MICROPROPAGATION, BIOCHEMICAL AND HISTOCHEMICAL STUDIES OF *GARCINIA INDICA* CHOISY (KOKUM)

A Thesis submitted to Goa University for the Award of the Degree of
DOCTOR OF PHILOSOPHY
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By

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STATEMENT

As required by the University Ordinance OB 9.9 (iv), I state that the present thesis "**Micropropagation, Biochemical and Histochemical Studies of *Garcinia indica* Choisy (Kokum)**", is my original contribution and the same has not been submitted on any occasion for any other degree or diploma of this University or any other University/ Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

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CERTIFICATE

As required by the University Ordinance OB 9.9 (vi), this is to certify that the thesis entitled "**Micropropagation, Biochemical and Histochemical Studies of *Garcinia indica* Choisy (Kokum)**", submitted by **Mr. M. Baskaran** for the award of the degree of Doctor of Philosophy in Botany, is based on his original and independent work carried out by him during the period of study, under my supervision.

The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institute.

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ABBREVIATIONS

BAP	6- Benzylamino purine
cm	Centimeter
DAF	Day after fertilization
°C	Degree centigrade
2, 4-D	2, 4-Dichlorophenoxyacetic acid
EDTA	Ethylenediaminetetraacetic acid
gms	Grams
HCl	Hydrochloric acid
hrs	Hours
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSR	Inter simple sequence repeat
Kg	Kilogram
KIN	Kinetin
L	Litre
µl	Microlitre
µM	Micro mole
M	Molar
mg	Milligram
min	Minutes
min	Minutes
ml	Millilitre
mm	Millimeter
mM	Millimolar

MS	Murashige and Skoog medium
ng	Nanogram
nm	Nanometer
N	Normal
NAA	Naphthalene acetic acid
OD	Optical density
ppm	Part per million
%	Percent
PGRs	Plant growth regulators
±	Plus or minus
psi	Pounds per square inch
RAPD	Random amplified polymorphic DNA
rpm	Revolutions per minute
RT	Room temperature
sec	Seconds
NaOH	Sodium hydroxide
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by volume
WPM	Woody plant medium

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INTRODUCTION

1. INTRODUCTION

Garcinia indica Choisy belongs to the family Clusiaceae (Guttiferae). The genus *Garcinia* comprising over 200 species in the world, of which about 30 different species are known to occur in India, 7 spp. are endemic to western Ghats, 6 spp. known to grow in Andaman and Nicobar islands and 4 spp. are in North East India (Krishnamoorthy *et al.*, 2007; Priya Devi *et al.*, 2012). About 10 species are found growing in Kerala (Nayar, 2011). Gunaga *et al.* (2010) documented ten species of *Garcinia* namely *Garcinia gummi-gutta* (L.) Robson., *Garcinia indica* Choisy, *Garcinia morella* (Gaerth.) Desr., *Garcinia rubroechinata* Koestern., *Garcinia spicata* (Wight & Arn) Hook. F., *Garcinia talbotii* Raizada ex Sant., *Garcinia travencoria* Bedd., *Garcinia wigthii* T. Andr., *Garcinia imbertii* Bourd. and *Garcinia xanthochymus* J. Hk. Ex T. And. in the entire stretch of Western Ghats.

Garcinia indica is an endemic tree species found distributed in tropical rain forest of Western Ghats of India and recorded as endangered medicinal tree species in Southern India (Chabukswar and Deodhar, 2006). A slender evergreen tree with dense canopy and drooping branches of pyramidal or conical shape, occurs up to an elevation of 800 meters from sea level. Its leaves are ovate or oblong, dark green above and pale beneath. It produces deep yellow sticky sap or latex called gamboge (Anonymous, 1956; Korikanthimath and Desai, 2012). The polygamodioecious tree flowers in November-February and fruits ripen in May-June. Fruits are globose or spherical 1-1.5 in diameter, dark purple when ripe; each fruit contain 5-8 seeds (Anonymous, 1956; Chabukswar and Deodhar, 2006).

Garcinia indica is commonly known as Kokum, Kokum butter tree, Mangosteen oil tree, Bhirind or Ansul in Marathi and Konkani, Kokam in Hindi, Kokan in Gujarati,

Murugal in Kannada, Punampuli in Malayalam and Murgal in Tamil (Abraham and Senthilkumar, 2007; Anonymous, 1956). Traditionally, the sun dried rind of Kokum is used for the preparation of several types of vegetarian and non-vegetarian preparations, especially fish curry in Konkan region. The dried rind is soaked in water and boiled into a soup called solkadi. The pulp of the fruits is agreeable flavor and has a sweetish acid taste. Kokum juice spiced and sweetened with jaggery, is a must for marriage feasts and functions in Uttara Kannada district of Karnataka and Goa (Korikanthimath and Desai, 2005). The normal shelf-life of fresh fruit is about five days (George *et al.*, 2002). Kokum fruits are chopped and steeped in sugar water to prepare amrutkokum, a healthy drink during the summer to relieve sunstroke (George *et al.*, 2002; Mishra *et al.*, 2006).

In home remedy, juice is used for heat strokes and it has been used in traditional medicine based Ayurveda. The fruit has many medicinal properties such as antihelmintic, cardio-tonic, digestive-tonic and treatment of piles, dysentery, skin allergies, scalds, chaffed, paralysis and tumors (George *et al.*, 2002; Mishra *et al.*, 2006; Korikanthimath and Desai, 2012). Kokum syrup and extract has antioxidant and antifungal properties and used as bio-preservative in food applications and neutraceuticals (Tamil Selvi *et al.*, 2003; Mishra *et al.*, 2006).

In Kokum, the genotype variability has been reported from Sindhudurg district of Maharashtra. Besides this, survey results revealed some of the elite genotypes namely MLDK-3 and MLDK-5. From Raigad district, the spreading genotypes, DVA-1, DVA-2 and DVA-3 have been collected for further evaluation at Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra (Korikanthimath and Desai, 2012; Gawankar *et al.*, 2012).

Kokum seed contains 23-26% edible butter called 'kokum better'. Kokum butter is extracted from seeds and it is considered nutritive, astringent, demulcent and emollient. This fat is rich in combined stearic and oleic acids. It contains about 75% of mono-oleodisaturated glycerides with low melting point and considerable brittleness (Anonymous, 1956; Nayak *et al.*, 2010b). It is suitable for use as confectionery butter. It is also suitable for making candles and soaps. The oil is suitable for ointments and pharmaceutical purposes. It is also used in local application of ulcers, fissures of lips and hand etc. The oil cake left after the extraction of oil can be used for manufacturing of industrial adhesives, as manure and cattle feed (Patil, 2006; Korikanthimath and Desai, 2012).

The fruit rind contains hydroxycitric acid (HCA) (1,2 dihydroxypropane-1,2,3-tricarboxylic acid) which is an important biologically active secondary metabolite used by pharmaceutical industries for the preparation of anti-obesity and anti-cholesterol drugs. Animal studies have revealed that HCA effectively suppresses food intake, curbs appetite, increases the rate of hepatic glycogen synthesis, reduces fatty acid synthesis and lipogenesis and decreases body weight gain (Jena, 2002a). HCA and its derivatives is used in combination with other health ingredients for the purpose of the enhancing body weight loss, cardio protection, correcting the conditions of lipid abnormalities and endurance in exercise (Jena, 2002a). It potentially inhibits ATP citric acid cleavage enzyme, an important enzyme in Kreb's cycle for conversion of carbohydrates into fat (Heymsfield *et al.*, 1998; Jena *et al.*, 2002a). As the demand for HCA is increasing, amongst the 200 species of *Garcinia,* only few species, namely *G. cambogia*, *G. indica*, *G. atroviridis*, *G. cowa* and *G. pedunculata* are known to contain HCA (Lewis and Neelakantan, 1956; Lewis, 1969; Lowenstein and Bruneugraber, 1981; Jena *et al.*, 2002b).

Garcinol is a polyisoprenylated benzophenone derivative, extracted and purified from the fruit rind of *Garcinia indica*. About 2-3% of garcinol occurs in the fruit rinds (Yamaguchi *et al.*, 2000a). It has important biological activities such as antibiotic and antiulcer (Yamaguchi *et al.*, 2000b), ability to suppress colonic aberrant crypt foci (ACF) formation and inhibit histone acetyltransferases (HATs) which modulate gene expression (Ciochina *et al.*, 2006), ability to induce apoptosis through cytochrome *c* release, activation of caspases (cysteine-aspartic proteases) in human leukemia HL-60 cells (Pan *et al.*, 2001), antioxidant, anti-inflammatory and anti-carcinogenic (Yamaguchi *et al.*, 2000a; Sang *et al.*, 2001; Hong *et al.*, 2006; Padhye *et al.*, 2009).

Anthocyanin pigments are extracted from ripened fruits of *Garcinia indica*. The chemical name of the pigment is cyanidin-3-glucoside and cyanidin-3-sambubioside confirmed by the analyses of HPLC, Mass and NMR spectroscopy. This is used as a potential source of natural red colorants for various industries like foodstuffs, beverages, pharmaceuticals. Kokum contains high amount of anthocyanin pigments as compared to other plant sources (Nayak *et al.*, 2010a, b). Recently, *Garcinia* excited the scientific world for possessing anit-obesity, anti-cholesterol, antibiotic and antiulcer activities (Heymsfield *et al.*, 1998; Yamaguchi *et al.*, 2000b; Jena *et al.*, 2002; Padhye *et al.*, 2009). Though commercially important, these species remained neglected and not much attention has been given for the research and development.

Kokum cultivation occupies an area of about 1000 hectares and annual production of fruits is about 4500 metric tonnes (Haldankar *et al.*, 2012; Patil *et al.*, 2012). However, there is a continuous increase in demand as it is evident by the market trends and export scenario. The estimated domestic demand was about 1040 tonnes in the year 2004-05 (Korikanthimath and Desai, 2012).

The propagation of kokum is usually by seeds. Due to recalcitrant nature of seed and less viability period (about a month), it is not possible to propagate by seed throughout the year (Malik *et al.*, 2005a). Vegetative propagation and air-layering is not successful, because of the yellow resin exudates from the stem that interferes with the formation of adventitious roots (Rema *et al.*, 1997). Softwood grafting is reported to be ideal for propagation. These propagation methods however, are dependent on season, requires space and rootstock (seedling). Conventional breeding is limited due to complex genetic system and extended juvenility (Malik *et al.*, 2005a).

The male and female plants can be identified only at the flowering stage (after 10 years of growth). The fruit ripening varies from April to June and yield depends on the female tree and genotype of the tree (Chabukswar and Deodhar, 2006). Farmers prefer the early ripening and high yielding genotypes because the harvesting and processing of fruits needs to be completed before the onset of monsoon i.e. end of May or beginning of June in the Western India (Chabukswar and Deodhar, 2006).

OBJECTIVES OF THE PRESENT INVESTIGATION

With the above background, the present study aimed at developing high yielding elite clones through *in vitro* regeneration and mass multiplication using excised seed segments as explants. During this study, protocols were developed and standardized for the induction of multiple shoots, elongation of shoots, initiation of roots and acclimatization of regenerated plants. Further, histological studies were carried out to understand the origin and development of multiple shoots from mature seed segments. Anatomical characterization of regenerated plants as well as corresponding mother plant (donor plant) and molecular studies were carried out to understand the genetic variation, if

any, amongst the regenerated plants in comparison with corresponding mother plant (donor plant).

Second part of study includes the extraction and estimation of hydroxycitric acid (HCA) and garcinol during various stages of fruit development (young to mature stages) to find out the stage of development at which the fruit has high quantity of these compounds. As a part of the study, we have also developed a method for extraction and estimation of garcinol and hydroxycitric acid (HCA) from various stages of development of fruits.

Histochemical and developmental studies were carried out from anthesis to maturation of fruit to understand the structural and developmental changes in the fruit and the time-course for deposition and distribution of various storage and other chemical compounds Hence, the present work was undertaken with the following specific objectives:

1. To develop and standardize the efficient protocol for *in vitro* regeneration and mass multiplication to produce elite clones.

2. Acclimatization of regenerated plantlets and characterization of regenerated plants.

3. To develop and standardize the protocol for extraction and estimation of garcinol and hydroxycitric acid (HCA) during various stages of development of fruit.

4. Histochemical and developmental studies in the fruit from anthesis to maturation to understand the structural and developmental changes and time-course deposition and distribution of various storage compounds (starch, protein, lipids) and other chemical compounds (tannin, lignin, garcinol, hydroxycitric acid) during various stages of development of fruit.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2. 1. Genus *Garcinia*

The genus *Garcinia* belongs to the family Clusiaceae (Guttiferae), which consists of about 40 genera and 1200 species around the world (Krishnamoorthy *et al.*, 2007). The genus *Garcinia* was named by Linnaeus for Laurent Garcin (1683-1757), a Swiss botanist with the Dutch Indies Company in India, who had published the first description of Mangosteen (Corner, 1988). *Garcinia* is a large genus of slow-growing evergreen tree or shrubs distributed in tropical Asia, Africa and Polynesia (Anonymous, 1956; Theodore, 1967). Botanical classification placed *Garcinia* in the family Guttiferae, now referred to as Clusiaceae (Krishnamoorthy *et al.*, 2007).

The genus *Garcinia* consists of evergreen trees or shrubs with straight trunk tapering to a conical canopy, branches in alternating pairs on the trunk at an acute angle becoming horizontal or drooping and very occasionally branches are modified to spines. Leaves are simple and entire, opposite and stipules absent or occasionally minute. Usually male or female flowers are on separate trees, but occasionally bisexual. Flowers bear in groups or singly in the axils of leaves but rarely terminal; unless solitary, they are imbricate or more or less compound umbellate or panicled; flowers regular with 4 persistent sepals and with 4 petals; rarely penta-merous; petals red, pink, yellow or white. Male flowers with seven to many stamens are inserted on a variously shaped receptacle, free or connate into one central column or 4-5 bundles. Female flowers with large hypo-gynous ovary mounted on a variously shaped receptacle; ovary many chambered; stigma very shortly stalked, but mostly sessile, plate-like, entire or lobed, often developed as a lobed disk; female flowers usually with staminodes. Fruits are fleshy berries with a thick, leathery pericarp and contain 1-4 flattened seeds in a pulpy mass; seeds large. Trees

possess vary hard timber and produce an abundant gummy latex (Ridley, 1967; Whitmore, 1973).

2. 2. Geographical Distribution of *Garcinia indica*

Garcinia indica Choisy (Kokum) is endemic to tropical evergreen forest of Western Ghats of Maharashtra, Goa, Karnataka and Kerala and it is mostly found distributed abundantly in Konkan region of Goa, Maharashtra, South Kanara and West Mumbai. This tree occurs up to an elevation of 800 meters above sea level (George *et al.*, 2002; Korikanthimath and Desai, 2006).

2. 3. Brief Description of *Garcinia indica*

Garcinia indica is a slender evergreen tree, dense canopy with drooping branches. Leaves are ovate or oblong, dark green above and pale beneath, 1-1.5 inches broad and 2.5-3.5 inches long. Flowers are axillary or terminal, solitary. Sepals are four, thick and fleshy, green in colour. Petals are four, thin, yellow to pink. Male flowers have numerous, short, filamentous anthers and two celled. Female flower having short pedicels, arranged two or three together. Ovary is four to eight celled with sessile stigma. Fruits are globose or spherical 2.5-3.0 cm in diameter, dark purple when ripe; each fruit contains 4-8 seeds (Anonymous, 1956; George *et al.*, 2002; Korikanthimath and Desai, 2006).

2. 4. Traditional Uses

Traditionally, the fruit rind is used for the preparation of different type of curries (vegetarian and non-vegetarian), cooling syrup and cool drinks. The dried rind mixed in water, is boiled into a soup called solkadi. The fruit has sour taste with pleasant flavor and steeped in sugar syrup to make 'amrutkokum', a healthdrink popular during summer. It is also a traditional home remedy in case of flatulence, heat strokes and infections. Many

therapeutic effects of the fruit have been described in traditional medicine based in Ayurveda. These include its usefulness as an infusion, in skin ailments such as rashes caused by allergies; treatment of burns, scalds and chaffed skin; remedy for dysentery and mucous diarrhea; an appetizer and a good liver tonic. Kokum improves appetite and allays thirst; it is a cardio tonic and provides relief from bleeding piles, dysentery, tumors and heart diseases. It is used as spice and sweetened with jaggery, it is a must for marriage feasts and functions in Uttara Kannada district of Karnataka and Goa (Korikanthimath and Desai, 2006; Mishra *et al.*, 2006).

2. 5. Propagation of *Garcinia indica*

Kokum is traditionally cultivated by raising through seeds and recently by softwood grafting.

2. 5. 1. Nature of seed and seed treatments

Seeds are collected from fully ripened fruits of high yielding trees. These seeds are of recalcitrant nature and the viability period is only for a month. Seeds are dried under shade and pre-sowing of seed with wet packing or drying with coal ash is recommended for better germination (Haldankar *et al.*, 2012).

2. 5. 2. Raising of Seedlings

After seed treatment, seeds are sowed directly in seed beds or in poly bags. Seeds sown in polythene bags containing soil and farmyard manure (FYM) germinated after 40-60 days. In seed beds, the top soil was mixed with FYM. The seeds were sown horizontally at 1.5-2.5 cm depth at 5 cm interval. After germination, seedlings were transplanted into large polythene bags containing soil and FYM in the ratio of 3:1.

Transplanted seedlings were kept under shade and watered as per requirement. 12-14 months old seedlings were preferred for planting in the field.

2. 5. 3. Softwood Grafting

Air layering, inarch grafting, veneer grafting, softwood grafting and root cutting were attempted previously (Rema *et al.*, 1997). Amongst the methods softwood grafting was successful. The scion was selected from high yielding trees. The terminal shoots of 0.5-0.6 cm thickness and 10-12 cm length, greenish brown were selected as scion. The mature scion of 5-6 month old branches contributed more success. For rootstocks, 22 weeks old Kokum seedlings of 10-12 cm height and 0.25 cm thickness at collar region with green apical softwood were selected for the grafting. The selected rootstock was cut and split vertically from the top to length about 4 cm with the help of sharp knife. The scion stock was cut about 4 cm both sides at lower end to make a wedge. The wedge was inserted into the cleft of the rootstock. The joints were tied tightly with polythene roll of 250 gauge (62.5 micron) and 1.5 cm width. Grafted plants were kept in shade house or poly-house. Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli released improved Kokum varieties namely Konkan Amruta and Konkan Hatis by softwood grafting. Yield and other characteristic of fruits of Konkan Hatis are better than Konkan Amruta (Haldankar *et al.*, 2012). It was found that October to November and March to August was suitable seasons for softwood grafting.

2. 5. 4. Effect of Tropism

When orthotropic shoots were used as scion for grafting the structure of the tree resembles mother tree, becomes tall and conical shape, occupies large area and produces more number of fruits (high yield). Plucking of fruits from such tall trees however, becomes very difficult and wastage of fruits (broken fruits) is more. When plagiotropic

shoots were used as scion the tree remains short and bushy. The harvest of the fruit is much easier with no damage to fruits.

2. 5. 5. Soil, Land Preparation and Planting

Kokum is grown in all types of soils having depth of 1.2 meter and pH of 6-7 with areas receiving over 250 cm rainfall. It requires warm and tropical humid climate. Kokum is planted as a monocrop or mixed crop in coconut and arecanut plantation. It also planted in home garden or kitchen garden. Spacing of planting is 6×6 meter, considered the growth and conical canopy of the Kokum trees. In well established coconut plantation, planting space of Kokum was 7.5 to 8 meters in between two coconut tree. Three hundred Kokum plants can be planted per hectare as a mixed crop with coconut plantation. Pits of 60 cubic centimeter are prepared before monsoon and filled with top soil, 10 kg FYM and 1 kg super phosphate and planting is done on the onset of monsoon. Initially growth of seedling and grafts is very slow. First one year shade is necessary to protect the plants during summer (Haldankar *et al.*, 2012).

2. 5. 6. Irrigation and application of manure

Irrigation is necessary for the growth and establishment of Kokum plants and its requirement depends on soil and environment conditions. For the first three years irrigation is essential. The modern method like drip irrigation are beneficial than conventional methods. In Konkan region, 2 kgs farmyard manure (FYM), 50 gms nitrogen, 25 gms of phosphorus oxide and 25 gms of potassium oxide is applied to one year old plant. It is increased in the same proportion every year up to ten years and thereafter 20 kilogram FYM, 500 gram nitrogen, 250 gram of phosphorus oxide and 250 gram of potassium oxide is applied. The application of manure in the month of August

after the heavy rain) is suitable. Manure is applied 30 cm deep into the soil around the plant.

2. 5. 7. Plant protection

No major diseases are noticed in Kokum plantation. Sometimes a pink disease is noticed on branches, that can be removed and bordeaux paste may be applied on the wound.

2. 5. 8. Yield and harvest

Plants start fruiting after 7 to 8 years of planting. In case of grafts, fruiting starts after 3 to 4 years. The fruits can be harvested after 120 days of fruit set. Most of the fruits are harvested by end of May and beginning of June. It has been found that the processing of Kokum fruits for various purposes become very difficult during the monsoon season. Hence, post-flowering foliar spray of potassium nitrate and monopotassiun phosphate has helped the early harvest of fruits (about 10-34 days early as compared to non-foliar spray). All fruits are not ripened at the same time and harvesting of fruits is generally carried out in 6-8 batches. Spraying of ethrel at 300 ppm enhances the early maturity and ripening of fruits, reduces the number of harvest and improves the yield as well as biochemical composition of fruits (Haldankar *et al.*, 2012).

2. 6. Production of Kokum

As per survey in 2010, production of Kokum fruits is about 4500 metric tonnes in the area of about 1000 hectares in the Konkan region. According to forest department, a total of about 46,600 trees are found growing in the state of Maharashtra (43,000 trees in Ratnagiri and Sindhudurg districts). Naturally growing scattered trees were observed in Raigad and Sindhudurg district. It is estimated that these trees are scattered over about 108

hectares along the riverbanks, streams, valleys, roadsides and backyard wastelands of Sindhudurg district. It was noted that in South Konkan region 1,674 metric tonnes of fruits are used for the production of dried Kokum rind, 757 metric tonnes for the preparation of Kokum syrup and 40 metric tonnes for production of Kokum butter (Haldankar *et al.*, 2012; Patil *et al.*, 2012). Kokum trees are found throughout Goa as natural population however, current data on area of cultivation and production of Kokum in Goa is not available (Priya Devi *et al.*, 2012).

2. 7. Promotion of Kokum Plantation

The Government of Maharashtra has been promoting the farmers to grow Kokum in a systematic manner under employment guarantee scheme and district rural development agency. Kokum plantation in Goa is increasing due to the effort of Forest Department, Government of Goa. Major importance is being given to Kokum and around 15-20 hectares of demonstration plots have been developed by the Forest Department. Besides, Department of Agriculture produces and distributes Kokum seedling every year to farmers to increase the area under Kokum cultivation (Priya Devi *et al.*, 2012).

2. 8. Kokum Products

2. 8. 1. Kokum Syrup (Amrit Kokum)

The ripened fruits are washed thoroughly in tap water and cut open to separate the pulp and rind. The rinds are cut in to small pieces and mixed with sugar in 1:2 (1 part of rind and 2 parts of sugar) in a stainless vessel and kept for a week. Every day, the mixture is mixed well twice. After a week, syrup is filtered through a stainless steel vessel and sodium benzoate is added at the rate of 610 mg/kg of the syrup. The syrup is mixed well and filled in the pre-sterilized glass bottles (Joshi, 2012).

2. 8. 2. Kokum Juice Agal

The ripened fruits are washed with water, cut open and the rind and pulp along with seed are separated. Pulp with seed is taken in a vessel and salt added at the rate of 160g/kg, mixed well. Mixture is stirred twice daily for seven days. After seven days, blinded juice is filtered through stainless steel vessel and filled in pre-sterilized bottles. It has been used as condiment in fish curry and for the preparation of solkadi. In Goa, Kokum agal has been mixed with coconut milk and spiced with green chillies and green coriander leaf pieces (Dalvi *et al.*, 2010; Joshi, 2012).

2. 8. 3. Ready to Serve Drink

Selected fruits are washed and boiled with water and crushed to make juice. The juice is filtered through suitable stainless steel vessel and sodium benzoate is added at the rate of 200 mg/kg juice and stirred well. The juice is boiled for about five minutes and final product is filled in pre-sterilized bottles (Joshi, 2012).

2. 8. 4. Kokum Squash

The ripened fruits are washed thoroughly with tap water, boiled with required amount of water and crushed in a vessel. The juice is filtered through a stainless steel vessel and sodium benzoate is added at the rate of 610 mg/kg of the filtered juice. The juice is mixed well and filled in the pre-sterilized glass bottles (Joshi, 2012).

2. 8. 5. Kokum Amsul (dehydrated salted rind)

Fresh ripen fruits are washed with water, cut open and separated into rind and pulp along with seeds. The pulp along with seeds is mixed with 10% salt and salt water leaching out from the mixture is filtered. The filtered salt water is used for dipping of the separated rind. The dipped rind is placed for sun drying/tray drying. Next day the dried

rinds are again dipped in salt water, which is leached on the second day from the salt-pulp mixture. The dipped rinds are again kept for drying. This process is repeated for 4-5 times to get the amsul. The Kokum amsul is used for the preparation of both vegetarian and non-vegetarian curries instant of tamarind and very popularly used for preparation for fish curries (Thakor *et al.*, 2012).

2. 8. 6. Kokum Rind Powder

Ripen fruits are washed with water, cut open to remove the inner pulp along with seeds. The rinds are dried using the drier at 50-55°C. After complete drying, the rinds are powdered using electrically operated grinder. Then the powder is sieved through 1 mm mesh sieve and packed as desired (Joshi, 2012).

2. 8. 7. Kokum Wine

Fresh Kokum juice with 4% sugar is fermented to produce wine. Kokum wine is prepared in Goa using the traditional methods with commercial baker's yeast. Selected fresh fruits are cut open and seeds are removed. Red rind and white pulp are taken in a vessels, boiling water is added to it and kept for 24 hrs. The yeast is activated in warm water and added to wheat grain at room temperature. Later, the wheat grain containing yeast is added to the juice and left for fermentation. The mixture is stirred twice every day. On the 9th day 1.5 kg of sugar is added to the mixture and kept for fermentation. After 21st day, the clear wine is decanted and racked in large bottles. Finally sugar is added as per requirement (Braganza and Rodrigues, 2012).

Improved method was developed by Braganza and Rodrigues (2012). Ripe Kokum fruit rind was cut in to small pieces, taken in a vessel and mixed with sugar. Later the juice was collected in another container and pectinase was added at the rate 2 g/L of juice. The

juice was allowed to settle and filtered. For sweet wine, sugar was added as per requirement and then pH adjusted to 3.1-3.4 by using citric acid. The yeast (red wine yeast for good quality of wine) was activated by placing in warm dilute sugar solution, added to the juice and mixed vigorously at periodic intervals. Once air bubbles appeared the juice was kept for fermentation for 10 -12 days. The fermented product is filtered, poured in to jars, corked and kept for another 15 days (anaerobic fermentation) for other particles to settle down. After 15 days, the wine is transferred in to bottles, sealed tightly and stored until used.

2. 8. 8. Kokum Butter

Kokum butter is prepared from the seeds and contains 25% edible fat. It is extracted from roasted kokum seeds. Ground seeds are placed in water, boiled and kept overnight. The top layer of fat is collected as Kokum butter. It is also extracted by solvent extraction method. Crude Kokum butter is yellowish which after refinement turns white. Refined Kokum butter is high quality of hydrogenated fats. Kokum butter is considered nutritive, astringent, demulcent and emollient. This fat is rich in combined stearic and oleic acids. It contains about 75% of mono-oleodisaturated glycerides. It is suitable for use as confectionery butter. Kokum butter is suitable for chocolate and cocoa industry. It improves the texture of chocolate without affecting the flavor. It is also suitable for making candles and soaps. The oil is suitable for ointments, suppositories and pharmaceutical purposes. It is also used as local application to ulcers and fissures of lips, cracks/cuts in chopped hands, etc. The oil cake left after the extraction of oil can be used for manufacturing of industrial adhesives, as manure and as cattle feed (Kshiragar *et al.*, 2003; Maheshwari and Yella Reddy, 2005).

