradual morphological development of the stigma and pistil in concurrence with decreasing stamen number and their grouping tendency.

3.2.3 Discussions

After conducting many collection tours to Western Ghats of Maharashtra, it can be concluded that though *Garcinia indica* is listed as endangered species in southern Western Ghats of India, the plants are well distributed in coastal regions of Maharashtra. But, the

population of female plants is less. Moreover, as the male plants do not bear fruits, the plants are being removed by farmers which reduce the pollination chances. Here comes the necessity for conservation of female plants by biotechnological approach.

The plants are distributed from 0 mtrs to 1132 mtrs altitude. Change in altitude results in change of other ecological parameters like temperature, rain fall, soil type, soil pH. All these parameters along with adaptation to changing environment results in variation in leaf shape, fruit shape, fruit bearing capacity of the plant and time of maturity of fruits according to different locations.

3.3 Genetic diversity studies

3.3.1 Introduction

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

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

Characterization and evaluation as applied specifically to plant genetic resources refers to the description of the material in a collection (Phenotypic characterization). Characterization usually involves taxonomic confirmation of the accessions and biochemical or molecular discrimination between germplasm accessions using highly heritable and easily visible traits. Classical methods of estimating genetic diversity or relatedness among the plants rely upon morphological characters. Several studies based on morphological characters were carried out to understand the degree of variation within the species. In *Gaultheria fragrantissima*, Paulsamy *et al.*, (2007) estimated the degree of variability present between the four ecological variants and reported the leaf thickness and oil content were highly correlated with the population density. Kaura *et al.*, (1998) studied the seed morphology (seed length and seed weight) and oil content in Neem from five provenances and reported that seed oil content from most of the provenances was not consistently and significantly correlated with the morphological parameters of the seed. Molecular and biochemical markers avoid many of the complications of environmental factors by looking directly at the variation controlled by genes or DNA fragments.

Hamrick *et al.*, (1992) concluded that tree species maintain more intraspecific variation than other life forms but generally display less variation between populations, particulary in the case of widespread species with out crossing breeding systems and wind or animal dispersed seeds.

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

Genetic polymorphism is classically defined as the simultaneous occurrence of two or more discontinuous variants or genotypes of a trait in the same population. Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is very expensive and laborious. A wide variety of techniques have, therefore been developed in the past few years for visualization of DNA sequence polymorphism.

The term DNA fingerprinting was introduced for the first time by Jeffery in 1985, to describe bar code-like DNA fragment patterns generated by hybridization with multilocus probes after eletrophoretic separation of genomic DNA fragments. The emerging pattern makes up a unique feature of the analyzed individual and is currently considered to be the ultimate tool for biological individualization.

Population genetics parameters are comparable across studies and are closely associated with various life history traits (e.g. Hamrick & Godt 1989, 1996), thus having the potential to produce information with important implications in evolutionary biology and ecology as well as in conservation biology. Nevertheless, practical constraints (e.g. insufficient amount of easily accessible plant tissue) as well as low levels of polymorphism have brought about an increasing interest in DNA-marker based methods. Among these, Polymerase chain reaction (PCR)-derived markers obtained with nonspecies specific primers have become exceedingly popular since they do not request sequence information for the target species. Consequently, these methods are especially suited to a situation where little or no molecular genetics research has been conducted previously, which is true for the majority of our wild plant species (Hilde Nybom, 2004).

Woody species maintain more variation within species and within populations than species with other life forms but have less variation among populations. Woody species with large geographic ranges, outcrossing breeding systems, and wind or animal-ingested seed dispersal have more genetic diversity within species and populations but less variation among populations than woody species with other combinations of traits. Although life history and ecological traits explain a significant proportion (34%) of the variation among species for the genetic parameters measured, a large proportion of the interspecific variation is unexplained. The specific evolutionary history of each species must play an important role in determining the level and distribution of genetic diversity (Hamrick *et al*., 1992).

Types and description of DNA markers

Single or low copy probes:

Restriction fragment length polymorphism (RFLP). RFLPs are simply inherited, naturally occurring, Mendelian characters. They have their origin in the DNA rearrangements that occur due to evolutionary processes, point mutations within the

restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over. RFLP markers were used for the first time in the construction of genetic maps by Botstein *et al*., 1980. RFLPs, being codominant markers, can detect coupling phase of DNA molecules, as DNA fragments from all homologous chromosomes are detected. They are very reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in an individual, an information highly desirable for recessive traits (Winter and Kahl, 1995)

However, their utility has been hampered due to the large amount of DNA required for restriction digestion and Southern blotting. The requirement of radioactive isotope makes the analysis relatively expensive and hazardous. The assay is time-consuming and labour-intensive and only one out of several markers may be polymorphic, which is highly inconvenient especially for crosses between closely-related species. Their inability to detect single base changes restricts their use in detecting point mutations occurring within the regions at which they are detecting polymorphism. RFLP markers converted to PCR based markers are Sequence Tagged Sites (STS), Allele Specific Associated Primers (ASAP), Expressed Sequence Tag markers (EST) and Single Strand Conformation Polymorphism (SSCP) (Orita *et al*., 1989).

Multilocus probes :

Microsatellites and minisatellites - The term microsatellites was coined by Litt and Lutty, (1989) while the term minisatellites was introduced by Jeffrey *et al*., (1985). Both are multilocus probes creating complex banding patterns and are usually non-species specific occurring ubiquitously. They essentially belong to the repetitive DNA family. Fingerprints generated by these probes are also known as oligonucleotide fingerprints. The methodology has been derived from RFLP and specific fragments are visualized by hybridization with a labelled micro- or minisatellite probe.

Inter simple sequence repeat markers (ISSR) - Reported by Zietkiewicz *et al.* (1994) in this technique, Primers based on microsatellites are utilized to amplify inter-SSR DNA sequences. Here, various microsatellites anchored at the 3' end are used for amplifying genomic DNA which increases their specificity. These are mostly dominant markers,

though occasionally a few of them exhibit codominance. An unlimited number of primers can be synthesized for various combinations of di-, tri-, tetra- and pentanucleotides [(4)3 = 64, (4)4 = 256] etc. with an anchor made up of a few bases and can be exploited for a broad range of applications in plant species.

ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Gupta *et al*., 1994; Tsumura *et al*., 1996; Joshi *et al*., 1999; Wang *et al*., 1998). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wang *et al*., 1998; Reddy *et al*., 2002). This technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility possibly due to the use of longer primers (16-25 mers) as compared to RAPD primers (10-mers) which permit the subsequent use of high annealing temperature (45-60º C) leading to higher stringency. This technique is found to be stable across wide range of PCR parameters (Bornet *et al*., 2001; Reddy *et al*., 2002) and the potential for integrating ISSR-PCR into programs of plant improvement is enormous.

ISSR have several advantages due to which it becomes more reliable than other techniques:

(1) the technology is easy to use (low cost and low amount of DNA required)

(2) DNA sequencing is not required to develop experiments
    (Ghariani *et al*., 2003).

(3) ISSR reveal a much higher number of polymorphic fragments per primer than
    RAPD (Fang and Roose, 1997) (Esselman *et al*., 1999)

(4) ISSR produce more reliable and reproducible bands compared with RAPDs
    because of the higher annealing temperature and longer sequence of ISSR
    primers (Tsumura *et al*.,1996; Nagaeko and Ogihara, 1997; Qian *et al*.,2001).

Garcinia indica: Use of ISSR markers

Being a polygamodioecious and cross pollinated plant, variation in the characters like height, spread, volume and physio-chemical parameters of fruit such as fruit shape, size, color, no.of fruits /plant (yield), variability in bearing habitat and quantity of HCA exists.

Maintenance of variation within the species is essential for their long-term survival and fitness. A good understanding of level and distribution of genetic variability within the species is vital for the development of strategies for their effective conservation and utility. Hence, there is a need to study the existing variation within the species. Furthermore, occurrence of genetic variation is a matter of great concern where commercial success in micropropagation depends solely on the maintenance of clonal uniformity (Bonga and Durzan, 1987).

The objective of this study is to determine the level of genetic variation among natural populations of *Garcinia indica* collected from Western Ghat regions of Maharashtra state.

3.3.2 Materials and Methods

The accessions used in the study consisted of 11 spontaneous populations collected from Maharashtra state during fruiting season i.e. March so as to collect the leaves of only female plants. The range of habitats is described in Table 3.2. Each population taken for this study is at a distance of minimum 50 kms from each other and consists of 5 plants each. The young leaves were collected for experiment and the location, altitude, latitude, longitude, soil pH, fruit size, shape, leaf size, shape etc. were also recorded. All sampled leaves were stored at -70^0 C in sealed polythene bags.

Morphological Characterization: Along with young leaves, fruits of the plants were also collected. Shape, size and color of the fruit were noted down.

DNA was isolated from fresh leaf material collected from 55 trees, using modified C- TAB method reported by Khanuja *et al*., 1999. The details of DNA isolation, checking of purity and PCR protocols are described in chapter 2.

Various parameters including (a) Sample DNA concentration (20ng to 80 ng/ reaction) and (b) *Taq* polymerase (0.4 to 0.8U/ reaction) were tested to optimize PCR conditions. These parameters were tested in three randomly chosen DNA samples using ISSR primer 857.

Hundred ISSR Primers of Set #9 (801…900) obtained from University of British Columbia (UBC), Canada, British Columbia, were screened for amplification using two randomly chosen DNA samples of *Garcinia indica.* Twelve (817, 827, 830, 835, 836, 840,

848, 855, 856, 857, 868, 880) 'UBC set #9' ISSR primers, which gave reproducible and unambiguous amplification products, were chosen for variability studies. DNA samples of trees were amplified using these 12 primers.

PCR amplification was done individually for each primer and the experiment was repeated twice. A low range (3 Kb) DNA ladder was used for band sizing. Presence/absence of each scorable fragment was recorded in a binary data matrix and the data were analyzed using Win Dist and Win Boot software programs.

3.3.3 Results

In the preliminary studies, repeatability of bands was examined by amplifying and running the PCR product of 55 plants from 11 populations with 5 plants from each population. As no significant differences were obtained in banding pattern of plants from same population (Figure 3.6), DNA of 1 plant from one population were taken as representative of particular location.

Table 3.2 Geographical parameters of *Garcinia indica* populations sampled

| No.	Location	District	Altitude (meters)	North Latitude	East Longitude
1	Pavas	Ratnagiri	0	$16^0 52^, .23$	$73^0 19^, .35$
2	Chaul	Raigad	2	$18^0 36^, .86$	$72^0 54^, .259$
3	Diveagar	Raigad	7	$18^0 19^, .82$	$72^0 57^, .41$
4	Murud	Raigad	9	$18^0 19^, .85$	$72^0 57^, .68$
5	Guhagar	Ratnagiri	10	$17^0 29^, .43$	$73^0 13^, .77$
6	Amboli	Sindhudurga	39	$15^0 56^, .11$	$73^0 54^, .53$
7	Rajapur	Ratnagiri	59	$15^0 56^, .11$	$73^0 54^, .53$
8	Kankauli	Sindhudurga	64	$17^0 34^, .15$	$78^0 22^, .12$
9	Nevre	Ratnagiri	78	$17^0 60^, .62$	$73^0 17^, .80$
10	Sukondi	Ratnagiri	135	$17^0 51^, .35$	$79^0 07^, .77$
11	Prtapgad	Satara	1132	$18^0 51^, .10$	$73^0 45^, .75$
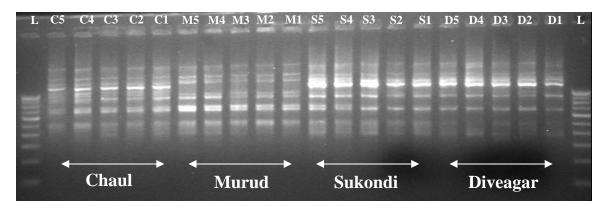
Figure 3.6 **Agarose gel electrophoresis pattern of PCR amplification products using ISSR primer UBC 857, Lane 1 to 20: Inter microsatellite finger prints of 4 populations with 5 plants/population**

DNA isolation:

Initially several protocols like these of Doyle and Doyle (1987), Murray and Thompson's (1980), Lodhi's CTAB (1994) and Khanuja *et al*., (1999) were tried to extract DNA from *G. indica* leaves. Difficulties were encountered from the stage of cell lysis to DNA separation in procedures described by Doyle and Doyle (1987), Murray and Thompson (1980) and Lodhi (1994). Low DNA yield and poor PCR amplification were also the problems encountered. Khanuja's DNA extraction protocol (1999) yielded better quality and quantity of DNA. DNA from Sukondi and Pratapgad populations were used as template for optimizing PCR conditions.

Optimization of PCR conditions:

The experiment was conducted to optimize DNA concentration to be used per reaction for obtaining clear banding pattern (20 to 80 ng/reaction). DNA at concentration 50 ng/reaction produced clear fingerprints (Figure 3.7). The concentration of 50ng DNA was therefore chosen for PCR. Different Concentrations of Taq polymerase (0.4 to 0.8 units/ reaction) were tested for optimum amplification in two DNA samples. The amplified products were as shown in Figure 3.8. There was no change in the banding pattern with enzyme concentration in any of these samples tested. Thus Taq polymerase concentration of 0.6 units/ reaction was considered optimum for PCR amplification of *G. indica* DNA. Based on these experiments finally 50ng genomic DNA and Taq polymerase 0.6 units/ reaction were used for PCR amplification of *G. indica* DNA.
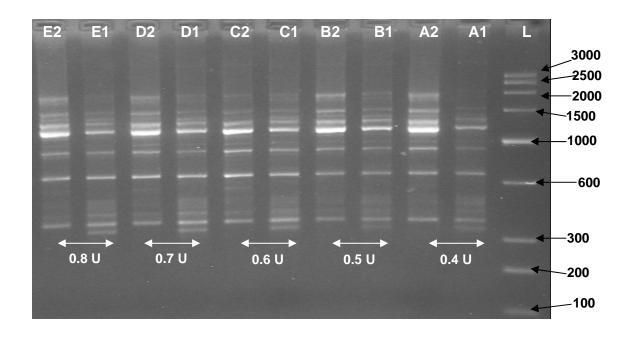
Figure 3.7 Agarose gel electrophoresis pattern of PCR amplification products using ISSR primer UBC 857

Lane 1 to 10: Inter microsatellite finger prints with various concentrations of Taq polymerase enzyme used, 1- Sukondi location   2 - Pratapgad location
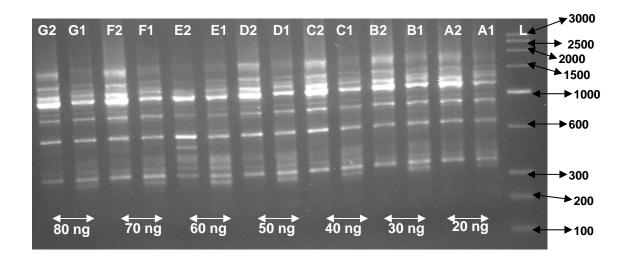

Figure 3.8 Agarose gel electrophoresis pattern of PCR amplification products using ISSR primer UBC 857

Lane 1 to 14: Inter microsatellite finger prints with various concentrations of DNA used, 1- Sukondi location   2 - Pratapgad location

Initially 72 ISSR primers (UBC primers set #9) were screened for amplification using two DNA samples of *G. indica*. Out of 72 primers, 51 primers did not produce any amplification product, 9 primers gave weak fragments and 12 primers produced clear, intense bands (Table 3.3). The results of the ISSR primer screening in *Garcinia indica* suggest that di nucleotide repeat motifs occur at high frequencies while tri, tetra and pentanucleotide appear less frequent along the *G. indica* genome. Even in the crops like bean, maize and soyabean, it was found that di and tri nucleotide SSR occur along the genomes at higher frequencies than tetra nucleotide repeats (Yu *et al*., 1999; Akagi *et al*., 1996; Chin et al., 1996). Out of 12 primers, ten were dinucleotide repeats (817, 827, 830, 835, 836, 840, 848, 855, 856, 857) and two were trinucleotide repeats (868, 880).

Table 3.3 - Nucleotide sequence of the primers producing clear amplification products

Primer No. (UBC)	Sequence (5'-3')
817	CAC ACA CAC ACA CAC AA
827	ACA CAC ACA CAC ACA CG
830	TGT GTG TGT GTG TGT GG
835	AGA GAG AGA GAG AGA GYC
836	AGA GAGAGA GAG AGA GYA
840	GAG AGA GAG AGA GAG AYT
848	CAC ACA CAC ACA CAC ARG
855	ACA CAC ACA CAC ACA CYT
856	ACA CAC ACA CAC ACA CYA
857	ACA CAC ACA CAC ACA CYG
868	GAA GAA GAA GAA GAA GAA
880	GGA GAG GAG AGG AGA

Analysis of polymorphism

The amplification products using 12 primers were separated on agarose gel for following locations -

1.Pavas, 2.kankauli, 3.Rajapur, 4.Diveagar, 5.Guhagar, 6.Sukondi, 7.Pratapgad, 8.Murud, 9.Nevre, 10.Chaul, 11.Amboli
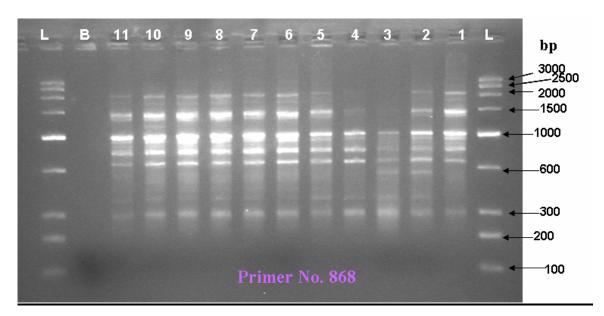
Figure 3.9 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 868. L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.
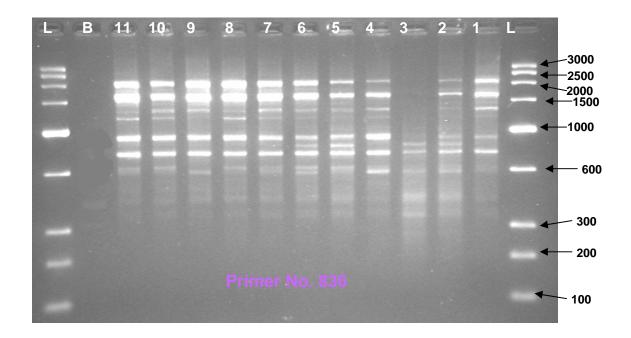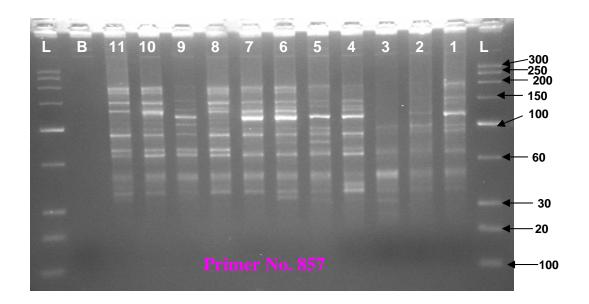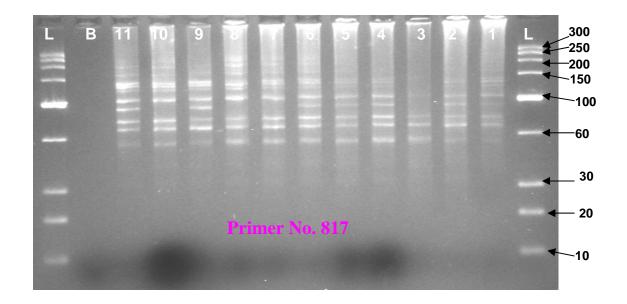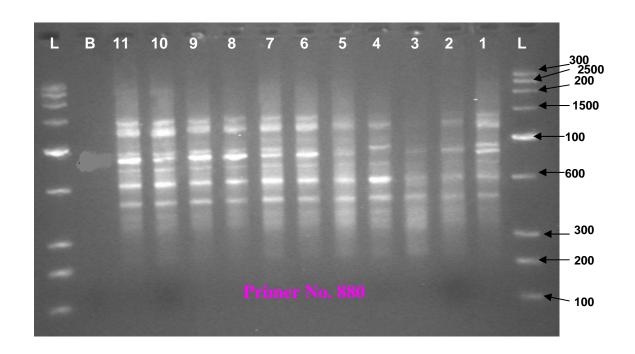

Figure 3.10 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 836. L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.
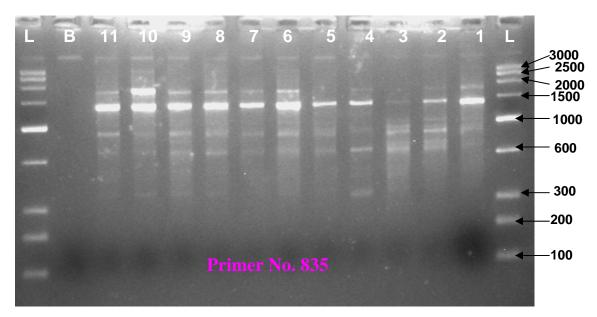
Figure 3.11 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 857 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.
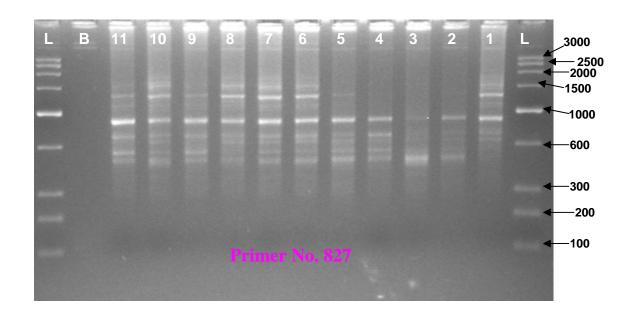

Figure 3.12 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 817 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.