2. 9. Plant Regeneration, Mass Multiplication and Conservation through Tissue Culture

Plant tissue culture plays an important role in mass multiplication and conservation of endangered and rare plant species (Seeni and Latha, 2000; Decruse *et al.*, 2003). *In vitro* conservation in controlled conditions (Martin and Pradeep, 2003; Pacheco *et al.*, 2009; Rani and Dantu, 2012) and *in vitro* propagation has proven to be a potential technology for mass production of medicinal plant species (Faisal *et al.*, 2003; Sharma *et al.*, 2010).

Lakshmanan *et al.* (1997) studied the hormonal regulation of *de novo* shoot bud formation in Mangosteen (*Garcinia mangostana* L.) from leaf explants. During the investigation, various developmental states, namely, morphogenic competence, caulogenic determination and organ differentiation were noticed. The age of the leaf had a profound influence on the expression of morphogenic competence. About 80-90% of the explants from 10-12 days old leaves were morphogenically competent, compared to only 28% and 12% of the explants derived from 6 and 15 days old leaves, respectively. In addition, 10-12 days old leaves produced significantly more shoot buds than the leaves of other developmental stages. The leaf explants required a minimum of 6 days of BA treatment (20 μM) for callus formation. The increased percentage of explants (23%) producing shoot buds as well as the number of shoot buds (5) formed per leaf at the end of 50 days of culture on BA at 20 μM.

Goh *et al.* (1997) evaluated the role of ethylene in shoot bud regeneration in Mangosteen using *in vitro* grown leaf explants. Leaf segments (2 mm) were cultured on WPM supplemented with 20 μM of BA, ethylene inhibitors aminoethoxyvinylglycine (AVG) or ethylene precursors 1-aminocyclopropane-1-carboxylic acid or silver nitrate

($AgNO_3$), under aerated or airtight conditions. Explants showed the emergence of shoot buds on midribs within 18 days of culture. Maximum percentage (92%) of the explants showed regenerated shoot buds after 42 days of culture under aerated condition. However, ethylene delayed the callus formation by at least 12 days and supported the regeneration of shoot buds. In contrast, addition of 1-aminocyclopropane-l-carboxylic acid delayed the shoot bud regeneration and enhanced callus proliferation. $AgNO_3$ and AVG under airtight conditions prevented the delaying of shoot regeneration and callus formation. Ethylene or ethylene inhibitors did not affect the percentage of regenerating explants after 49 days of culture. But, ethylene delayed the emergence of shoot buds and probably regulated some of the early developmental stages associated with shoot bud regeneration in Mangosteen leaf explants.

Nodular callus was induced using young, purple red, 5-15 mm long leaf lamina taken from *in vitro* grown plants of Mangosteen. The nodular callus was induced on Murashige and Skoog (MS) medium supplemented with 2.22-4.45 μM benzyladenine (BA), 0.45-4.50 μM thidiazuron (TDZ), 500 mg/L polyvinylpyrrolidone (PVP) and 3% sucrose. The best nodular callus induction was on the medium supplemented with 2.22 μM BA and 2.25 μM TDZ. A multiplication rate of 2-3 shoots was obtained by subculture of the nodular callus at 3-4 week intervals. Shoots formation from the nodular callus was achieved by transfer to woody plant medium (WPM) supplemented with 500 mg/L PVP, 0.4 μM BA and 3% sucrose. Elongated shoots were rooted to 100% when wounded at the base of shoot, dipped in 4.4 μM indole-butyric acid (IBA) solution in the dark for 15 min and cultured on WPM supplemented with 1.11 μM BA, 0.25% activated charcoal, 34.5 μM phloroglucinol (PG) and 3% sucrose (Te-chato and Lim, 1999).

Plant regeneration was achieved for Mangosteen using the leaf explants from *in vitro* grown plantlets. The leaf explants were cultured on MS medium supplemented with 0.44 μM to 4.44 μM BA and 0.45 μM to 4.50 μM TDZ. Amongst the treatments, TDZ at 2.25 μM, in combination with BA (2.22-4.44 μM) induced 67-69% of meristematic nodular callus formation within 3 weeks of culture. The lower (0.45 μM) and higher (4.5 μM) concentration of TDZ decreased the percentage of nodular callus induction. The callus sub-cultured on WPM supplemented with 0.44 μM BA exhibited 9.3 shoots per explant. 80-90% of shoot elongation was obtained on the same medium overlaid with half strength MS medium with 0.32 μM NAA and 0.13 μM BA. Successful rooting (68.2%) was achieved on WPM with 1.11 μM BA and 0.25% activated charcoal. Plantlets were successfully established in soil in pots after acclimatization (Te-chato and Lim, 2000).

Huang *et al.* (2000) studied the effect of temperature and photoperiod on formation direct shoot buds in *Garcinia mangostana* L. Cotyledon segments were cultured on MS medium supplemented with naphthaleneacetic acid (NAA), N^6-benzyladenine (BA) and N^6-isopentenyladenine (2-iP). Differentiating cotyledons and shoot bud formation were found in the medium supplemented with 13.3 mM BA after 8 weeks of culture. No differentiation was observed in cotyledon segments cultured on medium supplemented with 44.4 mM BA, with or without NAA. The optimum concentration of BA for primordial differentiation was 13.3 mM. Observations indicated that all combinations involving an 8 hrs photoperiod or a 30°C temperature produced significantly fewer shoots than the 16 hrs photoperiod with 26°C temperature treatment. 85% of rooting was achieved when shoots were first placed in an inductive medium containing 4921.3 mM IBA for 3 day or 492.1 mM IBA for 10 days and then transferred to a second medium without auxin. Amongst the treatments, 4921.3 mM IBA for 10 day showed the better rooting. Rooted plantlets were transplanted in pots containing an equal volume

vermiculite, peat and perlite. Plantlets were acclimatized by lowering the relative humidity in the controlled environment chamber from 95 to 85% in 5% decrease at weekly intervals, in the greenhouse observed 95% of survival rate.

Micropropagation system was developed for Mangosteen by Sirchi *et al.* (2008a) using leaf sections and mature seeds. They studied the effect of various modified Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) alone or in combination with naphthalene acetic acid (NAA) on multiple shoot induction from leaf and half seed explants. Amongst the treatments, leaf explants gave the highest percentage of shoot formation (70.07%), highest number of shoots formed per explants (46.5%) and high mean shoot height (0.48 cm) on medium supplemented with 2.0 mg/L (w/v) of BAP. It was observed that medium supplemented with 0.5 mg/L NAA did not induce shoot organogenesis, but induced 90% callus in leaf explants. Seed explants recorded the highest percentage (60.2%) of shoot formation and high mean number of shoots (9.5 per explants) on medium supplemented with 20 mg/L (w/v) BAP and 10 mg/L (w/v) NAA. However, highest mean shoot height (0.69 cm) was exhibited on medium supplemented with 10 mg/L (w/v) BAP and 10 mg/L (w/v) NAA. In most of the treatments seeds explants induced callus. Medium supplemented 20 mg/L (w/v) NAA noticed the highest percentage (83.36%) of callus formation. Rooting of regenerated shoots was tried in various concentration of indol-3-butyric acid (IBA) and NAA in various MS salt strengths. High percentage of root formation (90.4%) was obtained on quarter strength MS medium containing 0.1 mg/L NAA. Plantlets transplanted and acclimatized on media composed of soil, sand, organic matter and vermiculite (2:2:1:1) recorded 80% survival.

Sirchi *et al.* (2008b) developed the protocol for *in vitro* plantlet regeneration of Mangosteen (*Garcinia mangostana* L.). The shoot tip and stem explants were cultured on MS medium supplemented with different concentrations of BAP (0, 0.1, 1.0 and 2.0 mg/L) either alone or in combination with kinetin (KIN) (0, 0.05, 0.1 and 0.2 mg/L). For seed explants, the concentrations of BAP (0, 2, 4 and 8 mg/L) either alone or in combination with NAA (0, 0.1, 0.2 and 0.5 mg/L) were used. After 12 weeks of culture, shoot tip explants produced the highest mean number of shoots (73.3) per explant on media supplemented with 1.0 mg/L BAP and 0.05 mg/L KIN. The medium containing 0.1 mg/L BAP and 0.05 mg/L KIN showed the maximum shoot height (1.2 cm). The highest percentage (90%) of callus formed was induced in the medium containing 0.20 mg/L KIN. The medium supplemented with 4 mg/L of BAP with 0.2 mg/L of (NAA) produced the highest number of shoots (75) per half seed explant. The highest percentage (90%) of callus formation was obtained on medium containing 2 mg/L IBA and 0.5 mg/L of NAA. Root induction was tested on quarter strength MS medium supplemented with IBA (0.1, 1.0, 2.0 mg/L) and NAA (0.1, 0.5, 1 mg/L). The highest percentage (90.4%) of root formation was obtained on quarter strength MS medium containing 0.1 mg/L NAA. Rooted plantlets were acclimatized on medium composed of soil, sand, organic matter and vermiculite in the ratio of 2: 2: 1: 1.

Rostika *et al.* (2008) studied *in vitro* propagation of Mangosteen. Surface sterilized seeds was sliced to four pieces and cultured on MS medium supplemented with BA (1, 3, and 5 mg/l) for bud/shoot inductions. Highest percentage of seed growth (100%) with the highest number of shoots per seed (2.7) and highest leaf number (2.9) was obtained on medium supplemented with 5 mg/L BA. For root induction, combinations of two basal media (MS and WPM), two concentrations of media (full strength and ¼ strength) and two levels of IBA (5 and 10 mg/L) were used. Within MS media, 5 mg/L IBA showed best for

rooting (75%) with 1.6 cm root length. Transplantation and acclimatization was done on two different potting media, soil and soil & compost in glasshouse conditions. Plant acclimatization on soil & compost in the glasshouse, with 75% shading promoted fast plant growth. After 4 months, the primary roots developed secondary and tertiary roots.

Elviana *et al.* (2011) studied the morphological and histological changes during the somatic embryogenesis in Mangosteen. Leaf explants from young Mangosteen seedlings were cultured on MS medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP) and thidiazuron (TDZ). Highest percentage (40%) of explants producing globular structures were obtained from those cultured on MS with 0.7 mg/L BAP and 0.7 mg/L TDZ. For further growth and development, sub-culturing was done using various media supplemented with different concentrations and combinations of plant growth regulators, however, the globular structures did not develop into further stages of somatic embryogenesis.

Rineksane *et al.* (2012) studied the potential of seeds with seed coat and without seed coat for the formation of embryogenic callus in Mangosteen. Sterilized seed segments were cultured on Linsmaier and Skoog (1965) medium supplemented with IAA, IBA, 2, 4-D and NAA at various concentrations (0, 0.5, 1, 2, 4, 8, 16, 32 mg/L). The highest percentage (93.3%) of callus formation was recorded when seed without seed coat explants were cultured in basal LS medium containing 8 mg/L 2, 4-D. Amongst the combinations of cytokinins and 2, 4-D highest percentage of callus (80%) and lowest percentage of callus browning (53.53%) with yellowish and compact nodular calli were obtained on MS medium supplemented with 8 mg/L 2, 4-D and 0.1 mg/L BAP. Addition of glutamine (500 mg/L) on MS medium containing 8 and 0.1 mg/L BAP- indicated that glutamine did not increase the growth of calli, however, gave friable texture and yellowish

color to the callus as compared to the non-glutamine treatment. The growth and multiplication of cells in suspension cultures showed that the cells were able to divide and proliferate even in half strength MS liquid medium without 2,4-D. The BAP and TDZ stimulated cell growth into advanced embryogenic stages. After six months of culture, heart shaped embryogenic stage was observed only on medium supplemented with 1 mg/L BAP. Addition of TDZ in different concentrations such as 1, 3 and 9 mg/L into the medium induced the transition of single cell calli to more advanced embryogenic stages (torpedo) after five months of culture.

Rohani *et al.* (2012) developed protocol for somatic embryogenesis of Mangosteen using red young leaf segments from 1 to 3 year-old seedlings. Leaf segments were cultured on B5 or Murashige and Skoog (MS) basal media and supplemented with several plant growth regulators at different levels, including 2,4-D and kinetin or 2,4-D and BA or TDZ and BA. The highest percentage of globular structures (36%) was recorded on MS medium supplemented with TDZ (0.7 mg/L) and BA (0.7 mg/L). B5 medium with 0.5 mg/L 2,4-D and 3.0 mg/L BA also induced the highest percentage (53.3%) of nodular calli. However, it did not develop further into globular structures. They remained nodular or changed back into mass callus, blackened and finally died. Histological observation of sections of globular structures showed accumulation of dense meristematic cells, a common characteristic of globular formation during somatic embryogenesis.

Farzana *et al.* (2010) studied direct shoot bud formation on *Garcinia quaesita* Pierre. Leaf explants from *in vitro* grown plant were cultured on MS medium supplemented with 5.0 and 10.0 mg/L BAP showed direct shoot bud regeneration. The maximum average number of 9-12 shoot buds was produced on the medium supplemented with 10 mg/L BAP. Shoot elongation was achieved on medium supplemented with 20

mg/L BAP. Rooting could not be achieved on MS medium supplemented either with IBA (1.0, 5.0, 10.0 mg/L) or NAA (1.0, 5.0, 10.0 mg/L).

2. 10. Tissue Culture of *Garcinia indica*

Garcinia indica plants were regenerated using immature seed explants through direct somatic embryogenesis by Thengane *et al.* (2006a, b). Kulkarni and Deodhar (2002) developed protocol for plant regeneration using immature seeds of *Garcinia indica.* Malik *et al.* (2005a) developed protocol for plantlet regeneration from mature seed explants of *Garcinia indica. De novo* shoot regeneration from root culture of *Garcinia indica* was established by Deodhar *et al.* (2008).

Thengane *et al.* (2006b) reported plant regeneration via direct somatic embryogenesis. Immature seeds of *Garcinia indica* were cultured on WPM supplemented with 6-benzylaminopurine (4.44–22.19 μM) alone or in combination with *a*-naphthaleneacetic acid (2.69 μM). The combination produced somatic embryos within a period of 2-3 weeks with 80% frequency, without an intervening callus phase. Embryo induction was observed all over the surface of explants. Maturation of these embryos was achieved after 12 weeks of culture on a medium containing BAP (16.08 μM) in combination with indole-3-acetic acid (2.85–5.71 μM) and/or kinetin (4.65 μM). Rooting was achieved in half-strength WPM supplemented with NAA (5.37–10.74 μM) and IBA (4.90 μM). Origin of the embryo was confirmed by histological studies from sub-epidermal layer of the seed. About 75% of the regenerated somatic embryos converted into complete plantlets. The plantlets were transplanted in to sterilized potting mixture of sand and soil (1:1) and they showed 92% survival in greenhouse conditions.

Plantlets were regenerated via adventitious bud differentiation on leaf explants from *in vitro* raised seedling as well as mature trees. The explants were cultured on MS basal medium or MS medium supplemented with cytokinins 6-benzylaminopurine (BAP), kinetin and thidiazuron (TDZ) alone and in combination with auxin (α-napthaleneacetic acid (NAA), for direct shoot proliferation. Maximum number of shoots (2.67) was formed in 63.89% explants on 1.0 μM TDZ after 2-4 weeks of culture. Elongation of shoots was achieved on MS basal medium containing 0.2% activated charcoal. Rooting was observed in shoots cultured on half-strength MS medium supplemented with 10 μM IBA. Rooted plantlets were transferred to pots containing soil, vermiculite and farmyard manure (FYM) 1:1:1 and recorded 90% of survival rate in pots (Malik *et al.*, 2010).

A method was developed for regeneration of plantlets via adventitious bud differentiation in *Garcinia indica*. Mature seeds were cultured on MS medium supplemented with cytokinins like BAP, kinetin and TDZ alone and in combination with auxin (NAA). Multiple shoots were formed in all the combinations within 4-5 weeks of culture. The shoot forming capacity of the seed was influenced by the concentration of BAP and optimal responses were observed at different concentrations of BAP (12.5-50 μM) in different genotypes investigated. Significant differences were recorded in terms of percentage of response (27.78-100%). Average number of shoots varied per explants (3.49-57.67) among the four genotypes. Shoot elongation was achieved on MS basal medium containing 0.2% activated charcoal. Root induction was achieved on half-strength MS medium supplemented with IBA and NAA. Best rooting was observed on 10 μM IBA. The rooted plantlets were transferred to pots containing soil, vermiculite and FYM in 1:1:1 ratio and the plantlets showed 90% survival in the pots with normal growth and morphological characteristics (Malik *et al.* 2005a).

Potted plantlets (tissue cultured plantlets), cultured long time on half-strength MS medium supplemented with BAP (0.44-2.22 μM) showed *de novo* shoot formation. Roots attached to mother plants showed more number of shoots, while excised root segment produced lesser shoots. Maximum number of shoots (29.3 per root) was noticed on medium supplemented with BAP (2.22 μM). Elongation of shoots was achieved on WPM supplemented with BAP (4.44-22.69 μM), IAA (5.71 μM) and kinetin (4.65 μM). Root induction was observed on WPM supplemented with NAA (2.69-10.74 μM) and IBA (4.90 μM) within 20-25 days. Maximum rooting (77%) was recorded on medium supplemented with NAA (5.37 μM) and IBA (4.90 μM). Rooted plantlets were transplanted in pot containing mixture of sterilized sand and soil (1:1) in greenhouse and showed 90% survival rate (Deodhar *et al.*, 2008).

Tembe and Deodhar (2011) established a protocol for *in vitro* propagation of Kokum using apical and intercalary buds of the root as source explants. Multiple shoots were initiated in woody plant medium supplemented with benzylaminopurine (BAP) and thidiazuron (TDZ). Elongation of shoots was observed on half-strength WPM medium supplemented with BAP (0.22, 0.44, and 0.66 μM) with 0.5g/L activated charcoal. Better shoot elongation was observed on medium with 0.44 μM of BAP + 0.5 g/L activated charcoal. For rooting pulse, treatment of indole-3-butyric acid ranging from 4.9 to 19.6 μM for 30 seconds and 1 min was tried. About 65% root induction was obtained in shoots subjected to 19.6 μM of IBA for 30 seconds. Rooted plantlets were transplanted to pot containing coco-peat and it showed about 75% survival rate.

Kulkarni and Deodhar (2002) developed a protocol for multiple shoots and callus production. Multiple shoots were obtained from immature seed explants cultured on MS basal medium supplemented with NAA (2.69 μM), BAP (8.9 μM) and KIN (0.93 μM).

Shoot elongation was achieved on half-strength MS medium supplemented with NAA (0.54 μM), BAP (0.44 μM) and KIN (0.93 μM). Rooting was induced by treating with 4900 μM IBA for 30 seconds and cultured on half-strength MS basal medium. The regenerated plantlets were successfully transferred to soil in pots.

2. 11. Biochemical constituents

The genus *Garcinia* has been used in ayurvedic preparations to medicate various patho-physiological disorders. Bioactive molecules such as hydroxycitric acid (HCA), flavonoids, terpenes, polysaccharides, procyanidines and polyisoprenylated benzophenone derivatives like garcinol, xanthochymol and guttiferone isoforms have been isolated from the genus *Garcinia.* The species in the genus has been receiving the attention of pharmaceutical industries due to their immense remedial qualities. The hydroxycitric acid (HCA) has been known for its hypolipidemic property. The polyisoprenylated benzophenone and xanthone derivatives are known for their antioxidant, apoptotic, anti-cancer, anti-inflammatory, anti-bacterial, anti-viral, anti-fungal, antiulcer, anti-protozoal and histone acetyltransferase (HAT) inhibiting properties (Hemshekhar *et al.*, 2011).

Kokum fruit rind contains three major bioactive compounds, such as hydroxycitric acid (HCA), garcinol and anthocyanins. These compounds play a beneficial role in human health. According to Krishnamurthy *et al.* (1982) Kokum fruit rind contains 80 g/100g of moisture, 1% protein, 2.6% total ash, 1.7% tannin, 0.9% pectin, 4.1% total sugars, 1.4% crude fat, 5.9 % organic acid (as HCA) and 2.4% pigments. Other than that, Kokum seed yields edible butter and it is used for various purposes.

2. 11. 1. Hydroxycitric acid (HCA)

Hydroxycitric acid (HCA) is present in the genus *Garcinia*. HCA is found to be isolated from certain species of *Garcinia*, which include *G. cambogia*, *G. indica*, *G. atroviridis*, *G. cowa* and *G. pedunculata* (Jayaprakasha *et al.*, 2003). HCA is enriched in the calyxes of *Hibiscus subdariffa* and *H. rosa-sinensis*. HCA has also been produced from microbes like *Streptomyces* sp. and *Bacillus megaterium* (Yamada *et al.*, 2007).

2. 11. 1. 1. Chemistry of HCA

Hydroxycitric acid (1,2-dihydroxypropane-1,2,3-tricarboxylic acid) is a derivative of citric acid and comprises of a citric acid molecule with a hydroxyl group at the second carbon. Martius and Maue (1941) have synthesized the four possible stereoisomers of hydroxycitrate. One of these isomers occurs in *Garcinia* and another in *Hibiscus* species (Lewis, 1969). The absolute configurations of hydroxycitric acid lactones, hibiscus acid and garcinia acid, were determined to be (2*S*, 3*R*) and (2*S*, 3*S*)-2-hydroxycitric acid-2,5-lactone, respectively. The absolute configuration is determined from Hudson's lactone rule, optical rotatory dispersion curves, circular dichroism curves and calculation of partial molar rotations (Boll *et al.*, 1969). Glusker *et al.* (1969 & 1971) have reported the structure and absolute configuration of the calcium hydroxycitrate and HCA lactone by X-ray crystallography. Stallings *et al.* (1979) have reported the crystal structures of the ethylenediamine salts of diastereoisomeric hydroxycitrates.

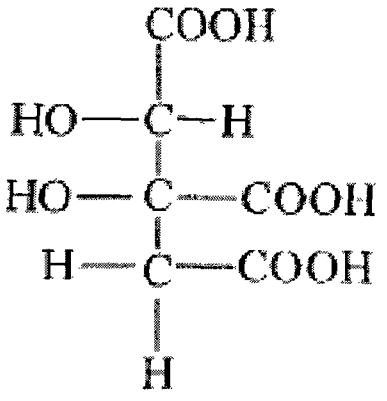


Fig. 1. Structure of Hydroxycitric acid (Jena et al., 2002a)

2. 11. 1. 2. Properties of HCA and Lactone

The equivalent weight of pure lactone is 69, as determined by alkali titration or silver salt decomposition. The IR spectra of the ethyl ester showed ester and hydroxyl groups at 5.41-5.76 and 2.74-2.79 μ, respectively (Lewis, 1965). The structure of the (-)-HCA lactone was further established by IR and ^{1}H NMR spectroscopy. The HCA lactone displayed strong IR bands at 3200, 1760, and 1680 cm^{-1}. ^{1}H NMR spectra of the HCA lactone showed two protons at the γ-carbon, which gave an AB quartet at δ 2.53 and δ 2.74 with J =17.1 Hz, and one proton at the R-carbon showing a singlet at δ 5.15 (Jayaprakasha and Sakariah, 2000).

2. 11. 1. 3. Extraction and estimation

Lewis and Neelakantan (1956) studied the extraction of HCA in the fruit rinds of *G. cambogia* and identified HCA on the basis of chemical and spectroscopic studies. The method consisted of extracting the acid by cooking the raw material with water under pressure (10 lb/inch^2 for 15 min). The extract was concentrated and pectin was removed by alcohol precipitation. The clear filtrate was neutralized with alkali and passed through cation exchange resin for recovery of the acid. Identification and separation of the hydroxycitric acid on Whatman No. 1 filter paper were performed using *n*-butanol/acetic

acid/water (4:1:5) and *n*-propanol/formic acid/water (4:1:5). The spots were identified by spraying with 5% metavanadate. The eluate noticed only one lower spot with Rf value of 0.34 and it corresponding to the free HCA. On concentration the eluate showed only one upper spot with Rf value of 0.46 and it is corresponding to the HCA lactone. Fruit extracts showed two predominant acid spots on chromatograms with two different solvent systems and this was clear that two spots on the chromatograms are those of hydroxycitric acid and its lactone.

Lowenstein and Brunengraber (1981) estimated the hydroxycitrate content of the fruit of *G. cambogia* by gas chromatography (GC). GC estimation involves the conversion of acid to volatile silyl derivative. They used an OV-17 GC column. The column was run at 145°C using nitrogen as the carrier gas (40 ml/min) with an injection port temperature of 250°C and a detector temperature of 300°C. HCA lactone was the major constituent of the extract and the re-crystallized compound contains <0.5% of impurities. Silylation requires completely dried samples, but HCA has a tendency for lactonization during drying. Highly hygroscopic nature of HCA makes it difficult to dry the sample. For this reason, the free HCA content cannot be estimated. Moffett *et al.* (1996) have developed a process for the extraction of HCA from *Garcinia* rinds using water. The extract was loaded into an anion exchange column for adsorption of HCA, and it was eluted with sodium/potassium hydroxide for the release of HCA. The extract was passed through a cation exchange column to yield a free acid.

Jayaprakasha and Sakariah (1998) developed a method for the extraction of HCA from *Garcinia cambogia* rind. 25 g of rind was autoclaved with 50 ml of water at 15 lbs/inch^2 pressure for 20 min and the extract was filtered. The rind was again autoclaved with above conditions and the extract was filtrated. The extraction was repeated twice for

complete extraction. The filtrate was combined and it was decolorized using activated charcoal and re-filtered. The decolorized extract was concentrated to 25 ml and was treated with 50 ml of ethanol to remove pectinaceous material and centrifuged. The supernatant was concentrated to 25 ml under reduced pressure and stored at 4°C until further use. The samples were analyzed using chromatographic system (Shimadzu LC-6A model with C_{18} column (300×3.9mm I.D.) and a multi-wavelength detector). The elution with 10 mM sulfuric acid was carried out at a flow-rate of 0.7 ml/min under isocratic conditions. The compounds were quantified using a Shimadzu C-R4A Chromatopak data processor. Amaximum of 18.0 g/100g and minimum of 16.0 g/100g of HCA was recorded in the sample.

Jayaprakasha and Sakariah (2000) studied the various methods of sample preparation for HPLC determination of HCA. In the first method, 0.1 g of *Garcinia cambogia* extract was added to 30 ml of 8 mM sulfuric acid and stirred for 15 min with magnetic stirrer. The supernatant was decanted and the residue was treated once again with 20 ml of 8 mM sulfuric acid, stirred for 15 min for complete desalting and the supernatant was collected. The supernatants were combined and made up to 100 ml, filtered and stored at 4°C until further use. In the second method, 0.5 g of *Garcinia cambogia* extract was suspended in 10 ml of water and 5 g of Dowex-50 [H+] was added, stirred for 20 min with a magnetic stirrer. The supernatant was decanted and the resin was washed to neutral pH and the supernatant were collected. The combined supernatant was made up to 250 ml using water, filtered and stored at 4°C until further use. The two stored samples were analyzed using chromatographic system (Shimadzu LC-6A model with C_{18} column (300×3.9 mm I.D.) and the detection at 210 nm by multi-wavelength detector). The injection was carried out with 20 μl sample loop and UV-visible Spectrophotometer was used for detection at 210 nm. The elution with 6 mM, 8 mM, 10 mM and 12 mM

sulfuric acid solutions was carried out at a flow-rate of 1.0 ml/min under isocratic conditions. HCA was resolved as a single peak in all samples analyzed with no interference of other compounds. The identity of the HCA peak was confirmed by determination of relative retention time and by spiking with standard HCA. 6 mM H_2SO_4 was found to be the best elution solvent for the separation of all peaks from commercial *Garcinia cambogia* samples.

Jayaprakasha and Sakariah (2002) determined organic acid from leaves and rinds of *G. indica*. 20 g of fresh leaves of *G. indica* were extracted with 250 ml of water at 15 lbs/inch^2 pressure for 20 min and the extract was filtered. The extraction and filtration was repeated twice for complete extraction and all filtrates were combined. The combined filtrates was concentrated to 50 ml under vacuum, and treated with 200 ml of ethanol and centrifuged to remove pectinaceous material. The supernatant was concentrated to 25 ml under reduced pressure and stored at 4°C until further use. 10 g of rind of G. *indica* was extracted with 50 ml of water at 15 lbs/inch^2 pressure for 20 min and the extract was filtered. The extraction and filtration was repeated twice for complete extraction. The combined filtrate was treated with activated charcoal and filtered for discoloration. The decolorized extract was concentrated to 25 ml under vacuum and was treated with 100 ml of ethanol to remove pectinaceous material by centrifugation. The supernatant was concentrated under reduced pressure to 25 ml and stored at 4°C until further use. Samples were analyzed using high performance liquid chromatographic system (Hewlett Packard HPLC model HP1100 Series with a C_{18} column (250×4.6 mm I.D)). The injection was carried out using 20μl sample loop and the detection was done at the wavelength of 210 nm by a HP1100 Series variable wavelength detector. The elution was carried out with 8 mM sulfuric acid and flow rate was 1.0 ml/min under isocratic condition. HP CHEMSTATIONS software was used for quantification of compounds. Amongst the rind

samples collected from different parts of coastal Karnataka, rinds-III recorded maximum (12.74g/100g) amount of HCA content and rinds-I recorded the minimum (10.27g/100g). In leaves, leaves-II showed maximum (4.64g/100g) while leaves-III showed the minimum (4.10g/100g).

Liquid chromatographic method for determination of organic acids in leaves, pulp, fruits and rinds of *G. pedunculata* was developed by Jayaprakasha *et al.* (2003). About 5 g of fresh leaves, pulp and fruits were used for extraction. Each sample was extracted with 50 ml water at 10 psi for 20 min and the extract was filtered through muslin cloth. The extraction and filtration were repeated once again for complete extraction. The filtrates were combined and concentrated to 20 ml under vacuum. They were treated with 100 ml ethanol to remove pectinaceous material, by centrifugation at 1200× *g* for 15 min. The supernatant was concentrated to 25 ml under reduced pressure and stored at 4°C until further use. Five grams of rinds was extracted with 100 ml water at 15 psi for 30 min. The extract was filtered through a glass filter. For complete extraction, extraction and filtration was repeated twice. The filtrate was concentrated to 30 ml under vacuum, treated with 120 ml ethanol to remove pectinaceous material and centrifuged at 1200 × *g* for 15 min. The supernatant was concentrated to 25 ml under reduced pressure and stored at 4°C until further use. The acid content was determined by acid-base titration. 10 grams of rinds were extracted with 100 ml acetone in a Soxhlet extractor for 8 hours and methanol separately for 8 hours. The extracts were filtered through Whatman No.1 filter paper and concentrated to 10 ml under vacuum. The concentrate was suspended in 20 mL water. After, the extract was filtered through a glass filter, the filtrate was diluted to 50 ml with water. The LC system of Hewlett-Packard LC Model HP1100 Series equipped with a quaternary LC pump, fitted with C_{18} (25 cm×4.6 mm ID) analytical column was used for the separation. 20 μl samples were injected into the system. . Detection was carried out

with HP1100 Series variable wavelength detector at the wavelength of 210 nm. The mobile phase used was 6 mM phosphoric acid with a flow rate of 1.0 ml/min. Quantification of the compounds was done by using HP CHEMSTATIONS software. Among the various samples, rinds recorded maximum quantity (20.11g/100g) of HCA followed by fruit (3.01g/100g). Leaf and pulp showed minimum quantity of 2.45g/100g and 0.82g/100g respectively.

2. 11. 2. Garcinol

Garcinol is a polyisoprenylated benzophenone derivative, extracted from *Garcinia indica* and other species. The garcinol molecule has phenolic hydroxyl groups and a β-diketone structure and it has molecular weight of 602 (Padhye *et al.*, 2009).

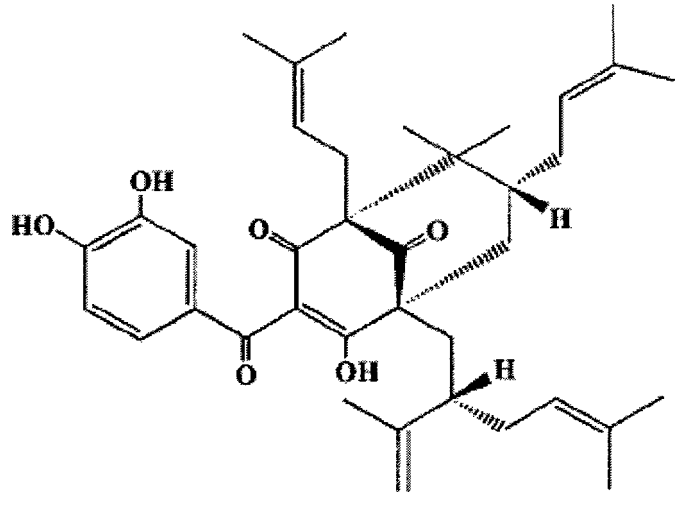


Fig. 2. Structure of garcinol

2. 11. 2. 1. Antioxidant Activity

Hong *et al.* (2006) have investigated the possible mechanisms of antioxidant action of garcinol and its derivatives on arachidonic acid metabolism and nitric oxide (NO) radical synthesis at concentrations (>1 μM), that may be achievable under in *vivo* conditions. The preliminary results indicated that peak plasma and urine plasma concentration levels of garcinol in female mice were 12 and 2.7 μM, respectively, after oral feed of garcinol (10 mg dose per mouse). Garcinol has been found to be possessing antioxidant activity in the H_2O_2-NaOH-DMSO system as well as the radical scavenging activity against superoxide anion, hydroxyl radical and methyl radicals. The emulsified garcinol suppresses superoxide anion to almost same extent as DL-α tocopherol by weight, while it exhibits nearly three times greater free radical scavenging activity against 2, 2, diphenyl-1-picrylhydrazyl (DPPH) radicals than DL-α tocopherol by weight (Yamaguchi *et al.*, 2000b).

The neuroprotective effects of garcinol were examined by Liao *et al.* (2005). They found that at 5 μM concentration it prevented NO radical accumulation in lipopolysaccharide (LPS), treated astrocytes and significantly reduced the expression of LPS-induced inflammatory mediators, such as nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). These results suggest that the neuroprotective effects of garcinol are associated with its antioxidant nature involving inhibition of iNOS induction in astrocytes. It has been suggested that the compound may be neuroprotective against brain injury through similar mechanism (Liao *et al.*, 2005). Sang *et al.* (2002) also planned the antioxidant mechanism of garcinol according to which the compound reacts with peroxyl radicals by a single electron transfer followed by deprotonation of the hydroxyl group from the enolized 1, 3-diketone to form a resonance pair. Depending on the position

of hydroxyl group (C-3 or C-5) which initiates the reaction, different compounds are formed.

Yamaguchi *et al.* (2000a) studied various pharmacological activities of garcinol including antioxidant activity, chelating activity, free radical scavenging activity and anti-glycation activity. They observed that garcinol exhibited reasonable antioxidant activity in the micellar linoleic acid peroxidation system and exhibited chelating activity at almost the same level as citrates. In a phenazine methosulfate/NADH-nitro blue tetrazolium system garcinol exhibited superoxide anion scavenging activity and suppressed protein glycation in a bovine serum albumin/fructose system. Thus, the compound may be useful as a glycation inhibitor under specified conditions.

2. 11. 2. 2. Anti-inflammatory activity

The nitric oxide radical moiety is involved in various physiological processes, including vasodilation, inhibition of platelet function, synaptic neurotransmission as well as host defence. The formation of NO radicals from arginine in the biological system is catalyzed by three different types of nitric oxide synthase (NOS) enzymes, viz. endothelial NOS, neuronal NOS and inducible NOS (iNOS). The enzyme iNOS is stimulated by inflammatory cytokines for NO production by macrophages and by many other cell types. It has been reported that garcinol inhibits the expression of iNOS and COX-2 in lipopolysaccharide (LPS)-activated macrophages. It was observed that garcinol strongly blocks the LPS induced activation of eukaryotic transcription nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This inhibition of NF-κB activation takes place through the suppression of phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α) and p38 Mitogen-Activated Protein Kinases (MAPK). Garcinol lowers the LPS-induced increase of intracellular

reactive oxygen species (ROS), which contribute to the activation of NF-κB (Liao *et al.*, 2004).

Aberrant arachidonic acid metabolism and generation of nitric oxide radicals (NO) have found to be involved in inflammation and carcinogenesis. Arachidonic acid is released by phospholipase A2 (cPLA2) from membrane phospholipids and is further metabolized by cyclooxygenase (COX), lipooxygenase (LOX) enzymes and Cytochrome P450 pathways. Modulation of arachidonic acid metabolism by inhibiting COX and LOX enzymes has been considered as an effective approach for treating inflammation and for cancer chemoprevention. Garcinol and its derivatives modulate the arachidonic acid metabolism by retarding the phosphorylation of cytosolic PLA2 (cPLA2) through the inhibition of the extracellular ERK1/2 kinase activation and suppression of iNOS expression through modulation of the Janus kinase (JAK)/ STAT-1 signaling pathway. When added prior to LPS, garcinol suppressed NF-κB activation and COX-2 expression through the interruption of LPS binding to toll-like receptors (Hong *et al.*, 2006).

Koeberle *et al.* (2009) reported that the garcinol significantly interferes with two enzymes that play crucial roles in inflammation and tumorigenesis, viz. 5-lipoxygenase and microsomal prostaglandin PGE2 synthase. In cell-free assays, garcinol inhibits the activity of purified 5-lipoxygenase and blocks the mPGES-1-mediated conversion of PGH2 to PGE2 with IC50 values of 0.1 and 0.3 μM, respectively. Garcinol was found to be suppress 5-lipoxygenase product formations in intact human neutrophils and reduced PGE2 formation in interleukin-1β-stimulated A549 human lung carcinoma cells as well as in human whole blood stimulated by lipopolysaccharide. Garcinol also interfered with isolated COX-1 enzyme (IC50=12 μM) and with the formation of COX-1-derived 12(*S*)-hydroxy-5-cis-8, 10-trans-heptadecatrienoic acid as well as thromboxane B2 in human

platelets. The high potency of garcinol in selectively suppressing PGE2 synthesis and 5-lipoxygenase product formation provides the molecular basis for its anti-inflammatory and anti-carcinogenic effects and rationalizes its therapeutic use.

2. 11. 2. 3. Anticancer activity

Tanaka *et al.* (2000) reported the modifying effects of dietary feeding of the compound on the development of azoxymethane (AOM) induced colonic aberrant crypt foci (ACF) in male F344 rats. The effects of garcinol on proliferating cell nuclear antigen (PCNA) index in ACF and activities of detoxifying enzymes such as glutathione S-transferase (GST) and quinone reductase (QR) in liver. It was observed that garcinol administration significantly lowers proliferating cell nuclear antigen (PCNA) index in ACF and significantly elevates liver glutathione *S*-transferase (GST). In addition, garcinol was found to be NO generation and expression of iNOS and COX-2 proteins. These observations suggest possible chemopreventive role of garcinol.

Garcinol suppresses the ACF formation in rats. The beneficial effects of garcinol against tumor prevention in human colorectal cancer cell line, HT-29 (Human colon adenocarcinoma grade II cell line) were investigated. Matrigel analysis showed that exposure of HT-29 cells to 10 μM garcinol inhibited cell invasion and decreased the dose-dependent tyrosine phosphorylation of focal adhesion kinase (FAK). Thus, garcinol reduces cell invasion and survival through the inhibition of FAK's downstream signaling (Matsumoto *et al.*, 2003).

The potent cytotoxic activity for the methanol extract of the fruit rinds of *Garcinia indica* against three human cancer cell lines, viz. colon (COLO-320-DM), breast (MCF-7) and liver (WRL-68) has been reported. Fractionation of the methanol extract into hexane-,

chloroform- and ethyl acetate-soluble portions was performed and their cytotoxic activity was evaluated. The ethyl acetate fraction was found to be the most effective as compared to the two other fractions. Thus, current results provide evidence for the potential of garcinol as a chemopreventive agent in carcinogenesis. Additionally, feeding garcinol-containing diets does not cause retardation of body weight gain and pathological alterations in liver and other organs including kidney, lung, heart, and esophagus, which is indicative of the low toxicity of the compound. This is a very attractive feature of any anti-cancer agent (Kumar *et al.*, 2007).

2. 11. 2. 4. Anti-HIV Activity

Histone acetylation is a diagnostic feature of transcriptionally active genes, which is responsible for the proper recruitment and function of histone acetyltransferases (HATs) and histone deacetylases (HDACs). These enzymes are key regulatory steps for gene expression and cell cycle. Functional defects of either of these enzymes may lead to several diseases, including cancer. It has been reported that garcinol is a potent non-specific inhibitor of histone acetyltransferases p300 (IC50 = 7 μM) and strongly inhibits HAT activity-dependent chromatin transcription, whereas transcription from DNA template is not affected (Balasubramanyam *et al.*, 2004).

2. 11. 2. 5. Anti-ulcer activity

Garcinol has potential free radical scavenging activity and it interacts with the three types of free radical generating systems. Its scavenging activity against hydroxyl radical has been found to be stronger than that of α-tocopherol (Yamaguchi *et al.,* 2000a). The other scavenging activities were found to be slightly less. Since, hydroxyl radical is regarded as the most damaging reactive oxygen species (ROS), garcinol is expected to be useful for preventing diseases caused by the hydroxyl radical damages such as stress-

induced gastric ulcer (Das *et al.*, 1998). In the water immersion stress model, Yamaguchi *et al.* (2000b) have shown that garcinol suppressed gastric injury to almost same extent as cetraxate hydrochloride as a positive control. It also prevented indomethacin-induced gastric injury. These results suggest that garcinol may have potential as an anti-ulcer drug. Although mechanism of its anti-ulcer activity is not yet understood, it may be speculated that the compound may scavenge reactive oxygen species on the surface of gastric mucosa, thus protecting cells from injury.

2. 11. 2. 6. Anthocyanin pigments in Kokum

Color is an important constituent of all types of food. Synthetic colors are very commonly used in medicines, food products, cloths and in other products. Consumers are aware of the toxicological effects associated with synthetic colors, so an increased usage of the natural colorants in various purposes is seen. The demand for natural pigments becomes high. The existing colorants and their methods of extraction and large scale production from natural sources are not able to meet this demand. The size of the color market on a global scale is estimated to be about US $940 million, out of which that of natural colors is estimated to be US $250 million (27% market share). This demand is met by betalains, anthocyanins, algal pigments, carotenoids and other pigments (Nayak *et al.*, 2010b).

The use of natural pigments for food colorants permitted by the regulatory authorities is very limited and the any new source that has a potential to be used as food colorant requires the approval of the Food and Drug Administration (FDA). Anthocyanins are present in nature as flavonoids. These are water-soluble and vacuolar pigments present in the epidermal cells and flowers of plants. Anthocyanins comprise of diverse groups of intensely colored pigments. These are range of natural colors such as orange, red, purple,

and blue and they are present in many fruits, vegetables, flowers, leaves, roots and other plant organs. These natural colorants have properties of solubility in particular solvent. The anthocyanins are easily soluble in water and it makes them the best candidate for the incorporation into aqueous food systems (Nayak and Rastogi, 2010b).

Ripened Kokum fruits contain 2 to 3 % of anthocyanins (red pigment) that is about 2400 mg/100g of fresh fruit. This is the highest concentration of anthocyanins as compared to other natural sources. Kokum is therefore regarded as a rich source of anthocyanin pigments. Anthocyanins are water soluble and possess antioxidant properties. Two major pigments characterized in Kokum as cyanidin-3-glucoside and cyanidin-3-sambubioside which are usually present in the ratio of 4:1. It was first identified by thin layer chromatography using acetic acid: HCl: water in ratio of 15:3:82. HPLC was used for identification of the types of anthocyanins. The respective sugars associated with these two pigments are glucose and xylose (Nayak *et al.*, 2010a, b).

The extracts of anthocyanins contain water, pigments and sugars. High water content in the anthocyanin extracts brings low shelf-life and the extracts need to be concentrated. The concentration of anthocyanins through membrane processes such as microfiltration, ultrafiltration or reverse osmosis are employed. However, these methods have few drawbacks such as need of high pressure, membrane clogging, maximum achievable concentration and reduction in the gradient. A novel, forward osmosis method is also developed for concentration of Kokum anthocyanins using semi-permeable non-porous active skin layer of cellulose triacetate embedded in a nylon mesh with NaCl solution as an osmotic agent. In case of forward osmosis, the anthocyanin extract was concentrated from 49.63 mg/L to 2.69 g/L in 18 hours; however, it was coupled with migration of sodium chloride (0.21 moles/m^2s). In case of osmotic membrane distillation

process, the concentration of anthocyanin was achieved up to 72 mg/L, at the same time without any transfer of osmotic agent. The transmembrane flux was low in case of osmotic membrane distillation as compared to forward osmosis (Nayak and Rastogi, 2010a).

Nayak and Rastogi, (2010c) studied the stability of anthocyanins. Stability of anthocyanins is influenced by various factors like temperature, pH, structure, concentration and the presence of light metallic ions, enzymes, oxygen or sulfur dioxide. Microencapsulation of anthocyanin pigments was carried out with maltodextrin of various dextrose equivalents (DE 06, 19, 21, and 33) and other additives such as gum *acacia* (*Acacia senegal*) and tricalcium phosphate, to enhance the stability of the pigment. The microencapsulated pigment containing 5% maltodextrin DE 21, 0.25% gum acacia, and 0.25% tricalcium phosphate was found to have lowest hygroscopic moisture content (4.38%), highest antioxidant activity (69.90%), and highest anthocyanin content (485mg/100g). The glass transition temperature was found to be below 44.59°C for storage. The adsorption isotherm for microencapsulated powder showed that the samples were stable up to water activity less than 0.43. The scanning electron microscope structures depicted that the particle size ranged from 5 to 50 μm with smooth spheres. Storage at 4°C increased the half-life two-fold compared to that of the spray-dried product kept at ambient temperature (25°C). This study showed that microencapsulated anthocyanin pigment powder belongs to a class of food products that is highly hygroscopic in nature.

2. 12. Antimicrobial activity of *Garcinia indica*

Tamil Selvi *et al.* (2003) studied the antimicrobial and antioxidant properties of *Garcinia* sp. Chloroform extract of rinds of *Garcinia indica* was inhibitory to *Aspergillus flavus*. Negi and Jayaprakasha (2004) studied the control of food borne pathogenic and

spoilage bacteria by *Garcinia indica* extracts, and their antioxidant activity. The extract was effective against *Bacillus* sp. and *Staphylococcus aureus* at very low concentration (2 ppm). Negi *et al.* (2008) also studied the antibacterial activity against some food borne pathogens and spoilage bacteria, such as *Bacillus cereus*, *B. coagulans*, *B. subtilis*, *Staphylococcus aureus* and *Escherichia coli,* using crude hexane and chloroform extracts from the fruit rinds of *Garcinia cowa* and *Garcinia pedunculata*. Extracts from both the species of *Garcinia* had a variable growth inhibitory effect. The hexane and chloroform extracts from the fruit rinds of *Garcinia cowa* exhibited marked inhibitory effect against all the test organisms and were more effective than that of *Garcinia pedunculata* extracts Higher antibacterial activity was exhibited by the chloroform extract from *Garcinia cowa* against *Bacillus cereus*, *B. coagulans* and *Escherichia coli* as compared to the hexane extract of the same species.

Varalakshmi *et al.* (2010) studied the antimicrobial properties of *Garcinia indica.* The fruit rind extract inhibited the growth of *Bacillus subtilis* with the highest zone of inhibition of 2.4 mm followed by *Enterobacter aerogenes, Escherichia coli.* Least zone of inhibition of 1.0 mm was observed for a strain of *Staphylococcus aureus*. The minimum inhibitory concentrations of the water extract against bacteria were, 0.5mg/ml for *E. coli*, 5 mg/ml for both *Bacillus subtilis* and *Enterobacter aerogenes* and 50mg/ml for *Staphylococcus aureus.* The extract also exhibited antifungal activity. Maximum inhibition of 17 mm was recorded against *Candida albicans* followed by *Penicillium* sp. Vishnu Priya *et al.* (2010) studied the anti- microbial activity of *Garcinia mangostana.* The pericarp powder extract was tested against *Staphylococcus aureus*, *S. albus* and *Micrococcus luteus* using the disc diffusion method. The zone of inhibition against *Staphylococcus aureus*, *Micrococcus lutus* and *Staphylococcus albus* was 12 mm, 14mm and 17mm, respectively. The minimum inhibitory concentration for *Staphylococcus*

aureus was 200µg/ml and that of *Micrococcus luteus* and *Staphylococcus albus* was 50µg/ml each. *Garcinia mangostana* is used as a phytomedicine in South East Asia for the treatment of trauma, diahorrea and skin infections.

2. 13. Molecular Characterization of *in vitro* Regenerated Plants

To understand the genetic stability within micropropagated plants, various DNA marker techniques were employed (Saker *et al.*, 2006; Feyissa *et al.*, 2007; Xing *et al.*, 2010). Molecular markers have come up as the most desirable tool for establishing genetic uniformity of the micropropagated plantlets. PCR (Polymerase Chain Reaction) based markers such as RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats) and SSRs (Simple Sequence Repeats) are used for characterization as well as comparison of genomic instability within the micropropagated population (Borchetia *et al.*, 2009) and these are the most widely used methods for DNA fingerprinting (Banerjee *et al.*, 1999; Taylor and Soliman, 1999; McGregor *et al.*, 2000). These techniques have been largely used in recent years for checking genetic uniformity of a number of micropropagated plant species since they are relatively simple, low cost, non-radioactive and requires only a small quantity of DNA. In these technique no specific prior primer sequence is required (random primers are used) (Joshi and Dhawan, 2007; Huang *et al.*, 2009).

Joshi and Dhawan (2007) studied the genetic fidelity of micropropagated plant *Swertia chirayita* by inter simple sequence repeat (ISSR) marker. DNA isolated from fifteen tissue cultured plants and sixteen ISSR primers were used for PCR amplification. ISSR primer banding pattern confirmed the clonal fidelity of the tissue cultured plantlets of *S. chirayita*. The assessment of genetic stability within the clonal propagation of *Zephyranthes grandiflora* using bulbs as explants is explained by Gangopadhyay *et al.*

(2010). Genomic DNA was isolated from randomly selected bulb tissues of *in vitro* regenerated. The study revealed that of the twenty ISSR primers used eight showed clear visible bands. The ISSR profiles of micropropagated plants showed monomorphic bands indicating genetic uniformity in the tissue culture derived plants.

ISSR molecular markers were used for assessment of the genetic stability of long-term micropropagated plantlets of London plane tree (*Platanus acerifolia).* Total DNA was extracted from fresh leaves of twenty micropropagated plantlets. Out of the 38 ISSR primers screened, 16 primers produced 103 distinct clear reproducible bands. Similarity indices were used to construct an UPGMA dendrogram and demonstrated that all 20 micropropagated plants grouped together in one major cluster with a similarity level of 91%. This indicated that the micropropagated plants of *Platanus acerifolia* was genetically stable (Huang *et al.*, 2009).

Clonal fidelity of micropropagated plants of *Chlorophytum borivilianum* was studied by Kumar *et al.* (2010) using RAPD and ISSR markers. Total genomic DNA was isolated from leaf material of randomly selected micropropagated plants. PCR amplification was performed using five RAPD primers and eight ISSR primers. The RAPD and ISSR analysis of the regenerated plants showed similarity with mother plant indicating the clonal nature. In all the 13 primers (5 RAPD and 8 ISSR) used for amplification, banding patterns in regenerated plants were similar to that in the mother plant which suggests that clonal fidelity of regenerated plants are of true-to-type.

Pati *et al.* (2008) assessed the genetic integrity of micropropagated plants of *Aegle marmelos.* Total genomic DNA was isolated from leaf of micropropagated plants and mother plants. Thirteen RAPD and three ISSR primers were used for the standard PCR

amplification. Banding profile obtained from all primers observed no difference between the tissue cultured plants and their mother plant, indicating the genetic integrity of micropropagated plants with that of mother plants. RAPD primers were used for the analysis of genetic stability in regenerated plants of *Solanum melongena*. Total of 42 RAPD primers were used for the analysis in 40 randomly selected regenerated plants. Out of 42 primers, 16 produced clear identifiable bands and yielded 91 countable bands with an average of 5.7 bands per primer. The results indicated that there exists almost no somaclonal variation in the regenerated eggplants (Xing *et al.*, 2010).

Genetic stability of the regenerated plants of spine gourd (*Momordica dioica*) was assessed using RAPD (Rai *et al.*, 2012). Genomic DNA was isolated from juvenile leaves of the mother plant and randomly selected *in vitro* regenerated plants. PCR amplification was carried out using a total of 16 RAPD random primers. All sixteen RAPD random primers produced reproducible bands. The number of amplified bands produced by each RAPD primer ranged from 3 to 9. RAPD banding pattern of genomic DNA of the *in vitro* grown plants was similar to the mother plant. All the 16 RAPD random primers produced only monomorphic bands confirming the genetic homogeneity of the *in vitro* raised plants. The results of this molecular study revealed that the micropropagated plants were genetically identical to mother plant and no variation is induced during clonal propagation.

Kumar *et al.* (2010) studied genetic fidelity of micropropagated date palm *Phoenix dactylifera* by RAPD and ISSR markers. Total genomic DNA was extracted from leaves of 27 micropropagated plants and a mother plant (control). Total of 160 RAPD and 21 ISSR primers were initially screened, amongst which 30 RAPD and 12 ISSR primers produced a total of 347 (246 RAPDs + 101 ISSRs) clear, distinct and reproducible band. These bands were monomorphic across all 27 micropropagated plants. All DNA of

micropropagated plants generated homogeneous banding patterns with both RAPD and ISSR markers indicating that the micropropagated plants were true-to-type.

Mohanty *et al.* (2011) assessed the genetic stability in micropropagated plantlets of *Zingiber rubens* through RAPD and ISSR markers. Total genomic DNA was isolated from leaves of both *in vitro* and *ex vitro* grown mother plants of *Z. rubens* . Eighteen selected RAPD primers gave a total of 109 bands. The number of bands for each primer varied from 3-9 with an average of 6 bands per primer. A total of 6540 bands were generated as monomorphic bands. In ISSR analysis, 8 selected primers produced a total of 42 bands. For each primer, the number of amplified bands varied from 2-8 and a total of 2520 bands were generated, all of them monomorphic in nature. Monomorphic banding pattern of RAPD and ISSR markers showed genetic stability in propagated plants

DNA fingerprinting profiles of regenerated plants of *Pithecellobium dulce* was reported by Goyal *et al.* (2012). Total genomic DNA was extracted from fresh leaves of 11 randomly selected regenerated plants and from the leaves of mother plants. Out of 20 random RAPD primers used, 12 produced distinct bands. A total of 1,116 bands were obtained and primer OPF-08 generated highest number of bands (10) bands. Fourteen ISSR primers were used, of which seven primers produced clear bands. A total of 550 bands were obtained and primer UBC 811 produced highly reproducible banding pattern with 12 bands. DNA fingerprinting profiles of regenerated plants revealed that there were no polymorphic DNA fragments and no variation amongst mother plants and tissue cultured plants.

Rai *et al.* (2012) studied the genetic homogeneity of guava (*Psidium guajava*) plants developed through *in vitro* somatic embryogenesis. Total genomic DNA was

extracted from juvenile leaves of 7 randomly selected *in vitro* regenerated plants and mother plant. A total of six simple sequence repeat (SSR) primers were used for PCR amplification. All six primers produced reproducible and clear bands. Ten ISSR primers were used for PCR amplification. Six ISSR primers were produced resolvable, reproducible bands. A total of 25 bands were obtained, with 3-6 banks per primer (average of 4.1 bands per primer). The amplification products were monomorphic across all the micropropagated plants. The monomorphic banding pattern in micropropagated plants and the mother plant confirmed the genetic homogeneity of the *in vitro* raised plants and demonstrated the reliability on *in vitro* propagation system for guava.

2. 14. Histochemical and Developmental Studies

2. 14. 1. Structure and Development of Fruit

The fruit development has a vital role in all plant species. The flowers develop into fruits to provide both, efficient protection and dispersal of seeds. The fruits develop from the ovaries or other flower parts (i.e. floral tube, petal sepal etc). After fertilization, there occurs rapid cell division and growth of cells, which determines fruit size, shape and strength of the fruit. For example, cell division may be completed 7-10 days after anthesis in tomato or extend up to 50 days after anthesis in orange. At the end of fruit growth, embryo matures and the fruit ripens. Changes occur in the hormonal concentrations, cell wall integrity, colour, aroma, flavour compounds and biochemical composition of fruits. These desirable characteristics have led commercial development and understanding of fruit crops like apple, grape, tomato, citrus and stone fruit. Many of these crops bear fruit with little resemblance to their wild relatives because of long period of domestication (Richardson *et al.*, 2011).

Krishnan and Dayanandan (2003) studied the structure and development of rice grain from anthesis to maturation and grain-filling in the caryopsis of rice (*Oryza sativa*). During the study, free-hand thin sections, wax and Spurr plastic-embedded thin sections of caryopsis at different developmental stages were stained with various bright-field and fluorescent dyes. The study revealed that the nutrients are transported to the endosperm through a single ovular vascular trace present on the ventral side of the ovary. During the early stage of development, solute enters through the chalaza into the nucellar projection and then into the endosperm. In the mature stage, transport of nutrients occurs through the nucellar epidermis, centripetally towards the endosperm. The cell walls of the nucellar epidermis are provided with rib-like thickenings.