Figure 3.13 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 880 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.
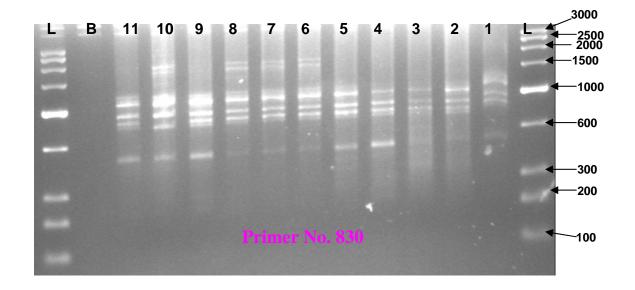

Figure 3.14 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 835 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.
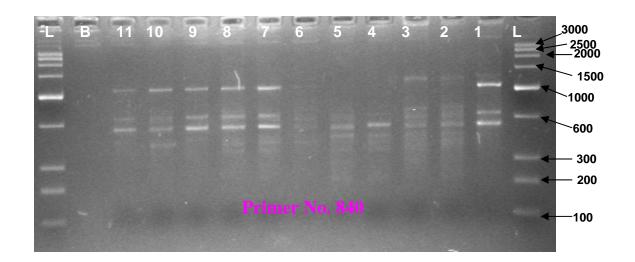
Figure 3.15 **Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 827 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.**

Figure 3.16 **Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 830 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.**
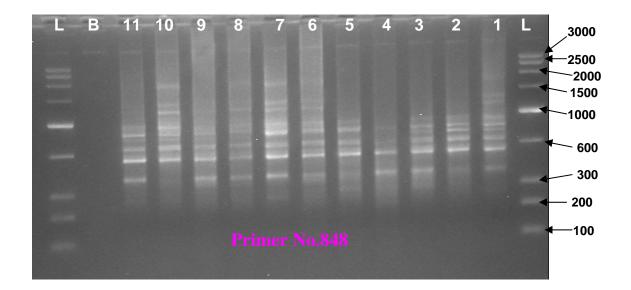
Figure 3.17 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 840 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.

Figure 3.18 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 848 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.
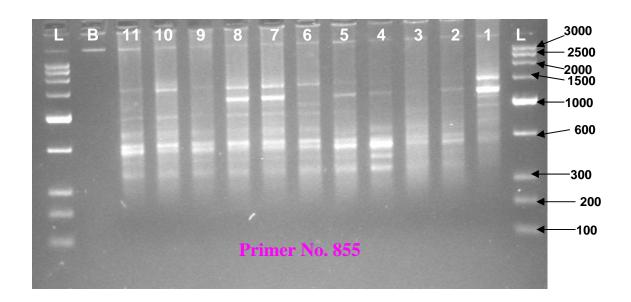
Figure 3.19 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 855 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.
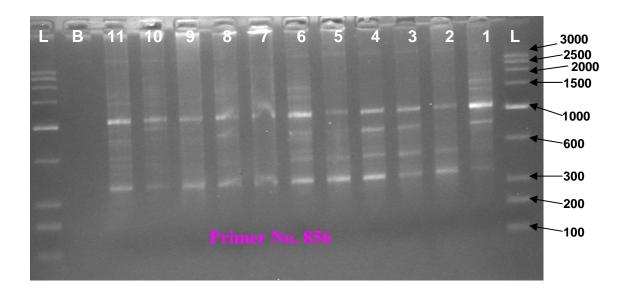

Figure 3.20 Agarose gel electrophoretic pattern of amplification products obtained with 11 populations using the primer 856 L: Low range DNA ruler (3 kb). B: Negative control of PCR amplification. Lane 1-11: Populations in order.

The data generated using these 12 primers were analyzed for variability studies. 12 ISSR primers gave rise to 207 scorable bands, out of which 181 bands were polymorphic. % polymorphism calculated was 87.43 %. 14-21 bands were obtained from each primer with an average of 17.2 bands/primer. The minimum being 12 bands for the primer UBC-840 and the maximum 21 bands for primer UBC-856. The approximate size of the largest fragment was 2930 bp (UBC-856), whereas the smallest recognizable fragment was about 370 bp in size (UBC-836) (Table 3.4).

Table 3.4 The nucleotide sequences of the eight selected primers and the number of bands scored

Primer No. (UBC)	Sequence	Amplification temperature	Total no. of bands		Band Size (bp)
			Amplified	Polymorphic	
817	CAC ACA CAC ACA CAC AA	52	20	17	560-3190
827	ACA CAC ACA CAC ACA CG	54	14	12	500-1640
830	TGT GTG TGT GTG TGT GG	57	20	20	520-2870
835	AGA GAG AGA GAG AGA GYC	50	14	12	380-2920
836	AGA GAGAGA GAG AGA GYA	50	19	15	370-2310
840	GAG AGA GAG AGA GAG AYT	54	12	10	390-2220
848	CAC ACA CAC ACA CAC ARG	48	17	15	300-2220
855	ACA CAC ACA CAC ACA CYT	53	15	13	400-2220
856	ACA CAC ACA CAC ACA CYA	53	16	15	390-2930
857	ACA CAC ACA CAC ACA CYG	50	21	18	390-2190
868	GAA GAA GAA GAA GAA GAA	48	19	15	200-3010
880	GGA GAG GAG AGG AGA	48	20	19	300-2850

ISSR markers used here proved to be able to assess the similarity / variability of *Garcinia indica* plants between populations. The source of polymorphisms may include deletion of a priming site. The objective of the study was to assess the genetic

similarity/distance within 11 populations tested. The estimate of pair wise genetic distance between 11 selected populations is given in Table 3.5.
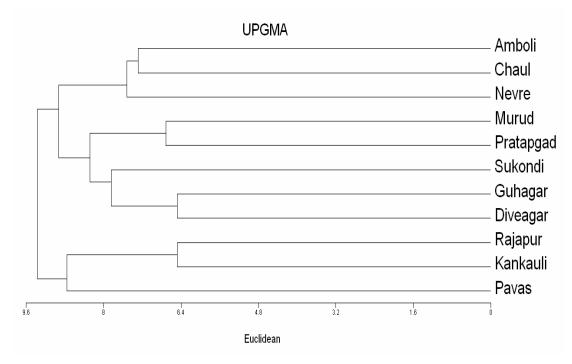
Table 3.5 Genetic Distance matrix among populations of *Garcinia indica* using UPGMA

	Amboli	Chaul	Nevre	Murud	Pratapgad	Sukondi	Guhagar	Diveagar	Rajapur	Kankauli	Pavas
Amboli	0.000										
Chaul	7.280	0.000									
Nevre	7.616	7.416	0.000								
Murud	8.602	8.426	7.211	0.000							
Pratapgad	9.220	9.165	8.185	6.708	0.000						
Sukondi	9.434	9.798	9.000	8.660	7.348	0.000					
Guhagar	9.592	9.539	8.718	8.602	7.937	7.141	0.000				
Diveagar	9.165	9.434	8.367	8.832	8.307	8.544	6.481	0.000			
Rajapur	9.381	9.747	8.718	9.487	9.220	9.220	8.602	7.483	0.000		
Kankauli	9.592	9.747	8.944	8.944	9.434	9.644	8.246	7.483	6.481	0.000	
Pavas	10.247	10.198	10.247	10.440	10.583	10.198	9.434	9.644	9.327	8.185	0.000
	Amboli	Chaul	Nevre	Murud	Pratapgad	Sukondi	Guhagar	Diveagar	Rajapur	Kankauli	Pavas

Distance matrix obtained exhibits distance range 6.48 to 10.58 with an average of 8.53. Smallest distance value of 6.48 is observed between Kankavli – Rajapur (64 MSL and 59 MSL respectively) and Guhagar – Diveagar (10 MSL and 7 MSL respectively) accessions suggesting high degree of similarities between these populations. Maximum genetic distance obtained is 10.58 between Pratapgad and Pavas (1132 MSL and 0 MSL) populations that represents maximum diversity between them (Table 3.5). It may be because the populations showing minimum genetic distance are situated at almost same altitude while the populations showing maximum genetic distance are at diverse altitude i.e. 0 mtrs and 1132 mtrs.

The distance matrix was used to generate dendrogram. It showed overall genetic relatedness among the selected trees.

Figure 3.21 Dendrogram showing genetic relationship among the 11 populations of *Garcinia indica* generated by ISSR data using UPGMA method.

Dendrogram obtained using UPGMA exhibits three main clusters: First consist of Amboli, Chaul and Nevre location; Second consist of Murud, Pratapgad, Sukondi, Guhagar and Diveagar and Third consist of Rajapur, Kankavli and Pavas (Figure 3.21).

3.3.4 Discussions

ISSR markers successfully identified genetic variation among the selected trees of *Garcinia indica.* High polymorphism (87.43 %) was observed among 11 populations. Efforts were made to correlate banding pattern with the morphological characters. Fruit morphology was mainly considered for the variation studies.

To the best of our knowledge, this is the first report of genetic diversity studies in *Garcinia indica*. Based on the present investigations, the natural populations of *G. indica* possess fairly high level of polymorphism. These natural populations of *G. indica* often occur on sea shore as well as mountain regions with extremely complex topography including plateaus and valleys with wide range of climatic conditions. When populations are small and isolated from one another, genetic drift influences genetic structure and increase differentiation among populations (Barett and Kohn, 1991; Ellstrand and Elam, 1993)

The closest geographical distances between the sampled *G. indica* populations in the study were over 50 kms apart that should be beyond the ranges both of seed and pollen dispersals. Therefore it is reasonable that a greater proportion of genetic diversity resides among populations of *G. indica*.

Several studies have indicated that morphological variation is apparently the result of an adaptive response to the environment. The samples from Pavas, Diveagar, Guhagar, Amboli and Pratapgad populations showed morphological differences in shape of fruit and leaf along with change in season of fruit maturity suggesting that these genotypes are better adapted to its environment.

G. indica is a cross pollinated species. Cross pollination increases gene exchange between different individuals and populations, leading to high genetic diversity.

The use of ISSR technology in *G. indica* has permitted the confirmation of molecular markers suitable for exploration of the polymorphism. We first assayed a great number of primers with (AG), (GA), (CT), (TC), (AC), (CA) repeats in order to select suitable polymorphic ISSR markers for the present study. The investigation showed that (AC) repeat primers produce a great number of polymorphic bands. The result therefore suggests that the (AC) microsatellite repeats and their adjacent regions are highly polymorphic in the genome of *G. indica*. (AC) repeat primers are abundant in tropical tree genomes and could provide large number of polymorphic markers for studies of plant population genetics (Condit and Hubbell, 1991).

3.4 Analysis of - (-) HCA content

3.4.1 Introduction

Tropical plants are a rich source of valuable secondary metabolites including alkaloids, terpenoids, and polyphenols; some of which are useful as medicines and food additives. Hydroxycitric acid (HCA) is a major acid component in the fruit rind of *Garcinia* species, including *G. cambogia, G. indica* and *G. astroviridis*. The rinds are traditionally used in combination with salt for curing fish in South and Southeastern Asian countries. Extracts of *Garcinia* fruits have become popular worldwide as an ingredient in dietary supplements with a market value exceeding US $400 million annually. 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 weight loss, cardioprotection, correcting conditions of lipid abnormalities. Hydroxycitric acid is proposed to be one of the active components in various weight-loss formulations and appetite-suppressor products. In recent years, (-)-HCA has received considerable attention because of its putative weight reduction effects in animal and human studies.
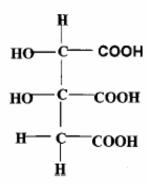

Figure 3.22 Structure of Hydroxycitric acid

Hydroxycitric acid has 2 assymetrical carbon atoms and has 4 different isomers are possible. Out of the 4 isomers (-)-HCA is found naturally in *Garcinia* species and (+)-HCA is found in *Hibiscus* species. (-)-HCA has been reported in the fruit rinds of *Garcinia indica*, *Garcinia cambogia*, *Garcinia artroviridis*, *Garcinia cowa* and *Garcinia*

pedunculata. Some *Streptomyces* species have also been found to produce (+)-HCA (Yamada *et al*., 2007).

BIOCHEMISTRY OF (-)-HCA

Inhibition of Citrate Cleavage Enzyme by (-)-HCA.

Citrate cleavage enzyme, that is, ATP:citrate lyase (ATP:citrate oxaloacetate lyase, EC 4.1.3.8), catalyzes the extra mitochondrial cleavage of citrate to oxaloacetate and acetyl-CoA. Watson *et.al*., (1969) encountered the powerful inhibition of ATP:citrate oxaloacetate 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 *â*-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 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 catalyzes the following reaction: citrate + ATP + CoA f acetyl-CoA + ADP + Pi + oxaloacetate (Watson and Lowenstein, 1970; Lowenstin JM, 1968; Lehninger Al, 1983; Stryer L, 1988). 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 and haft, 1956). 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 lowenatein, 1965; Bressler and Brendel, 1966). 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 of a high-carbohydrate diet elevated levels of ATP: citrate lyase were detected (Linn and Srere,1979).

It has been suggested that because HCA inhibits the formation of acetyl-CoA in the cytoplasm, it may also inhibit the formation of the next compound (malonyl-CoA) in the pathway of fatty acid synthesis. Malonyl-CoA is the ''base'' from which fatty acids (and thus fat) and cholesterol are formed from carbohydrates. Malonyl-CoA, in turn, inhibits the enzyme, carnitine acyltransferase, which is required for the oxidation of fat. Hence, reduction in the formation of malonyl-CoA with HCA might stimulate fat metabolism leading to a decrease in body weight. Decreased levels of malonyl-CoA suggest that carnitine acyltransferase can transport existing fat into the mitochondria where it can be more easily burned.

It is also suggested that other metabolic effects of HCA could contribute to the wasting of ATP and therefore promote the increased consumption of calories in the presence of hydroxycitrate. Gluconeogenesis consumes ATP; if molecules are converted to glucose and glycogen, instead of fat, this may consume ATP. Clouatre and Rosenbaum (1994) also suggested that inhibition of ATP-citrate lyase by HCA consumes a significant amount of energy. In a related hypothesis, it has been suggested that hydroxycitrate increases thermogenesis (metabolism of fat or other compounds to generate heat rather than metabolic energy in the form of ATP).
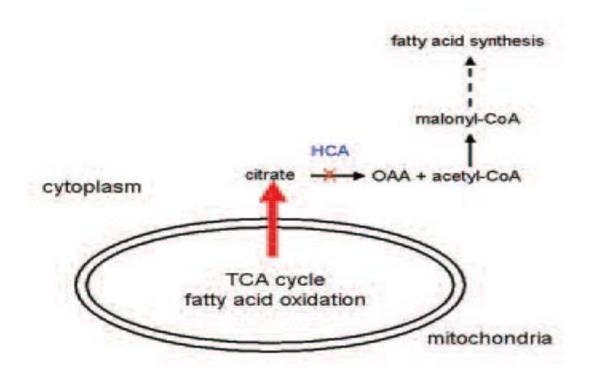
Figure 3.23 Simplified metabolic pathway showing the proposed site of action of L-hydroxycitric acid (HCA), which is proposed to act by inhibiting ATPcitrate lyase reducing the amount of acetyl-CoA available for fatty acid synthesis.
TCA: tricarboxylic acid, OAA:oxaloacetate, CoA :coenzyme A.

In summary, several mechanisms of action of HCA are proposed in the published literature. The primary mechanism appears to be related to the action of HCA as a competitive inhibitor of the enzyme ATP-citrate lyase. In the cytosol, ATP-citrate lyase catalyzes the conversion of citrate and coenzyme A to oxaloacetate and acetyl coenzyme A (acetyl-CoA). Acetyl-CoA is used in the synthesis of fatty acids, cholesterol and triglycerides and also in the synthesis of acetylcholine in the central nervous system. Oxaloacetate may enter the gluconeogenic pathway, which can lead to the production of glucose and glycogen. The putative weight reduction effect of HCA is due to suppression of fatty acid and fat synthesis. In addition, HCA is thought to suppress feed intake via loss of appetite by stimulation of liver gluconeogenesis.

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 to act as an inhibitor of this enzyme (Garland *et al*.,1963; Paramaggiani and Bowman, 1963; Zammit, 1979) It has also been seen that AMP contents drop in the presence of (-)-HCA. This can also be expected to reduce the activity of phosphofructokinase, because AMP is an activator of this enzyme (Bloxham and Lardy, 1995*).* 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 catalyzed by phosphofructokinase in glycolysis is irreversible and controls the glycolysis.

Chronic metabolic acidosis increases proximal tubular citrate uptake, causing hypocitraturia associated with an increase in cortical ATP:citrate lyase activity and protein abundance. Hypokalemia, which causes only intracellular acidosis, also caused hypocitraturia and increased renal cortical ATP:citrate lyase activity. Inhibition of this enzyme with (-)-HCA significantly abated hypocitraturia and increased urinary citrate excretion in chronic metabolic acidosis and in K+ depletion (Melnick *et al*., 1996). These results suggest an important role of ATP:citrate lyase in proximal tubular citrate metabolism. (-)-HCA has been reported to alter the pyruvate metabolism by tumorigenic cells (Board and Newsholme, 1996). Pyruvate consumption by tumor cells declined, but the mean percentage of oxidation increased with (-)-HCA.