A comparison of grain-filling in C_3 and C_4 cereals suggests that rice has structural features close to wheat, however, with significant differences. Krishnan *et al.* (2009) reported the structure of rice caryopsis in relation to distribution of micro-nutrients in polished and unpolished rice grains of *indica* and *japonica* rice varieties. Little *et al.* (2009) investigated the anatomy and development of fruits of Lauraceae. Study revealed that the youngest fruits are found attached to floral remnants. During the stages of development the receptacle enlarges, fruit wall thickens and development of large number of sclereid clusters occurs. The mature fruits have an endocarp with palisade layer of radially elongated cells and stellate outlines, an inner mesocarp of radially arranged sclereid clusters and a fleshy outer mesocarp with numerous idioblasts. The matured fruit contains a single seed with outer integument and innermost radially elongated transfusion cell layer. Mature seeds contain a cellular embryo with idioblasts.

Rancic *et al.* (2010) reviewed the anatomy of tomato fruit in comparison with fruit pedicel. The study revealed the developmental stages of fruit and fruit pedicel at different

anatomical parameters. The study further discussed the anatomical bases the water and solute transport during fruit development and the factors affecting fruit development. Ferrandiz *et al.* (1999) studied the structure and development of fruit of *Arabidopsis thaliana.* The structure, development and histochemical localization of major storage compounds, minerals and other compounds was carried out in the seed (nut) and pseudocarp (apple) of *Anacardium occidentale* (Krishnan *et al.*, 2011).

2. 14. 2. Histochemical Localization of Major Storage and Other Compounds

Histochemical techniques were used for the localization of trace quantities of compounds present in the biological tissues and to characterize the structure of cells, tissues and organs (Pearse 1972; Krishnamurthy 1998). Fluorescence microscopic methods were used to study the plant structure and detect minute quantities of compounds in various biological tissues (O'Brien and McCully, 1981; Ploem and Tanke, 1987).

Krishnan *et al.* (2001) localized major storage compounds such as protein, lipid and starch during various developmental stages and minerals like calcium, potassium and iron in mature stages of rice grain using specific fluorescent, non-fluorescent dyes and biochemical techniques. The bright-field dye Sudan IV and a fluorescent dye Nile blue were used for the localization of lipids in the aleurone, embryo and cuticle over the nucellar epidermis. Proteins were localized by using a fluorescent dye barbituric acid. Iron, calcium and potassium were localized in aleurone layer of rice caryopsis using Prussian blue technique. Starch during various developmental stages was detected using iodine-potassium iodide staining reaction. The longitudinal sections of caryopsis 10 days after fertilization (DAF) stained with Sudan IV observed the lipids present in the aleurone cells, nucellar epidermis and pericarp. The phytin granules in aleurone were localized by

using a fluorescent dye Alizarin red. The sodium cobalti-nitrite reagent was used for the localization of potassium associated with phytin in aleurone.

Histochemical localization of storage components in caryopsis of rice (*Oryza sativa*) was demonstrated by Krishnan *et al.* (2001). Transverse sections of pre-anthesis ovary stained with I_2KI at various stages of development showed the presence of starch in the pericarp and nucellus. The maximum level of starch storage was noticed on nucellus and endosperm, not in pericarp, five days after fertilization (DAF). Mature caryopsis at 30 DAF, observed that starch was completely filled in endosperm cells. Starch started to disappear in the pericarp on 10 DAF, while it completely disappeared on 14 DAF. At this stage starch was seen only in the endosperm cells. Transverse sections of mature seed (1 day after imbibitions) stained with I_2KI and Sudan IV showed the presence of starch in the endosperm and lipids in the aleurone layer and the embryo. Presence of lipids in the aleurone and in the embryo was exhibited in the transverse sections of mature seed (1 day after imbibitions) stained with Nile blue A and excited with blue light. Aleurone peel from germinated seed (one week after germination) noted the presence of lipids and protein when simultaneously stained with Sudan IV and Coomassie brilliant blue. Localization of proteins in sub-aleurone region was detected by barbituric acid under blue excitation.

Krishnan *et al.* (2003) tissue specifically localized iron and β-carotene in transgenic and non-transgenic rice grains. The study revealed that in non-transgenic rice grain iron is present only in the aleurone and embryo regions, while in transgenic rice grain iron was localized in the entire endosperm other than in aleurone and embryo indicating the expression of transgene in the endosperm.

For histochemical detection of iron in the rice grain, the thin transverse sections of non-transgenic and transgenic rice grains were treated with freshly prepared 2% potassium ferrocyanide and 2% hydrochloric acid mixture, for 20-30 min. The acid was used to release ferric ions from the tissue and potassium ferrocyanide reacts with ferric ions to produce a blue insoluble compound of ferric ferrocyanide. Transverse section of mature transgenic rice grains at the embryo region showed high amounts of iron deposition in embryo and endosperm as compared to non-transgenic rice which showed less amount of iron in embryo and aleurone layer (Sivaprakash *et al.*, 2006; Krishnan *et al.*, 2009).

Histochemical studies showed the pattern of distribution of lipids, protein, starch, iron, ascorbic acid and phenols in cotyledons of *Anacardium occidentale.* The dye movement study using a phloem specific fluorochrome 5(6)-carboxyfluorescein revealed that the vascular bundles of pseudocarp and placenta are important transport tissues and nutrient supply from pseudocarp to developing cotyledons occurred only through the placenta (Krishnan *et al.*, 2011).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study was carried out as a part of Ph.D. programme of the Department of Botany, Goa University during the period from July 2008 to June 2012. Details of the methodology used for the study is presented in this chapter.

3. 1. Study Area

Goa is a small state with an area of 3,702 sq. km. It covers a length of 105 km. and width of 60 km. It is located between 14° 49'-15° 52' N latitudes and 73°38'-74° 24' E longitudes. Mandovi and Zuari are the two major rivers of Goa. River Mandovi covers a distance of 61.6 km and river Zuari meanders through 34 km in Goa (Naithani *et al.*, 1997).

The climate in Goa is tropical with high percentage of atmospheric humidity. Three distinct seasons are exhibited in Goa. The south-west monsoon starts from early June and ends by middle of October. The winter is a short spell between November to January. The summer starts in late February and ends in May. The maximum temperature is 32°C and minimum is 24°C. The humid climate in Goa is mainly due to the Arabian Sea and the mountainous barriers of Sahyadri (Naithani *et al.*, 1997).

3. 2. Survey and Collection of Plant Materials

During this study, periodic field surveys were carried out in all parts of Goa for identification of the elite trees of *Garcinia indica*. Plant material such as leaves, shoot tips, nodal cuttings and fruits were collected from the identified elite trees for tissue culture studies.

3. 3. Sterilization of Glassware and Culture Vials

The glassware and culture vials were soaked in 1% sulfuric acid and detergent solution overnight and then washed with tap water and there after rinsed with distilled water. Glassware and culture vials were kept in a hot air oven at 60°C for 12 hrs.

3. 4. Sterilization of Inoculation Accessories

Instruments used during inoculation were sterilized by wet sterilization method. Inoculation accessories, such as forceps, surgical blades, surgical blade holders and cotton were wrapped in aluminum foil and subjected to wet sterilization in an autoclave at 121°C and 15 psi pressure for 20 min. After sterilization they were immediately transferred to laminar air-flow chamber.

3. 5. Culture Room

The culture room was maintained in sterile condition by fumigating the area once in a month. Fumigation was done by vaporizing formaldehyde solution and the vapors were allowed to remain in the culture room for 20-24 hrs. The formaldehyde vapors were absorbed by placing ammonia solution in a Petri plate for 10-12 hours or overnight or until formaldehyde smell disappeared.

3. 6. Laminar Air-flow System

The inoculation of explants was carried out in a laminar air-flow system. Prior to inoculation the inner surface of the laminar air-flow system was swabbed with dettol followed by alcohol (commercial spirit) using absorbent cotton or white tissue paper. Autoclaved surgical blades, blade holders, sterile forceps, cotton, glass vessels (conical flask, beaker, Petri plates) were brought in to the laminar air-flow system. Then the laminar air-flow chamber was exposed to germicidal UV (253.7 nm) light for about 15-20

min. Later, culture medium, explants etc were brought to the laminar air-flow chamber and then inoculation of explants was carried out.

3. 7. Media Stock Preparation

3. 7. 1. Murashige and Skoog (MS) Medium

MS medium (Murashige and Skoog, 1962) consisted of macronutrients, micronutrients, vitamins and iron source. The detailed composition of MS medium is provided in Table 1. All the required chemicals for the preparation of MS were weighed using digital electronic weighing balance. Iron stock solution of MS was prepared by weighing desired amount of sodium ethylenediaminetetra acetic acid (Na_2 EDTA) and ferrous sulphate ($FeSO_4$ $7H_2O$) and dissolved separately in warm double distilled water. After complete dissolving, the ferrous sulphate solution was poured into sodium ethylenediaminetetraacetic acid solution by gentle stirring and then the final volume was made. All stock solutions were kept in amber coloured bottles in the refrigerator. The details of MS stock solution are given in Table 2.

3. 7. 2. Woody Plant Medium (WPM)

WPM consisted of macronutrients, micronutrients, vitamins and iron source (Lloyd and McCown, 1981) (Table 3). All the required chemicals for the preparation of WPM were weighed using digital electronic balance. Iron stock solution of WPM was prepared as mentioned above in MS medium. All stock solutions were kept in amber coloured bottles in the refrigerator. The details of WPM stock preparation are provided in Table 4.

3. 8. Preparation of MS Medium

To prepare one litre of MS medium, 400 ml of warm double distilled water was taken in 2 litre beaker and 100 ml of stock solution of A, followed by 10 ml of stock

Table 1. The Composition of MS Medium (Murashige and Skoog, 1962).

Nutrients	Constituents	(mg/L)
Major salts	Ammonium nitrate (NH_4NO_3)	1650
	Potassium nitrate (KNO_3)	1900
	Calcium chloride ($CaCl_2.2H_2O$)	440
	Magnesium sulfate ($MgSO_4.7H_2O$)	370
	Potassium dihydrogen orthophosphate ($KH_2 PO_4$)	170
Minor salts	Boric acid ($H_3 BO_3$)	6.2
	Manganese sulfate ($MnSO_4.4H_2O$)	22.3
	Zinc sulfate ($ZnSO_4.7H_2O$)	8.6
	Potassium iodide (KI)	0.83
	Sodium molybdate ($Na_2MoO_4.\ 2H_2O$)	0.25
	Cobalt chloride ($CoCl_2.6H_2O$)	0.025
	Copper sulfate ($CuSO_4.5H_2O$)	0.025
Iron sources	Ferrous sulfate ($FeSO_4.7H_2O$)	27.84
	Sodium ethylenediaminetetraacetic acid (Na_2 EDTA.$2H_2O$)	37.24
Organic supplements	Thiamine HCl	0.10
	Nicotinic acid	0.50
	Pyridoxine HCl	0.50
	Glycine	2.00
	Myo-inosital	100

Table 2. Stock Solutions of MS Medium.

Stocks	Constituents	Amount (g/L)	Strength of Stocks	Stocks to be taken for 1 litre medium
A	NH_4NO_3	16.5	10X	100 ml
	KNO_3	19.0		
B	$MgSO_4.7H_2O$	37.0	100X	10 ml
	$MnSO_4.4H_2O$	2.23		
	$ZnSO_4.7H_2O$	0.86		
	$CuSO_4.5H_2O$	0.0025		
C	$CaCl_2.2H_2O$	44.0	100X	10 ml
	KI	0.083		
	$CoCl_2.6H_2O$	0.0025		
D	$KH_2 PO_4$	17.0	100X	10 ml
	$H_3 BO_3$	0.62		
	$Na_2MoO_4.2H_2O$	0.0025		
E	$FeSO_4.7H_2O$	2.784	100X	10 ml
	Na_2 EDTA. $2H_2O$	3.724		
F	Thiamine HCl	0.010	100X	10 ml
	Nicotinic acid	0.050		
	Pyridoxine HCl	0.050		
	Glycine	0.200		
	Myo-inosital	10.0		

solution B, C, D, E and F was added (Table 2). Then 30 grams of sucrose (carbon source) was added and dissolved by stirring using a glass rod. Media volume was made to 900 ml using distilled water. The pH of the medium was adjusted to 5.7 ± 0.2 using 0.1N NaOH or 0.1 N HCl after adding required amount of plant growth regulators (auxins or cytokinins) alone or in combinations. After addition of agar (8 grams), the volume was made to one litre with double distilled water. In order to dissolve agar, the media was boiled, allowed cool to 60°C, dispensed into culture vessels and plugged with non-absorbent cotton wrapped in single layer of muslin cloth. The culture vessels containing medium was autoclaved at 121°C and 15 psi pressure for 20 min. 15-20 ml of media was then dispensed into sterile culture tubes (150×25 mm). All culture tubes and vessels were obtained from Borosil Glass Works Pvt. Ltd., Mumbai, India. Basal medium without any plant growth regulators was used as control. IAA was filter-sterilized using Millex syringe driven filter unit (0.22 μm, 33 mm) obtained from Millipore Corporation, Bedford, U.S.A. IAA was added just before solidification of the medium.

3. 9. Preparation of Woody Plant Medium (WPM)

To prepare one litre of MS medium, 200 ml of warm double distilled water was taken in 2 litre beaker and to that was added 100 ml of stock solution of A, B, C, D and E, followed by 10 ml of stock solution F, G and H, by continuous stirring with glass rod (Table 4). Then, 30 grams of sucrose (carbon source) was added and dissolved by stirring using glass rod. Media volume was made to 900 ml using distilled water. The pH of medium was adjusted to 5.7 ± 0.2 using 0.1N NaOH or 0.1 N HCl, after adding required amount of plant growth regulators (auxins or cytokinins) alone or in combinations. After addition of agar (8 grams), the volume was made to one litre with double distilled water. In order to dissolve agar, the media was boiled, allowed cool to 60°C, dispensed into culture vessels and plugged with non-absorbent cotton wrapped in single layer of muslin

Table 3. The Composition of Woody Plant Medium (Lloyd and McCown, 1981).

Nutrients	Constituents	(mg/L)
Macronutrient	Ammonium nitrate (NH_4NO_3)	400
	Calcium nitrate ($Ca(NO_3)_2.4H_2O$)	556
	Calcium chloride ($CaCl_2.2H_2O$)	96
	Potassium dihydrogen orthophosphate ($KH_2 PO_4$)	170
	Magnesium sulfate ($MgSO_4.7H_2O$)	370
Micronutrient	Boric acid (H_3BO_3)	62
	Manganese sulfate ($MnSO_4.4H_2O$)	22.3
	Zinc sulfate ($ZnSO_4.4H_2O$)	86
	Copper sulfate ($CuSO_4.5H_2O$)	0.25
	Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25
Iron sources	Ferrous sulfate ($FeSO_4.7H_2O$)	27.84
	Sodium ethylene diamine tetra acetic acid (Na_2 EDTA. $2H_2O$)	37.24
Organic supplements	Thiamine HCl	1.00
	Nicotinic acid	0.50
	Pyridoxine Hcl	0.50
	Glycine	2.00
	Myo- inositol	100

Table 4. Stock solutions of Woody Plant Medium (WPM).

Stocks	Constituents	Amount (g/L)	Strength of Stocks	Stocks to be taken for 1 litre medium
A	NH_4NO_3	40.0	10X (Macronutrient)	100 ml
B	$Ca(NO_3)_2.4H_2O$	55.6	10X (Macronutrient)	100 ml
C	$CaCl_2.2H_2O$	9.6	10X (Macronutrient)	100 ml
D	$KH_2 PO_4$	17.0	10X (Macronutrient)	100 ml
E	$MgSO_4.7H_2O$	37.0	10X (Macronutrient)	100 ml
F	H_3BO_3	0.620	100X Micronutrient	10 ml
	$MnSO_4.H_2O$	2.230		
	$ZnSO_4.4H_2O$	0.860		
	$CuSO_4$ $5H_2O$	0.025		
	$Na_2MoO_4.2H_2O$	0.025		
G	$FeSO_4.7H_2O$	2.780	100X (Iron source)	10 ml
	$Na_2EDTA.2H_2O$	3.740		
H	Thiamine HCl	0.10	100X Organic supplements	10 ml
	Nicotinic acid	0.05		
	Pyridoxine HCl	0.05		
	Glycine	0.20		
	Myo- inositol	10.0		

cloth. The culture vessels containing the medium was autoclaved at 121°C and 15 psi pressure for 20 min. 15-20 ml of media was then poured into culture tubes (150×25 mm). Basal medium without any plant growth regulators was used as control. IAA was filter-sterilized as mentioned above and added just before solidification of the medium.

3. 10. Preparation of Stock Solution of Plant Growth Regulators (PGRs)

All plant growth regulators, cytokinins such as 6-benzylaminopurine (BAP), kinetin (KIN), and auxins such as indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) were prepared as mg/ml stock and the details of same is provided in Table 5. Plant growth regulators were obtained from Sigma-Aldrich, St. Louis, USA. 100 mg of the plant growth regulator was weighed and dissolved in 1-2 ml of 1 M NaOH and then the volume was made to 100 ml using sterile double distilled water. Stocks of plant growth regulators were stored in amber coloured bottles in the refrigerator for further use.

3. 11. Explants

3. 11. 1. Source of Explants

Various types of explants such as leaves, shoot tips, nodal and inter nodal segments, flower buds, flowers, various developmental stages of fruits and seeds, including completely ripened fruits were used for tissue culture. Leaf, shoot tips, nodal and inter nodal cuttings were collected throughout the year from trees with high yielding and early ripening genotypes located at Marcel, Priol and Ponda. Flower buds, flowers, various developmental stages of fruits and seeds including ripened fruits were collected from the above mentioned trees during the months of November to May (flowering and fruiting season), for tissue culture studies.

Table 5. Plant growth regulators (PGRs) used for *in vitro* regeneration and mass multiplication.

Plant growth regulators	Molecular weight	Solubility	Stock (mg/100ml)
6-Benzylaminopurine (BAP)	225.25	1M NaOH	100
Kinetin (KIN)	215.22	1M NaOH	100
Indole-3-acetic acid (IAA)	175.19	1M NaOH	100
Naphthalene acetic acid (NAA)	186.21	1M NaOH	100
Indole-3-butyric acid (IBA)	203.24	1M NaOH	100
2,4-dichlorophenoxyacetic acid (2,4-D)	221.04	1M NaOH	100

3. 11. 2. Isolation and Surface Sterilization of Explants

Leaf, shoot tips, nodal cuttings and fruits were washed thoroughly with clean tap water. In case of fruits, they were dissected out to separate the seeds. Later, the seed coat was removed from the seeds. Further steps were carried out under sterile condition in a laminar air-flow chamber. The leaves, shoot tips and nodal segments were washed with sterile distilled water, followed by rinsing with 70% ethanol (v/v) for 1-2 min and then with sterile distilled water 3-4 times. Later, they were surface sterilized using 0.1% (w/v) aqueous mercuric chloride for 1 min followed by rinsing with sterile distilled water 3-4 times. The leaves and nodal segment were cut into small pieces and used as explants. Each surface sterilized seed was cut into three pieces and used as explant.

3. 12. Inoculation

Prior to inoculation, pre-autoclaved forceps, surgical blades, surgical blade holders, cotton, Petri dishes, culture vials and culture vials with medium were kept inside the laminar air-flow chamber in the presence of UV light for about 15-20 minutes. The surface sterilized explants were carefully transferred to the culture medium. Prior to inoculation, forceps and surgical blade holders were dipped in 95% ethyl alcohol,exposed to the heat of the flame, cooled and used. After use, again the tools were dipped in ethyl alcohol and re-flamed to be reused.

3. 13. Culture Conditions

The cultures were incubated at 25 ± 2°C, a relative humidity of 50-60% and 16 hours photoperiod (50 μ mol m^{-2} s^{-1}; cool white fluorescent tubes). The results were recorded on the basis of visual and microscopic observations.

3. 14. Shoot Bud Induction Medium

WPM supplemented with various plant growth regulators at different concentrations alone or in combination, were used for shoot bud induction (Table 6, 7). Total of six treatments, which includes (i) 6-benzylaminopurine (BAP); (ii) kinetin (KIN); (iii) 2,4-dichlorophenoxy acetic acid (2,4-D) alone at various concentrations, and combinations of (iv) 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA); (v) 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA); (vi) 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA), were used. Seed segments were inoculated on the shoot induction medium (15-20 ml), made slanted in rimless culture tubes of size 150×25 mm (Borosil Glass Works Limited, Mumbai, India).

3. 15. Shoot Elongation Medium

WPM supplemented with 1 mg/L BAP were used for shoot proliferation and elongation. The cultures regenerated on shoot induction medium were sub-cultured on 100-125 ml of WPM medium containing 1 mg/L BAP, prepared in 250 ml wide mouth conical flasks (Borosil Glass Works Limited, Mumbai, India) and 250 ml phyta jar containers (Hi-media, Mumbai, India). All sub-cultures were carried out at the intervals of about 20-25 days.

3. 16. Root Induction

The elongated shoots of 3-5 cm height containing 3-4 internodes and 6-8 leaves were carefully cut at the basal region and transferred to half-strength WPM supplemented with IBA or NAA at concentrations of 0.5-5 mg/ml with 1.5% sucrose. The media was solidified with 0.75% agar (Table 8, 9). After one month of culture, the percentage of root induction, number of roots per shoot and length of roots were recorded.

Table 6. Various concentration of plant growth regulators used for regeneration.

Treatment (i)	Treatment (ii)	Treatment (iii)	Parameters studied
BAP mg/L	KIN mg/L	2,4-D mg/L	
0.5	0.5	0.5	
1.0	1.0	1.0	(i) Number of days taken for shoot bud induction
1.5	1.5	1.5	(ii) Number of days taken for callus formation
2.0	2.0	2.0	(iii) Percentage of callus induction
2.5	2.5	2.5	(iv) Average number of shoots
3.0	3.0	3.0	(v) Average length of shoots
3.5	3.5	3.5	
4.0	4.0	4.0	
4.5	4.5	4.5	
5.0	5.0	5.0	

Table 7. Various combination of plant growth regulators used for regeneration.

Treatments	BAP mg/L	IAA mg/L	NAA mg/L	IBA mg/L	Parameters studied
(iv)	0.5	1.0	-	-	(i) Number of days taken for shoot bud induction (ii) Percentage of culture regeneration (iii) Percentage of callus induction (v) Average number of shoots (vi) Average length of shoots
	1.0	1.0	-	-	
	1.5	1.0	-	-	
	2.0	1.0	-	-	
	2.5	1.0	-	-	
	3.0	1.0	-	-	
	3.5	1.0	-	-	
	4.0	1.0	-	-	
	4.5	1.0	-	-	
(v)	0.5	-	1.0	-	
	1.0	-	1.0	-	
	1.5	-	1.0	-	
	2.0	-	1.0	-	
	2.5	-	1.0	-	
	3.0	-	1.0	-	
	3.5	-	1.0	-	
	4.0	-	1.0	-	
	4.5	-	1.0	-	
(vi)	0.5	-	-	1.0	
	1.0	-	-	1.0	
	1.5	-	-	1.0	
	2.0	-	-	1.0	
	2.5	-	-	1.0	
	3.0	-	-	1.0	
	3.5	-	-	1.0	
	4.0	-	-	1.0	
	4.5	-	-	1.0	

Table 8. Effect of IBA on rooting of regenerated shoots of *Garcinia indica*.

Medium	Plant growth regulators (mg/L)	Parameters studied
½ WPM	0.5	(i) Number of days taken for root induction (ii) Percentage of rooting (iii) Average number of roots (iv) Average length of roots
½ WPM	1.0	
½ WPM	2.0	
½ WPM	3.0	
½ WPM	4.0	
½ WPM	5.0	

Table 9. Effect of NAA on rooting of regenerated shoots of *Garcinia indica*.

Medium	Plant growth regulators (mg/L)	Parameters studied
½ WPM	0.5	(i) Number of days taken for root induction (ii) Percentage of rooting (iii) Average number of roots (iv) Average length of roots
½ WPM	1.0	
½ WPM	2.0	
½ WPM	3.0	
½ WPM	4.0	
½ WPM	5.0	

3. 17. Root Hardening

Well rooted plantlets were carefully washed with sterile distilled water to remove the medium adhered to the roots. Later the plantlets were transferred to culture tubes containing 15-20 ml quarter-strength liquid WPM without sucrose, for 10-12 days.

3. 18. Data Collection and Statistical Analysis

The cultures were observed at regular intervals of every 4 days under Stereo-zoom light microscope to understand the morphogenetic changes. The experiments were made in a complete randomized design (CRD). Ten replications were maintained for each treatment and each experiment was repeated three times. The effect of plant growth regulators were evaluated on the basis of percentage of cultures showing shoot bud formation and/ or morphogenetic responses. After 42 days inoculation, the percentage of regeneration, number of shoots and length of shoots buds were recorded for each treatment. During rooting, the percentage of root induction, number of roots per shoot and length of roots were recorded after one month of culture. All the data were subjected to statistical analysis using WASP-2.0 (Web Based Agricultural Statistics Software Package, Developed by ICAR (http://icargoa.res.in/wasp2.0/index.php), Goa, India) and processed data was presented as mean ± SD along with level of significance. Macro-photographs of cultures were taken using *Nikon Coolpix4500* digital camera or *Nikon D90 SLR* digital camera attached with 60 mm macro lens (Nikon Corporation, Tokyo, Japan).

3. 19. Acclimatization of regenerated plantlets

For hardening, rooted plantlets were carefully washed in sterile distilled water to remove the traces of agar, and transferred to culture tubes containing quarter-strength liquid WPM without sucrose, for 10-12 days. The plantlets were then carefully planted to seed trays containing the mixture of different potting media combinations (Table 10). Seed

Table 10. Different potting mixture used for the acclimatization.

Sl. No	Composition of potting mixture
1	Cocopeat
2	Vermicompost
3	Garden soil
4	Cocopeat + Sand (1:1)
5	Vermicompost + Sand (1:1)
6	Garden soil+ Vermicompost (1:1)
7	Garden soil + Sand (1:1)
8	Cocopeat + Garden soil (1:1)
9	Vermicompost + Garden soil (1:1)
10	Cocopeat + Vermicompost + Garden soil (1:1:1)
11	Cocopeat + Vermicompost + Sand (1:1:1)

trays along with plantlets were kept under high relative humidity conditions in a growth chamber for two weeks. The plantlets were then transplanted into plastic pots (13×13cm) containing garden soil and sand (3:1), and transferred to greenhouse. After 6 months of growth, regenerated plants grown to the height of about 1 feet were transferred to large black plastic bags containing garden soil: manure: sand (2:1:1), for further plantation.

3. 20. Characterization of Regenerated Plants

3. 20. 1. Histological and Anatomical Characterization

Seed segments after 1-3 weeks of culture in woody plant medium (WPM) supplemented with plant growth regulators, were used for histological studies, to understand the shoot bud initiation and regeneration. Seed segment explants after one week of culture were fixed on to the specimen holder of the cryo-microtome (Leica CM1800, Leica Instruments, Nussloch, Germany). Specimen was placed in required direction and water was added drop by drop surrounding the specimen to support ice formation, so that the specimen is fixed properly on the specimen holder. The specimen holder along with the specimen was fixed at right direction and position in the cryo-microtome. The cryo-microtome temperature was set at -6°C and thin transverse sections of 8-10 μm were obtained, which were collected in a Petri plate containing water.

Leaf, stem and roots were collected from regenerated plants in poly-house condition and from corresponding mother plants (donor), and washed with clean tap water. Thin cryo-microtome sections were obtained using the above mentioned methods. Free-hand thin sections were also taken during this study.

The intact sections were selected and stained with safranin (1%) and toluidine blue (0.1%), for 2-3 min. One gram of safranin was dissolved in 100 ml of distilled water and 0.1 gram of toluidine blue was dissolved in 100 ml of distilled water as described by Krishnan and Dayanandan (2003). An excess stain was removed by placing the sections in water for a minute. After washing, the sections were transferred to a clean glass slide, mounted in a drop of dilute glycerin (5%) and covered with a cover glass without trapping any air bubbles. Prepared specimen slides were examined under compound research microscope to understand the shoot bud induction and general anatomical structural and developmental variation, if any, in the *in vitro* regenerated plantlets in comparison with mother plants. The observation and photographs were obtained using *Nikon E800* Research Microscope attached with Nikon Cool Digital Camera and Nikon Image Analyzing System (NIS-Elements basic research version 3.00) or using *Nikon Coolpix4500* Camera fitted using C-mount adapter, directly to the microscope (Nikon Corporation, Tokyo, Japan).