Possible mode of action on reduction in food intake

The *in vitro* studies showed that (-)-HCA can depress acetylcholine synthesis in nerve tissues, however 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 has been shown to reduce appetite, inhibit fat synthesis, and decrease body weight without stimulating the central nervous system (Triscari & Sullivan, 1984; Ohio *et al.*, 2001; Ohio *et al.*, 2002). Furthermore, HCA does not cause nervousness, rapid heart rate, high blood pressure, or insomnia, symptoms that are often associated with dietary stimulants such as ephedra (Ma-Huang), caffeine or phenylpropanolamine (Ohia *et al.*, 2002). Thus, HCA may prove to be a safe alternative to these popular diet aids. Studies by Ramos *et al.*, (1995) demonstrated that 500 mg HCA taken three times per day before meals for 8 weeks resulted in 215% greater weight loss in twenty overweight adults than those taking a placebo. No adverse events were reported. Of added importance, a significant reduction was also observed in cholesterol and triglyceride levels. Mattes and Borman (2000) demonstrated that a daily dose of 1.2 gm HCA along with a daily diet of 5,020 kJ (1,200 kcal) for 12 weeks resulted in a significant difference in weight loss (3.7±3.1 vs 2.4 ±2.9 kg) compared to placebo.

(-)-HCA is available commercially in the brand names of SuperCitrimax (Inter Health Nutraceuticals), Ayurslim (Himalaya) and Bioslim (Sunova). 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 *Garcinia indica* located in Maharashtra and to study the effect of the ecological factors on the (-)-HCA content.

3.4.2 Materials and Methods

Extraction and Estimation of (-)-HCA

Fresh fruits of *Garcinia indica* were collected from 11 different locations in Maharashtra in the month of March. Around 35-40 fruits/plant were collected so that the fruit rind will be 25 gm each. 5 plants from each location were taken for chemodiversity studies. Mean HCA content of these 5 plants from one location was taken as representative of the particular location.

The method followed for the extraction as well as estimation of (-)-HCA was as

reported by Jayaprakasha and Sakariah (1998) with some modifications. 25 gm 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 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 the 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.

3.4.3 Results

(-)-HCA extracted from the fruits of *Garcinia indica* varied in colour from light brown to dark brown as shown in Figure 3.24.
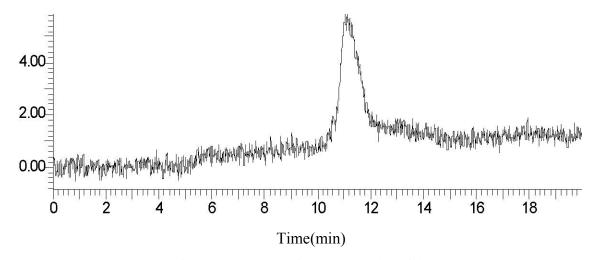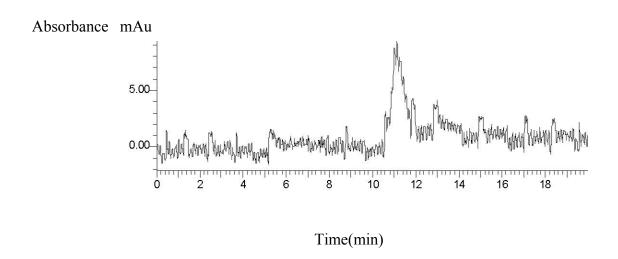
Figure 3.24 - (-)-HCA extracts of *Garcinia indica*

Table 3.6 - % (-)- HCA content in fruit rind of *Garcinia indica* according to altitude

Location	Altitude (in MSL)	% (-)- HCA (± SE)
Pavas	0	7.38 ± 1.7
Chaul	2	6.46 ± 1.5
Diveagar	7	8.45 ± 0.5
Murud	9	7.27 ± 1.2
Guhagar	10	10.63 ± 1.4
Amboli	39	3.13 ± 1.8
Rajapur	59	1.39 ± 1.2
Kankauli	64	1.41 ± 1.4
Nevre	78	2.52 ± 1.4
Sukondi	135	1.71 ± 0.7
Pratapgad	1132	0.76 ± 1.5
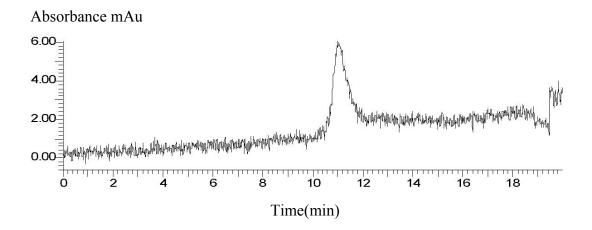
Absorbance mAu
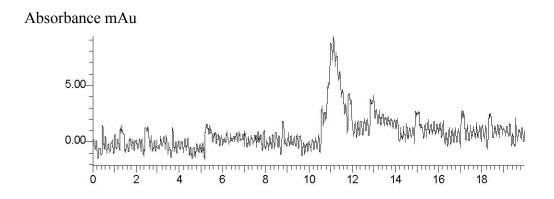
3.25 Chromatogram of standard (-)-HCA

Absorbance mAu

Time(min)

3.26 Chromatogram of fruit extract of *Garcinia indica* (Guhagar)

Absorbance mAu

3.27 Chromatogram of fruit extract of *Garcinia indica* (Diveagar)

Absorbance mAu

Time(min)

3.28 **Chromatogram of fruit extract of *Garcinia indica* (Pavas)**

Among the accessions collected along Maharashtra state, % (-)-HCA varies from 0.76 to 10.63. The (-)-HCA content in the fruit rinds of *Garcinia indica* have been reported to be between 10.3-12.7% (Jayaprakasha and Sakariah, 2002). In the present study, Maximum (-)-HCA content (10.63%) was found in plants growing at 10 MSL (Guhagar) while lowest HCA, 0.76%, was obtained in plants at Pratapgad growing at altitude of 1132 MSL.

3.4.4 Discussions

HCA content can be correlated with the altitude at which the plant is growing. Plants growing at sea shore (0-10 MSL) show more (-)-HCA content than the plants growing at higher altitude. The effect might be because of variation in rainfall or humidity. Similar effect of altitude on chemical composition of plant was observed by Phillip *et al*., (2006) in blacktea.

The existing method of assay of HCA in the fruit of *Garcinia cambogia* consists of titration of fruit extract against standard sodium hydroxide. This method has the limitation of interference by other organic acids present in the samples. A gas chro-matographic (GC) method has been reported by Brunengraber *et al*., 1978, which involves de-rivatization of the acid before analysis. Determination by means of GC involves the conversion of the acids to volatile silyl derivatives. For silylation the sample should be dried completely and the HCA has the tendency to undergo cyclization to the g-lactone during drying. Secondly, due to the highly hygroscopic nature of HCA it is rather difficult to dry the sample completely. In contrast, in HPLC dilute extracts can be quantified without concentration, drying and derivatization. The existing methods for the determination of HCA use an acid–base titration which gives the total acidity of fruit extracts.

Jayaprakasha *et al*., (2003) reported 3% (-)-HCA content in fresh fruits of *Garcinia peduncalata* and in *Garcinia cowa* it 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% and in *Garcinia cowa* to be 12.7% (Jayaprakasha and Sakariah 1998).

3.5 Correlation between genetic and chemo diversity

If dendrogram obtained by genetic diversity and Table (3.6) showing % HCA content will be compared, it can be seen that Rajapur (59 MSL) and Kankauli (64 MSL) locations which are present in one cluster in dendrogram shows almost same % HCA (1.39 and 1.41 respectively). Similarly Diveagar (7 MSL) and Guhagar (10 MSL) from one cluster shows 8.45% and 10.63% HCA respectively. Exceptionally, Murud (9 MSL) and Pratapgad (1132 MSL) forms one cluster where % HCA varies from 0.76 in Pratapgad and

7.27 in Murud. Thus it can be concluded that locations showing similarity at genetic level also shows similarity in its chemical constituents.

4.1 Introduction

Somatic embryogenesis refers to the remarkable ability of nonzygotic plant cells (including haploid cells) to develop through characteristic embryological stages into an embryo capable of developing into a mature plant. Somatic embryogenesis is an expression of totipotency and the associated differential gene expression. It is a process through which embryos develop from somatic cells, without the fusion of gametes and later differentiates into plants through characteristic developmental pattern, a phenomenon, which is not observed in zygotic embryogenesis (Tisserat *et al*., 1979; Williams and Maheshwaran, 1986; Zimmerman, 1993; Merkle *et al*., 1995)**.**

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.

The first report of SE appeared when Reinert (1958) demonstrated the phenomenon using carrot callus. It is a pathway of *de novo* regeneration from *in vitro* cultured tissues either from callus or from cells of an organized structure such as stem, leaf, hypocotyl or zygotic embryo (Williams and Maheshwaran, 1986). 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) or proliferative embryogenesis (Stamp and Meridith, 1988). The direct somatic embryogenesis is advantageous over indirect pathway because embryos produced via callus phase may not produce true to type plants. However, useful variants could be

isolated through indirect embryogenesis due to somaclonal variation. It has also been 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.

The somatic cells within a plant contain the genetic information necessary to form a complete and functional plant (Merkle *et al.*, 1995). The somatic embryos are bipolar structures, lacking a vascular connection with the mother tissue and resemble the zygotic embryo in giving rise to a new plant. They consist of complete propagules (contains both the root and shoot pole) requiring only a single step for the formation of an entire plant (Stuart and Redenbaugh, 1987; Parrott *et al.*, 1995). The initiation of somatic embryogenesis occurs with the termination of the existing gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression program in those cells of the explant, from which somatic embryos arises (Merkle *et al.*, 1995).

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. The process of embryo initiation and development 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 tissue (Table 4.1).

Table 4.1 Classification of embryos (Bhojwani and Razdan, 1997)

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.

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). 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 the presence of a lowered concentration of auxin. 2,4-Dichlorophenoxyacetic acid is the most commonly used auxin. Reduced nitrogen in the form of NH_4 is also required (Kohlenbach, 1978; Wetherell, 1979).

The induction of somatic embryogenesis is also associated with modulations of auxins by TDZ (Capelle *et al.,* 1983, Visser *et al*., 1992, Hutchinson *et al*., 1996). In general TDZ at very low levels induces direct somatic embryogenesis eg. Geranium (Qureshi and Saxena, 1992), alfalfa (Iantcheva *et al*., 1999), water melon (Compton and Gray,1992) and muskmelon (Gray *et al*., 1992). The association between TDZ-induced responses and endogenous plant growth regulators of dicotyledonous plants has been documented by means of the quantification of their profiles. TDZ promotes the synthesis & accumulation of purines (Capelle *et al*., 1983) and also alters cytokinin metabolism (Mok *et al*., 1982).

The induction of direct somatic embryogenesis and organogenesis in the presence of BAP (alone or in combination with auxin) has been observed in intact seedlings of bean (Malik and Saxena, 1992), in cotyledon explants from *Medicago truncatula* (Trieu and Harrison, 1996) and leaf explants from *Helianthus smithii* (Laparra *et al*., 1997). In the case of Oil Palm, the addition of 6-benzyladenin during the development of somatic embryos was found to induce shoot apex differentiation and thus increased germination rates, by up to 70% (Aberlenc-Bertossi *et al*., 1999).

<u>**4.1.1 Applications of Somatic Embryogenesis may be summarized as follows: -**</u>

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 liter suspension culture has been reported in carrot (Drew, 1980).

☛ 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 meristem. Thus conversion to seedling can be obtained in a single step.

2. Synthetic seed production

It is an applied technology which capitalizes on the capacity for plant multiplication via somatic embryogenesis. As the techniques for somatic embryogenesis improved, the possibility of encapsulation for synthetic seed production has been explored. Artificially encapsulated somatic embryos can be sown under *in vitro* or *ex vitro* conditions, producing uniform clones (Aitken-Christie *et al*., 1995). According to Bornman (1993), synthetic seed may provide the only technology realistically amenable to the extensive scale-up required for the commercial production of some clones. Synthetic seeds have multiple advantages over organogenesis for propagation, including ease of handling, potential long-term storage, higher scale-up potential and low cost of production (Ghosh and Sen, 1994). The potential for automation of the whole production process is another advantage because the commercial application of somatic embryogenesis requires high volumes (Ziv, 1995). Cold storage of encapsulated embryos is also important for post-storage survival and germination. Since medium- or long-term storage is one of the aims of synthetic seed production, their storage life is a critical parameter (Janeiro *et al*., 1995). Kitto and Janick (1985) encapsulated somatic embryos of carrot by mixing equal volumes of embryo suspension and a 5% (w/v) solution of a polyethylene oxide homopolymer. Redenbaugh *et al.* (1986) also reported the encapsulation of somatic embryos of alfalfa as single embryo beads. Synthetic seed research has since been extended to numerous other crops, including conifers (Fowke *et al*., 1994), high-value vegetable crops like celery, lettuce (Sanada *et al*.,

1993) and pistachio (Onay, 1996), but only very few woody species (Gray, 1987; Bapat and Rao, 1988; Rao and Bapat, 1993).

3. 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. Standardization of optimal doses for ionizing radiation and chemical mutagens to plant tissue culture and their response on *in vitro* mutation efficiency has been reported in many major crops such as tobacco (Hell, 1983), rice (Gao *et al*., 1992), groundnut (Venkatachalam and Jayabalan, 1996), sugarcane (Khan *et al*., 1999), potato (Al-Safadi *et al*., 2000) and *Chrysanthemum* (Mandal *et al*., 2000). This steady supply can be of economic 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 may be rescued by culturing them for secondary somatic embryogenesis and simultaneously the plant can be multiplied. This has been exploited as a 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 expresses 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 culturing 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) and *B. campestris* (Aslam *et al.*, 1990) 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.

4. Disease elimination

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 nuceller or nucellus callus would also be free of pathogens including viruses (Janick, 1993).

5. Transformation

More number of regenerants can be obtained originating from few or single cells, which increase the likelihood of achieving, transformed cells. Chimeric transformants cycled in repetitive systems can lead to wholly transformed individuals (Baker and Wetzstein, 1992). Somatic embryogenesis is the most often used tissue culture system in woody plants for genetic transformation. 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). 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). Each of these plant system has used SE for transformation. The advantages in using such system are, more number of regenerates can be obtained originating from few or single cells which increase the likelihood of achieving transformed cells.

6. 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.,* 1997). 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)

7. 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 cryogenic 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).

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

4.1.2 Physiological and Biochemical aspects of Somatic embryogenesis-

For the formation of somatic embryos, certain criteria have to be fulfilled before initiation. The species has to have the genetic potential to form embryo from somatic cells and one or a few cells of the plant/explant have to be competent to receive a signal (endogenous or exogenous) that triggers the pathway of embryogenic development leading

to embryo formation even in the absence of further signals.

For *in vitro* forms of somatic embryogenesis, the condition (potential, competence, induction, commitment) have to be experimentally optimized.

Embryogenic potential - The potential for somatic embryogenesis is first of all determined at the level of genotype. It is clearly proved by the successful transfer of the embryogenic capability between embryogenic and recalcitrant genotypes via sexual crossing (Kielly and Bowley, 1992, Moltrasio *et al*., 2004). In spite of the continuously increasing number of species where the conditions for somatic embryo induction have been established, there are number of species that are still recalcitrant to form a somatic embryo. Highly embryogenic and recalcitrant genotypes exist even within a given species. It has to be emphasized however, that in many instance 'recalcitrance' could be resolved by optimizing growth conditions of plants or by proper explant selection (Krishna Raj and Vasil, 1995). Genetic determinants therefore may only serve to define the conditions when and where embryogenic competence can be expressed. Thus, the embryogenic potential is largely defined by the developmental program of the plant as well as by environmental cues.

In most plant species, embryogenic competence is restricted to certain tissues of a given genotype. Tissue culture experiments support the view that there exists a kind of gradient in the embryogenic response among the various plant organs. The embryogenic potential is highest in tissues with embryogenic origin and decreases towards the hypocotyl, petiole, leaf and root. But even if embryogenic competence seems to be lost in somatic plant cells, it can potentially be regained.

Cellular competence – Embryogenic competence is expressed finally at the level of single cell. According to definition, embryogenic competent cells are those cells which are capable of differentiating into embryos if they receive inducers of differentiation (Halperin, 1969). However, embryogenic competence itself needs to be induced in many cases and the signal inducing competences are not easy to separate. Cellular competence is associated with the differentiation of somatic cells that allows them to respond to new developmental signals.

Embryogenic competent cells can be morphologically recognized as small, rounded cells with rich cytoplasm and small vacuoles. In this respect they are very similar to meristematic cells or zygotes and this similarity is further emphasized by their asymmetric

division. Embryogenic competent cells can also be characterized by central position of nucleus and by prominent radiating particular microtubules and action filaments. These types of cells either originate from embryogenic/meristematic tissues or can be formed from elongated, vacuolized cells under specific conditions eg. after treatment with different hormones. Expression of somatic embryogeness receptor kinase (SERK1) gene is indeed linked to the embryogenic cells fate (Schmidt *et al*., 1997).

Alfalfa leaf protoplast also represent a rather homogenous and synchronized system that allows detailed investigations both at that single cell and at the cell population level (Feher *et al*.,2003). The comparisons made between embryogenic and non embryogenic cells revealed that the two types exhibit not only characteristic morphological differences but that their physiology is also altered. Among other differences, the embryogenic competent cells have higher cyoplasmic vacuolar pH values and an altered metabolism. These protoplast derived cells were shown by faster medium acidification and earlier BrdU/thymidin incorporation into their genomic DNA as well as by earlier cell divisions (Pasternak *et al*., 2002).

A PCR based complementary DNA (cDNA) subtraction approach shows that the most of the proteins identified are released at cellular reorganization, including stress responses, intracellular membrane transport and secretion, protein synthesis and nuclear functions. The genes had distinct expression patterns during somatic embryogenesis, indicating their participation in various processes underlying the embryo formation from protoplast derived cells.

4.1.3 Somatic embryogenesis in woody plants

The induction of Somatic embryogenesis 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 (Tulecke, 1987).

In many woody plants, somatic embryogenesis is a circular process; a somatic embryo develops upto a certain stage and than reverts to earlier stages of embryogenic pathway. At a certain moment cells of a more or less developed embryogenic structure

separate from group control and reenter the embryogenic pathway, resulting in a culture which continuously proliferates new embryogenic tissue.

In spite of these limitations somatic embryogenesis 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 somatic embryogenesis is rare. Problems and potentials of tissue culture in forest tree improvement has been discussed extensively by 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.

Well established procedures for somatic embryogenesis are available in many woody species. Many woody species are used in a forestation programs or as plantation crops. This requires a large number of plants. This can be produced via multiple shoot culture, which requires several steps and is labour intensive and thus is expensive. Producing plants via somatic embryogenesis can reduce labor input. Clonal material can be used for the initiation of somatic embryogenesis in some woody species and thus allow the large scale multiplication of selected elite genotypes. This is of special interest in outbreeding woody species or in species with low seed production. Technology such as production of somatic embryos in bioreactor and its encapsulation as synthetic seeds might reduce the cost associated with the use of somatic embryogenesis for plant multiplication (Gray, 1995).

Somatic embryogenesis has been described in numerous woody species. In many of these species embryogenesis can be maintained for prolonged period of time. Based on the morphology, the embryogenic tissue in such cultures can be divided into three types

   a.   Small embryogenic callus aggregates or embryogenic suspensor mass

   b.   Secondary somatic embryos

   c.   Nodular callus

The first two types are most commonly seen. In several woody species, embryogenesis can be maintained in several ways and by using selection and/or specific culture regimes the embryogenic tissue can shift from one group to the other (Raemakers *et al.*, 1999).

4.2 Material and Methods

Green immature fruits of *Garcinia indica* were collected in the month of March from the semievergreen forest of Diveagar (18º10.238´ N, 72º59.377´ E, 5 m) in Maharashtra, India. Plant selected from Diveagar shows high fruit bearing capacity. So the plant can be considered as elite for fruit yield.

Figure 4.1 - Fruiting twig

of *Garcinia indica*

Figure 4.2 – T.S. of immature fruits

showing seed explant

The fruits were surface sterilized by the following method.