3. 20. 2. Molecular characterization

3. 20. 2. 1. Genomic DNA Extraction and Purification

Genomic DNA was extracted from frozen leaf material using the method described by Ramage *et al.* (2004) with some modifications. During this study, instead of 2% PVP, 4% PVP (Polyvinylpyrrolidone) was used while the centrifugation speed was altered to 12,000 rpm instead of 8000 rpm. Activated charcoal was not used during the extraction of DNA. Five grams of leaf tissue were taken from *in vitro* regenerated plantlets or their corresponding mother plants, cut into small pieces and ground to a fine powder using liquid nitrogen. Before thawing, the fine powder was transferred to 50 ml centrifuge tubes and 25 ml of pre-warmed (65°C) extraction buffer was added [Extraction buffer contained

2% (w/v) CTAB, 4% (w/v) PVP, 2% (v/v) β-mercaptoethanol, 2M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0)]. The content of the centrifuge tubes were mixed by inverting the tubes three to four times. The tubes were incubated at 60°C for 30 minutes. After 30 minutes, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inverting the tube three to four times and centrifuged at 12,000 rpm for 20 minutes, at 4°C. Supernatant was carefully transferred to a new centrifuge tube and 0.6 volume of ice cold isopropanol was added. The tubes were incubated at -20°C for 2 hours. The DNA pellet was obtained by centrifugation at 12,000 rpm for 20 minutes at 4°C. After centrifugation, the supernatant was discarded and the pellet containing DNA was dissolved in 700 µl of TE buffer (10 mM Tris-HCl and 1mM EDTA, pH 8.0).

The genomic DNA was purified using equal volume of phenol: chloroform (1:1). 300 µl each of phenol and chloroform was added to the tube and then centrifuged at 8000 rpm for 10 minutes at room temperature (RT). Then the transparent upper layer was transferred to a new Eppendorf tube and equal volume of chloroform was added. Mixed gently by inverting the tube three to four times and centrifuged at 8000 rpm for 10 minutes at RT (repeated this step for two times). The transparent upper layer was transferred to a new Eppendorf tube and 1/10th volume (40µl) of 3M sodium acetate and three volumes (1 ml) of ethanol (100%) were added and then incubated at -20°C for overnight followed by centrifuged at 8000 rpm for 20 minutes at RT. Discarded the supernatant and 1 ml of 70% ethanol was added to the pellet, gently vortex and centrifuged at 8000 rpm for 10 minutes at RT. The DNA pellet was air dried or incubated at 37°C for 15 min till no water droplets present in the pellet. Then the DNA pellet was then dissolved in 200 µl of TE buffer and stored at -20°C for further use.

3. 20. 2. 2. Determination of Purity of DNA by Spectrophotometer

The purity of the DNA was determined by using spectrophotometer method. The absorbance ratio of A260 nm and A280 nm provides an estimate of the purity of DNA. 15 µl of DNA sample was taken in a cuvette and 735 µl of TE buffer was added and gently mixed well. Absorbance reading was taken at 260 nm and 280 nm by using spectrophotometer (UV-2450, Shimadzu Corporation, Kyoto, Japan). TE buffer was used as a blank. The purity of the DNA was calculated by taking the absorbance ratio of 260 nm and 280 nm. A ratio of 1.8-2.0 indicates that the DNA is pure without any contamination. A ratio below 1.8 denotes that the DNA is contaminated with protein and or other UV absorbers in the sample. A ratio higher than 2.0 indicates that the sample may be contaminated with chloroform or phenol (CIMMYT, 2005).

3. 20. 2. 3. Determination of Quality of DNA by Agarose Gel Electrophoresis

The quality of DNA was determined by using agarose gel containing ethidium bromide. 0.8 g of agarose was added to 100 ml of 1X TBE buffer and gently melted. On slight cooling 3 µl of ethidium bromide (stock 10 mg/ml) was added to the agarose solution, mixed well, poured in to gel-forming tray with proper comb setting and kept for 20-30 minutes for solidification of the gel. Agarose gel was carefully taken in to gel-running tray with 1X TBE buffer and the comb was slowly removed. 1 µl of 6X gel loading dye was taken in 0.5 ml PCR tube and placed in ice box, 10 µl of DNA sample was added and mixed well. The DNA samples were carefully loaded into the wells and run at 50 volt for 5 minutes. The voltage was increased to 100 volt. When the DNA samples reached three-fourth the length of the gel, the electrophoretic unit was put off. The gel was then visualized and photographed using the gel documentation system (Alpha DigiDoc™, Alpha Innotech Corporation, California, USA).

3. 20. 2. 4. Quantification of DNA

DNA yield was determined by the method described by CIMMYT (2005). DNA concentration was measured by taking absorbance at 260 nm using spectrophotometer (UV-2450, Shimadzu Corporation, Kyoto, Japan). An A260 value of 1 corresponds to 50 μg DNA per ml. During the analysis, 15 μl of DNA sample was taken in a cuvette and 735 μl of TE buffer was added and gently mixed well. Absorption value was taken at 260 nm in spectrophotometer by using TE buffer as a blank. Calculations were made as follows:

Volume of DNA sample = 15 μl

Dilution = 15 μl of DNA sample + 735 μl of TE

$$\text{Concentration of DNA sample } (\mu g/\mu l) = \frac{A_{260} \times 50\ \mu g/ml \times \text{dilution } (50)}{1000}$$

3. 20. 2. 5. Random Amplified Polymorphic DNA (RAPD) Analysis

3. 20. 2. 5. 1. RAPD Primers

RAPD analysis of genomic DNA of regenerated plants and their corresponding mother plants (donor) was carried out using universal random oligonulceotide primers of 10 base pairs obtained from Metabion International AG, Martinsried, Germany and the list of primers are summarized in Table 11.

3. 20. 2. 5. 2. Amplification Reaction

Polymerase chain reaction (PCR) amplification was carried out in final volume of 25 μl, containing about 30-50 ng of genomic DNA, 2.5 μl of 10X Taq assay buffer, 3 μl of

25 mM $MgCl_2$, 2.5 µl of 10 mM dNTPs mix, 15 ng of primers and 0.5 units of Taq polymerase (Merck Specialities Pvt. Ltd., Bangalore, India).

3. 20. 2. 5. 3. Amplification Condition and Scoring of Bands

The PCR amplification was performed as described by Parab and Krishnan. (2008), in a Mastercycler gradient (Eppendorf AG, Hamburg, Germany). Initial denaturation step of 3 min at 94°C, followed by 40 cycles of 94°C for 1 min (denaturation) and 72°C for 2 min (extension) and 10 min for final extension step at 72°C. The amplified products were analysed on 1.4% agarose gels in 1X TBE buffer. The gel was stained with ethidium bromide, visualized under UV and photographed using Gel Documentation System (AlphaDigiDoc™, Alpha Innotech Corporation, California, USA). The number of bands present in the regenerated plants and corresponding mother plants were visually scored.

3. 20. 2. 6. Inter Simple Sequence Repeat (ISSR) analysis

3. 20. 2. 6. 1. ISSIR Primers

ISSR analysis of genomic DNA of regenerated plants and their corresponding mother plants (donor) was carried out using universal random oligonulceotide primers obtained from Metabion International AG, Martinsried, Germany and the list of primers are summarized in Table 12.

3. 20. 2. 6. 2. Amplification Reaction

Polymerase chain reaction (PCR) amplification was carried out in final volume of 25 µl, containing about 30-50ng of genomic DNA, 2.5 µl of 10X Taq assay buffer, 3 µl of 25 mM $MgCl_2$, 2.5 µl of 10 mM dNTPs mix, 15 ng of primers and 0.5 units of Taq

Table 11. List of RAPD random primers used the analysis of *in vitro* regenerated plants and mother plants.

Sl. No	Primer name	5'-3' Sequence	Annealing temperature
1	OPA 1	CAGGCCCTTC	32°C
2	OPA 2	TGCCGAGCTC	32°C
3	OPA 9	GGGTAACGCC	32°C
4	OPC 2	GTGAGGCGTC	32°C
5	OPD 16	AGGGCGTAAG	27.9°C
6	OPD 20	ACCCGGTCAC	32°C
7	OPF 10	GGAAGCTTGG	27.9°C
8	OPF 13	GGCTGCAGAA	27.9°C
9	OPF 16	GGAGTACTGG	27.9°C
10	OPF 20	GGTCTAGAGG	27.9°C
11	SB 12	AGTCAGCCAC	27.9°C
12	SB 19	CAGCACCCAC	32°C
13	OPH 12	ACGCGCATGT	27.9°C
14	OPH 13	GACGCCACAC	32°C
15	OPH 18	GAATCGGCCA	27.9°C
16	AB 16	CCCGGATGGT	32°C
17	AE 11	AAGACCGGGA	27.9°C
18	AO 12	TCCCGGTCTC	32°C
19	AP 20	CCCGGATACA	27.9°C
20	AV 03	TTTCGGGGAG	27.9°C

Table 12. List of ISSR random primers used for the analysis of *in vitro* regenerated plants and mother plants.

Sl. No	Primer name	5'-3' Sequence	Annealing temperature
1	IB 1	AGAGAGAGAGAGAGAGT	49.4°C
2	IB 2	GAGAGAGAGAGAGAGAT	49.4°C
3	IB 3	TCTCTCTCTCTCTCTCC	51.8°C
4	IB 4	ACACACACACACACACC	51.8°C
5	IB 5	TGTGTGTGTGTGTGTGA	49.4°C
6	ISSR-ST2	GAGAGAGAGAGAGAGAYG	51.8°C
7	ISSR-810	GAGAGAGAGAGAGAGAT	49.4°C
8	ISSR-808	AGAGAGAGAGAGAGAGC	51.8°C
9	ISSR-834	AGAGAGAGAGAGAGAGYT	51.8°C
10	ISSR-812	GAGAGAGAGAGAGAGAA	51.8°C

polymerase. All PCR chemicals were obtained from Merck Specialities Pvt. Ltd., Bangalore, India.

3. 20. 2. 6. 3. Amplification Condition and Scoring of Bands

The PCR amplification was performed as described by Parab *et al.* (2008) in a Mastercycler gradient (Eppendorf AG, Hamburg, Germany). Initial denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 1 min (denaturation) and 72°C for 2 min (extension) and 10 min for final extension step at 72°C. The amplified products were analysed on 1.4% agarose gels in 1X TBE buffer. The gel was stained with ethidium bromide, visualized under UV and photographed using Gel Documentation System (AlphaDigiDoc™, Alpha Innotech Corporation, California, USA). The number of bands present in the regenerated plants and corresponding mother plants were visually scored.

3. 21. Biochemical Study

3. 21. 1. Plant Materials

For the extraction of hydroxycitric acid (HCA) and garcinol, the different stages of development of fruits of *Garcinia indica* were collected from high yielding trees grown in various locations of Goa. Collected fruits were separated up to 7 different developmental stages depending on the size of the fruits (Table 13). The stalk and sepals were removed from the fruits and the fruits were thoroughly washed with tap water followed by a rinse with distilled water.

3. 21. 2. Extraction

100 g of fruits were cut open to remove seeds. After removing the seeds, the pulp along with rind were cut into small pieces and ground with the help of mortar and pastel.

Table 13. Different developmental stages of fruits of *Garcinia indica* used for hydroxycitric acid (HCA) analysis.

Trees and Locations	Samples
Tree No. 1 (Marcel)	Stage 1 (20 days after fertilization)
	Stage 2 (35 days after fertilization)
	Stage 3 (50 days after fertilization)
	Stage 4 (60 days after fertilization)
	Stage 5 (75 days after fertilization)
	Stage 6 (80 days after fertilization)
	Stage 7 (85 days after fertilization)
Tree No. 2 (Priol)	Stage 1 (20 days after fertilization)
	Stage 2 (35 days after fertilization)
	Stage 3 (50 days after fertilization)
	Stage 4 (60 days after fertilization)
	Stage 5 (75 days after fertilization)
	Stage 6 (80 days after fertilization)
	Stage 7 (85 days after fertilization)
Tree No. 3 (Ponda)	Stage 1 (20 days after fertilization)
	Stage 2 (35 days after fertilization)
	Stage 3 (50 days after fertilization)
	Stage 4 (65 days after fertilization)
	Stage 5 (75 days after fertilization)
	Stage 6 (80 days after fertilization)

They were then taken in pre-washed and dried thin cotton bag and the bag with its contents was put inside the extractor (1 litre) of Soxhlet apparatus (Vensil Glass, Bangalore, India). 1.3 litre of methanol was taken in a boiling flask (2 litre) of Soxhlet apparatus. Slowly methanol was boiled to get vapor for the extraction. After 8 hours of complete hot methanol extraction, the Soxhlet apparatus was put off and waited for 20-30 minutes for the temperature of the extract to come down. The extract was carefully removed from the Soxhlet apparatus in a glass beaker.

3. 21. 3. Filtration and Concentration of Extract

Extract was carefully filtered through laboratory filter paper to remove pectic substances and the filtrate was kept overnight at room temperature to allow the pectinaceous material present in the filtrate to settle down. The filtrate was again filtered through laboratory filter paper and further filtered through Whatman No.1 filter paper. Finally the filtrate was collected in a glass beaker and reduced to a volume of up to 15 ml by using a vacuum rotary evaporator at 20-25°C. The reduced extract was taken in a small (100 ml) beaker. The extract was kept in hot air oven at 40°C overnight to get solid extract. The solid extract was kept in refrigerator for further analysis.

3. 21. 4. Thin Layer Chromatography (TLC) for Confirmation of HCA

20×20 cm TLC aluminium sheets (Merck KGaA, Darmstadt, Germany) were used for confirmation of HCA from the samples. Samples were dissolved in methanol and few drops of sample was loaded at a respective loading point with the help of a fine capillary tube and kept for 5 minutes. Butanol: Formic acid: Water in the ratio 4:1:5 were used as solvent system for running of TLC. 1% Sodium metavanadate (Hi-Media Laboratories, Mumbai, India) was used as a spray for detection of HCA (Jena *et al.*, 2002).

3. 21. 5. Quantitative Estimation of Hydroxycitric Acid (HCA) by HPLC

3. 21. 5. 1. HPLC System

The high performance liquid chromatographic system (HPLC) SpectraSYSTEM AS3000 Auto-sampler (Thermo Fisher Scientific, California, USA) with UV1000 detectors was used for the analysis. HPLC analysis was carried out at Neucon Pharma Pvt. Limited, Verna, Goa. An assay was performed on C_{18} Reversed phase ODS column (150×4.6 mm I.D, MZ- Analysentechnik GmbH, Mainz, Germany). All solvents used for analysis were of HPLC grade and obtained from Nice Chemicals (P) Ltd., Kerala, India.

3. 21. 5. 2. Preparation of HCA Standard and Calibration

Hydroxycitric acid calcium salt standard was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. 100 mg of Hydroxycitric acid calcium salt was dissolved in 50 ml of perchloric acid: water mixture (HPLC grade; pH adjusted 2.2 with water) and filtered through 0.45 μm membrane filter. After filtering, 10 μl of standard HCA was taken into vials of auto-sampler of the HPLC instrument. The mobile phase was perchloric acid: water mixture (HPLC grade; pH adjusted 2.2 with water), filtered through 0.45 μm membrane filter and flow rate was 0.8 ml/min. Detection was done at wavelength of 210 nm. The above analysis was repeated three times to get an average peak area of standard HCA.

3. 21. 5. 3. Preparation of Samples for HPLC Analysis

100 mg of solid samples at each stage of development of fruit was dissolved in 100 ml of perchloric acid: water mixture (HPLC grade; pH adjusted 2.2 with water). 10 μl of this sample was taken into vials of auto-sampler of the HPLC instrument after filtering through 0.45 μm membrane filter. The mobile phase was perchloric acid: water mixture

(HPLC grade; pH adjusted 2.2 with water), filtered through 0.45 μm membrane filter and flow rate was 0.8 ml/min. The respective peak area was obtained at wavelength of 210 nm.

3. 21. 6. Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis of Hydroxycitric Acid (HCA)

3. 21. 6. 1. LC-MS System

The LC-MS analysis of HCA was carried out using Agilent 6530 accurate Mass Quadrupole time-of–flight (Q-toff) (Agilent technologies, CA, USA) with UV-Visible detectors. The LC-MS analysis was done at Neucon Pharma Pvt., Ltd., Verna, Goa. An assay was performed using Nucleosil C_{18} column (3μm×2.15mm ×4.6mm, MZ-Macherey-Nagel GmbH & Co. KG, Duren, Germany).

3. 21. 6. 2. Sample Preparation and LC-MS Condition

50 mg of solid sample was dissolved in 100 ml of 0.1% perchloric acid in water. 5 μl of sample was loaded in LC-MS system after filtering through 0.45 μm membrane filter. The mobile phase A consisted of 0.1% perchloric acid in water while mobile phase B consisted of methanol and flow rate was maintained at 0.8 ml/min and detection was done at the wavelength of 210 nm.

3. 21. 7. Quantitative Estimation of Garcinol by HPLC

3. 21. 7. 1. Extraction of Garcinol from Fruit Samples

100 g of fruits were weighed, cut open, removed the seeds and pulp were cut into small pieces and chopped well with the help of mortar and pastel. Then they were taken in pre-washed and dried thin cotton bag. The sample with cotton bags was put inside the extractor (1 litre) of Soxhlet apparatus (Vensil Glass, Bangalore, India). The 1.3 litre of

methanol was taken in boiling flask (2 litre) of Soxhlet apparatus. Slowly methanol was boiled to get vapor for the extraction. After 8 hours complete hot methanol extraction, the Soxhlet apparatus was put off and waited for 20-30 minutes to temperature of extract to come down. The extract was carefully removed from the Soxhlet apparatus in a glass beaker.

3. 21. 7. 2. Concentration of Extracts

Each extract was carefully filtered through laboratory filter paper to remove the pectin substances and the filtrate was kept for overnight at room temperature allowed to settling down the pectinaceous material present in the filtrate. The filtrate was again through laboratory filter paper and further filtered through Whatman No.1 filter paper, finally the filtrate was collected in a glass beaker. The filtrate was concentrated and dried by using a vacuum rotary evaporator at 40-50°C. The solid residue was dissolved in 50 ml of hexane in a 100 ml beaker and kept at 4°C for 2 days in refrigerator. The yellow amorphous solid was settled down of the beaker and hexane was removed. The yellow amorphous solid was dissolved in 20 ml of cold hexane and kept at RT for 24 hrs. Pale yellow crystal was settled down in beaker was carefully removed and taken in vials, kept at -20°C for further analysis.

3. 21. 7. 3. HPLC System

The high performance liquid chromatographic system (HPLC) SpectraSYSTEM AS3000 Autosampler (Thermo Fisher Scientific, California, USA) with UV1000 detectors was used for the analysis. HPLC analysis was carried out at Neucon Pharma Pvt., Limited, Verna, Goa. An assay was performed on C_{18} Reversed phase ODS column (150×4.6 mm I.D, MZ- Analysentechnik GmbH, Mainz, Germany).

3. 21. 7. 4. Preparation of Garcinol Standard and Calibration

Garcinol standard was obtained from Biomol International, USA. 2 mg of garcinol was dissolved in 2 ml of methanol (HPLC grade) and filtered through 0.45 μm membrane filter. After filtering, 10 μl of standard garcinol was taken into vials of auto-sampler of the HPLC instrument. The mobile phase was methanol, filtered through 0.45 μm membrane filter and flow rate was at 1 ml/min. Detection was done at wavelength of 280 nm. This analysis was repeated three times to get average peak area of standard garcinol.

3. 21. 7. 5. Preparation of Samples for HPLC Analysis

100 mg of pale yellow crystal of each stage of development of fruit was dissolved in 50 ml of methanol. 10 μl was taken into vials of auto-sampler of the HPLC instrument after filtered through 0.45 μm membrane filter. The mobile phase was methanol, filtered through 0.45 μm membrane filter and flow rate was at 1 ml/min. Detection was done at wavelength of 280 nm.

3. 21. 8. Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis of Garcinol

3. 21. 8. 1. LC-MS System

The LC-MS analysis of garcinol was carried out using Agilent 6530 accurate Mass Quadrupole time-of–flight (Q-toff) (Agilent technologies, CA, USA) with UV-Visible detectors. The LC-MS analysis was done at Neucon Pharma Pvt., Ltd., Verna, Goa. An assay was performed using Nucleosil C_{18} column (3μm×2.15mm ×4.6mm, MZ-Macherey-Nagel GmbH & Co. KG, Duren, Germany).

3. 21. 8. 2. Sample Preparation and LC-MS condition

50 mg of solid samples was dissolved in 2 ml of hexane and 5 μl of sample was loaded in LC-MS after filtering through 0.45 μm membrane filter. The mobile phase A consisted of formic acid while mobile phase B consisted of methanol and flow rate was maintained at 1 ml/min. Running time was set as 3 min and detection was carried out at the wavelength of 280 nm.

3. 22. Developmental and Histochemical Studies

3. 22. 1. Developmental Studies

Different stages of development of the fruits (from anthesis to maturation) were collected from the Kokum tree. The collected fruits were separated and classified to 7-8 developmental stages depending on the size of the fruits (including flower stage). Free-hand and microtome thin transverse sections were obtained and placed in a Petri plate containing distilled water. Intact sections were selected and stained with safranin and toluidine blue, washed, mounted in dilute glycerin (5%) and observed under bright-field microscope (Krishnan and Dayanandan, 2003). Both safranin and toluidine blue stains were prepared, as mentioned above, for anatomical characterization of regenerated plants.

3. 22. 2. Histochemical Studies

Localization of major storage compounds and bio-active ingredients were done using various fluorescent, non-fluorescent dyes and biochemical reactions. Fluorescent and non-fluorescent dyes used during this study were obtained from Sigma-Aldrich, St. Louis, USA. Standard histochemical procedures were used to carry out the histochemical staining reactions (Pearse, 1972; Krishnamurthy, 1988; Pearse, 1988; Krishnan *et al.*, 2001; Krishnan and Dayanandan, 2003; Krishnan *et al*, 2009). Hydroxycitric acid (HCA)

was localized in the fruits by biochemical reaction using 2% sodium metavanadate (Jena *et al.*, 2002).

3. 22. 2. 1. Localization of Starch

Iodine Potassium Iodide (I_2KI): I_2KI was prepared by dissolving 2 gms of potassium iodide and 0.2 gms of iodine in 100 ml of water and stored in amber coloured bottle. Free-hand thin sections were placed in water, stained with I_2KI solution for 1 minute, mounted in dilute glycerin and observed under bright-field microscope. Starch appeared brownish black.

3. 22. 2. 2. Localization of Protein

8-anilino-1-naphthalenesulfonic acid (ANS): 100 mg of ANS was dissolved in 50 ml of distilled water and stored in amber coloured bottle. Free-hand thin sections were stained with ANS for 15 minutes. Sections were thoroughly washed with distilled water, mounted in dilute glycerin and observed under fluorescent microscope under UV excitation (330-380 nm). Protein fluoresced whitish blue.

3. 22. 2. 3. Localization of Lipids

Nile blue: One gram of Nile blue stain was dissolved in 100 ml of distilled water and stored in amber coloured bottle. Thin sections were stained with Nile blue for 30 seconds, washed thoroughly with water, mounted in dilute glycerin and observed under fluorescence microscope under blue excitation (450-490 nm). Lipids fluoresced yellow.

3. 22. 2. 4. Localization of Lignin

Phloroglucinol: One gram of phloroglucinol was dissolved in ethanol and stored in amber coloured bottle. Sections were placed in phloroglucinol for 2-3 minutes in small Petri dish and then add few drops of concentrated hydrochloric acid (HCl) added. Slide was gently heated using sprit lamp, sections were carefully transferred to clean slide, mounted with dilute glycerin and observed under bright-field microscope. Lignin appeared red.

3. 22. 2. 5. Localization of Tannins

Ferric Chloride: Two grams of ferric chloride was dissolved in 10% formalin and stored in amber coloured bottle. Free-hand thin sections were placed on a clean slide and few drops of ferric chloride solution were added. After one minute ferric chloride solution was drained and the section was mounted with dilute glycerin and observed under bright-field microscope. Tannins appeared brown to yellow.

3. 22. 2. 6. Localization of HCA

Sodium metavanadate: One gram of sodium metavanadate was dissolved in 100 ml of distilled water and stored in amber coloured bottle. Free-hand thin sections of the fruit was taken and placed on a clean glass slide, few drops of sodium metavanadate solution was added. After few minutes HCA appeared orange red in color.

3. 23. Observation and Photography

Both for developmental and histochemical studies, prepared slides were observed and photographed using bright-field and fluorescent microscopy, depending on the staining reactions. The observation and photographs were made using *Nikon E800* Research Microscope attached with Nikon Cool Digital Camera and Nikon Image Analyzing System (NIS-Elements basic research version 3.00) and also using *Nikon*

Coolpix4500 camera fitted using C-mount adopter directly fitted to the microscope. Macro-photographs were taken using *Nikon D90 SLR* digital camera attached with 60 mm macro lens. The stereo-zoom micro-photographs were taken using stereo-zoom microscope (*Leica EZ4D*) attached with inbuilt camera using Image Analyzing Software (Leica application suite version 1.5.0).

RESULTS

4. RESULTS

The results reported here are based on the investigation of micropropagation, biochemical and histochemical studies of *Garcinia indica* Choisy (Kokum). (i) During this study, protocol was developed and standardized for the induction of multiple shoots, elongation of shoots, initiation of roots and acclimatization of regenerated plants. Further, histological studies were carried out to understand the origin and development of multiple shoots from mature seed segments. As part of study, regenerated plants were characterized anatomically in comparison with the corresponding mother plant (donor plant). Molecular studies were also carried out to understand the genetic variation if any amongst the regenerated plants in comparison with corresponding mother plant (donor plant); (ii) Extraction and estimation of hydroxycitric acid (HCA) and garcinol was carried out during various stages of fruit development (young to mature stages) using HPLC and LC-MS systems; (iii) Histochemical and developmental studies were carried out from anthesis to maturation of fruit, to understand the structural and developmental changes of fruit and time-course for deposition and distribution of major storage compounds (starch, protein, lipids) and other chemical compounds (hydroxycitric acid and garcinol) during various developmental and mature stages of fruit.

During this study, regular field trips were made to different locations in Goa for the identification of *Garcinia indica* genotypes having characteristics such as high yield, large fruit size and early ripening. Kokum trees with elite characteristics were selected from Marcel village, for the regeneration, mass multiplication and production of elite clones. The tree at Marcel is about 40 years old, 10-15 meter in height with drooping branches and round fruits (Plate 1).

Plate 1. High yielding *Garcinia indica* tree bearing fruits, located at Marcel, Goa. Insert, a branch with fruits.

The different types of explants such as leaves, shoot tips, nodal region, inter nodal cuttings, flower buds, flowers, various stages of fruits (un-ripened and ripened) and various developmental stages of seeds (immature and mature) were collected from above mentioned tree at Marcel and used for the tissue culture studies. All explants were sterilized using standardized sterilization methods mentioned in materials and methods. Various stages of leaves (juvenile, young, mature) were used as explants. These leaves were cut in to squares (5×5 mm, 1×1cm) and discs and used for tissue culture studies. They however did not respond in culture and remained same after 5-7 subsequent sub-cultures. The wounded areas became black (death of the cells), while the other portions remained alive for more than six months. No regeneration of plants however, occurred. Similar kinds of observations were made in shoot tips and nodal and inter nodal cuttings.

Flower buds, flowers and pericarps at various developmental stages of fruits were cultured using WPM and MS medium supplemented with various plant growth regulators, however, they did not show any response after 3-5 sub-culture in same medium. The explants became necrotic and died. Amongst the explants, seed segments (each seed cut in to three pieces) from ripened fruits showed fast responses. These showed better results as compared to the full seed and half seed explants and were therefore selected for the regeneration and mass multiplication studies.

MS medium and WPM supplemented with various concentrations and combinations of plant growth regulators were used during the study. All types of explants mentioned above were cultured on both MS medium and WPM. While culturing it was found that WPM was more efficient in culture regeneration and shoot bud formation as compared to MS medium. Since, the study mainly aimed at developing an efficient

protocol for regeneration and mass multiplication, WPM was selected for the *in vitro* regeneration and mass multiplication and production of elite clones.