Initially fruits were kept in running tap water for 1 hr to remove dust from the surface of fruits. After that they were cleaned with few drops of detergent and treated with 10% savlon for 3-5 min as it acts as surfactant. They were then soaked in 0.1% (w/v) insoluble Polyvinylpyrrolidone (PVP) (Sigma, USA) for 30min and later in 1% (w/v) Bavistin (BASF, India) for 60min on rotary shaker. Bavistin removes fungal spores from surface of fruits. 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 fruits were dissected to recover the immature seeds, which were first rinsed with 70% ethanol (v/v) and washed 3-4 times with sterile distilled water (SDW) followed by treatment with 0.05% $HgCl_2$ (w/v)(Qualigens, India) for 10min and again washed thoroughly 4-5 times with SDW.

These surface sterilized seeds after removing the seed coat were inoculated on somatic embryo induction media where basal media was Woody plant medium (Loyd and McCown, 1980) supplemented with 6-Benzyl amino purine (BAP) (2.22-22.19μm) alone

or in combination with α-Naphthalene acetic acid (NAA) (2.69μm)/2,4-Dichlorophenoxyacetic acid (2,4 D) (4.52 – 22.62 μm) or Indole-3-acetic acid (IAA)(2.85 – 28.54 μm). These media were supplemented with 2% sucrose (Qualigens, India) and 0.85% agar (Qualigens, India) after adjusting the pH to 5.7-5.8. All the growth regulators were incorporated into the medium before autoclaving. The media were autoclaved at 120^0C for 20 min at 15psi. The cultures were incubated at 25 ± 1^0C at 16/8 hrs photoperiod with $35\mu E^{-2}m^{-2}s^{-1}$ illumination provided by white cool fluorescent tubes. Seeds were incubated on these media for 8 weeks. The frequency of explants inducing the embryos were scored after 30 days based on 20 replications for each experiment.

The explants showing primary somatic embryogenesis on induction medium were subcultured on WPM medium supplemented with BAP (16.08μm), Indole-3-acetic acid (IAA) (2.85-5.71μm) and/or kinetin (4.65μm) (Step I media) which were later transferred to media composed of half strength WPM medium without any growth hormones (Step II). The embryos showing poor root pole were shifted to the rooting medium consisting of half strength WPM supplemented with NAA (5.37-10.74μm) with Indole -3- butyric acid (IBA) (4.90μm) or Indole-3-propionic acid (IPA) (5.29μm) and solidified using Phytagel 0.25% (Himedia, India). For elongation the germinated embryos were subsequently shifted to half strength MS medium (Murashige and Skoog, 1962) supplemented with 0.89μm BAP. Well-developed plantlets were later hardened in pots containing a mixture of sterilized soil and sand (1:1).
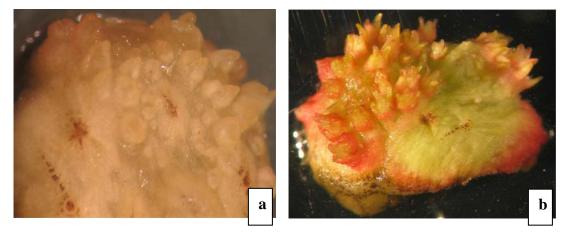
Histological studies:

For histological studies the explants were fixed in glacial acetic acid and ethanol (1:3) for 24 hrs and later stored at 4^0C in 70% ethanol. For dehydration, the tissue was passed through different grades of water: ethanol: tertiary butyl alcohol (TBA) (Sharma and Sharma, 1980) and embedded in paraffin wax (Melting Point 59-60^{0}C). Serial sections of 10μm size were cut at and stained with Hematoxylene (1% w/v) - Eosin (1% w/v), mounted in DPX [2 chloro N (4 methoxy 6 methyl–1,3,5-triazin-2-yl amino carbonyl) benzene sulfonamide] (Qualigens, India) and observed microscopically.

4.3 Results

4.3.1 Primary somatic embryogenesis

Immature seeds were inoculated on series of media containing BAP (2.22-22.19 μm) alone or in combination with NAA (2.69μm) / 2,4D (4.52–22.62 μm) / IAA (2.85–28.54 μm). The explants on all the concentrations of BAP alone or with NAA containing media showed response with swelling within one week and initiation of small protuberances within 2–3 weeks of culture (Figure 4.3a). Somatic embryos were visible all over the seed surface within a period of 5 weeks (Figure 4.3b).

Media incorporated with BAP (2.22-22.19 μm) 2,4 D (4.52–22.62 μm) / IAA (2.85–28.54 μm) induced white friable callus on seed surface (Figure 4.3 c). This callus turned brown after subculture with no further growth.
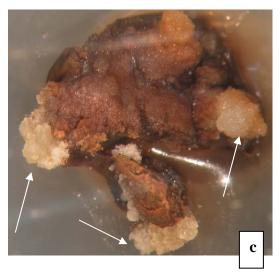

Figure 4.3 a and b. Induction of somatic embryogenesis on BAP (22.1 μm),

c. Callus induction on BAP+2,4 D containing media

Table 4.2 Influence of growth hormones on induction of primary somatic embryogenesis (PSE) in *Garcinia indica*.

Medium No.	Medium for induction of PSE WPM* + PGRs (μm)		Average percent explants showing PSE	Av. Mean no. of PSE per explant
	BA	**NAA**		
1	2.22	--	45.0 (42.1)**	07.0
2	4.40	--	70.0 (56.9)**	11.0
3	16.00	--	66.6 (54.7)**	20.3
4	22.19	--	80.0 (63.4)**	16.2
5	2.22	2.69	28.0 (31.9)**	17.2
6	4.40	2.69	08.0 (16.4)	21.0
7	16.00	2.69	12.0 (20.2)**	23.0
8	22.19	2.69	16.0 (23.5)**	15.0

Data was scored after 6 wk of culture.

   *Basal medium: WPM + sucrose 2% + agar 0.85%

LSD (p=0.05) = 17.7; LSD (p=0.01) = 18.3.

*, ** Significant at 5% and 1% level, respectively.

Figures in the parenthesis are angular transformation values of percentage of response.

The mean number of somatic embryos per explant increased with an increase in BAP from 2.22-16.00 μm, while with further increase in concentration, there was a decrease in the number of primary embryos (Table 4.2). However, percent somatic embryogenesis was highest i.e., 80% (significant at p = 0.01) with 22.1 μm BAP alone. In the combination of BAP (16.00 μm) supplemented with NAA (2.6 μm), there was an increase in the mean number of somatic embryos per explant, but the percentage of somatic embryogenesis reduced drastically. On the basis of statistical analysis it was observed that the percentage of primary somatic embryo induction was more in medium supplemented with BAP alone as compared to the medium with a combination of BAP and NAA. So, WPM supplemented with BAP (22.1 μm) only is significantly superior (p = 0.01) over the other media compositions. The results from table 4.1 highlight the

redundancy of NAA in the induction of somatic embryogenesis from immature seed explants of *Garcinia indica*. It is also note worthy that the inclusion of high NAA in the BAP containing medium results in a drastic reduction in the average percentage of the explants showing embryogenic response. The average mean number of primary somatic embryo's formed per responsive explant remain unchanged at higher BAP concentration.

Embryogenic masses were incubated on induction media for next 3 weeks with subculture on same media after every 15 days so as to obtain maturity of embryos. After 3 weeks embryogenic masses showed profuse growth in shoot pole of embryos still attached to seed explant.

Figure 4.5 a-c Embryogenic masses showing profusely growing shoot pole of embryo on WPM media containing BAP (22.1 µm) after 7 weeks culture

Proliferation of somatic embryos occurred in two ways:

(1) Multiplication of somatic embryos from the explant through primary somatic embryogenesis; and (2) Proliferation of secondary somatic embryos from already formed somatic embryos through repetitive embryogenesis.

Bunches of 6-9 Primary Somatic Embryos (PSE) were separated and incubated on WPM media containing BAP (16.0 μM) + IAA (2.8-5.7 μM) + Kinetin (4.6 μM) to induce Secondary Somatic Embryogenesis (SSE).

4.3.2 Secondary somatic embryogenesis

When primary somatic embryos were shifted to Step I WPM media containing BAP (16.0 μM), IAA (2.8-5.7 μM) and /or kinetin (4.6 μM), induction of secondary somatic embryogenesis was observed after 12 wk of incubation.

Maximum secondary somatic embryogenesis was noted on primary somatic embryos which were induced on 22.1 μm BA, i.e., 54% (significant at p = 0.01) (Table 4.3). Thus, it appears that high BA concentration at the time of induction of primary embryogenesis promotes subsequent induction of more secondary somatic embryos. Induction media containing only BAP (16.0 μm) and BAP (2.2 μm) with NAA (2.6 μm) showed no Secondary Somatic Embryo induction. In Step I, the mean number of secondary somatic embryos increased in the range of 25-30 in the best treatments (Table 4.3).
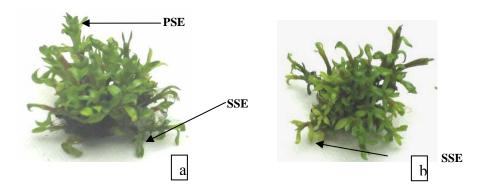

Figure 4.6 Induction of Secondary somatic embryos on WPM media
containing BAP (16.0 μM) + IAA (2.8-5.7 μM) + Kinetin (4.6 μM)

Table 4.3 Effect of step I and II media on secondary somatic embryo (SSE) induction in *Garcinia indica*.

Medium for induction of PSE WPM + PGRs (µM)		Mean No. of PSE per explant	Induction of secondary somatic embryos			
			Step I [z]		Step II [y]	
BA	NAA		Ave. % SSE	Mean No. of SSE (± SE)	Av. % SSE	Mean No. of SSE (± SE)
2.22	--	07.0	17.1 (24.4)** [e]	8.2 ± 0.5	30.0 (33.2)** [g]	9.1 ± 0.2
4.40	--	11.0	22.0 (27.9)** [d]	13.5 ± 0.6	31.0 (33.8)** [e]	14.5 ± 0.3
16.00	--	20.3	00.0 [g]	20.3 ± 0.5	21.0 (27.2) [h]	24.6 ± 0.4
22.19	--	16.2	54.0 (47.3)** [a]	25.1 ± 0.3	64.0 (53.1)** [b]	41.3 ± 0.7
2.22	2.69	17.2	00.0 [g]	17.2 ± 0.4	57.0 (49.0)** [c]	28.0 ± 0.5
4.40	2.69	21.0	42.0 (40.4)** [b]	30.0 ± 0.7	66.0 (54.3)** [a]	35.0 ± 0.6
16.00	2.69	23.0	32.0 (35.4)** [c]	30.0 ± 0.9	52.0 (46.1)** [d]	35.0 ± 0.9
22.19	2.69	15.0	13.0 (21.1) [f]	17.0 ± 0.2	26.0 (30.6)** [f]	19.0 ± 0.4

PSE = Primary somatic embryo.   SSE = Secondary somatic embryo.

SE = Standard error

[z] **Step I media:** WPM + BA (16.0 µM) + IAA (2.8-5.7 µM) + kinetin (4.6 µM) + sucrose 2% + agar 0.85 %. LSD (p=0.05) = 22.7; LSD (p=0.01) = 23.3.

[y] **Step II media:** Half strength WPM + sucrose 2% + agar 0.85%.

LSD (p=0.05) = 27.7; LSD (p=0.01) = 27.9. *, ** Significant at 5% and 1% level, respectively. Values in parentheses are angular transformation values of percentage of response.

a-h: Duncan's Multiple Range notation at 5% level

As all primary somatic embryos did not produce secondary somatic embryos, the embryogenic masses were then shifted to Step II half-strength WPM medium without any growth hormone, so as to facilitate the germination of embryos. On this medium, further increase in the number of secondary somatic embryos was observed up to a maximum of 41.3 (Table 4.3). Percentage of secondary somatic embryo induction was high upto 66% (significant at p = 0.01) on step II medium. Thus, higher numbers of repetitive somatic

embryos were observed in the second cycle of secondary somatic embryogenesis than in the first cycle.

4.3.3 Histological Studies

To confirm the origin of embryo induction, histological studies were conducted. The initiation of direct somatic embryos was observed from the subepidermal layer of seed explant without an intervening callus phase (Figure 4.4a). Differentiated meristematic pockets were seen at the periphery of explants that further developed into embryos. During further growth, the epidermal layer of primary somatic embryos became meristematic and produced secondary somatic embryos (Figure 4.4b, c, d). Induction of secondary somatic embryos was seen from the entire surface of primary somatic embryos. However, mostly these secondary somatic embryos were observed from the base of primary somatic embryos, especially near the suspensor (Figure 4.4e). Well-differentiated cotyledonary stage primary embryos can be seen still attached to an explant at the time of secondary somatic embryo induction. These primary embryos later got detached from the mother tissue, and a well-developed germinating embryo with shoot-root pole along with suspensor was seen.
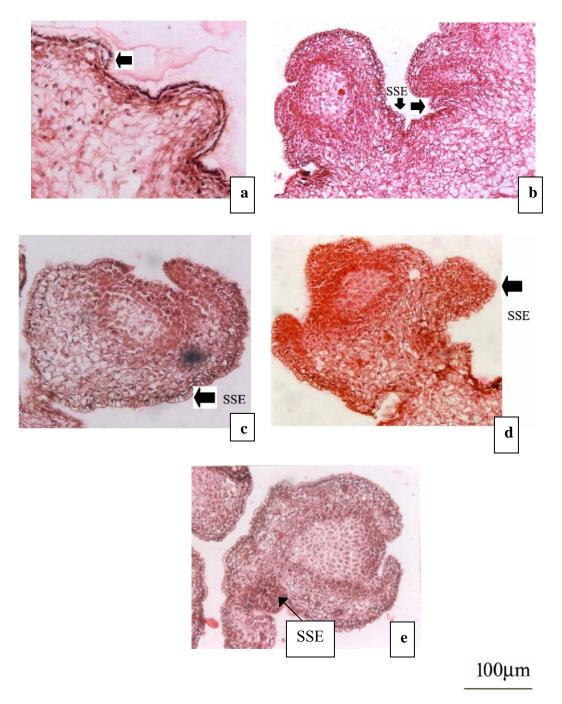
Figure 4.4 Histology of Primary and Secondary somatic embryogenesis in *Garcinia indica* Choiss. Longitudinal sections through various stages of embryo development.
a. Induction of primary somatic embryos from the sub-epidermal layer of explant,
b. Late heart shaped primary somatic embryo with initiation of secondary somatic embryos, c & d. Primary somatic embryos showing production of secondary somatic embryo from its epidermal layer, e. Mature primary somatic embryo with suspensor.
SSE = Secondary Somatic Embryo

4.3.4 Maturation

It was seen that most of the embryos showed precocious germination where the shoot pole was highly active. The secondary somatic embryos were seen to be growing from the base of primary somatic embryos. The shoots of primary embryos showed growth and elongation without root formation. Thus, in one explant, all stages of embryo development were seen simultaneously.

Along with induction of secondary somatic embryos in Step I, the primary somatic embryos also showed maturation (Table 4.4). This frequency of embryo maturation varied from 60-90% with 19.3-21.8 mature embryos per explant. Few of these embryos developed further on these media and germinated precociously (Figure 4.7).

Primary Somatic Embryos which do not show Secondary Somatic Embryogenesis from their bases were separated individually and shifted to rooting media. These PSE shows further growth like that of SSE which is mentioned further in the chapter.

On maturation medium shoot pole grow vigoursaly but root pole frowth was poor. Therefore, SE derived shoots were shifted to root induction medium.

Table 4.4: Effect of different growth hormones [z] on maturation of primary and secondary somatic embryos

BA (μM)	IAA (μM)	kinetin (μM)	Ave. percentage embryo maturation (%)	Mean No. of mature embryos per explant (± SE)
16.00	5.71	-	80.0	20.6 ± 1.7
16.00	2.85	4.6	60.0	19.3 ± 1.3
16.00	5.71	4.6	90.0	21.8 ± 2.5

SE = Standard error

[z] Basal medium: WPM + sucrose 2% + agar 0.85%
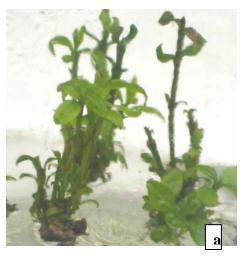
data score after 12 weeks of incubation

Figure 4.7 Maturation of somatic embryos on WPM media with

BAP (16.0o μM) + IAA (5.71 μM) + kinetin (4.60 μM)

4.3.5 Root induction

The germinating embryos with a well-developed shoot pole were separated and shifted to rooting medium containing different auxins: NAA (5.37-10.74 μM) and IBA (4.90 μM) or IPA (5.29 μM). Root development was observed within 15-20 days of incubation (Figure 4.8). The maximum of 76% rooting was observed on medium containing a combination of NAA (10.74 μM) and IBA (4.90 μM) with 15.3 roots per explant (Figure 4.8). A combination of NAA (5.37 μM) and IPA (5.29 μM) also gave similar results with 69.7% rooting (Figure 4.9 a-c). However, the roots induced on the NAA and IBA combination showed faster growth than those on medium containing IPA. Along with media containing phytagel, liquid media were also tried as it facilitates early rooting. But the explants on this media showed formation of callus at the base of shoots and do not show root induction (Figure 4.9 d).

Figure 4.8 Frequency of root induction on varying auxin combinations

Auxin composition: A- NAA (5.37μm), IBA (4.90 μm), Media B - NAA (10.74μm), IBA (4.90μm),

Media C - NAA (5.37μm), IPA (5.29μm)

*Basal medium: ½ strength WPM salts + sucrose 2% + phytagel 0.25%.
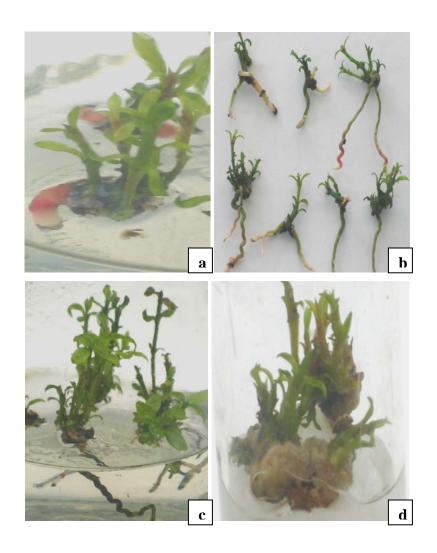
Data scored after 20 days of culture

Figure 4.9 a-c. Root induction on WPM media fortified with

NAA (10.74 μm) + IBA (4.90 μm),

d.Growth of callus at the base of shoots on IPA containing media

4.3.6 Hardening

The well-developed plantlets, when shifted to half-strength MS medium with 0.8 μM BA, showed further elongation. These plantlets of height 3-4 cm were later shifted to potting mixture of sterilized sand and soil (1:1) for acclimatization and transferred to greenhouse, where 90% survival was achieved (Figure 4.10).
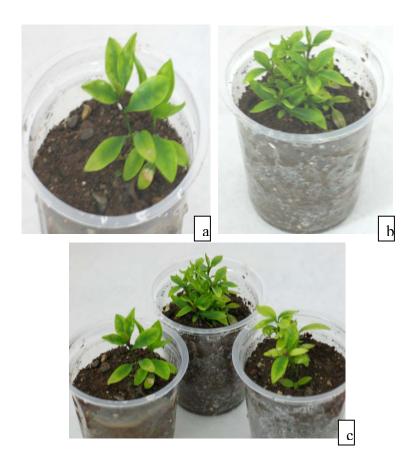
Figure 4.10 Somatic embryo derived hardened plants

4.4 Discussions

Direct somatic embryogenesis without intervening callus phase has been obtained during the study. Somatic embryos developed in cluster and multiplied rapidly.

BAP induced direct somatic embryogenesis in *Garcinia indica.* The similar role of BAP in induction of Somatic embryogenesis has also been reported in Sugarcane (Geetha and Padmanabhan, 2001), *Solanum* (Swamy *et al.*, 2005), *Citrus* (Mukhtar *et al.*, 2005) and date palm (Zouine and Hadrami, 2007). BAP acts as mitotic trigger and starts the process with activation of meristematic cells (Mujib and Samaj, 2006). Similar to direct embryogenesis the efficiency of secondary somatic embryogenesis is dependent on the medium used for the induction of primary embryos (Vasic *et al.*,2001).In this system also the secondary somatic embryo induction was highest on primary somatic embryos induced on maximum BAP. This may be the carryover effect of BAP which continues from induction of primary somatic embryos to secondary somatic embryos.

The effect of BAP to stimulate secondary somatic embryogenesis could be partly explained by the stimulation of cell division on the apical part of primary somatic embryos. This stimulating effect could induce the development of the globular embryos on the surface of the primary somatic embryos suppressing their normal germination.

Addition of NAA (ineffective when used singly) in BAP supplemented media accelerated the frequency of embryogenesis and embryo number in culture of *Citrus aurantifolia* and *Citrus nensis* (Mujib and Samaj, 2006). But in *G. indica* inclusion of NAA in the BAP containing medium results in a drastic reduction in the average percentage of the explants showing embryogenic response. Similar inhibitory effect of NAA on induction of somatic embryogenesis has been reported by Pania *et al*. (2004) and Onay *et al*. (2004).

The auxin and cytokinin ratio had an influence on the frequency of embryo formation, germination and the capability of regenerated plants. Percentage of regenerated plants was higher when embryos were induced on high cytokinin containing medium.

Secondary somatic embryogenesis is the process of induction of new somatic embryos from preexisting embryo. Primary embryos have shown to be an excellent source of secondary embryos, where some cells break away from group control and start initiating new somatic embryos (Williams and Maheswaran, 1986). Secondary somatic embryos usually, proliferate from superficial cells of primary embryos (Merkle *et al*., 1995). Cyclic somatic embryogenesis that is the basis of plant cloning has been reported in many species such as *Dianthus caryopyllus* (Karami *et al*.,2007), *Piper nigrum* L. (Nair and Gupta, 2006), *Morus alba* L. (Agarwal *et al*.,2004), *Coffea sp.*(Giridhar *et al*.,2004), *Phalaenopsis amabilis* (Chen and Chang, 2004), *Arachis hypogea* (Little *et al*.,2000), *Medicago sp.* (Neves *et al*.,1999), *Myrtus communis* L. (Parra and Amo-Marco, 1998) and in *Dalbergia sisso* (Das *et al*.,1997).

Since new embryos are continually formed from existing embryo, secondary somatic embryos has the potential to produce many plants and once initiated may continue to produce embryos over a long period of time (Te Chato and Hilae, 2007). The effect of BAP to stimulate secondary embryogenesis could be partly explained by the stimulation of cell division on the apical part of primary somatic embryos. This stimulating effect could induce the development of the globular embryos on the surface of the primary somatic embryos suppressing their normal germination (Trigiano, 1997).

Selection of the developmental stage of somatic embryos at the time of subculture was the most important factor for conversion to viable plantlets. Secondary embryogenesis requires no growth regulators in species with cytokinin driven primary embryogenesis (Raemakers *et al*., 1995). In this system also formation of Secondary somatic embryos was greatly stimulated by transfer of primary embryos to step II media which is without growth hormones.

As the germination of primary somatic embryos and induction of secondary somatic embryogenesis was simultaneous, it seems that secondary somatic embryogenesis negatively affected the somatic embryo conversion to plantlets by arresting root development. In *Garcinia indica*, secondary somatic embryo induction started from the heart-shaped primary embryos until their conversion into plantlets. A similar phenomenon was observed by other workers in a variety of species (Das Nerves *et al*., 1990; Daigny *et al*., 1996).

The maturation, germination, and conversion of somatic embryos into plants are difficult tasks during somatic embryogenesis (Sutton *et al*., 1999). For woody species especially, the efficiency of conversion is relatively low, hampering the application of somatic embryogenesis systems for the purposes of commercialization. In comparison to root cuttings and micropropagation, somatic embryos offer the potential for high volumes large scale propagation system that can be translated into significant labor savings.

Maturation process is a critical step in somatic embryogenesis which leads to the complete plantlets formation (Swamy *et al*., 2005). Here in this study maturation of primary somatic embryos and induction of secondary somatic embryos occurs simultaneously. Though secondary somatic embryos get induced at this stage, 80% primary somatic embryos shows maturation. Thus, secondary somatic embryogenesis is beneficial for the system and do not affect conversion rate of primary somatic embryos.

Using this protocol 40-45 plants can be produced from a single seed in 35-40 weeks period.

5.1 Introduction

Clonal propagation is a true-to-type propagation of a selected genotype using *in vitro* culture techniques. The word 'Clone' was first used by Webber for cultivated plants that were propagated vegetatively. The word is derived from Greek word Clone which means twig. It signifies that plants grown from such vegetative parts are not individuals in the ordinary sense, but are simply transplanted parts of the same individual and such plants are identical. Thus clonal propagation is the multiplication of genetically identified individuals by asexual reproduction while clone is a plant population derived from single individual by asexual reproduction (Chawla, 2002).

The significant advantages offered by the aseptic methods of clonal propagation over the conventional methods is that in a relatively short span of time and space, a large number of plants can be produced starting from single individual. It has been estimated that axillary bud proliferation approach typically results in an average, 10 fold increase in shoot number per monthly culture passage. The method is also used for obtaining disease free clones.

5.1.1 Clonal propagation via axillary bud proliferation

For clonal propagation generally apical and axillary buds are used. It is the stimulation of axillary buds, which are usually present in the axil of each leaf to develop into a shoot. All the buds contain quiescent or active meristem depending upon the physiological state of the plant. Each of this meristem is capable of growing into a shoot that is identical to main axis. The technique exploits the normal ontogenetic route of branch development by lateral (axillary) meristem. In nature these buds remain dormant for various periods depending upon the growth pattern of the plant.

In some species removal of terminal bud is necessary to break the apical dominance and stimulate the axillary bud to grow into shoot. Due to continuous application of cytokinine in the culture medium *in vitro* apical shoot formed by the bud produces axillary buds if apical dominance is removed. The shoots are then separated individually and rooted to produce plants. Such *in vitro* grown shoots can be used as propagules for further propagation. The merit of using axillay bud proliferation is that the incipient shoot has already differentiated *in vivo*.

5.1.2 Applications of clonal propagation

1. The method can be used to produce, maintain, multiply and transport pathogen free plants safely and economically.

2. Shoot multiplication cycle is very short and each cycle results in logarithmic increase in the number of shoots. Therefore large number of plants can be produced in short time.

3. Plant multiplication can be continued throughout the year irrespective of season.

4. A large number of horticultural, plantation and forest species are being propagated *in vitro* on commercial scale.

5. *In vitro* conservation and propagation of endangered plants is another important application.

6. Clonal propagation is extremely important in case of dioecious species where seed progeny yields 50% males and 50% females but plant of one of the sexes are more desirable commercially.

5.1.3 Clonal propagation can be divided in four stages

Stage I - Initiation of culture:

The purpose of stage-I is to initiate axenic culture. This stage begins with the explant selection. For enhanced axillary branching only such explants are suitable which carry a preformed vegetative bud. Generally subterminal and older segments withstand the toxic effect of sterilizing agents much better than the terminal cuttings. The physiological state of the parent plant at the time of explant excision has a definite influence on the response of buds. Explant from actively growing shoots at the beginning of growing season generally gives best results.

Sterilization – To survive and grow properly, *in vitro* plant cultures need to be free of fungal and bacterial infections. Contamination in tissue culture can originate from two sources, through carry over of microorganisms on the surface and/or in the tissues of explants or through faulty procedures in the laboratory. Special precaution need to be taken when explants are derived from field grown materials. The growing branches of trees may be cut and kept in polythene bags to protect from wind-borne contamination. The explants can be treated with antimicrobial chemicals to remove endogenous or exogenous contamination. These include Bavistin/Benomyl for eradicating fungal contamination.

Many kinds of bacteria cause systemic infection in plants or occur interstitially between cells and are difficult to detect *in vitro*. Their presence within cultured tissues can result in slow growth and an altered morphogenic potential (De Fossard, 1977). Addition of antibiotics in nutrient media may solve the problem associated with bacterial contamination.

In general the ingredients of the culture medium in this stage are decided by the kind of response desired e.g. axillary shoot formation or adventitious shoot formation or callusing etc. The success of plant tissue culture as a means of plant propagation is generally influenced by the nature of culture media used. These culture media includes major salts, minor salts, vitamins and carbohydrate source. Depending on the mineral requirements of different plant species, several media compositions have been formulated. The most commonly used basal medium is MS medium (Murashige and Skoog, 1962). Details of this medium composition are described in chapter 2.

Supplementation of cytokinines like BAP, kinetin, TDZ in the media is desired for apical and axillary shoot formation rarely auxins IAA, IBA can also be used. Carbohydrate as carbon source is one of the important component of the plant tissue culture medium. Sucrose is the most commonly used carbon source in the media. Most of the earlier studies strongly suggested the use of sucrose as a carbon source.

A rapidly proliferating culture is established under optimum levels of common white cool fluorescent tubes having $35\mu E^{-2}m^{-2}s^{-1}$ illumination and incubated at $25 \pm 1^{0}C$ at 16/8 hrs photoperiod. In rare cases higher temperature is also maintained as per tissue requirement.

Stage II - Multiplication of shoots in culture:

Once the sterile cultures are established the next goal is its multiplication. This is the most crucial stage where most of the failure occurs. Rate of shoot multiplication may be initially low but with each passage the number of shoots increase logarithmically and within a year astronomical figures can be achieved. Proliferated shoots 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 multiplication, shoots from the

cultures maintained for several passages and having higher subculture numbers is avoided to ensure genetic fidelity.

Media composition can be manipulated so as to induce the multiplication of axillary buds. Addition of hormones like TDZ, Kinetin in the media induces multiple shoots. These multiple shoots obtained at each passage interval can be separated after elongation and inoculated individually to obtain more shoots.

Stage III – Rooting:

Adventitious shoots developed in the presence of cytokinin generally lack roots. To obtain complete plants the shoots must be transferred to rooting medium which is different from shoot multiplication medium particularly in its hormonal and salt composition. For root induction, individual shoots measuring about 2 cm in length are excised and transferred to rooting medium. Shoots can be cultured either in growth regulator free medium or subjected to pulse treatment of auxin prior to transferring to an auxin free medium for root induction. The duration of auxin treatment varies from few hours to few days depending upon the tissue and concentration of hormone used for the treatment. Single auxin or mixture of auxins is used for root induction.

In some woody species rooting of microcuttings may require a high concentration of auxin. Prolonged exposure to high auxin level, however, has some undesirable effect such as callusing, leaf chlorosis, inhibition of root elongation and quiescence or dormancy in the shoot tip results in the difficulty during acclimatization (Maynard *et al*., 1991). A possible solution to this problem is to treat such shoots with a high concentration (100-500 mg/l) of a suitable auxin for a short period (4-5 hrs) and then transfer them to auxin free medium *in vitro*/*ex vitro*.

It is a common practice to induce *in vitro* rooting however, many commercial laboratories prefer to treat the *in vitro* multiplied shoots as microcuttings and root them *in vivo*. In this method shoots are excised as small cuttings (micro cuttings), treated with commercial rooting mixture and planted in a suitable mixture of sand and cocopeat. These are then placed in a high humidity chamber for rooting induction.

In vivo rooting offers many advantages –

1. *In vitro* rooting is labor intensive and expensive accounting for 35-75% of the total cost of micropropagation (Debergh and Maene, 1981). *In vivo* rooting combines the rooting and acclimatization stage and thus reduces aseptic handling.

2. *In vivo* formed roots are structurally and functionally of better quality than those developed *in vitro*. The *in vitro* formed roots are often thick and lack root hairs and good vasculature. These roots frequently die or collapse after the plantlets are removed from cultures and new, functional lateral and adventitious roots are formed during acclimatization. Under *in vivo* conditions, only rarely callusing at the base of shoots occurs ensuring a continuous vascular connection between root and shoot.

3. *In vitro* developed roots may get damaged during transplantation.

4. For difficult to root species it is easier and cheaper to create good rooting condition *in vivo* than *in vitro*.

Stage IV – Hardening of tissue culture raised plants:

The ultimate success of commercial micropropagation depends on the ability to transfer plants to fields on a large scale at low cost and with high survival rates. The plants multiplied *in vitro* are exposed to unique set of growth conditions viz. high level of inorganic and organic nutrients and growth regulators, sucrose as carbon source, high humidity, low light and poor gaseous exchange which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants, rendering them unfit for survival under *in vivo* conditions. The two main deficiencies of *in vitro* grown plants are

1. Poor control of water loss
2. Heterotrophic mode of nutrition

Therefore, gradual acclimatization is necessary for these plants to survive transition from culture to greenhouse or field.

During acclimatization the *in vitro* formed leaves do not recover but the plant develops normal leaves and functional roots. Thus it is necessary to acclimatize plantlets gradually to ensure survival until they develop new leaves that are more adapted to the ambient conditions under which plants are normally grown. High relative humidity has to be

maintained during hardening process to protect the plants from desiccation and enable them to initiate new roots and shoots.

5.1.4 Factors affecting clonal propagation

1. Developmental stage of the explant - The explant must be physiologically competent to survive the initial shock of culture environment and elicit the appropriate response. In general younger tissues, such as terminal or axillary shoot buds regenerate better than older and mature tissues. The age of the stock plant, physiological age of the explant and its developmental stage as well as its size can determine the success of the procedure (Franclet *et al.*, 1987).

2. Explant position on medium –Vertically placed explants gives early bud break than that of horizontaly positioned ones.

3. Along with plant growth regulators, certain antioxidants like ascorbic and citric acid and some additives like adenine sulphate, arginine, glutamine, ammonium citrate are also used.

4. Incubation conditions – like temperature, humidity, intensity of light.
   The majority of work arbitrarily adopts incubation temperature at 25 ± 2^0C to support growth of cultures. This is not necessarily optimum. Sometimes the optimum temperature can be elevated to 27-30^{0}C or the species occurring in cold region or season requires the temperature range of 17-22^{0}C.

5. Subculturing period – Early subculturing on the fresh media at regular intervals enhances shoot proliferation.

6. Browning of medium – A serious problem with the culture of some plant species is the oxidation of phenolic substances leached out from the cut surface of the explant which turns the medium yellowish to dark brown. It is often toxic to the tissue.

5.1.5 Clonal propagation of tree species

Conventional method of asexual propagation (vegetative propagation) like grafting, budding, layering etc. for tree species are either slow or fail completely. Tree species have long generation cycle with the problem of heterozygosity as a result of wide crossing. Clonal propagation technology overcomes all these problems.

Shoot tip culture has been used as a tool for propagation of various tree species such as *Acacia salinga* (Barakat and El-Lakany, 1992), *Tamarindus indica* (Kopp and Nataraja, 1991), *Trichopus zeylanicus* (Krishnan *et al.*,1995), *Quercus* species (Ostrolucka and Bezo,1994) and *Faidherbia albida* (Ruredzo and Hanson ,1993).

Tissue culture of trees unlike other horticultural plants is associated with very special problems. Some of these include the physiological nature of the material (Juvenile and mature phases), general recalcitrant response of the explants according to different medium, browning of explants, fungal and bacterial contamination, inadequate rooting of the regenerated shoots and the associated problems of poor transfer ratio of established plants into soil. Most of the problems arising at tissue culture level can indeed be sorted out but commercial exploitation of techniques developed for tree tissue culture into a technology calls for concentrated efforts of tissue culturists, foresters and tree breeders (Surendran *et al.*, 2000)

Juvenile tissues as a source of explant

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

Juvenile explant has been extensively employed for clonal propagation of woody plants. Frequency of sprouting is always higher in the buds taken from juvenile tissue than from mature plant during its vegetative phase. Clonal propagation from juvenile explants is useful for differentiation studies. Propagation using juvenile tissues can serve as a model system for standardization of protocol with mature tissues (Pierik, 1987) as well as provides a better understanding on the requirement of the plant for its growth and maturity. Reports of successful clonal propagation of various leguminous species are reviewed

extensively (Trigiano *et al*., 1992; Parrott *et al*., 1992). In forest tree species, the success has been largely restricted to seedling materials (Rodriguez & Vendrame, 2003).

Browning of medium

This problem is especially common with the adult tissues from woody species. The oxidative products of phenols can be phytotoxic and can cause necrosis and eventually death of explants. Several different methods have been used to overcome this problem like quick transfer of explants to fresh medium at short intervals, dark incubation at the initiation of callus, use of antioxidants like ascorbic acid, PVP and charcoal also helps in eliminating the interfering phenolic exudates.

Contamination

Endogenous microbial contamination in field grown plants is a major hurdle in clonal propagation of tree species. Removal of these contaminations is often difficult or impossible and high contamination rates are common. External treatment of different antifungal and antibiotic agents or incorporation of these agents in media can reduce the contamination.

Season of explant collection

The season of explant harvesting varies according to flowering period of the plant and geographical conditions in which the plant is growing. Explants collected in rainy season generally show maximum contamination. Maximum sprouting response with least contamination can be obtained when explants collected during natural sprouting.

Root induction

Many tree species, most conifers and woody cuttings from mature trees are generally difficult to induce root. Some plants may lack root initials because of which rooting becomes difficult.

Lot of work has been carried out on successful clonal propagation of various medicinal plants for rapid propagation such as *Baliospermum montanum* (Sasikumar *et al*.,2009, George *et al*.,2008), *Spilanthes acmella* (Haw and Keng, 2003), *Curculigo orchioides* (Wala and Jasrai, 2003), *Azadirachta indica* (Joshi and Thengane, 1996) etc.

5.2 Material and Methods –

Plant material

Explants from mature female trees were collected in different seasons of the year from Pratpgad area of Western Ghats of Maharashtra. Apical and axiliary buds were collected from healthy 10-12 year old mature trees.

Explants were kept in running water for 1 hour before surface sterilization. They were then washed with labolene (Qualigens, India) as a surfactant followed by washing with 10% savlon (v/v) (Johnson and Johnson Ltd., India) as an antiseptic. This was followed by 0.1% (w/v) polyvinyl polypyrolidone (Sigma, USA) for 30 min and later by 1% (w/v) Bavistin (BASF, India) for 60 min. After each treatment explants were washed thoroughly with single distilled water. All further operations were carried out in a laminar air flow chamber under sterile conditions. Buds were than rinsed with 70% ethanol (v/v) and washed with sterile distilled water (SDW) followed by treatment with 0.1% $HgCl_2$(w/v) (Qualigens, India) for 7 min and again washed thoroughly 4-5 times with SDW.

Establishment of sterile cultures

Fungal Contamination was a major hurdle in establishment of *Garcinia indica* cultures. Therefore attempts were made for establishing sterile cultures.

Surface sterilized buds of 3-4 cm size were inoculated on Woody Plant Medium (WPM; Lloyd and McCown, 1980) supplemented with only one concentration of 6-benzylamino purine (BAP; 8.87 µM). Medium was fortified with 2% sucrose (Qualigens, India) and solidified with 0.75% agar (Qualigens, India) after adjusting the pH to 5.8-6. All growth regulators were incorporated into the medium before autoclaving. The medium was autoclaved at 120° C for 20 min at 105 kPa. The cultures were incubated at 25 ± 1^0C at 16/8 hrs photoperiod with $35\mu E^{-2}m^{-2}s^{-1}$ illumination provided by cool white fluorescent tubes.