4. 1. *In vitro* Shoot Bud Induction using Benzylaminopurine (BAP)

Mature seed segments cultured on WPM supplemented with 0.5-5 mg/L BAP showed swelling of seed segment after a week of inoculation. Shoot buds were initiated on the outer portion of cut regions (Table 14; Plate 2A). The explants showed 36.6-100% of regeneration frequency after four weeks. In the case of BAP, maximum regeneration (100%) was observed at a concentration of 3, 3.5, 4 and 4.5 mg/L and minimum regeneration (36.6) was noticed at 0.5 mg/L followed by 1 mg/L (50%) and 1.5 mg/L (53.3%). Maximum number of shoot buds (27) was recorded in 4.5 mg/L BAP with 100% regeneration after 31-35 days of culture (Plate 2 F), 4 mg/L of BAP gave the second best regeneration number of 21 shoot buds per explant, after 31-35 days of inoculation (Table 14; Plate 2D).

The medium containing 3.5 mg/L BAP counted 19 shoot buds after 31-35 days of inoculation of explants (Plate 2 B). Medium fortified with 3 mg/L BAP accounted for 17 shoot buds per seed segment, after about a month of culture with 100% regeneration (Plate 2 E) while and 2.5 mg/L BAP produced 15 shoot buds per explant, after 30-33 days of culture.(Plate 2 G). Minimum number (4) of shoot buds were noted on medium containing 0.5 mg/L BAP followed by medium fortified with 1 mg/L BAP (5 shoot buds/explant; Plate 2 C) and 1.5 mg/L BAP (9 shoot buds/explants), after 27-35 days of incubation. Minimum number of shoot buds (4) and as well as minimum regeneration rate (36.6%) was recorded on the medium supplemented with 0.5 mg/L BAP. Moreover, with increase in the concentration of BAP from 0.5 to 4.5mg/L, there was an increase in the number of shoot buds. Concentration of BAP beyond 4.5 mg/L however, did not increase the number

Table 14. Effect of BAP on regeneration of *Garcinia indica.*

Conc. of BAP (mg/L)	Days taken for shoot bud formation	Culture regeneration (%)	Avg. no. of shoots	Avg. Length of shoot (mm)
0.0	-	-	-	-
0.5	27-30	36.6	04.5±0.70[i]	5.3±0.91[b]
1.0	27-30	50.0	05.0±1.41[i]	5.6±0.56[a]
1.5	27-35	53.3	09.1±0.75[h]	5.0±0.38[c]
2.0	28-30	86.6	10.0±0.53[g]	5.1±0.74[c]
2.5	30-33	93.3	15.0±0.81[f]	4.5±0.52[d]
3.0	31-34	100	17.0±0.81[d]	4.4±0.51[d]
3.5	31-35	100	19.4±0.69[c]	3.7±0.42[e]
4.0	31-35	100	21.0±0.81[b]	3.5±0.52[e]
4.5	31-35	100	27.0±0.94[a]	3.6±0.51[e]
5.0	31-36	90.0	16.0±0.66[e]	2.1±0.56[f]
Critical difference (CD) (5%)			0.699	0.483
Coefficient of variation (CV)			4.672	13.513

Means ± SD followed by different letters differ significantly at 5%.

Plate 2. Different stages of development of direct multiple shoot formation from mature seed segment explants of *Garcinia indica* cultured on WPM supplemented with BAP.

A. Seed segments cultured on BAP 2 mg/L showing the shoot buds formation after 4 weeks of culture.

B. Multiple shoot buds induced on BAP 3.5 mg/L.

C. Initiation of leaf on shoot buds cultured on elongation medium BAP 1 mg/L after 5 weeks of culture.

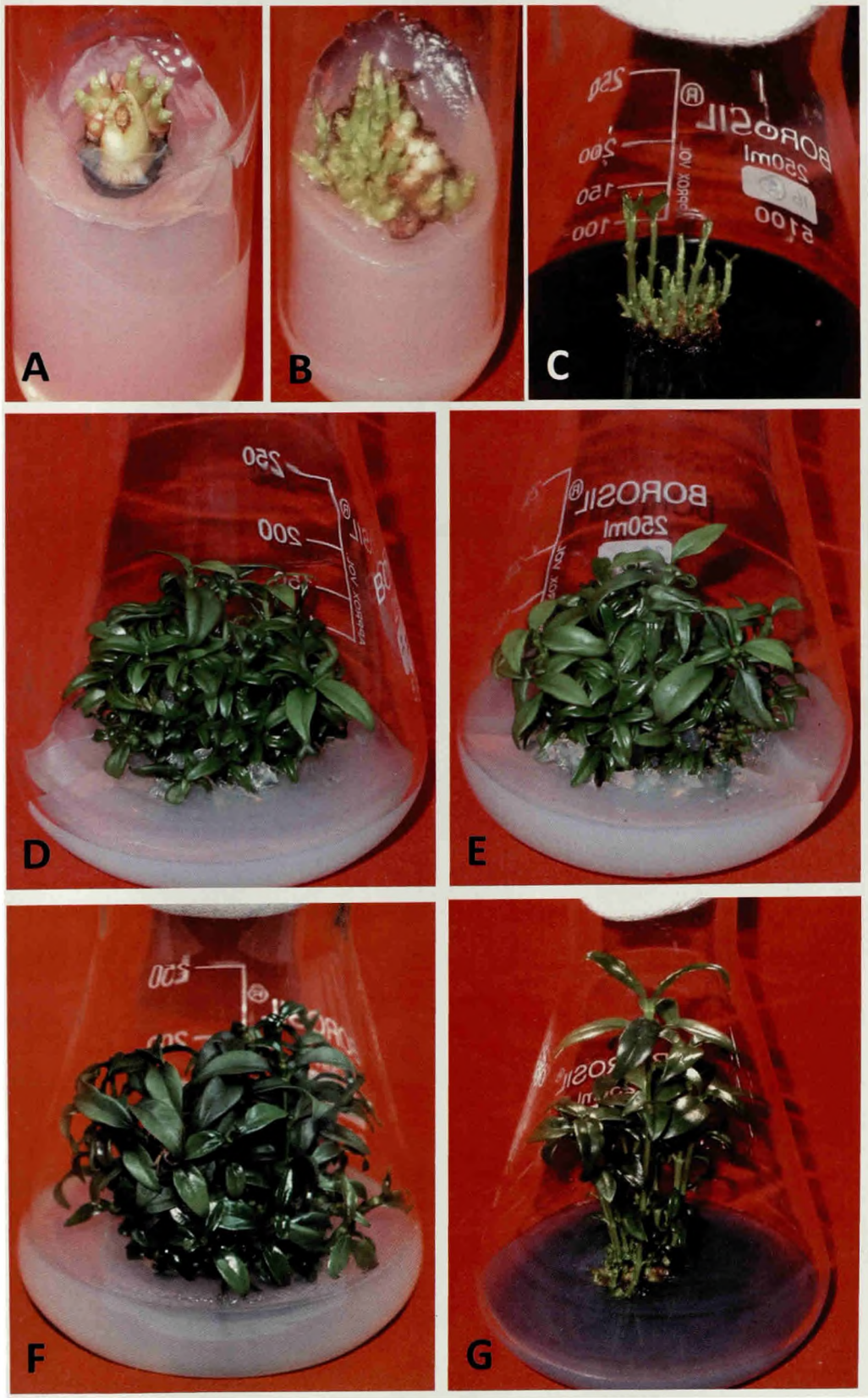
D. Growth of shoot buds from BAP 4 mg/L after 8 weeks of culture.

E. Elongation of shoots induced from the medium supplemented with BAP 3 mg/L.

F. Well grown shoots developed from BAP 4.5 mg/L about 9 weeks of culture.

G. Elongated and fully grown shoots (ready for rooting) on WPM supplemented with BAP 2.5 mg/L (9-10 week of culture).

Plate - 2



of shoot bud formation. Hence, concentration of BAP at 4.5 mg/L is assigned to be optimal concentration for the shoot bud induction (Table 14).

The lower concentration of BAP (0.5, 1, 1.5, 2 mg/L) showed the initiation of shoot bud primordial structures on cut portion of the seed segments and a regeneration rate between 36.6-86.6% with 4 to10 shoot buds induced after 27-30 days of culture. These shoot buds showed faster growth when compared to the shoot buds regenerated from higher concentrations (2.5, 3, 3.5, 4, 4.5, 5 mg/L). This may be due to the availability of high nutrition for few shoot buds. The BAP concentrations more than 2.5 mg/L up to 4.5 mg/L in the medium showed prominent swelling all over the explants after one week of culture (Table 14) as compared to low concentrations (0.5, 1, 1.5, 2 mg/L).

Induction of multiple shoot primordial structures was noted after 21-36 days of culture, without any distinct callus formation. The multiple shoot bud primordium were slowly grew in to shoots, when sub-cultured same medium. The length of shoots varied with varying concentrations, and the shoots length ranged from 2.1-5.1 cm, after 42 days of culture. BAP concentration beyond 4.5 mg/L did not support more number of shoot buds, so BAP at 4.5 mg/L concentration served as the optimum concentration (Table 14). The shoot buds induced on medium supplemented with BAP more than the optimal concentrations (5mg/L), showed stunted growth and development.

4. 2. *In vitro* Shoot Bud Induction using Kinetin (KIN)

Mature seed segment explants were cultured on WPM supplemented with different concentrations of KIN (0.5 to 5 mg/L) (Table 15). After 28 days, the explants showed shoot bud primodium formation (Plate 3A). Maximum of 100% regeneration was obtained on medium supplemented with 4.5 and 5.0 mg/L KIN. Maximum number of shoot buds

Table 15. Effect of KIN on regeneration of *Garcinia indica.*

Conc. of KIN (mg/L)	Days taken for shoot bud formation	Culture regeneration (%)	Avg. no. of shoots	Avg. Length of shoot (mm)
0.0	-	-	-	-
0.5	35-42	40.0	02.5±0.57[e]	1.6±0.18[de]
1.0	35-42	40.0	03.0±0.81[e]	1.6±0.12[d]
1.5	35-42	43.3	03.2±0.95[e]	1.7±0.08[cd]
2.0	33-38	46.6	05.7±0.50[d]	1.8±0.09[b]
2.5	33-38	56.6	07.0±0.70[c]	1.7±0.10[bc]
3.0	30-35	60.0	10.8±0.83[a]	2.0±0.15[a]
3.5	30-33	70.0	06.7±0.95[c]	1.6±0.14[de]
4.0	30-33	83.3	08.6±0.84[b]	1.5±0.09[e]
4.5	28-30	100	05.0±0.81[d]	1.8±0.10[bc]
5.0	28-30	100	07.0±1.05[c]	2.0±0.12[a]
Critical difference (CD) (5%)			0.771	0.108
Coefficient of variation (CV)			13.533	16.041

Means ± SD; SD followed by different letters differ significantly at 5%.

Plate 3. Different stages of shoot buds formation from mature seed segment explants of *Garcinia indica* cultured on WPM supplemented with kinetin (KIN).

A. Formation of shoot bud primordium and shoot buds on KIN 4 mg/L after 6 weeks of culture.

B. Multiple shoot bud formation and initiation of leaves on KIN 3 mg/L after 7 weeks of inoculation.

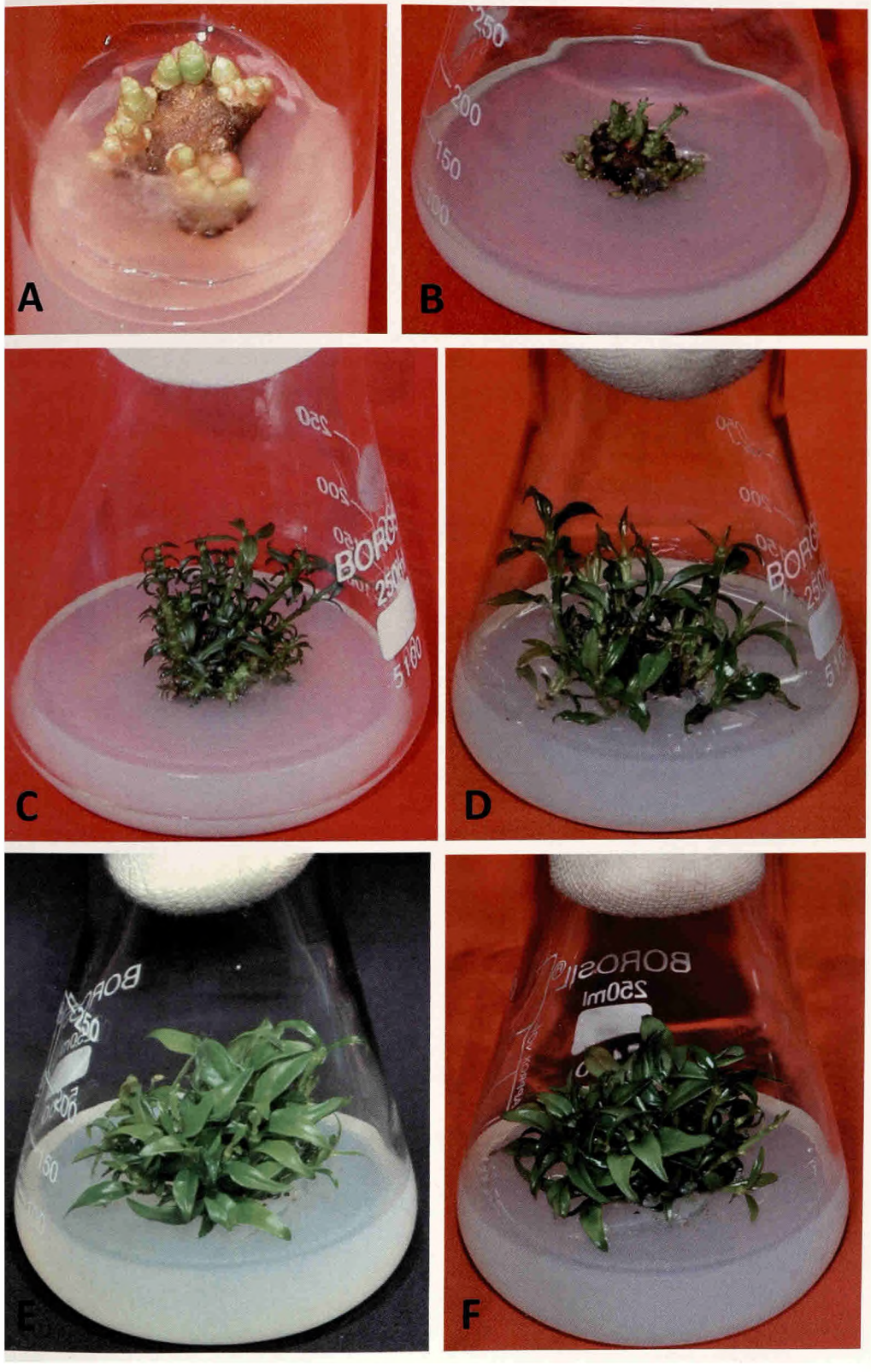
C. Elongation of shoots and shoot buds from KIN 2.5 mg/L after 8 weeks of culture.

D. Elongation shoots induced from medium supplemented with KIN 4 mg/L after 9 weeks of culture.

E. Elongated shoots formed on WPM supplemented with BAP 3 mg/L (11 weeks of culture).

F. Elongated shoots formed on WPM supplemented with BAP 5 mg/L (12 weeks of culture).

Plate - 3



per explant (10.8) and 60% culture regeneration was recorded on medium containing 3 mg/L KIN, after 30-35 days of culture (Plate 3B). 8.6 shoot buds per explant were observed on 4 mg/L with 83.3% culture regeneration, after 30-33 days (Plate 3D) while 2.5 mg/L KIN showed 0.7 shoot buds per explant and 56.6% culture regeneration (Plate 3C). While KIN concentrations increased from 0.5 up to 5 mg/L, a gradual increase in the percentage of culture regeneration (40-100%) was observed, however, no any significant increase in number of shoot buds formation (Table 15).

Minimum culture regeneration of 40% and minimum number shoot buds per explant (2) and (3) was noticed on medium supplemented with 0.5 and 1 mg/L of KIN, respectively, after 35-45 days of incubation. The length of regenerated shoots ranged from 0.5-1 cm after 42 days of culture. The highest length (1 cm) of shoots was obtained on medium fortified with 3 (Plate 3E) and 5 mg/L KIN (Plate 3F). While lowest length (0.5 cm) was observed with 4 mg/L KIN. The shoots that regenerated from KIN treatments showed very slow growth and development after two months. The results obtained from kinetin treatments were not significant in terms of number of shoot bud induction, culture regeneration and length of shoots (Table 15).

4. 3. Effect of 2, 4-D on Regeneration

Seed segments inoculated on WPM containing 2, 4-D at 0.5-5 mg/L showed callus formation after 7-10 days of culture (Table 16). Callus development was prominently seen at the cut portion of the explants at all the concentrations of treatments. Amongst the concentrations, 2, 4-D at 3 mg/L recorded 90% callus formation after 10 days of inoculation (Plate 4A), followed by 80% callus induction at 2 mg/L concentration (Plate 4B). Minimum (53.3%) callus formation was observed at 4 mg/L (Plate 4F), followed by 56.6% at 0.5 mg/L and 73.3% at both 1.0 and 1.5 mg/L of 2, 4-D (Table 16; Plate 4C, D).

Table 16. **Effect of 2,4-D on callus induction of *Garcinia indica.***

Conc. of 2,4-D (mg/L)	Days taken for callus induction	Callus induction (%)
0.0	-	-
0.5	8-10	56.6
1.0	8-10	73.3
1.5	8-10	73.3
2.0	8-10	80.0
2.5	7-10	76.6
3.0	7-10	90.0
3.5	7-10	60.0
4.0	7-10	53.3
4.5	7-10	60.0
5.0	7-10	70.0

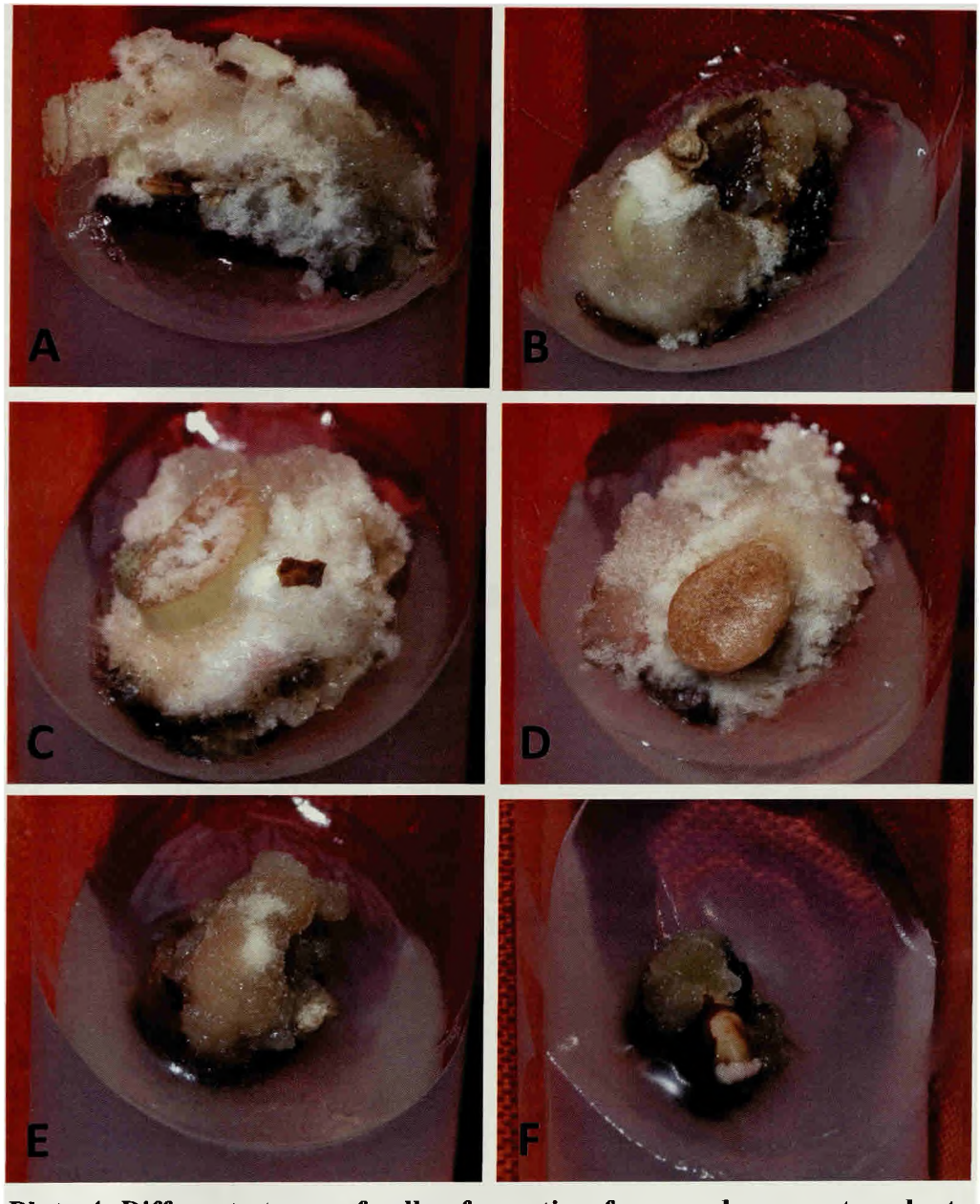


Plate 4. Different stages of callus formation from seed segment explants cultured on WPM supplemented with different concentrations of 2,4-D. A. Callus formed all over the seed segment on 2,4-D 3 mg/L; B. Callus induced on 2 mg/L 2,4-D after 2 weeks of culture; C & D. Callus formed from cut basal portion of seed segment on 2,4-D 1 mg/L and 1.5 mg/L respectively; E. Brown callus induced all over explants on medium containing 4.5 mg/L 2,4-D; F. Callus formed from portion of explants cultured on 4 mg/L 2,4-D after 3 weeks of culture.

60% callus induction was observed in 3.5 and 4.5 mg/L 2, 4-D (Plate 4E). The callus was not compact, looked very spongy and was whitish brown. The callus was sub-cultured after 20-22 days interval in fresh identical media composition or BAP supplemented media. The callus did not respond further, turned black and lost their potential for regeneration.

4. 4. *In vitro* Shoot Buds Induction with BAP and IAA

Mature seed segments cultured on WPM supplemented with BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 mg/L) and addition of 1 mg/L IAA, showed swelling on cut surface of the seed segments after a week of inoculation (Table 17; Plate 5A). The swelling was observed all over the seed segments after two weeks of culture. Initiation of shoot bud primordium was seen at peripheral (cut region and surrounded areas) of explants, later it was seen all over the explants (Plate 5B). After 42 days of culture, distinct shoot buds were observed that formed leaf pairs (Plate 5C).

Maximum of 86.2 shoot buds per seed segment was recorded in the medium supplemented with combination of 2 mg/L BAP and 1 mg/L IAA (Plate 5D, G) followed by 58 shoot buds (2.5 mg/L BAP + 1 mg/L IAA) (Plate 5E), 47.2 shoot buds per explant (3 mg/L BAP + 1 mg/L IAA) (Plate 5F) and 40 shoot buds per explants (1.5 mg/L BAP + 1 mg/L IAA). Minimum number of shoot buds (9) was observed in medium supplemented with 0.5 mg/L BAP + 1 mg/L IAA. Shoot buds were initiated after 40-42 days of culture. BAP at 2, 2.5, 3 and 4 mg/L concentrations, exhibited 100% regeneration of seed segments while low regeneration (56.6) was observed at low concentration of BAP treatments (0.5-1 mg/L). Maximum length (3.2cm) of shoots were recorded on both 4 and 4.5 mg/L BAP, followed by 3 cm which was noticed on both 1 and 3.5 mg/L BAP. 2.4 cm shoots length was measured on 3 mg/L BAP while lowest shoots length (2 cm) was noted

Table 17. **Effect of BAP and IAA on regeneration of *Garcinia indica.***

Conc. of BAP (mg/L)	Conc. of IAA (mg/L)	Days taken for shoot bud formation	Culture regeneration (%)	Avg. no. of shoots	Avg. Length of shoot (mm)
0.0	0.0	-	-	-	-
0.5	1.0	40-42	56.6	09.0±0.81[i]	2.5±0.57[bc]
1.0	1.0	40-42	56.6	13.0±1.41[h]	3.0±0.65[ab]
1.5	1.0	39-42	63.3	40.0±0.83[d]	2.0±0.92[c]
2.0	1.0	40-42	100	86.2±0.78[a]	2.1±0.60[c]
2.5	1.0	39-42	100	58.0±0.66[b]	2.3±0.42[c]
3.0	1.0	38-40	100	47.2±0.63[c]	2.4±0.69[bc]
3.5	1.0	38-40	96.6	30.0±0.81[e]	3.0±0.81[ab]
4.0	1.0	35-38	100	27.2±0.63[f]	3.2±0.63[a]
4.5	1.0	35-38	93.3	22.2±0.78[g]	3.2±0.63[a]
Critical difference (CD) (5%)				0.710	0.606
Coefficient of variation (CV)				1.940	25.798

Means ± SD; SD followed by different letters differ significantly at 5%

Plate 5. Different stages of growth, development and elongation of direct multiple shoots from mature seed segment explants of *Garcinia indica* cultured on WPM containing BAP and IAA.

A. Seed segment showing swelling after a week of inoculation in a media supplemented with BAP 1 mg/L and IAA 1 mg/L.

B. Induction of multiple shoot buds primordium on seed segment cultured on BAP 2.5 mg/L and IAA 1 mg/L after how many weeks of culture.

C. Leaf emerging from the shoot buds on BAP 1.5 mg/L and IAA 1 mg/L after 7 weeks of culture.

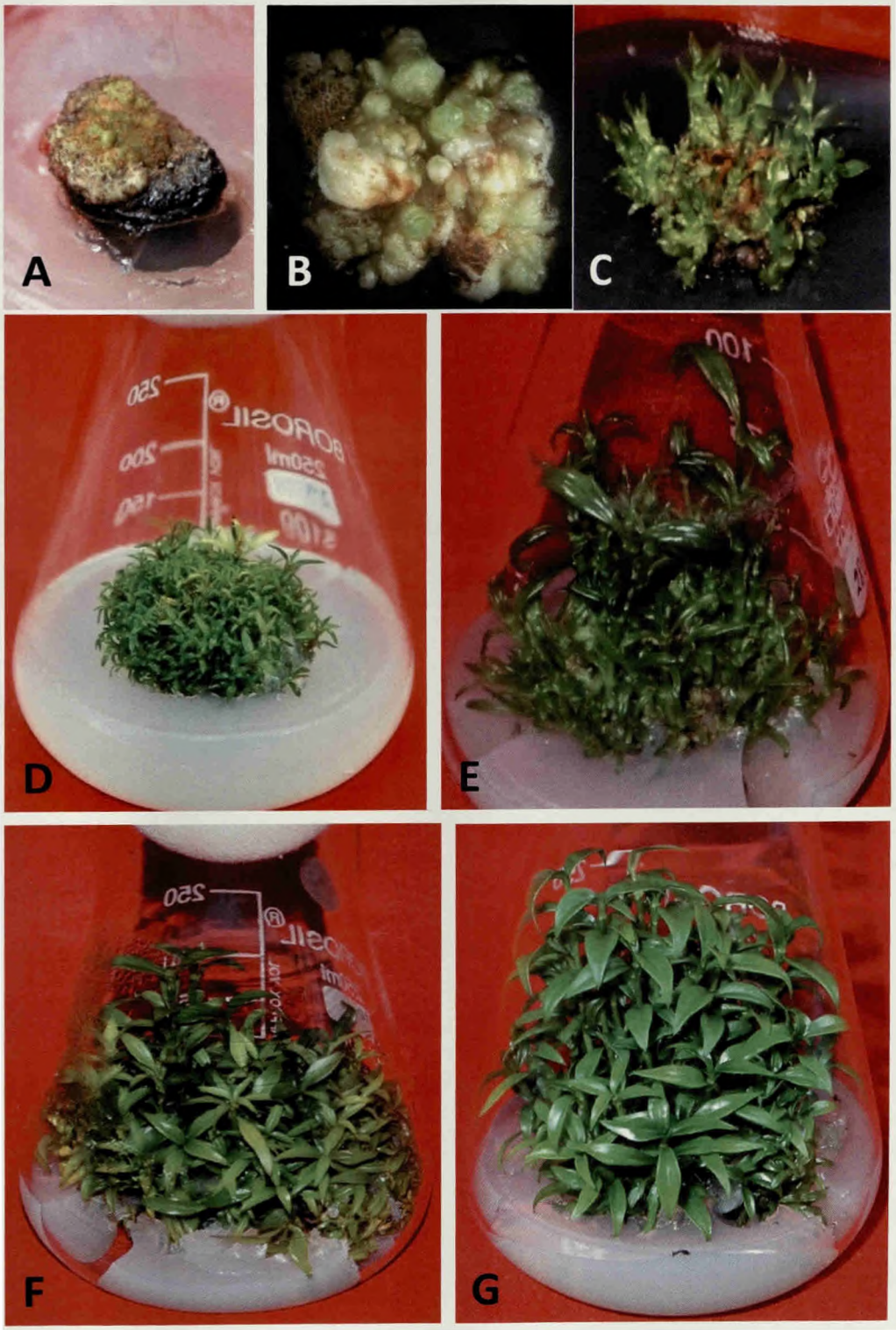
D. Multiple shoot induced from the medium supplemented with BAP 2 mg/L and IAA 1 mg/L after 9 weeks of culture.