To avoid contamination different antifungal and antibacterial agents were added in the medium-

- Bavistin (65ppm, 75ppm, 85 ppm)
- Bavistin (65ppm, 75ppm, 85 ppm) with antibiotic cefotaxim (300 ppm, 400 ppm, 500 ppm)
- Plant Preservative Mixture (Patented product)

<u>Selection of basal media for spouting</u>

WPM (Woody plant media) and MS (Murashige and Skoog) media were tried for bud break. Both these media were incorporated with BAP (8.87 µM), 2% Sucrose and 0.75% Bavistin and effect of these basal salts on bud sprouting were studied. Effect of charcoal on sprouting of buds was also studied. 0.2% charcoal was incorporated in above mentioned media before autoclaving.

<u>Optimization of hormones for bud break</u>

After obtaining sterile cultures, different combinations of plant hormones were tested for % response. WPM media with different combinations of BAP (4.44-8.8.7 µM) kinetin (2.32-4.65 µM), Indol-3-Acetic Acid (5.71 µM) and Indol-3-Butyric Acid (4.90 µM) were used for sprouting of buds.

<u>Selection of culture vessel for shoot induction</u>

To identify the appropriate culture vessel both cotton plugged culture tubes and plastic capped jam bottles containing 20 and 80ml medium respectively were used in this experiment. Single bud was cultured per vessel. For each medium formulation 20 tubes and 15 bottles were used. The experiment was repeated thrice.

<u>Multiple shoot induction</u>

After 6 weeks, sprouted buds were shifted to WPM media containing BA (8.87 µM) with Thidiazuron (TDZ) (2.27-4.54 µM) for multiplication without any antifungal and antibacterial agents. Explants were subcultured once in two weeks to fresh medium.

Multiplied shoots obtained were separated and inoculated individually on WPM medium with 0.88 µM BA for elongation.

<u>Rooting of shoots</u>

Shoots with approximately 4.4-5 cm length were transferred to liquid WPM medium fortified with IBA (245-490 µM) containing half strength macro salts with 1% sucrose to test rooting response. Shoots were incubated in this media for 48 hrs and then shifted to plane WPM media with half strength macro salts and 1% sucrose and solidified with 0.2% phytagel (Sigma, USA).

<u>Hardening of plantlets</u>

After 6 weeks in rooting media, rooted plantlets were hardened in pots containing a mixture of sterilized soil and sand (1:1) and maintained in controlled growth chamber

conditions (25 ± 2 °C, 16-h photoperiod, 80 % relative humidity and irradiance of 50 μmol m^{-2} s^{-1}).

Ex vitro rooting of shoots

In vitro raised shoots of _G. indica_ were also tested for _ex vitro_ rooting response. Shoots were treated with aqueous solution containing IAA (342 and 684 μM) and IBA (295 and 588 μM) for 2 hrs, planted in plastic pots containing sand and coco peat (1:1) and covered with polythene bags to minimize loss of moisture and maintain high humidity. Pots were maintained at 25 ± 1°C in 24 hrs light with watering after every 7days. The established plants were transferred to greenhouse.

5.3 Results

Nodal segments have been demonstrated to be useful material for mass propagation of woody plants because of high regeneration ability of the explants (Kaur _et al_., 1998, Kumar _et al_., 1998, Quraishi and Mishra, 1998). It is considered that shoots originated from axillary buds are preferable for vegetative propagation because of reduced risk of genetic instability (Hussey, 1986). Since the explants were obtained from field grown trees, establishment of axenic cultures was a major hurdle in the development of a regeneration protocol from mature trees. Major constraint encountered is the extent of contamination and exudation of phenolics associated with culture initiation.
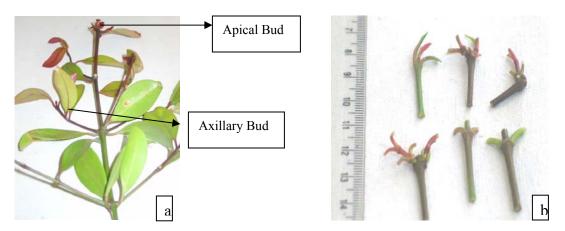

Figure 5.1 a. Vegetative twig of _Garcinia indica_, b.Explants

The inoculated buds began to sprout after 10-12 days in culture on induction media. The initial responses were tissue browning, contamination, bud growth along with shoot elongation. Exogenous and/or endogenous contamination was the main factor limiting culture establishment. Explant contamination was evident after fourth day of inoculation. Contamination frequency varied from 10 to 70%.

Another limiting factor in culture establishment was browning of explants. The explants that were not contaminated turned brown and subsequently died. Similar limitations in micropropagation have been observed by Yu and Reed (1995) and Nas (2004).

5.3.1 Establishment of sterile cultures

To avoid the contamination problem effect of antifungal and antibiotic agents were tested. External treatment of these agents during surface sterilization does not give any significant reduction in % contamination. Therefore these antifungal and antibiotic agents were incorporated into WPM media containing BAP (8.87µM) with 2% Sucrose and 0.75% Agar.

Media incorporated with only Bavistin (75 ppm) showed 57% contamination where along with fungal, bacterial contamination was also very high. Addition of 400 ppm Cefotaxim along with 75 ppm Bavistin gave maximum i.e. 86% sterile cultures. Niedz (1998) reported that PPM at 0.2% concentration could prevent post culture contamination in plant culture vessels. Babaoglu and Yorgancilar (2000) reported similar observation in the regeneration of salad burnet, PPM was also used to establish axenic cultures in wych elm (Biroščíková *et al*., 2004). In case of *G.indica* media containing Plant Preservative Mixture (PPM) gave 52.5-62.5% sterile cultures but these explants turned brown after sprouting. On the contrary sterile buds on bavistin and cefotaxim containing media were healthy and green with formation of new leaves on repeated subculture after 15 days (Table 5.1).

Table 5.1 Effect of antifungal and antibiotic agent on % sterile cultures of
Garcinia indica

Media	% sterile	% contamination
BAP (8.87µM) + Bavistin (75 ppm)	42.5	57.5
--- ''---+ --- ''---+ Cefotaxim (300 ppm)	30	70
--- ''---+ --- ''---+ Cefotaxim (400 ppm)	86	14
--- ''---+ --- ''---+ Cefotaxim (500 ppm)	40	60
BAP (8.87µM) + 1 ml PPM	52.5	47.5
BAP (8.87µM) + 2 ml PPM	62.5	37.5

Thus 75 ppm Bavistin and 400 ppm Cefotaxim were found to be optimum for establishment of sterile *G. indica* cultures.

Figure 5.2 a.Effect of Bavistin , Cefotaxim and PPM on contamination control of
Garcinia indica **cultures, b. Healthy and sterile bud on media containing 75ppm**
Bavistin and 400ppm Cefotaxim

5.3.2 Effect of explant collection season

The rate of contamination or bud break was highly dependent on the season during which the material was collected. By the best sterilization procedure described above, the cultures initiated in January – May showed higher bud break and less contamination than those raised in June – November (Table 5.2). Since later period coincides with the rainy season in India, 100% explants showed contamination. Another reason may be that new sprouting started in January. These new sprouts give less contamination.

Table 5.2 Effect of collection season on establishment of sterile cultures
of *Garcinia indica*

Month of collection of explant	Phynology of Plant	75 ppm Bavistin		75 ppm Bavistin+ 400 ppm Cefotaxim	
		% Sterile cultures	% Contamination	% Sterile cultures	% Contamination
January	Flowering	42.5	57.5	86	14
April	Fruiting	46	55	34	63
May	Fruiting	31	57.5	37	61
September	Vegetative	17.5	72	13	96
November	Vegetative	8	92	4	96

Data scored after 6-8 days

Two basal media WPM and MS were tried in the initial experiments to decide the basal media. It was found that WPM media was more suitable for the growth of this species as MS media gave stunted growth with small leaves. Similar results has been observed in *Arbutus unedo* L. in which the MS salts resulted in oxidative browning and death of the explants in 3 weeks (Mereti *et al.*, 2002). The inhibitory effects of MS salts can be due to the high concentration of salts especially NH_4NO_3 in MS medium (Dalal *et al.*,1991).

5.3.3 Effect of culture vessel on bud sprouting

High frequency of response in tubes than in bottles on WPM media containing 8.87µM BAP suggests that culture vessel with cotton plug provides more supportive microenvironment for growth of *G. indica* cultures. In bottles, the leaves were small and only partially opened and leaf fall was seen after 4-5 days of incubation. On the contrary the leaves were fully opened and green in tubes.

If the lid of vessel is sealed totally (in case of bottles) and no leakage could be found, the rate of gaseous exchange in culture vessels becomes zero (Chen and Chen, 2002). In such cases, inside gas concentrations; such as those of CO_2 and C_2H_4 accumulate to the danger level. Ethylene has also been found to affect the growth of plantlets in vessel (Matthys *et al.*, 1995). The ethylene concentration inside the vessel can be decreased by the gaseous exchange between inside microenvironment and outside air. However, if the culture vessel has more gaseous exchange rate, more water will evaporate from medium. The hardness of the medium increases as the moisture content decreases and the growth of plantlet may be retarded. Higher gaseous exchange rates also enhances the incidence of contamination. Cotton plugs in tubes permit exchange of gases and the moisture produced from evaporation of medium escapes through plugs creating a drier microenvironment for the tissue. On the contrary in bottles the moisture is trapped and the microenvironment of the culture remains saturated with moisture. This is reflected in condensation of moisture on the inner surface of the bottle. This study confirms the requirement of drier environment for the growth of this tissue.

5.3.4 Axillary bud proliferation

Nodal explants cultured on WPM medium with different concentrations of BAP, Kinetin, IBA and IAA alone or in combination, facilitated axillary bud sprouting after 2 weeks.

Charcoal in general reduced the sprouting percentage of *Garcinia indica*. If explants were continued to grow in charcoal media leaf fall occurred. Incorporation of activated charcoal in tissue culture media may either have a beneficial or adverse effect on growth and development, depending on the species and tissue used. Previous studies indicated that the effect of activated charcoal can be attributed to establishing a darkened

environment, adsorption of undesirable substances or growth regulators, or both (Pan and Van Staden, 1998). Activated charcoal enhanced the nitrogen source uptake to improve the proliferation of *Lagerstroemia indica* (Eymar *et al*., 2000), but reduced growth and inhibited bud proliferation in *Morus latifolia* (Lu, 2002). The inhibitory effect of activated charcoal on sprouting in *Garcinia indica* may be attributed to the absorption of plant growth regulators or other organic compounds in the medium.
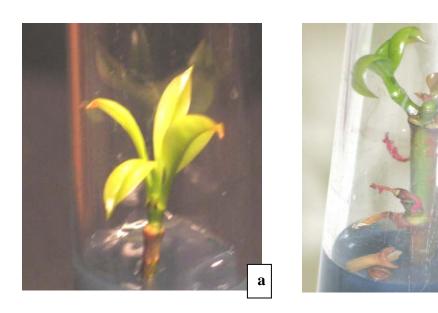
Figure 5.3 a. Bud sprouting on WPM media containing BAP (8.87 μM) with charcoal after 15 days incubation, b. Bud showing leaf fall on charcoal containing media

% response varied with the type of growth regulator used and its concentration. Among the various auxins and cytokinins tested BAP alone in concentration 8.87 μM resulted in maximum % of explants showing sprouting (82%) (Table 5.3). Similar effect of BAP on axillary bud proliferation is observed by Martin (2003) and Hiregoudar *et al*., (2006). BAP at higher concentrations not only reduced the response but also resulted in stunted growth of shoots. This result shows that the increase in BAP concentration above certain level does not correlate with enhancement of % response. Shoots were incubated on these particular media for 45 days with shifting to fresh media after every 15 days.

With the addition of kinetin in media 65-75% sprouting was observed. Though the % response is noticeable these buds on further subculture showed leaf fall with blackening of tissue. Shifting of these sprouted buds on best hormonal concentration (8.87 μM BAP in

this case) after 15 days did not showed any significant change. This means incorporation of kinetin in media during initial sprouting of buds affects its growth and the effect get carried over further.

Along with BAP and kinetin, effect of IBA (4.90 µM) and IAA (2.85 µM) on bud proliferation was also tested where both of these auxins do not affect significantly. Only 21-37 % sprouting was observed upon addition of these auxins.

Table 5.3 Effect of different hormones on % response in *Garcinia indica*

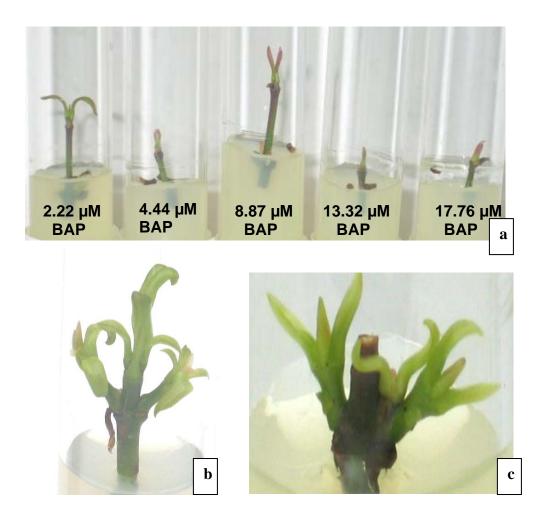| BAP (µM)	Kinetin (µM)	IBA (µM)	IAA (µM)	% response
2.22	-	-	-	55
4.44	-	-	-	79
8.87	-	-	-	82
13.32	-	-	-	68
17.76	-	-	-	62
2.22	2.32	-	-	65
4.44	2.32	-	-	72
8.87	2.32	-	-	75
13.32	2.32	-	-	71
17.76	2.32	-	-	68
2.22	4.64	-	-	31
4.44	4.64	-	-	52
8.87	4.64	-	-	48
13.32	4.64	-	-	41
17.76	4.64	-	-	32
4.44	2.32	4.90	-	21
8.87	2.32	4.90	-	25
13.32	2.32	4.90	-	28
4.44	2.32	-	2.85	31
8.87	2.32	-	2.85	35
13.32	2.32	-	2.85	37

Figure 5.4 a.Sprouting of buds on different concentrations of BAP after 8
Days of inoculation
b. Sprouted axillary bud on WPM media containing BAP 8.87
µM BAP after 20 days culture
c. Sprouted bud on BAP 8.87 µM BAP after 40 days

5.3.5 Multiplication of shoots

For multiple shoot induction the sprouted buds were transferred to different concentrations of TDZ (0.22-4.54 µM) with constant BAP (8.87 µM) with repeated subculture.

G. indica tissue was exposed to TDZ for 45 days with subculture on fresh media after every 15 days. This was followed by addition of low BAP concentration.

The shoots were sub cultured on WPM media containing 8.87 µM BAP constant with different concentrations of TDZ (0.22-4.45 µM).

Table 5.4 Effect of TDZ concentration on no. of multiple shoots and length of shoots in nodal explants of *Garcinia indica*

Medium	S1		S2		S3	
	No.of shoots/ explant	Length of shoots (± SE)	No.of shoots/ explant	Length of shoots (± SE)	No.of shoots/ explant	Length of shoots (± SE)
TB_1	1.11	1.1±0.06	1.5	1.3±0.8	2.22	1.5±0.5
TB_2	1.63	0.9±0.5	2.11	1.2±0.7	2.44	1.4±0.8
TB_3	1.78	0.8±1.2	2.64	1.2±0.5	2.85[**]	1.3±0.1
TB_4	2.75[**]	1.4±0.8	3.62[*]	2.3±1.1	3.75[*]	2.4±1.1
TB_5	2.66	1.3±0.01	3.33[*]	2.6±0.9	3.33[*]	2.8±0.7
TB_6	2.75[**]	1.4±0.5	3.87[*]	2.9±0.9	3.87[*]	3.4±0.9

Basal media WPM + 2% sucrose + 0.7% agar

TB_1- BA (8.87 µM) + TDZ (0.22 µM),   **TB_2**- BA (8.87 µM) + TDZ (0.91 µM)

TB_3- BA (8.87 µM) + TDZ (1.82 µM),   **TB_4**- BA (8.87 µM) + TDZ (2.72 µM)

TB_5- BA (8.87 µM) + TDZ (3.63 µM),   **TB_6**- BA (8.87 µM) + TDZ (4.54 µM)

**, * Significant at 5% and 1% level, respectively

Initiation of multiple shoots was observed after 20 days on TB_1 to TB_6 media. Significantly higher multiplication rate were recorded under the combined treatment of 8.87 µM BAP and 4.54 µM TDZ (significant at 1% level) where 3.87 shoots/explant were induced at third subculture with shoot length of 3.40 cm (Table 5.4). The shoots were subcultured on the same media after about 15 days. At every stage of subculture on same media shoot multiplication was observed.

These shoots elongates further with repeated subculture on respective media. After 40-45 days these multiple shoots can be separated. Transferring to fresh media after 15

days was found to be essential to prevent defoliation and culture deterioration for sustained shoot growth. The multiple shoots which proliferated in culture were harvested and the original explant was repeatedly transferred to fresh TB_1 to TB_6 media. Transferring of original explant to TB_1 to TB_6 media did not induce any additional multiple shoots. New shoots harvested at each subculture were inoculated individually on same media to obtain further multiplication.

The phenomenon of fasciated shoots has been reported to be associated with the use of TDZ (Huetteman and Preece, 1993). The regenerated plants produced in this study exhibited neither fasciated shoots nor any other abnormal characters. For micropropagation of *Garcinia indica* TDZ was found to be best without any adverse effect.

High concentration of TDZ resulted in decreased mean number of shoots in cultures with considerable callusing.
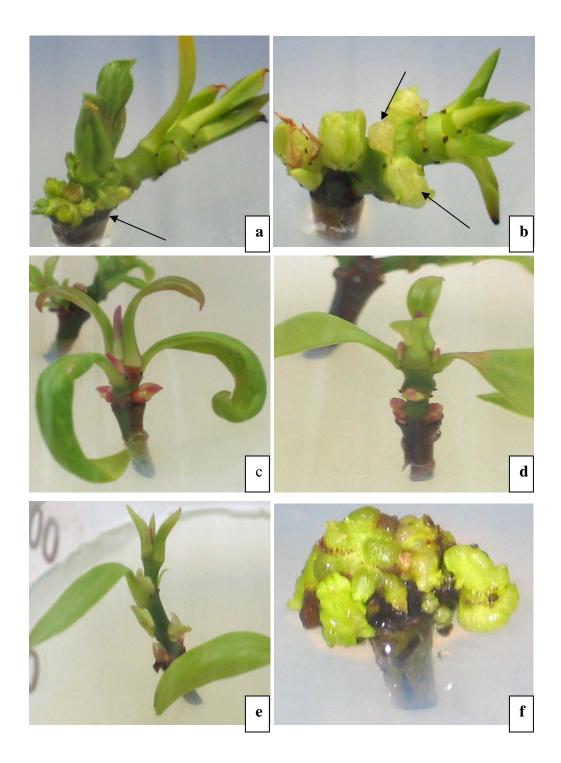
Figure 5.5 Effect of TDZ on multiple shoot induction in *G. indica*

a and b.Initiation of multiple shoots on TB_6 media (data scored after 12 days of incubation), c and d.Multiple shoot induction onTB_5 media, e.Effect of TB_4 media on multiple shoot induction (data scored after 25 days of incubation),

f. Callusing effect in higher concentration of TDZ

Shoots remained very small on multiplication media and required an elongation step to obtain shoots suitable for rooting. Therefore WPM media with a lower level of BAP (0.89 µM) was used for shoot elongation. Reduced concentration of BAP stimulates shoot elongation. Similar effect of BAP is observed by Rodriguez (1982) in *Castanea sativa* and by Chalupa (1987) in *Tila cordata.* At higher concentration of BAP shoot elongation gets inhibited (Fukui *et al.*, 1987; Bahrany, 2002).

Elongated shoots of 4.4-5.0 cm length was observed within 15–20 days after transfer on BAP containing media. These shoots were then used for rooting (Figure 5.6)
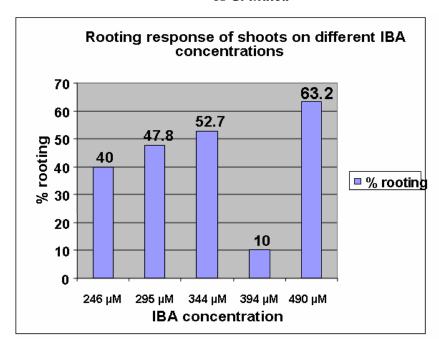

Figure 5.6 Elongated shoots of height 4.4-5.0 cm on WPM media with BAP (0.89 µM)

5.3.6 *In vitro* rooting

Shoots of 4.4-5.0 cm were transferred to WPM media containing half strength macro stock and IBA (245-490 µM) supplemented with 1% sucrose without agar to check their rooting competence. These shoots were incubated on these media for 48 hrs and then shifted to WPM media containing half strength macro salts with 1% sucrose and 0.2% phytagel without any hormones.

Root induction was observed within 20-25 days from the base of shoots at nodal portion (Figure 5.8 a-c). Maximum 63.2% rooting was noted on 490 µM IBA with 3.4 cm length of shoots. Rooting % was very less (10%) on 394 µM IBA containing media while 344 µM IBA showed 52.7% rooting but these roots did not grow further (Figure 5.7).

Figure 5.7 Effect of different IBA concentrations on % rooting of adventitious shoots of *G. indica*

Rooting response of shoots on different IBA concentrations

Basal media – WPM ½ Macro + 1 % Sucrose (liquid media)

Similar effect of IBA on rooting of woody plants was observed in wych elm (Biroscikova *et al*., 2004), neem (Chaturvedi *et al*., 2004), Quercus (Purohit *et al*., 2002), licorice (Thengane *et al*., 1998) etc.
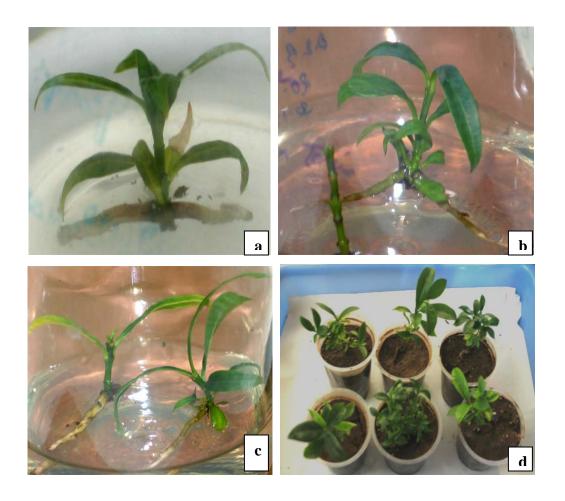
Figure 5.8 a-c. Rooting of shoots on WPM media containing IBA 490 μM

d. Hardened plants in green house

The well developed plantlets were later shifted to potting mixture of sterilized sand and soil (1:1) for acclimatization. These pots were initially covered with polythene bags for 15 days to maintain humidity and then removed gradually. Acclimatized plants were then transferred to green house, where 70% survival was achieved (Fig.5.8 d).

5.3.7 *Ex vitro* rooting

Ex vitro rooting was also induced where *in vitro* grown shoots of 4.4-5.0 cm length were dipped in IAA (342 & 684 μM) and IBA solutions (295 &588 μM) for 2 hrs and then shifted to potting mixture containing sterilized sand and cocopeat (1:1) and covered with

polythene bags to maintain moisture. These pots were maintained in hardening room with 25^0C temperature and continuous light. Watering was done after every 8 days.

Table 5.5 Effect of different auxin concentrations on % *ex vitro* rooting

Auxin concentration	% rooting
IBA (295 µM)	10
IBA (588 µM)	10
IAA (342 µM)	40
IAA (684 µM)	20

Figure 5.9 *Ex vitro* root induction

a. Shoots shifted to potting mixture of sand and cocopeat (1:1) after dipping in auxin solution for 2 hrs, b-c. Rooting of shoots after 55 days on IAA (342 µM) (b,c)

40 % rooting was noted in IAA treated shoots within 50-60 days. These plantlets were then shifted to greenhouse where 60% survival was obtained (Figure 5.9 a-c).

Ex vitro rooted plantlets survived better in greenhouse with higher elongation of plantlets and more leaf area.

5.4 Discussions

The experiment was conducted with an objective to generate clonal propagation protocol for female plants of *G.indica.*.

Serious limitations due to microbial contamination especially endophytic fungi were encountered during standardizing the protocol. To obtain sterile cultures and to avoid recurrence of fungal contamination after bud sprouting was a major hurdle. In order to establish sterile cultures several factors like the concentration of basal media, broad spectrum antibiotic like cefotaxim and antifungal Bavistin, season of explant collection were tested. Finally after incorporation of cefotaxim and bavistin in WPM basal media, 86% sterile cultures were obtained. % contamination varied with the season of explant collection. Bavistin in the culture medium has been reported to reduce fungal contamination in Litchi (Das *et al*., 1999). Cefotaxime has been found effective in controlling bacterial growth in plant tissue cultures of *Chrysanthemum* (Jaime and Teixeira, 2003), *Lycopersicon* (Raj *et al*., 2005), Cucumber (Sapountzakis and Tsaftaris, 2002) etc. Therefore this antibiotic was tested to raise microbe free culture.With the standardized sterilization treatment maximum sterile cultures can be obtained from explants collected in the month of January. Maximum contamination (96%) was observed in the month of September – November as this is monsoon period in India. Similarly seasonal effect on culture establishment has been reported in apple (Hutchinson, 1984), papaya (Litz and Conover, 1981), sweet gum (Sutter and Barker, 1985) and guava (Amin and Jaiswal, 1987).

Cultures maintained in cotton plugged culture vessels i.e. test tubes and conical flasks responded better than cultures in screw capped bottles. Higher gaseous exchange in cotton plugged cultures may be the reason for this. Cotton plugged cultures permits exchange of gases and moisture produced from evaporation of medium escapes through plugs thus creating drier microenvironment for the tissue. Influence of culture vessels on

growth and development of culture has been emphasized in woody species (McClelland and Smith, 1990) cultures.

Effect of different hormone composition on % sprouting was studied where maximum (82%) sprouting was observed on WPM basal media with 8.87 µM BAP. Addition of TDZ at different concentration in media keeping BAP (8.87 µM) constant showed multiple shoot induction. Concentration of 4.45 µM TDZ was found optimum for proliferation of sprouted buds. Such microshoots upon transfer to low concentration of BAP showed elongation upto 4.4-5.0 cm length. TDZ, is a phenylurea derivative known to simulate cytokinine like action. It is reported that an optimum exposure of explants in TDZ supplemented medium followed by the withdrawal of GR effectively triggers shoot multiplication in species like *Psoralea corylifolia (*Faisal *et al.*, 2006), *Tamarindus indica* (Mehta *et al.*, 2005), *Saintpaulia ionantha* (Mithila *et al.*, 2003), *Medicago truncatula* Gaertn. cv. Jemalong and *Medicago truncatula* ssp. Narbonensis (Neves *et al.*, 2001) and *Hibiscus cannabinus* (Srivatanakul *et al.*, 2000). On the contrary, cultures continuously grown on TDZ containing media resulted in the formation of fasciated and distorted shoots. Deleterious effect of continued presence of TDZ was reported on growth and multiplication of *Cicer arietinum* (Murthy *et al.*, 1996), *Pisum sativum* (Bohmer *et al.*, 1995) and *Rauwolfia tetraphylla* (Faisal *et al.*, 2005).

To induce rooting to these shoots were one of the major hurdles to develop complete protocol. After planning various experiments, 61.2% rooting was obtained in 490 µM IBA with 3.4 cm length of shoots within 20-25 days. *Ex vitro* rooting was also induced where on treatment with IAA (342 µM) 40% rooting was observed. But the time period required here varied from 50 to 60 days.

The results demonstrated the feasibility of the application of tissue culture technique for raising female and elite trees of *G. indica* - a tree of great economic importance. The rate of multiplication is low since the explants are derived from mature woody trees. Woody plants have been found to be in general recalcitrant to *in vitro* regeneration. Tissue culture of trees unlike other horticultural plants is beset with very special problems. Some of these include the physiological nature of the material (Juvenile and mature phases), general recalcitrant response of the explants vis a vis medium, inadequate rooting of the regenerated shoots and the associated problems of poor transfer to survival ratio for establishment of plants into soil.

Clonal multiplication of superior selected trees is desirable in economically important species. In *G. indica* same can be achieved by softwood grafting, seed germination and micropropagation. *G. indica* is a cross pollinated and of polygamodioecious tree where differentiation between male and female can be done only after flowering stage (7-8 years). Tree growth is very slow and in horticultural practices propagation is done by seeds and soft wood grafting (George *et al.*, 2002). The seeds are recalcitrant due to high sensitivity to desiccation and freezing with shelf life of only 4 weeks (Malik *et al.*, 2005). Thus, it is not possible to raise seedlings throughout the years. Provided in an orchard established with seedlings, 50% plants are generally male. Grafting is another method used for clonal propagation of *G. indica* (Nawala and Karmarkar, 1997; Haldankar *et al.*, 1993) which is season dependent, space requiring and grafted plants showed change in the canopy which reduces fruit yield. Thus it has limited applications. Availability of root stock is another limitation.

Micropropagation is the best method for propagating the plants clonally as clonal progenies are generally desired. Though the plant is distributed in konkan region of Maharashtra, number of female plants is less. The plant is listed as endangered in southern Western Ghats of India. So conservation of female plants are necessary. Moreover, being a horticultural crop, female plants are more important. Clonal propagation offers a potential method for mass multiplication of female *Garcinia indica* plants on a large scale for conservation purposes. Along with this the elite female plants having maximum % of HCA can also be propagated by this method.

Using this protocol 4-5 plants from single apical bud can be produced within 130 135 days in case of *in vitro* rooting and 165-170 days in case of *ex vitro* rooting.

6.1 Introduction

In nature, stem, leaf and root pieces of several taxa are able to differentiate shoots and roots leading to the establishment of new individuals. *De novo* morphogenesis is 'the *de novo* production of plant organs (buds, shoots, roots) or structures resembling zygotic embryos from non meristematic organized tissues or callus'. Capacity of cultured plant tissues and cells to undergo *de novo* morphogenesis, resulting in the formation of discrete organs or whole plants, has provided opportunities for numerous applications of *in vitro* biology, viz. in studies of basic botany, biochemistry, propagation, breeding and development of transgenic crops (Phillips, 2004).

De novo morphogenesis *in vitro* occurs in two different pathways i.e. organogenesis and embryogenesis. It can occur directly on explant or indirectly via callus. Primary advantage of *de novo* morphogenic methods over axillary shoot methods is a potential for high frequency plantlet production in a shorter period. Advantage of these systems over the axillary shoot methods lies in the amenability to different gene delivery techniques. Moreover any plant part can be used for the propagation. Method of propagation via buds depends on the season but this is not a case with *De novo* morphogenesis.

Organogenesis is the production of organs, either directly from an explant or indirectly from callus culture. Direct organogenesis can be either from pre existing meristem like apical bud, axillary bud or embryo or it can be from fully differentiated tissues like cotyledons, hypocotyls, epicotyl, root or leaf. According to Thorpe (1994), *De novo* organogenesis is the process by which cells and tissues are manipulated to undergo changes, which lead to *de novo* production of unipolar structure namely a shoot or root primordium, whose vascular system is often connected to the parent tissues. It can occur directly on explant or indirectly via callus.

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 then can be rooted relatively simply. The earliest report on the formation of *de novo* 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, such as plant modification and improvement, clonal propagation and germplasm storage (Banerjee and de Langhe, 1985; Levin *et al*., 1988; Brown and Thorpe, 1995; Jain, 2001; Chaturvedi *et al*., 2004), but also provides an 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, had been successfully regenerated from hundreds of plant species (Tran Thanh Van, 1973; Hicks and McHughen, 1974; Lu *et al*., 2000; Li *et al*., 1999, 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.

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

of *Albezzia lebbeck, Cassia fistula* and *C. siamea* (Gharyal and Maheswari, 1990) and root segment of *Citrus aurantifolia* (Bhat *et.al*., 1992).

6.1.1 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). In case of indirect organogenesis 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 can be induced by regulating the ratio between auxin and cytokinin. A relatively high ratio of auxin to 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*. 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.

6.1.2 Molecular basis of Organogenesis

Thorpe and Meier (1975) 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. Gibberellins 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.

6.1.3 Genes involved in organ identity

Organ identity genes play a key role during organogenesis in plants. Molecular analysis of *de novo* organogenesis shows that inductive expression of organ identity genes are 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). During shoot regeneration, number of genes are required for shoot apical meristem (SAM) formation, such as *SHOOT MERISTEMLESS* (*STM*), and *CUP-SHAPED COTYLEDON 1* and *2* (*CUC1* and *CUC2*) (Cary *et al*., 2002; Gordon *et al*., 2007). Cytokinin can stimulate the expression of *STM* which is involved in shoot meristem formation in tissue culture (Rupp *et al*., 1999; Teo *et al*., 2001). *CUC1* and *CUC2* are upregulated during callus formation on CIM (Callus Induction Medium) (Cary *et al*., 2002; Gordon *et al*., 2007). *CUC2* is also upregulated after the callus is transferred onto SIM (Shoot Induction Medium), and its expression reaches a peak at 6 d. At this time, clusters of small dividing *CUC2*-positive cells, which will develop into new shoot meristems with high frequency, were observed in some regions of the callus while absent from others (Cary *et al*., 2002;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. The induction of TFL1 is critical to *Arabidopsis* inflorescence regeneration. 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 *in vitro* organogenesis. However, the pathway by which the organ identity genes are activated by phytohormones remains unclear.

Until recently, standardization of successful regeneration methods has proceeded empirically. The literature abounds 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.

Organogenesis from different explants have been reported in many medicinal plants like *Arnebia euchroma* (Manjkhola *et al*., 2005), *Eryngium foetidum* (Martin, 2005), *Hypericum perforatum* (Zobayed and Saxena, 2003), *Embelia ribes* (Raghu *et al*., 2006), *Oldenlandia umbellate* (Siva *et al*.,2006). Shoots developed from root segments of same plant have been suggested to be genetically uniform (Bud, 1973). Moreover, root explants are advantageous over the other explants in terms of their easy manipulation and high regeneration potentials (Frankin *et al*., 2004).

In the present study, protocol for *de-novo* shoot regeneration from embryogenically derived plants of *Garcinia indica* is reported.

6.2 Materials and Methods

Shoot Induction: 8 months old somatic embryo derived plantlets served as the explants source. Three experiments were conducted to optimize conditions for *de-novo* shoot differentiation from root explants.

Expt A- Somatic embryo derived plantlets were kept for prolong incubation for about
60 days on defined media.

Expt B- Roots of somatic embryo derived plantlets showing bulging were separated from
mother plant and inoculated on defined media.

Expt C- Root segments cut from somatic embryo derived plantlet were used as an explant.

Half strength MS medium (Murashige and Skoog, 1962) supplemented with 6-benzyl amino purine (BAP) (0.44-2.22μM) containing 2% sucrose (Himedia, Mumbai, India) and 0.8% agar (Qualigens, Mumbai, India) were used in all the experiments. The growth regulators were incorporated into the medium before autoclaving. Medium pH was adjusted to 5.7-5.8 with 1N NaOH. The media were autoclaved at 120^0C for 20 min at 15psi. Each treatment had 20 replicates and experiments were repeated three times. The cultures were incubated at 25 ± 1^0C under 16/8 hr photoperiod with $35\mu E^{-2}m^{-2}s^{-1}$

illumination provided by cool white fluorescent tubes. The frequency of shoot formation on root explants was scored at 15 days intervals for 60 days.

For histological studies, root explants were fixed in glacial acetic acid and absolute ethanol (1:3) for 24 hrs. and then stored at 4^0C in 70% ethanol till further use. Fixed tissues were dehydrated through different grades of water: ethanol: tertiary butyl alcohol (TBA) series (Sharma and Sharma, 1980) and then embedded in paraffin wax (melting point 59-60^0C). Serial sections were cut at 10μm, stained with hematoxylene (1% w/v)-Eosin (1% w/v), mounted in DPX [2 chloro N (4 methoxy 6 methyl–1, 3, 5-triazin-2-yl amino carbonyl) benzene sulfonamide] (Qualigens, India) and observed microscopically.

Elongation: Cultures were maintained on the respective media for 60 days with transfer to fresh media at 20 days interval. Regenerated shoots 0.5-1 cm height were separated from each other and transferred to Woody Plant Medium (Lloyd and McCown, 1980) (WPM) containing combinations of BAP (4.44-22.69μM), IAA (5.71μM) and Kinetin (4.65μM) for elongation.

Rooting: After 45 days the elongated shoots were transferred for rooting to WPM basal medium along with auxin combination α-napthalene acetic acid (NAA) (2.69-10.74μm) and 4.90μm indole-3-butyric acid (IBA). In this medium phytagel 0.25 % was used as gelling agent.

Well developed plantlets of approximately 4-5 cm length and 4 leaf stages were transferred to potting mixture of sterilized sand and soil (1:1) and shifted to green house for acclimatization.

6.3 Result

In Experiment A and B, along the length of roots bulging at the intervals was observed which further showed shoot regeneration within 15-20 days on half strength MS media containing BAP (0.44-2.22μM) (Figure 6.1 a and b). Shoot bud differentiation always occurred at the distal region (away from mother plant) of the roots in bunches of 4 to 20 shoots/bunch.
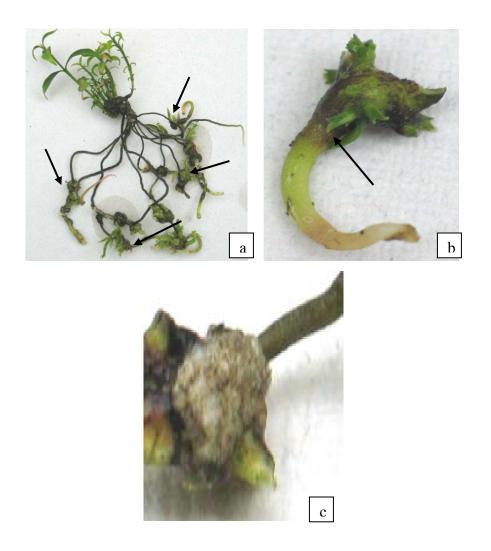
Figure 6.1. Explants showing bulging and initiation of shoot growth root explant a: Expt A Roots still attached to mother explant, b: Expt B Roots separated from mother explant after bulging, c: Expt C Explant showing callus after bulging

However, in most of the isolated root segments inoculated separately (Expt.C) bulging was followed by production of white callus which failed to show any shoot growth (Figure 6.1 c). Only rarely shoot induction was noticed where number of shoots were only 1-2. These shoots did not show any further growth.