E. Multiple shoot development and elongation of shoot and active growth of leaves on medium containing 2.5 mg/L BAP and 1 mg/L IAA.

F. Elongated and well grown shoots induced on BAP 3 mg/L and IAA 1 mg/L.

G. Well grown multiple shoots of about 3-5 cm developed from BAP 2 mg/L and IAA 1 mg/L supplemented medium, 15 weeks of culture.

Plate - 5



on 1.5 mg/L, followed by 2 mg/L (2.1 cm) and 2.5 mg/L BAP (2.3 cm). It was found that both BAP and IAA treatment enhanced the number of shoot bud formation (Table 17).

4. 5. *In vitro* Shoot Buds Induction with BAP and NAA

Mature seed segment cultured on WPM supplemented with combinations of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 mg/L) and NAA at 1 mg/L showed swelling on cut portion of the seed segments after a week of inoculation (Table 18). In the third week, whitish green callus was seen along with the formation of shoot bud primordium (Plate 6A). The maximum callus (60%) formation was seen on medium supplemented with 0.5 mg/L BAP + 1 mg/L NAA, while minimum callus formation (10-30%) was recorded in high (2.5 to 4.5 mg/L BAP) concentration of BAP in the medium. These concentrations showed 90-100% regeneration of seed segments and the number of shoot buds ranged from 37-61.2 (Table 18).

The maximum number (61.2) of shoot buds was induced on medium supplemented with combination of 4.5 mg/L BAP and 1 mg/L NAA (Plate 6F), followed by 42.5 shoot buds per seed segment (4 mg/L BAP + 1 mg/L NAA) (Plate 6E), 40 shoot buds per explant (3.5 mg/L BAP + 1 mg/L NAA) (Plate 6B), 38.5 shoot buds on medium containing 3 mg/L BAP + 1 mg/L NAA (Plate 6D), 37 shoot buds per explant on medium with 2.5 mg/L BAP + 1 mg/L NAA (Plate 6C) and 35 shoot buds per explant (2 mg/L BAP + 1 mg/L NAA). Minimum number (6) of shoot buds per segment was induced on medium supplemented with 0.5 mg/L BAP and 1 mg/L NAA. All treatments took 36 to 42 days for the formation of shoot buds. Highest length of shoots (3.2 cm) was recorded on medium supplemented with 2 mg/L BAP and 1 mg/L NAA while lowest length of shoots (2.1 cm) was noted on medium supplemented with 4.5 mg/L BAP and 1 mg/L NAA (Table 18).

Table 18. Effect of BAP and NAA on regeneration of *Garcinia indica.*

Conc. of BAP (mg/L)	Conc. of NAA (mg/L)	Days taken for shoot bud formation	Culture regeneration (%)	Callus induction (%)	Avg. no. of shoots	Avg. Length of shoot (mm)
0.0	0.0	-	-	-	-	-
0.5	1.0	38-42	43.3	60.0	06.0±1.41[i]	2.5±1.29[cd]
1.0	1.0	38-42	53.3	40.0	11.0±0.70[h]	3.0±0.53[bc]
1.5	1.0	38-42	76.6	36.6	12.0±1.06[g]	3.0±0.70[bc]
2.0	1.0	39-42	83.3	36.6	35.0±0.66[f]	3.2±0.63[a]
2.5	1.0	39-41	90.0	30.0	37.0±0.94[e]	2.9±0.31[bc]
3.0	1.0	37-40	100	20.0	38.5±0.52[d]	3.1±0.87[bc]
3.5	1.0	36-40	100	30.0	40.0±0.81[c]	3.0±0.81[bc]
4.0	1.0	40-42	96.6	26.6	42.5±0.52[b]	2.9±0.87[bc]
4.5	1.0	40-42	93.3	10.0	61.2±0.78[a]	2.1±0.56[d]
Critical difference (CD) (5%)					0.723	0.659
Coefficient of variation (CV)					2.295	25.243

Means ± SD; SD followed by different letters differ significantly at 5%

Plate 6. Different stages of multiple shoot formation and shoot elongation of *Garcinia indica* mature seed segment explants cultured on WPM supplemented with BAP and NAA.

A. Emergence of shoot bud primordial structures from seed segment explants cultured on BAP 3 mg/L after a week of inoculation.

B. Multiple shoots formed on the medium containing BAP 3.5 mg/L and NAA 1mg/L.

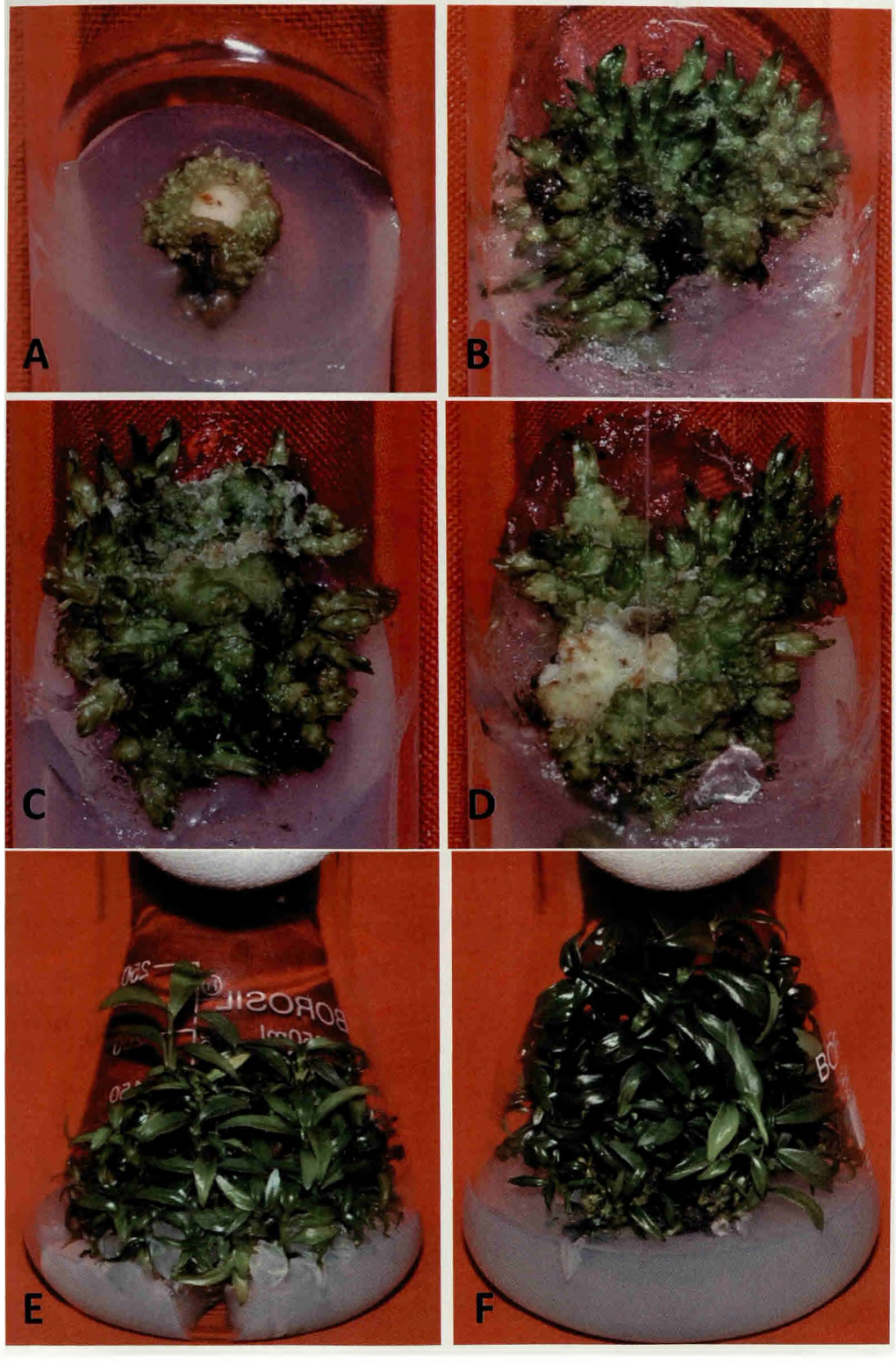
C. Multiple shoot induced on BAP 2.5 mg/L and NAA 1 mg/L after 6 week of culture.

D. Multiple shoots along with callus developed on medium containing BAP 3 mg/L and NAA 1 mg/L after 6 week of culture.

E. Shoot growth and elongation on BAP 1 mg/L after 10 weeks of culture, but before subculture in elongation medium, shoots were induced on medium supplemented with BAP 4 mg/L and NAA 1mg/L.

F. Elongated and well grown shoots induced from the BAP 4.5 mg/L and NAA 1 mg/L after 15 weeks of growth.

Plate - 6



4. 6. *In vitro* Shoot Bud Induction with BAP and IBA

Explants were cultured on WPM supplemented with combination of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 mg/L) and IBA at 1 mg/L (Table 19). After a week of culture, seed segments showed swelling. Prior to first sub-culture, explants were observed with small shoot bud primordium. Initially, shoot bud primordium and surrounding tissues turned pink in color (Plate 7A). About 42 days of culture, all shoot bud primordial developed in to shoots with small filamentous leaves. Maximum number shoot buds (42.7) were recorded on medium containing 4.5 mg/L BAP and 1 mg/L IBA with 100% of culture regeneration after 36-39 days of culture (Plate 7F). Second highest number of shoot buds (38.5) with 100% of culture regeneration after 36-40 days of culture, was obtained on medium supplemented with 4.0 mg/L BAP and 1 mg/L IBA (Table 19; Plate 7B).

Medium containing 3.5 mg/L BAP and 1 mg/L IBA exhibited 37.4 shoot buds with 100% of culture regeneration after 35-40 days of incubation (Plate 7E). BAP at 3.0 mg/L in combination with 1 mg/L IBA showed 96.6% of culture regeneration with 35.7 shoot buds per seed segment after 36-41 days of culture, followed by 32 and 30 shoot buds, respectively, induced from 2.5 mg/L BAP + 1 mg/L IBA and 2.0 mg/L BAP + 1 mg/L IBA, after 36-42 days of culture (Plate 7D). Minimum number of shoot buds (9.5) with 50% of culture regeneration were observed on medium supplemented with 0.5 mg/L BAP and 1 mg/L IBA, after 38-40 days, followed by medium supplemented with 1 mg/L BAP + 1 mg/L IBA (12.5 shoot buds) and 1.5 mg/L BAP + 1 mg/L IBA (16.3 shoot buds), after 36-41 days of culture (Plate 7C) and 56.6 and 86.6% regeneration, respectively. Highest length of shoots (3.5 cm) was measured on medium supplemented with combination of BAP (4 mg/L) and IBA (1 mg/L) and lowest (2.2 cm) was noted on 3 and 3.5 mg/L BAP + IBA (1 mg/L) after 42 days of culture (Table 19).

Table 19. Effect of BAP and IBA on regeneration of *Garcinia indica.*

Conc. of BAP (mg/L)	Conc. of IBA (mg/L)	Days taken for shoot bud formation	Culture regeneration (%)	Avg. no. of shoots	Avg. Length of shoot (mm)
0.0	0.0	-	-	-	-
0.5	1.0	38-40	50.0	09.5±0.54[i]	2.6±0.54[b]
1.0	1.0	36-41	56.6	12.5±0.53[h]	3.2±0.75[a]
1.5	1.0	36-41	86.6	16.3±0.86[g]	3.2±0.66[a]
2.0	1.0	36-42	90.0	30.0±0.81[f]	2.5±0.52[b]
2.5	1.0	36-41	90.0	32.0±0.66[e]	2.3±0.48[b]
3.0	1.0	36-41	96.6	35.7±0.48[d]	2.2±0.42[b]
3.5	1.0	35-40	100	37.4±0.84[c]	2.2±0.63[b]
4.0	1.0	36-40	100	38.5±0.70[b]	3.5±0.51[a]
4.5	1.0	36-39	100	42.7±0.48[a]	3.2±0.42[a]
Critical difference (CD) (5%)				0.611	0.495
Coefficient of variation (CV)				2.287	19.991

Means ± SD; SD followed by different letters differ significantly at 5%

Plate 7. Different stages of multiple shoot formation and shoot elongation of *Garcinia indica*. The mature seed segment explants cultured on WPM supplemented with BAP and IBA.

A. Initiation of shoot buds from seed segment cultured on BAP 2 mg/L and IBA 1 mg/L after 5 weeks of inoculation.

B. Multiple shoot buds on all over the explants when cultured on BAP 4 mg/L and IBA 1 mg/L after 6 weeks.

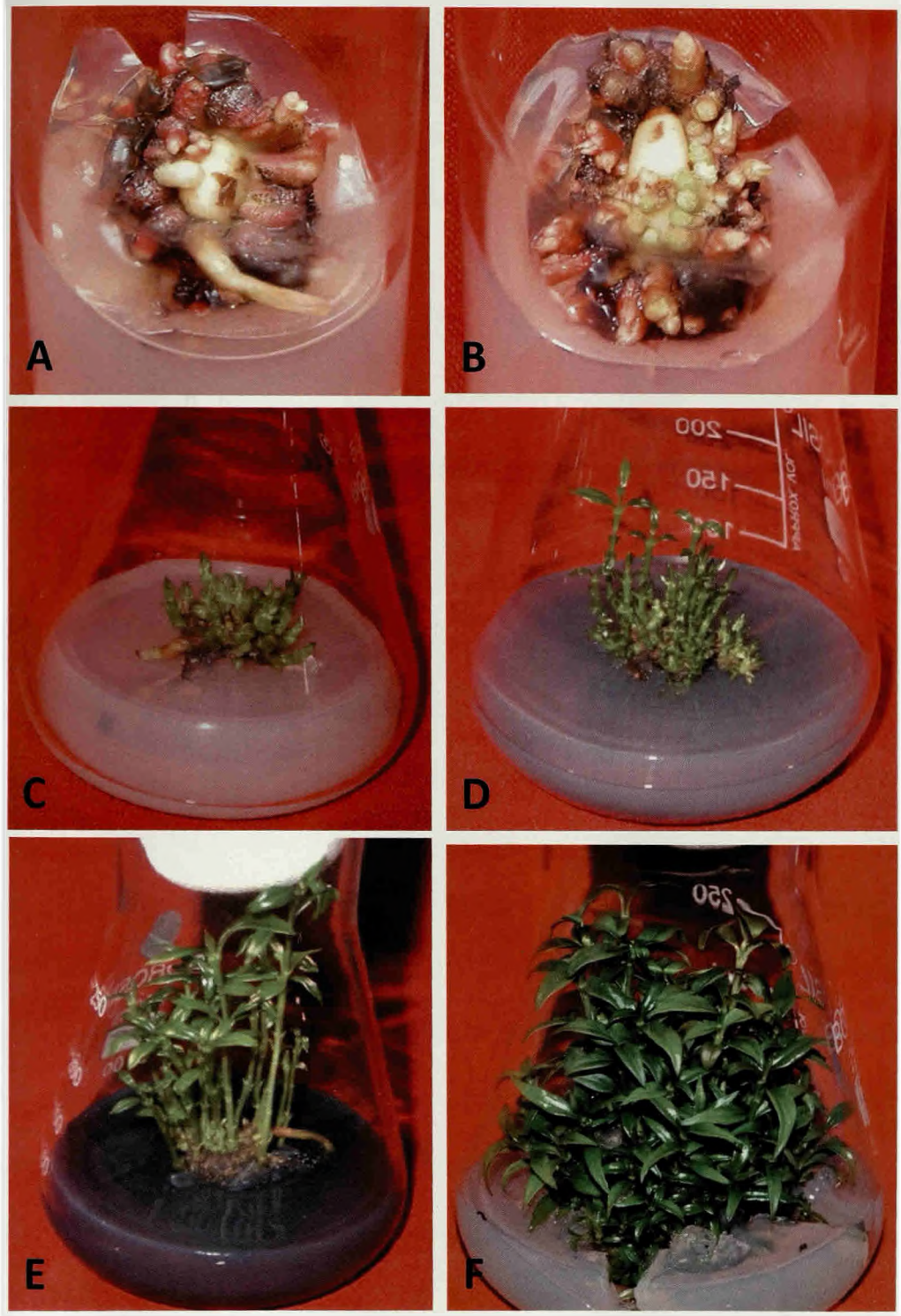
C. Multiple shoots buds along with few roots observed on BAP 1.5 mg/L and IBA 1 mg/L after 6 weeks.

D. Leaf formation from shoots, on medium supplemented with BAP 2mg/L and IBA 1 mg/L after 8 weeks of culture.

E. Elongation of shoots with 2-3 pairs of leaves on elongation medium BAP 1 mg/L about 11 weeks of culture, before sub-culturing on elongation medium, shoots were induced on BAP 3.5 mg/L and IBA 1 mg/L.

F. Elongated and well grown shoots on elongation medium BAP 1 mg/L of about 15 weeks of culture, but multiple shoots were originally induced on medium supplemented with BAP 4.5 mg/L and IBA 1 mg/L

Plate - 7



The present study revealed that the PGR BAP found to be more potential cytokinin as compared with KIN for morphogenic responses. Shoot bud induction capacity of BAP depended on their concentration used in the media. Among the BAP and KIN treatments, BAP 4.5 mg/L showed maximum numbers shoots. When BAP combined with IAA, NAA and IBA, BAP 2 mg/L + IAA 1 mg/L recorded the maximum number of shoot bud formation. Further, seeds were cut into three segments and used during this study for the regeneration on WPM supplemented with various concentrations and combinations of BAP, KIN, IAA, NAA and IBA, it was found that the regeneration ability of all seed segments were almost similar, they did show any significant difference in term of percentage of regeneration, number of multiple shoot buds per segment and the length of the shoots.

4. 7. Elongation of Shoots

Media used for the formation of shoot buds was not supportive for growth of shoots. Hence, the shoots developed from all treatments (after 42 days) were sub-cultured on to WPM containing 1 mg/L BAP with activated charcoal (0.1%) or without activated charcoal (elongation medium). Shoots showed fast growth and development on WPM containing 1 mg/L BAP (without activated charcoal) as compare to activated charcoal (0.1%) medium. The shoot elongation resulted in the formation of leaf and increase in leaf area. At the end of second sub-culture, almost all shoots elongated to 3-5 cm height. Shoot buds formed in KIN treatments showed further growth and development only on medium supplemented with 1 mg/L BAP. Subsequent sub-culturing carried out on same BAP medium, showed better growth and development of shoots. The shoot buds induced from BAP and NAA treatments not only produced shoot buds but also produced some callus. These shoot buds along with callus when sub-cultured on to elongation medium (WPM

supplemented with 1 mg/L BAP), developed in to shoots and elongated. No further growth of the callus however, was observed.

4. 8. Root Induction

Elongated shoots of about 3-5 cm were carefully cut at the basal region of shoot and separated from the core of regenerated explants. Each elongated shoots consisted of 3-4 internodes and 3-4 pairs of leafs. These were sub-cultured by placing the plant straight about 1 cm depth on the centre of the half strength WPM medium supplemented with different concentrations of IBA and NAA for root initiation. The results of the same are summarized in Table 20 & 21. Root formation was observed in all combinations of IBA and NAA after 2-3 weeks of culture. Roots were induced without any interference of callus formation and root formation was observed just above the basal cut end. However, differences in efficiency of rooting were observed among IBA and NAA. After the removal of elongated shoots, the core of regenerated explants along with un-elongated small shoots were sub-cultured on to fresh shoot elongation medium for further growth and development of shoots.

IBA treatments recorded 33.3-100% rooting (Table 20). Highest frequency of rooting (100 and 93.3%) was induced at a concentration of 3 and 4 mg/L IBA, respectively (Plate 8A) while minimum rooting (33.3%) was observed on 0.5 mg/L IBA. Maximum number of roots (2.8) as well as maximum length of roots (3.65 cm) was recorded at 3 mg/L IBA (Plate 8B). In case NAA treatment, 56.6-90% of rooting and maximum percentage (90%) of rooting was noticed at 5 mg/L NAA. Highest number of roots (5) per shoot with 86.6% rooting was observed at 4 mg/L NAA, after 15 days (Table 21). Amongst the IBA and NAA treatments, IBA at 3 mg/L concentration supported acclimatization process and high rate of plant survival as compared to NAA. Even though

Table 20. Effect of IBA on rooting of regenerated shoots of *Garcinia indica*.

Medium	Conc. of IBA (mg/L)	Days taken for root induction	% of root induction	Avg. No. of roots	Avg. Length of roots (cm)
½ WPM	0.0	-	-	-	-
½ WPM	0.5	15-25	33.3	1.03±0.05[c]	0.60±0.00[d]
½ WPM	1.0	10-15	70.0	1.28±0.48[bc]	3.14±0.37[a]
½ WPM	2.0	10-15	86.3	1.42±0.53[bc]	3.57±0.53[a]
½ WPM	3.0	12-15	100	2.80±0.42[a]	3.60±0.51[a]
½ WPM	4.0	11-13	93.3	1.70±0.48[b]	1.70±0.48[c]
½ WPM	5.0	15-22	80.0	1.50±0.53[bc]	2.62±0.53[b]
Critical difference (CD) (5%)				0.482	0.484
Coefficient of variation (CV)				27.100	17.551

Means ± SD; SD followed by different letters differ significantly at 5%

Table 21. Effect of NAA on rooting of regenerated shoots of *Garcinia indica*.

Medium	Conc. of NAA (mg/L)	Days taken for root induction	% of root induction	Avg. No. of roots	Avg. Length of roots (cm)
½ WPM	0.0	-	-	-	-
½ WPM	0.5	15-25	56.6	2.0±0.37[d]	1.20±0.10[d]
½ WPM	1.0	10-15	60.0	2.0±0.14[d]	1.81±0.16[a]
½ WPM	2.0	10-15	73.3	2.1±0.52[d]	1.84±0.05[a]
½ WPM	3.0	12-15	80.0	3.8±1.24[b]	1.21±0.14[d]
½ WPM	4.0	11-13	86.6	5.0±0.53[a]	1.33±0.09[c]
½ WPM	5.0	15-22	90.0	2.9±0.63[c]	1.60±0.10[b]
Critical difference (CD) (5%)				0.667	0.109
Coefficient of variation (CV)				22.653	7.616

Means ± SD; SD followed by different letters differ significantly at 5%

Plate 8. Root induction, root-hardening and acclimatization of regenerated plantlets of *Garcinia indica*. on half-strength WPM (basal medium) supplemented with IBA.

A. Induction of roots on IBA 3 mg/L after 15 days of sub-culture.

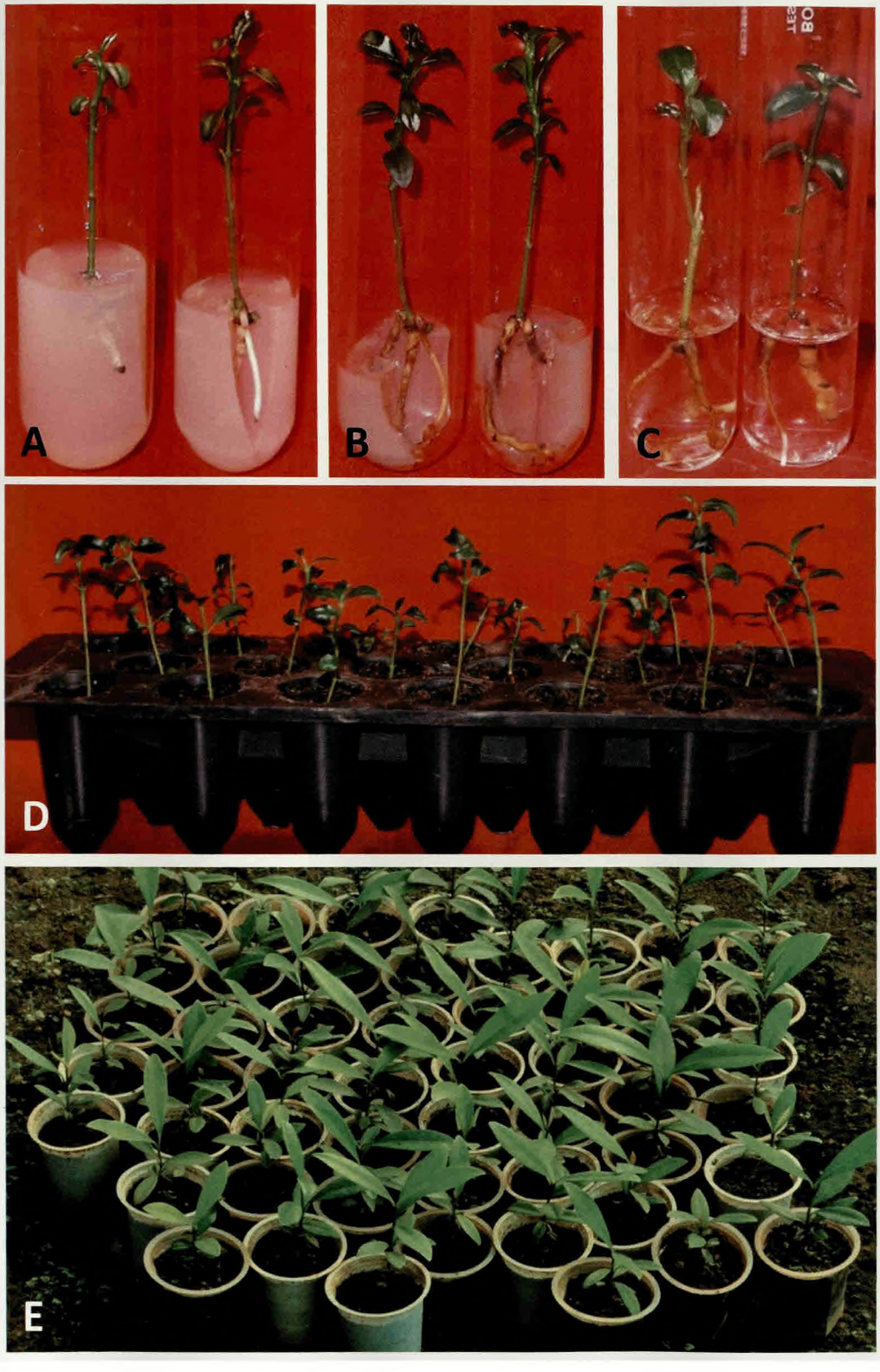
B. Well grown root on medium containing IBA 3 mg/L after 4 weeks of sub-culture.

C. Root hardening in quarter-strength WPM (basal medium).

D. Root hardened plants transferred to seed tray containing garden soil and sand (1:1).

E. After 20 days of growth in seed tray, plantlets were transplanted into small plastic pots containing garden soil and vermicompost (3:1).

Plate - 8



the number of roots were higher (5) in 4 mg/L NAA as compared to IBA, those roots remained stunted, swollen, greenish white and did not support acclimatization process.

4. 9. Root Hardening

Well rooted plantlets of about 20-25 days of rooting were carefully washed with sterile distilled water to remove the trace of rooting medium on root surface. Then, the plantlets were transferred to culture tube containing quarter strength WPM (liquid medium) without sucrose (Plate 8C). The culture tubes along with the rooted plantlets were incubated at room temperature for 10-12 days, for the roots to get harden. During this process the roots, which were more or less white on rooting medium, turned brown. At this stage, roots especially the outer surface, i.e. epidermal and adjacent cell layers harden. This process supports the plantlets for survival during acclimatization process.