Histological examination indicated that shoot induction originated from root cortical tissue. Enlargement of the roots occur with production of meristematic pockets in the cortical tissue (Figure 6.2b). Some of the cells from cortex becomes meristematic and produce shoot primordia, which were pushed outside the epidermal layer (Figure 6.2c).
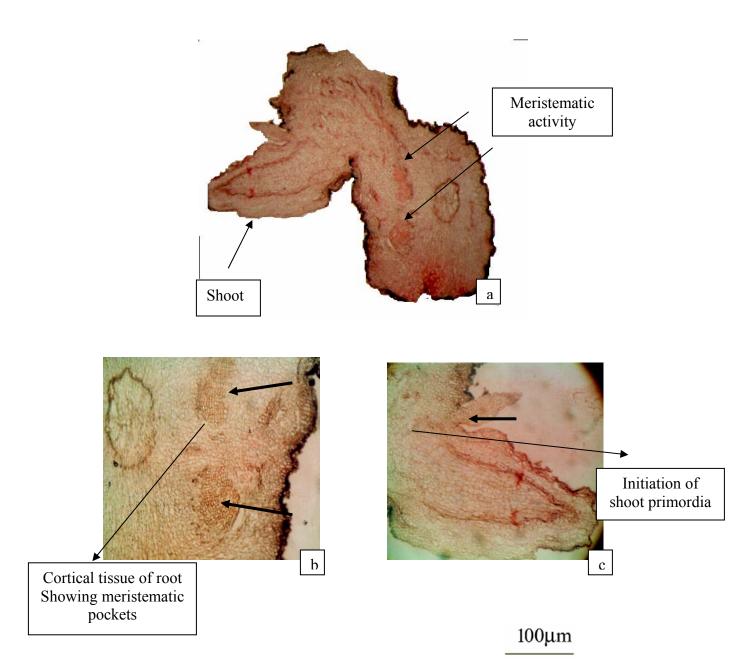
Figure 6.2.Histological studies

a. **T.S. of root showing initiation of shoots from cortical region along with**
meristematic activity, b. T.S. of root showing cortical tissue with meristematic
pockets, c. Section showing initiation of shoot primordia from root cortex pushed out
from subepidermal region of root

In experiment A, where the roots were still attached to the mother plant increase in concentration of BAP increases the number of shoots produced per root explant. Maximum number (29.3) was obtained on medium containing 2.22 µM BAP after 60 days (Table

6.1). While in experiment B, reduced concentration of BAP promoted shoot production. In this case maximum shoot production (19.50) was observed in media containing 0.44 µM BAP while higher concentration of BAP had adverse effect. In excised root segment (experiment C), 1-2 shoots per root explant were observed irrespective of BAP concentration (Table 6.1).

Table 6.1 Effect of BAP concentration on production of number of shoots/root explant

Medium composition (MS half strength + BAP)	Average number of shoots per root		
	Expt A	Expt B	Expt C
0.44 µM	9.75	19.50*	0.35
0.88µM	21.30	17.14	0.59
1.33µM	22.76	13.01	1.31
1.78µm	13.31	9.22	0.88
2.22µM	29.27**	10.88	0.32
LSD<0.05	10.44	10.48	10.48

Data scored 60 days after inoculations

Basal medium:1/2 strength MS + sucrose 2% + agar 0.85%

*, ** Significant at 5% and 1% level, respectively.

Expt A- Somatic embryo derived plantlets kept for prolong incubation for about 60 days on defined media.

Expt B- Roots of somatic embryo derived plantlets showing bulging were separated from mother plant and inoculated on defined media.

Expt C- Root segments cut from somatic embryo derived plantlets were used as an explant.

Figure 6.3 Increase in number of shoots produced on root explant on half strength MS media containing 2.22µM BAP after 45 days

In all the experiments increase in shoot numbers were observed till 60 days culture on all media combinations. After that no further increase in shoot number was noted. After 60 days shoot length ranges from 0.5-0.8 cm. However, the shoots induced in experiment A were healthier with respect to length and number of leaves than in experiment B. All the shoots produced on excised root segments (Experiment C) turned brown after 40-45 days.

After 60 days as no further increase in number of shoots and elongation were observed these shoots were transferred to elongation WPM media containing BAP (4.44-22.19µM) + IAA (5.71µM) + Kinetin (4.65µM) where effects of increased BAP concentration keeping IAA and Kinetin constant was studied. Maximum elongation (2.9 cm) was observed on 22.19µM BAP in 45 days (Table 6.2) (Figure 6.4 a, b).

These elongated shoots still attached to root explant were then separated from explant and inoculated separately on rooting media.

Table 6.2 Effect of supplementation of different growth hormones on elongation of shoots

BAP (μM)	IAA (μM)	Kinetin (μM)	Mean length of shoots (in cm) (± SE)
4.44	5.71	4.65	1.59±1.22
8.87	,,	,,	1.04±1.23
13.30	,,	,,	2.15±1.10
17.80	,,	,,	0.79±1.91
22.19	,,	,,	2.89±0.98

Basal medium: WPM + sucrose 2% + agar 0.85%

Data scored after 45 days

Figure 6.4 Elongated shoots on WPM media supplemented with BAP (22.19 μM)+ IAA (5.71 μM) + Kinetin (4.65 μM)

For rooting induction WPM basal media with NAA (2.69-10.74µM) keeping IBA (4.90µM) constant were tried where maximum 77% rooting (Figure 6.5) was obtained on NAA (5.37µM) and IBA (4.90µM) in about 20-25 days (Figure 6.6 a, b).

Figure 6.5- Effect of auxin composition on *in vitro* rooting

Media A- NAA (2.69 µM)+(IBA 4.90 µM)

Media B- NAA (5.37 µM)+IBA (4.90 µM)

Media C- NAA (10.74 µM)+IBA (4.90 µM)

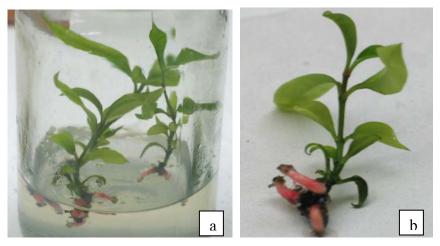Basal medium: WPM + sucrose 2% + Phytagel 0.25%

Data scored after 23 days

Figure 6.6 Rooting of shoots on WPM media fortified with

NAA (5.37 µM) and IBA (4.90 µM)

The plantlets of height 4-5 cm and 4 leaf stage were later shifted to potting mixture of sterilized sand and soil (1:1) for acclimatization and transferred to a greenhouse. Temperature of greenhouse was maintained to 24 ± 2^0C. Hardened plants showed 90% survival in greenhouse (Figure 6.7).

Figure 6.7 Hardened plants in green house

6.4 Discussions

In vitro micropropagation via organogenesis is essential for plant genetic resource management and has become increasingly important for conservation of rare plant species. Shoots developed from root segments of same plant have been suggested to be genetically uniform (Bud, 1973). Moreover, root explants are advantageous over the other explants in terms of their easy manipulation and high regeneration potentials (Frankin *et al.*, 2004). In *Brassica* species shoot differentiation has been reported from root explants, seedling root explants and isolated root segments (Sharma and Thorpe, 1989). While in *Citrus aurantifolia* (Bhat *et.al.*, 1992) root developed from nodal cuttings on liquid half MS media containing 22.2 µM BAP showed shoot differentiation. Rate of shoot regeneration was 16.3% with 1.25 mean shoots per root explant. In most of these studies physiological status of mother explants influenced the morphogenetic response.

In plants auxins and cytokinins are the two main growth regulators involved in the regulation of division and differentiation and have been proved to play an important role in the induction of shoot organogenesis (Feher *et al.*, 2003). In dicots, cytokinins alone are usually required for the induction of shoot organogenesis (Tang *et al.*, 2000; Nikolic' *et al.*, 2006). In the present study also, addition of BAP in the media showed bud differentiation directly from root explants without any callus formation. Callus formation was a characteristic of the BA supplemented medium in case of root organogenesis of *Eryngium foetidum* L. (Martin, 2005). No such effect was observed in this study. Similar effect of BAP on bud differentiation from root explants has been observed in many species like *Citrus mitis* (Sim *et.al.*, 1989), *Populas tramula* (Vinocur *et.al.*, 1979) etc.

It appears that in addition to external supply of cytokinines some other factors supplied by mother plant were responsible for shift in the morphogenetic pattern from root to shoot. The effect seems to be regulated by intrinsic factors, which may get produced during prolonged culturing on the media (Chaturvedi and Mitra, 1975). In conclusion, this study has shown that root explants of *Garcinia indica* can be efficiently used for direct shoot regeneration. The method is potentially useful for faster and large scale multiplication of this important tree species and can be extrapolated for female to female plants and can be useful for germplasm conservation. Results also shows that type of explant and concentration of cytokinines in the medium are crucial for plant regeneration from root explants.

Using this protocol, starting from single root 20-25 plants can be produced within 3 to 4 months time.

7.1 Summary and conclusion

Garcinia indica Choiss. from family Clusiaceae, is a medicinally important potentialy underexploited oil seed crop from Western Ghats of India. The species is currently in focus due to presence of (-)-HCA in its fruit rind. HCA is reported to posses an ability to inhibit the action of Citrate cleavage enzyme (Shara *et al.*, 2004; Preuss *et al.*, 2004; Jena *et al.*, 2002; Watson *et al.*, 1969) which is involved in lipidogenesis in human body. HCA causes the body to use existing body fat for energy during prolong exercise and ultimately reduces the cholesterol in the body. Because of this property *G.indica* is considered as the most potent antiobesity herbal drug in the market. With the purpose of speedy weight loss, cardioprotection, correcting conditions of lipid abnormalities and endurance of exercise, derivatives of HCA are incorporated into wide range of pharmaceutical preparations in combination with other ingredients.

Along with HCA Kokum fruit rind also contains 2-3% Garcinol, a polyisoprenylated benzophenone derivative. It has some antibiotic properties and has been found to be a potent inhibitor of histone acetyltransferase and is considered as a potential anti-cancer agent.

Tree is cross pollinated and of polygamodioecious nature where differentiation between male and female can be done only after flowering stage (7-8 years). Tree growth is very slow and in horticultural practices propagation is done by seeds and soft wood grafting (George *et al.*, 2002). The seeds are recalcitrant due to high sensitivity to desiccation and freezing and have shelf life of only 5 days. Thus, it is not possible to raise seedlings throughout the year. Provided in an orchard established with seedlings, 50% plants are generally male.

Garcinia indica represent a substantial variability in phenotype as well as in HCA content level. This needs well documentation for establishing phylogenetic relationships and unique marker profiles at DNA level. To date, there is no literature available regarding the molecular scaffold of this tree.

The present study was designed with the following objectives:
a) Study the ecological distribution of *Garcinia indica* in Maharashtra.
b) Development of micropropagation protocol by clonal propagation /

organogenesis / embryogenesis for large scale plant production.

c) Study chemo diversity among the populations of *Garcinia indica* occurring in Maharashtra with respect to HCA content using fruit rind.

d) Study Genetic Diversity among population of *Garcinia indica* occurring in Maharashtra using ISSR markers

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. The plants are distributed in large number from 0 mtrs to 1132 mtrs altitude. In Maharashtra the plant is distributed widely in Konkan region and the Western Ghat region covering 6 districts-Raigad, Ratnagiri, Sindhudurg, Kolhapur, Satara and Pune. Though the plant is listed as endangered in southern India, it is cultivated in large number in coastal regions of Maharashtra. Ecological data of 69 locations covering 6 districts of Maharashtra were studied during course of work. Lot of differences between shape, size of fruit and leaf were observed. Season of maturity of plants also varied according to different locations. This morphological variation may be the result of an adaptive response to the environment.

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

11 populations covering 5 plants from each location were studied. High polymorphism (87.43 %) was observed among these 11 populations. As the plants are growing from 0 mtrs to 1132 mtrs altitude, this vast difference in altitude and ultimately the geographical parameters may be the reason behind this high polymorphism. Cross pollinated nature of the plant may be the another reason for high genetic diversity. Distance matrix obtained exhibits distance range 6.48 to 10.58 with an average of 8.53. Smallest distance value of 6.48 is observed between Kankavli – Rajapur (64 MSL and 59 MSL respectively) and Guhagar – Diveagar (10 MSL and 7 MSL respectively) accessions suggesting high degree of similarities between these populations. Maximum genetic

distance obtained is 10.58 between Pratapgad and Pavas (1132 MSL and 0 MSL) populations represent the maximum diversity. Dendrogram obtained using UPGMA exhibits three main clusters: First consist of Amboli, Chaul and Nevre location; Second consist of Murud, Pratapgad, Sukondi, Guhagar and Diveagar and Third consist of Rajapur, Kankavli and Pavas. No perfect clustering according to altitude at which plants are growing was obtained.

% HCA contents were determined from fresh fruits of *Garcinia indica* collected from 11 different locations in Maharashtra in the month of March. 5 plants from each location were taken for chemodiversity studies. Mean HCA content of these 5 plants from one location was taken as representative of the particular location. The method followed for the extraction as well as estimation of (-)-HCA was as reported by Jayaprakasha and Sakariah (1998) with some modifications. Maximum (-)-HCA content (10.63%) was found in plants growing at 10 MSL (Guhagar) while lowest HCA, 0.76%, was obtained in plants at Pratapgad growing at altitude of 1132 MSL. Plants growing at sea shore show more HCA content than higher altitude grown plants. Ecoclimatic conditions like rain fall, humidity, soil conditions may be the reason for this variation. Locations showing similarity at genetic level also shows similarity in its chemical constituents except Pratapgad and Murud population.

Thus the plant show high adaptability to climate conditions. Considering the economic importance of the species conservation efforts must be taken to protect this species by using *in vitro* techniques.

Direct and repetitive somatic embryogenesis of the plant using seed explant was induced in *Garcinia indica* on WPM basal media with BAP. BAP activates the meristematic activity and starts the process of primary somatic embryogenesis. Secondary somatic embryo induction was also obtained and it was highest on primary somatic embryos induced on high BAP. Maximum 64% secondary somatic embryos with 41.3 mean embryos per explant were obtained on step II media consisting of ½ strength WPM without any growth hormones. It was seen that most of the embryos showed precocious germination where the shoot pole was highly active. Maximum of 76% rooting was observed on medium containing a combination of NAA (10.7 μM) and IBA (4.9 μM)

within 15-20 days of incubation. Well developed plantlets showed 90% survival in green house.

Somatic embryogenesis offers an alternative and efficient means for plant multiplication. Enhanced proliferation of meristems, followed by rooting is a two-stage process which can be practiced for higher multiplication of plants. Somatic embryogenesis can also offer advantages in crop improvement programmes. This is a first report for the induction of somatic embryogenesis in *Garcinia indica* where 40-45 plants can be produced from a single seed in 35-40 weeks period.

As *Garcinia indica* is a polygamodiecious plant and differentiation between male and female can be done only after flowering (after 7-8 years), propagation of female plants by clonal propagation becomes important. A regeneration protocol for clonal propagation from apical and axillary buds was established. The major constraint encountered in the study was to obtain sterile cultures under aseptic condition. Several factors were tested to obtain microbe free cultures. Incorporation of antifungal and antibacterial agents like bavistin and cefotaxim directly into the media gave 80% sterile cultures. Another limiting factor in culture establishment was browning of explants. The rate of contamination or bud break was highly dependent on the season during which the material was collected. By the best sterilization procedure described above, the cultures initiated in January–May showed higher bud break and less contamination than other seasons.

Optimum shoot proliferation from nodal explants was achieved in WPM medium supplemented with 8.87μM BAP, 2% sucrose along with 75ppm bavistin and 400 ppm cefotaxim where 82% buds showed response. TDZ is a phenylurea derivative known to stimulate cytokinin action. Effect of TDZ was studied in *Garcinia indica* for multiplication. Significantly higher multiplication rate were recorded under the combined treatment on WPM media supplemented with 8.87 μM BAP and 4.54 μM TDZ where 3.87 shoots/explant were induced after 45 days with shoot length of 5.92 cm. For elongation of these shoots many problems were encountered for root induction. After conducting many experiments, root formation was achieved within 20-25 days from the base of shoots at nodal portion. Maximum 63.2% rooting was obtained on 490 μM IBA with 3.4 cm length

of shoots. Efforts were also made to induce *ex vitro* rooting where 40 % rooting was obtained in IAA treated shoots within 50-60 days.

As the species is woody, it is recalcitrant to *in vitro* regeneration. Using the protocol female and elite plants of *Garcinia indica* can be raised. The protocol would greatly help in afforestation programmes as the plant is of great economic value. This is the first protocol for clonal propagation of female plants. Taking into consideration the medicinal importance and polygamdioecious nature of the plant, the protocol developed offers a potential system for mass multiplication and conservation of female plants of *Garcinia indica.*

In nature the plant of *G. indica* get propagated by root suckers. Taking into consideration this natural property of the plant an experiment for root organogenesis was planned. Roots of somatic embryo derived plantlets were used as explant for the experiment. Roots developed from root segments of same plant have been suggested to be genetically uniform. Prolong incubation of somatic embryo derived plantlets on ½ strength MS media supplemented with BAP gives direct organogenesis on roots without intervening callus phase. Root segment separated from mother plant do not show any organogenesis. Addition to external supply of cytokinines with some other factors supplied by mother plant is responsible for shift in the morphogenetic pattern from root to shoot. The effect seems to be regulated by intrinsic factors, produced during prolonged culturing on the media. The method is potentially useful for faster and large scale multiplication of this important tree species especially of female plants and can be useful for germplasm conservation. Using this protocol starting from single root 20-25 plants can be produced within 3 to 4 months time.

Garcinia indica plants are recalcitrant to *in vitro* responses. Along with this, being a tree species they are extremely slow growing. The plant is listed as endangered species in South Western Ghats of India. Though the plant is distributed in large number in coastal region of Maharashtra, number of female plants is less. The traditional methods used in horticultural practices like seed germination or soft wood grafting has their own limitations. *In vitro* techniques developed here can be used for the conservation and large

scale multiplication of this important medicinal plant containing antiobesity drug. Elite plants giving high yield and maximum – (-) HCA content can be multiplied using *in vitro* techniques. As the species can be regenerated by clonal propagation/organogenesis/embryogenesis, improvement of the plant by genetic transformation can also be done.

Epidemiological data show that the frequency of obesity in human beings is rising steeply, probably because of reduced physical activities and bad eating habits (Katsilambros, 2002).. Several synthetic antiobesity drugs like orlistat, fluoxetine etc. are available in market but side effects like heart diseases have been reported from these drugs (Lian *et. al.*, 2005). Moreover, though the weight loss of 2 to 10 kg is reported upon use of these drugs, the weight regain after terminating drug therapy is a matter of concern (Bray, 1993). There are no side effects reported on use of natural HCA as antiobesity drug. Though the extract of fruits of *Garcinia cambogia* is used in large scale in antiobesity drugs, fruits of *G. indica* can also be used as a additional source.

From the chemical and genetic diversity studies it can be seen that the plants growing at 0-10 MSL are the elite plants. The climatic conditions in this region can be the reason. These climatic conditions are found mainly in coastal region of Maharashtra. So for the conservation purposes, plants growing in this region should be conserved *in situ*. *In vitro* grown plants should also be acclimatized in this area for commercial purposes. As India has a large sea coast the geographical condition of the country can be beneficial for the conservation purposes.

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1. S.R.Thengane, **S.R.Deodhar**, S.V.Bhosle, S.K.Rawal, 2006, Repetitive somatic embryogenesis and plant regeneration in *Garcinia indica* Choiss., In vitro Cellular and Developmental Biology – Plant, 42(3), 256-261

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3**. Swapna R. Deodhar**, R.J.Thengane and S.R.Thengane, 2008, De novo shoot regeneration from root culture of *Garcinia indica* Choiss., Indian Journal of Experimental Biology, 46, 482-486

4. **Swapna Deodhar,** Varsha Dawande, R.J.Thengane, S.R. Thengane, 2009, Genetic diversity of *Garcinia indica* Choiss. in Maharashtra revealed by ISSR markers,
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5. **Swapna R. Deodhar**, Nitasha Singh, R.J.Thengane, S.R.Thengane, 2010, Clonal propagation of female plants of *Garcinia indica* Choiss: a tree species of high medicinal value,
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- **S. R. Deodhar**, S.K.Rawal, S.R.Thengane, 'A shift in morphogenetic pattern due to prolonged cultures of *in vitro* grown plants of *Garcinia indica'*, in National Symposium held on the occasion of 27th meeting of the Plant Tissue Culture Association of India organized by Center for medicinal and Aromtic Plants (CIMAP) Lucknow, 18th –20th November 2005.

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