4. 10. Acclimatization of Regenerated Plantlets

Root hardened and actively growing plantlets, consisting of 2-3 roots and 6-8 leaves, were transplanted into seed trays filled with different potting mixtures, such as, cocopeat, vermicompost, garden soil independently, and combination of cocopeat + sand (1:1), vermicompost + sand (1:1), garden soil + sand (1:1), cocopeat + garden soil (1:1), vermicompost + garden soil (1:1), cocopeat + vermicompost + garden soil (1:1:1) and cocopeat + vermicompost + sand (1:1:1) (Table 22). The trays with the plantlets were placed, at 12 h photoperiod, 27±1°C temperature and about 60-70% relative humidity, in a plant growth chamber. Survival rate of transferred plantlets ranged from 13.3 to 96.6%. Maximum survival rate of 96.6% was recorded in the combinations of garden soil + sand (1:1), followed by garden soil (93.3%) (Plate 8D), garden soil + vermicompost (76.6%), cocopeat + garden soil (60%), vermicompost + garden soil (36.6%) and 20% survival was noted in cocopeat + vermicompost + garden soil and vermicompost + sand. Minimum

Table 22. Regenerated plants transplanted in different potting mixture and their responses.

Sl. No	Composition of potting mixture	Number of plants transferred	Number of plants survived	Percentage of survival
1	Cocopeat	30	-	-
2	Vermicompost	30	-	-
3	Garden soil	30	26	93.3
4	Cocopeat + Sand (1:1)	30	-	-
5	Vermicompost + Sand (1:1)	30	-	-
6	Garden soil + Vermicompost (1:1)	30	23	76.6
7	Garden soil + Sand (1:1)	30	29	96.6
8	Cocopeat + Garden soil (1:1)	30	18	60.0
9	Vermicompost + Garden soil (1:1)	30	11	36.6
10	Cocopeat + Vermicompost + Garden soil (1:1:1)	30	6	20.0
11	Cocopeat + Vermicompost + Sand (1:1:1)	30	4	13.3

Ten plants were transplanted in each time per treatment (three times).

survival rates were observed in cocopeat + vermicompost + sand (13.3%) (Table 22). Among the potting mixture, garden soil and sand (1:1) showed the maximum survival rate, hence, garden soil and sand (1:1) was selected for further acclimatization of all regenerated plants during the entire study (Plate 9A). After 20 days of growth in seed trays with garden soil and sand (1:1), the plantlets were transplanted into small plastic pots containing garden soil and vermicompost (3:1) (Plate 8E). After 6 months of growth, the regenerated plants, grown to the height of about 1 feet (Plate 9B), were transferred to large black polythene bags containing garden soil: manure: sand (2:1:1) for further growth. The regenerated plants, after 9 months of growth in poly-house showed 2 feet in height (Plate 10A), and after 12 months reached to the height of about 1 meter (Plate 10B).

4. 11. Characterization of Regenerated Plants

4. 11. 1. Histological and Anatomical Characterization

4. 11. 1. 1. Histological Studies

Histological studies were carried out to determine the origin and development of shoot bud primordium and shoot buds. Transverse section of seed segments after one week culture showed the presence of large number of meristematic zones, mostly present in central region (arrow) (Plate 11A, B). After two weeks of inoculation, the explants showed more number of meristematic zones toward the peripheral region. In three week cultures, the meristematic zones further developed into shoot primordium (Plate 11C). Later, the shoot primordium developed in to shoot buds after four weeks of culture (Plate 11D). The sections also revealed the initiation of pair of leaves from the shoot buds (Plate 11E, F).

Plate 9. Acclimatization of regenerated plantlets in poly-house condition. A. Different stages of growth of regenerated plants; B. Regenerated plants after 6 months of transplantation.

Plate 10. Regenerated and acclimatized plants in poly-house condition. A. Regenerated plants transplanted in to large poly-bags after 9 months of growth; B. Regenerated plants after 12 months of growth and height of the plants reached to 1 meter (Note, a one meter scale vertically placed in the middle).

Plate 11. Histological studies of origin and development of shoot bud primordial structures and shoot buds in mature seed segment explants of *Garcinia indica* cultured on WPM supplemented with different plant growth regulators.

A. Transverse sections seed segment after one week of culture showing zone of meristematic regions stained with toluidine blue, (X20).

B. Safranin, (X20).

C. Seed segment after 3 weeks of culture showing early stage of development of shoot primordial structures (X20).

D. Seed segment after 4 weeks of culture showing shoot primordium with leaf development (X20).

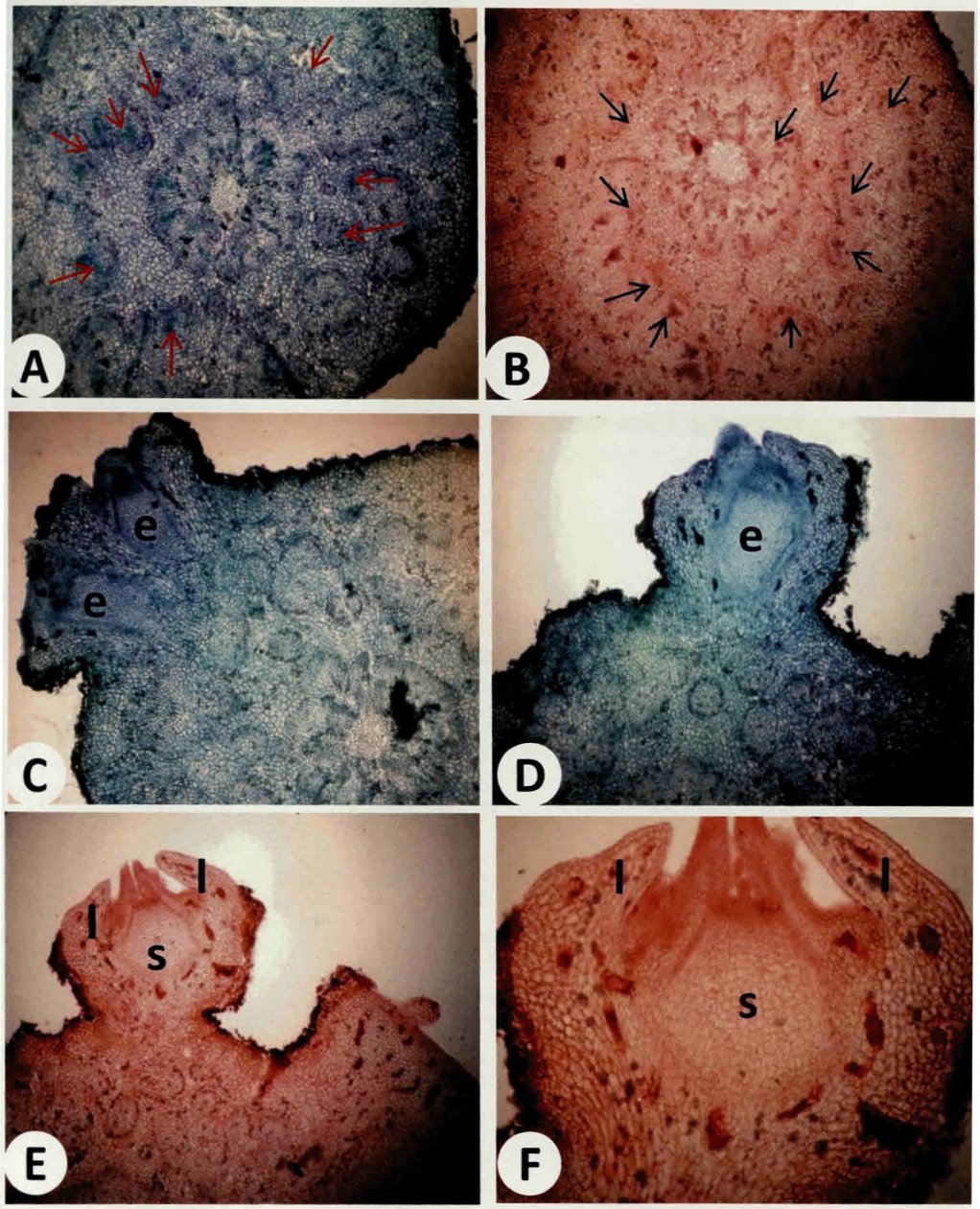
E. Seed segment after 5 weeks of culture formed shoot primordium with a pair leaf, (X20).

F. Enlarged view of shoot primordium with leaves, (X50).

G. Early stage of shoot primordium.

e, early stage of shoot primordium; s, shoot primordium; l, leaf.

Plate - 11



4. 11. 1. 2. Anatomical Characterization

Free-hand thin transverse sections of leaf, stem and root of mother plants as well as *in vitro* regenerated plantlets were taken and stained with safranin and toluidine blue to compare variation if any in the anatomical structure and development, between the *in vitro* regenerated plants in comparison with corresponding mother plants (donor).

4. 11. 1. 3. Transverse Sections of Leaf

Thin transverse sections of regenerated plant and mother plant, stained with safranin, shows a single layer of upper and lower epidermis with a cuticle. The epidermal cells were rectangular in shape and arranged in a compact manner, it was observed that the upper epidermal cells were larger than the lower epidermal cells. Just below the epidermis were present few layers of densely packed parenchymatous cells, known as chlorenchyma, which contains chloroplast to perform photosynthesis. Single major vascular bundle is located in the central region of the leaf (midrib), and it consists of xylem surrounded by phloem. Below the vascular bundle homogenous mass of parenchymatous cells were observed. When the leaf of regenerated plants and mother plants was compared, no any significant difference was noted (Plate 12 A, B).

4. 11. 1. 4. Transverse Sections of Stem

Thin transverse sections of stem of regenerated plant and mother plant, stained with toluidine blue, shows no prominent epidermal structures, since periderm formation was already started. Below the periderm, homogenous mass of parenchymatous cortex was observed containing gum ducts. At the end of the cortex region, patches of sclerenchyma cells were observed. The vascular bundle of the stem is collateral type, in which the xylem is surrounded by the phloem. Both in regenerated plant and mother plant,

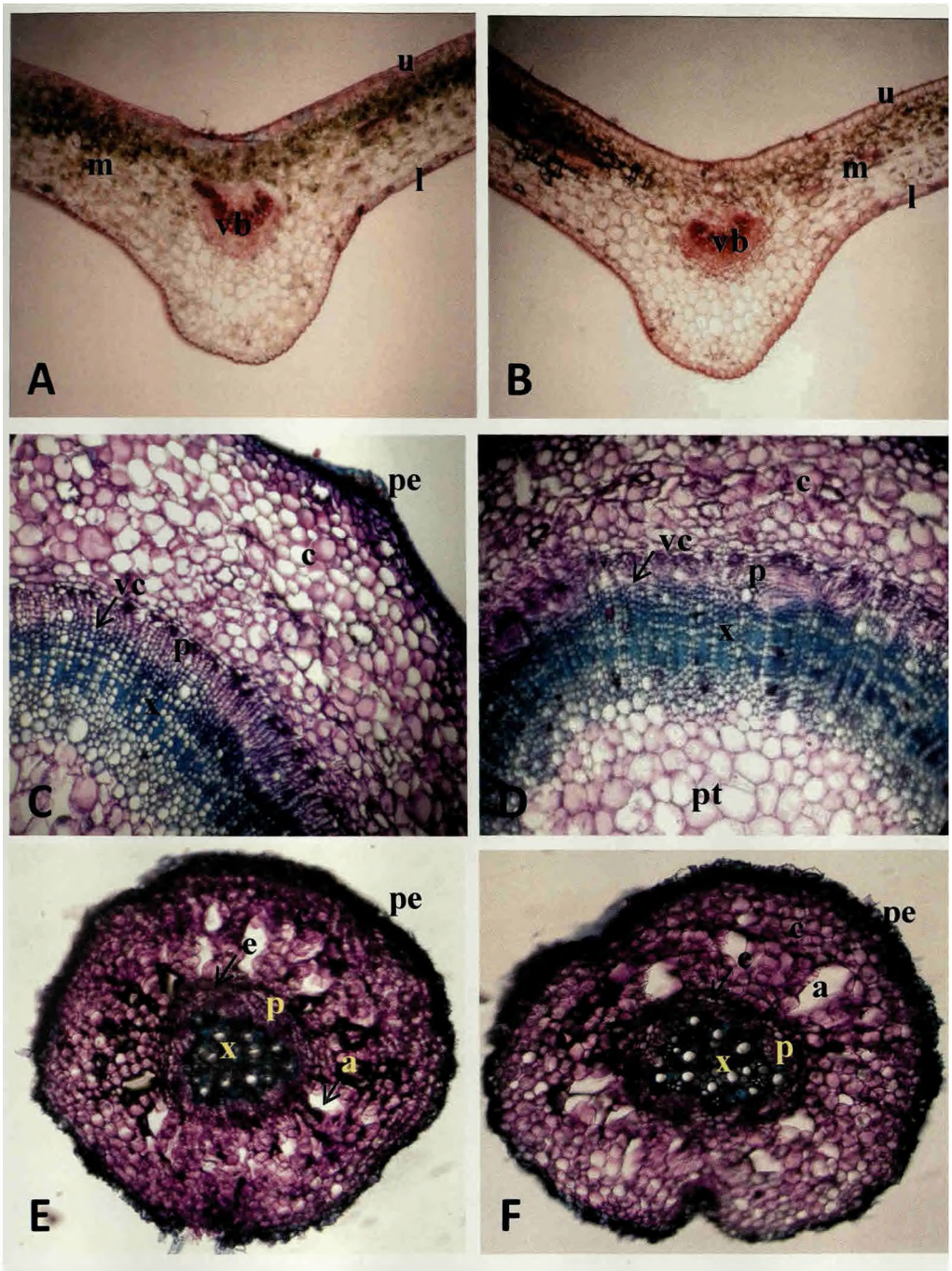


Plate 12. Anatomical characterization of *in-vitro* regenerated plantlets in comparison with mother plant of *Garcinia indica*. Free-hand transverse section of leaf stained with safranin (a, b) and toluidine blue (c - f). a. Leaf of mother plant; b. Leaf of regenerated plant; c. Stem of mother plant; d. Stem of regenerated plant; e. Root of mother plant; f. Root of regenerated plant. a, b, e, f. X20; c, d. X50. a, air-space; c, cortex; e, endodermis; l, lower epidermis; m, mesophyll cells; p, phloem; pe, periderm; pt, pith; vb, vascular bundle; vc, vascular cambium; x, xylem.

the stem had already undergone secondary growth. In between the secondary xylem and secondary phloem, vascular cambium was observed. As xylem is a complex tissue, xylem vessels, xylem tracheids, xylem fibres and xylem parenchyma cells were observed. In few places vascular ray cells were also observed. Overview of the stem distinctly showed the presence of central pith, vascular bundle and cortex. It was observed that the pith parenchyma cells in the central region were larger than the peripheral region. The comparison of stem of regenerated plant and mother plant revealed no any significant difference in their structures (Plate 12 C, D).

4. 11. 1. 5. Transverse Sections of Root

Transverse sections of root of regenerated plant and mother plant, stained with toluidine blue, showed three distinct regions, central vascular bundle, cortex and periderm. The root of both the regenerated plant and the mother plant had undergone secondary growth. In comparison with stem, the characteristic of root was absence of pith. Secondary xylem was located at the central region of root. Patches of phloem were observed in the surrounding region of xylem. Outer region of phloem had a single layer of pericycle followed by a single layer of endodermis. Cortex was with loosely arranged parenchymatous cells with large number of air-spaces. Outer side of the cortex was with periderm tissue. When the root of regenerated plant and mother plant was compared, no any significant difference was observed (Plate 12 E, F).

4. 11. 2. Molecular Characterization

Genomic DNA was extracted from the regenerated plants as well as corresponding mother plants (donor). Quantitative and qualitative estimation of genomic DNA from regenerated plants and corresponding mother plants are summarized in Table 23. PCR was performed using RAPD and ISSR random primers to understand the genetic variation,

Table 23. Quantitative and qualitative estimation of genomic DNA from regenerated plants and corresponding mother plants.

Sl.No.	DNA extracted from	Absorbance at 260 nm	Absorbance at 280 nm	Absorbance ratio 260/280	Quantity of DNA (µg/µl)
1	Regenerated plants	1.212	0.610	1.98	3.03
2		1.142	0.592	1.92	2.85
3		1.078	0.596	1.80	2.69
4		1.101	0.590	1.86	2.75
5		1.222	0.670	1.82	3.05
6		1.180	0.660	1.78	2.95
7		1.214	0.620	1.95	3.03
8		1.340	0.720	1.86	3.35
9		1.165	0.650	1.79	2.91
10		1.371	0.693	1.97	3.42
11	Corresponding mother plants	0.920	0.510	1.80	2.30
12		0.992	0.560	1.77	2.48

if any, between the regenerated plants and corresponding mother plants (donor), by counting the number of bands and appearance of the bands in the agarose gel.

4. 11. 2. 1. RAPD Banding Pattern of PCR Products

Twenty RAPD random primers were obtained from Metabion International AG, Martinsried, Germany (Table 24). All 20 primers were initially screened with mother plant (donor) DNA for reproducibility bands. Amongst 20 primers, eighteen showed clear visible banding pattern with mother plant. The details of results are summarized in the Table 24. Primer OPA 2 and SB 19 did not produce any bands. After testing of primers, eighteen primers were used for PCR amplification of DNA from regenerated plants as well as mother plant. Maximum number (9) of bands was obtained using the primer OPC 2 (Plate 13B) and eight bands each were produced by OPA 1 (Plate 13A), OPD 20 (Plate 14A) and AB 16 (Plate 14B). Seven bands were noted from DNA amplified with primer OPF 13. Primers OPA 9 (Plate 14A), OPH 18 and AV 03 produced six bands each after PCR amplification of DNA from both regenerated plants and mother plants (donor). Five amplified bands each were produced by primer AO 12 (Plate 15B), OPH 13 and primer SB 12. Primer OPF 16, OPF 20, OPH 12 and AP 20 formed four bands each. Three bands were produced by primer OPD 16 and AE 11 while minimum number of bands (2) was produced by primer OPF 10. Moreover, the RAPD primers recorded 2 to 9 bands during the RAPD analysis of DNA of regenerated plants as well as corresponding mother plants.

4. 11. 2. 2. ISSR Band Pattern of PCR Products

Ten ISSR random primers were obtained from Metabion International AG, Martinsried, Germany (Table 25). All 10 primers were initially evaluated for reproducibility of banding pattern with mother plant (donor) DNA. Amongst the 10 primers, seven produced reproducible clear banding pattern and three primers namely IB

Table 24. RAPD analysis of *in vitro* regenerated plants and mother plants.

Sl. No	Primer name	5'-3' Sequence	Number of amplified bands of mother plants	Number of amplified bands of regenerated plants
1	OPA 1	CAGGCCCTTC	8	8
2	OPA 2	TGCCGAGCTC	-	-
3	OPA 9	GGGTAACGCC	6	6
4	OPC 2	GTGAGGCGTC	9	9
5	OPD 16	AGGGCGTAAG	3	3
6	OPD 20	ACCCGGTCAC	8	8
7	OPF 10	GGAAGCTTGG	2	2
8	OPF 13	GGCTGCAGAA	7	7
9	OPF 16	GGAGTACTGG	4	4
10	OPF 20	GGTCTAGAGG	4	4
11	SB 12	AGTCAGCCAC	5	5
12	SB 19	CAGCACCCAC	-	-
13	OPH 12	ACGCGCATGT	4	4
14	OPH 13	GACGCCACAC	5	5
15	OPH 18	GAATCGGCCA	6	6
16	AB 16	CCCGGATGGT	8	8
17	AE 11	AAGACCGGGA	3	3
18	AO 12	TCCCGGTCTC	5	5
19	AP 20	CCCGGATACA	4	4
20	AV 03	TTTCGGGGAG	6	6

Table 25. ISSR analysis of *in vitro* regenerated plants and mother plants.

Sl. No	Primer name	5'-3' Sequence	Number of amplified bands of mother plants	Number of amplified bands of regenerated plants
1	IB 1	AGAGAGAGAGAGAGAGT	14	14
2	IB 2	GAGAGAGAGAGAGAGAT	15	15
3	IB 3	TCTCTCTCTCTCTCTCC	-	-
4	IB 4	ACACACACACACACACC	10	10
5	IB 5	TGTGTGTGTGTGTGTGA	9	9
6	ISSR-ST2	GAGAGAGAGAGAGAGAYG	-	-
7	ISSR-810	GAGAGAGAGAGAGAGAT	11	11
8	ISSR-808	AGAGAGAGAGAGAGAGC	9	9
9	ISSR-834	AGAGAGAGAGAGAGAGYT	-	-
10	ISSR-812	GAGAGAGAGAGAGAGAA	12	12

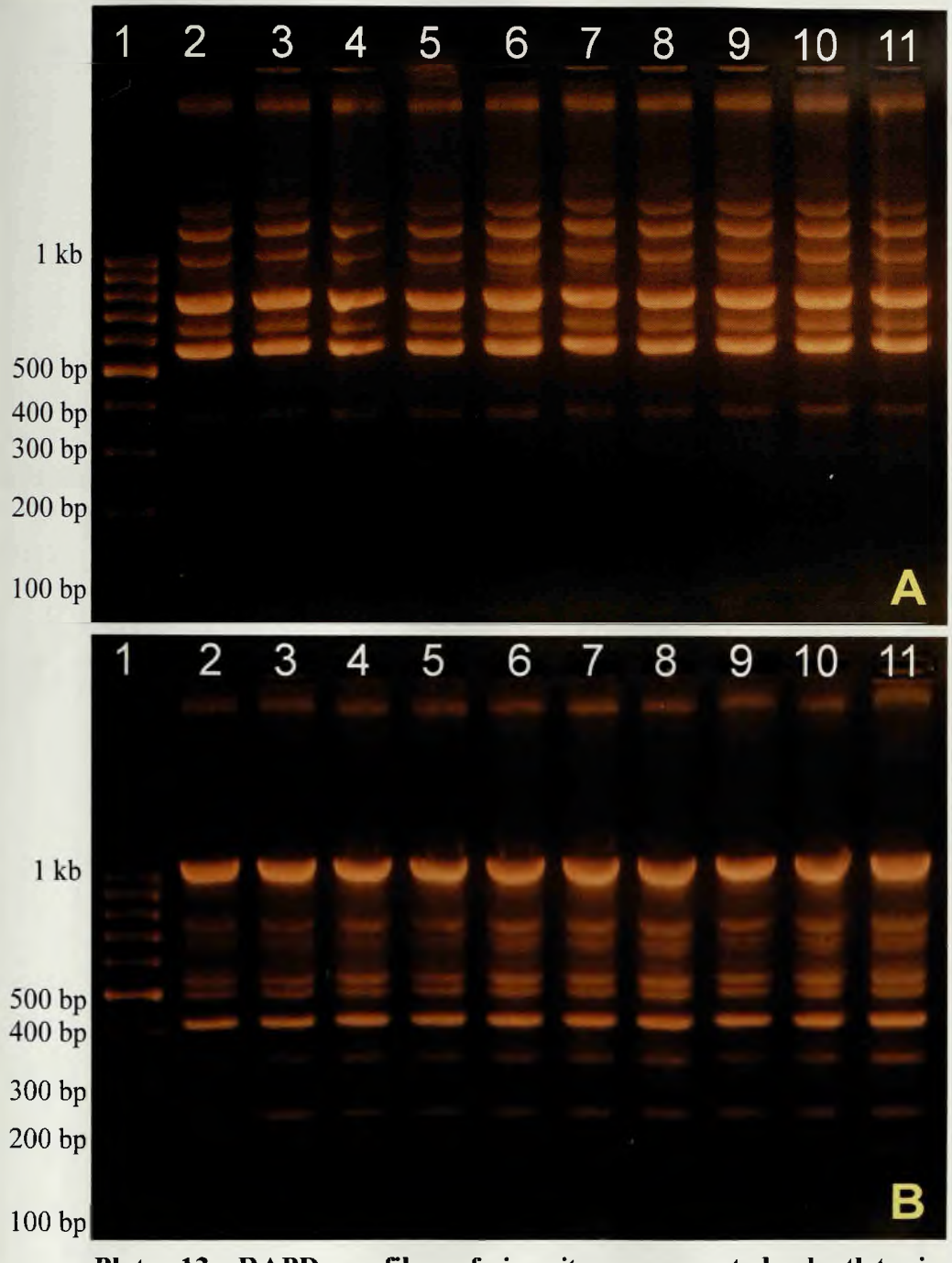


Plate 13. RAPD profiles of *in vitro* regenerated plantlets in comparison with mother plants of *Garcinia indica.* A. Primer OPA 1; B. Primer OPC 2; Lane 1, 100 bp ladder; Lane 2 & 3, DNA from mother plants; Lane 4-11, DNA from *in vitro* regenerated plants.

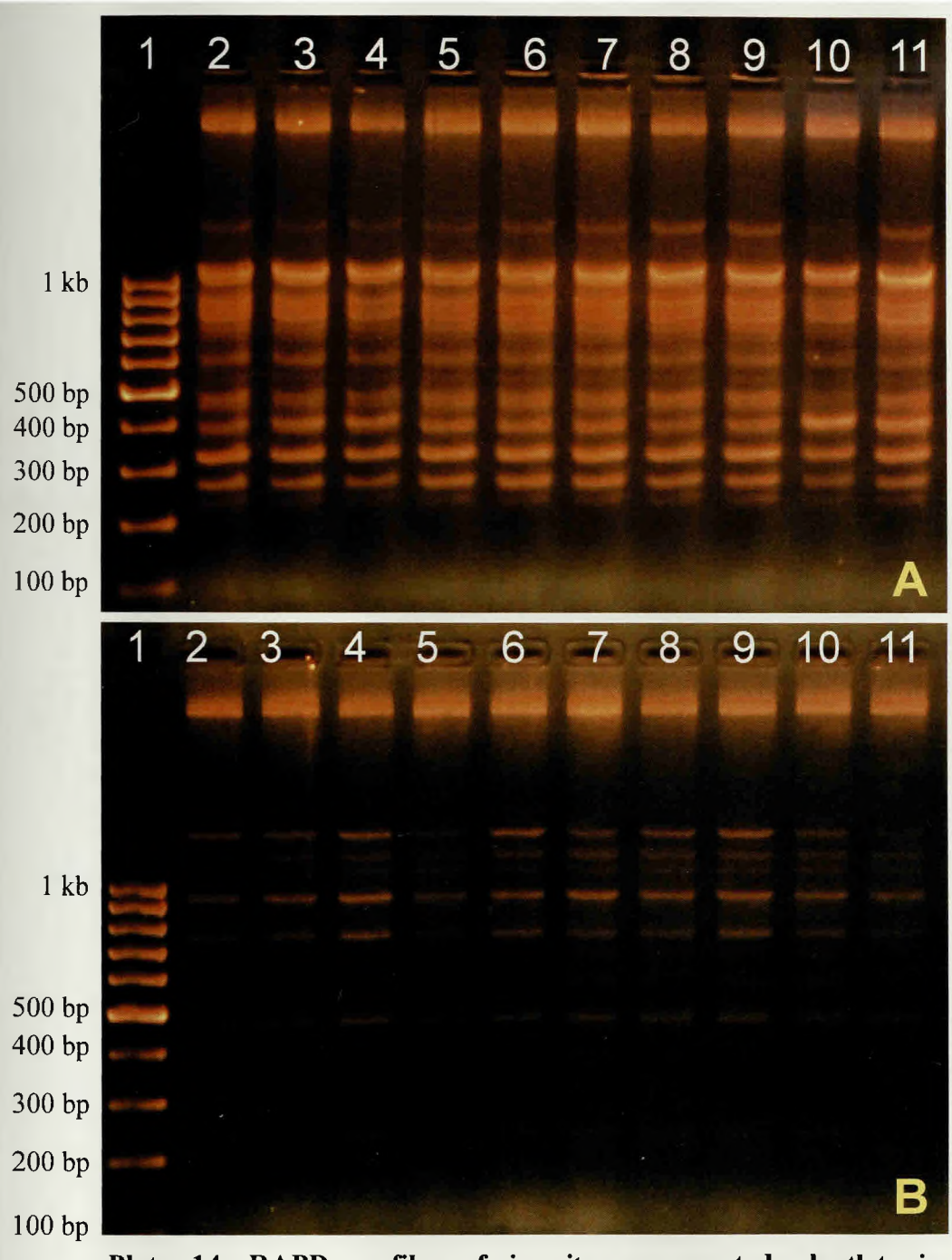


Plate 14. RAPD profiles of *in vitro* regenerated plantlets in comparison with mother plants of *Garcinia indica*. A. Primer OPD 20; B. Primer AB 16; Lane 1, 100 bp ladder; Lane 2 & 3, DNA from mother plants; Lane 4-11, DNA from *in vitro* regenerated plants.

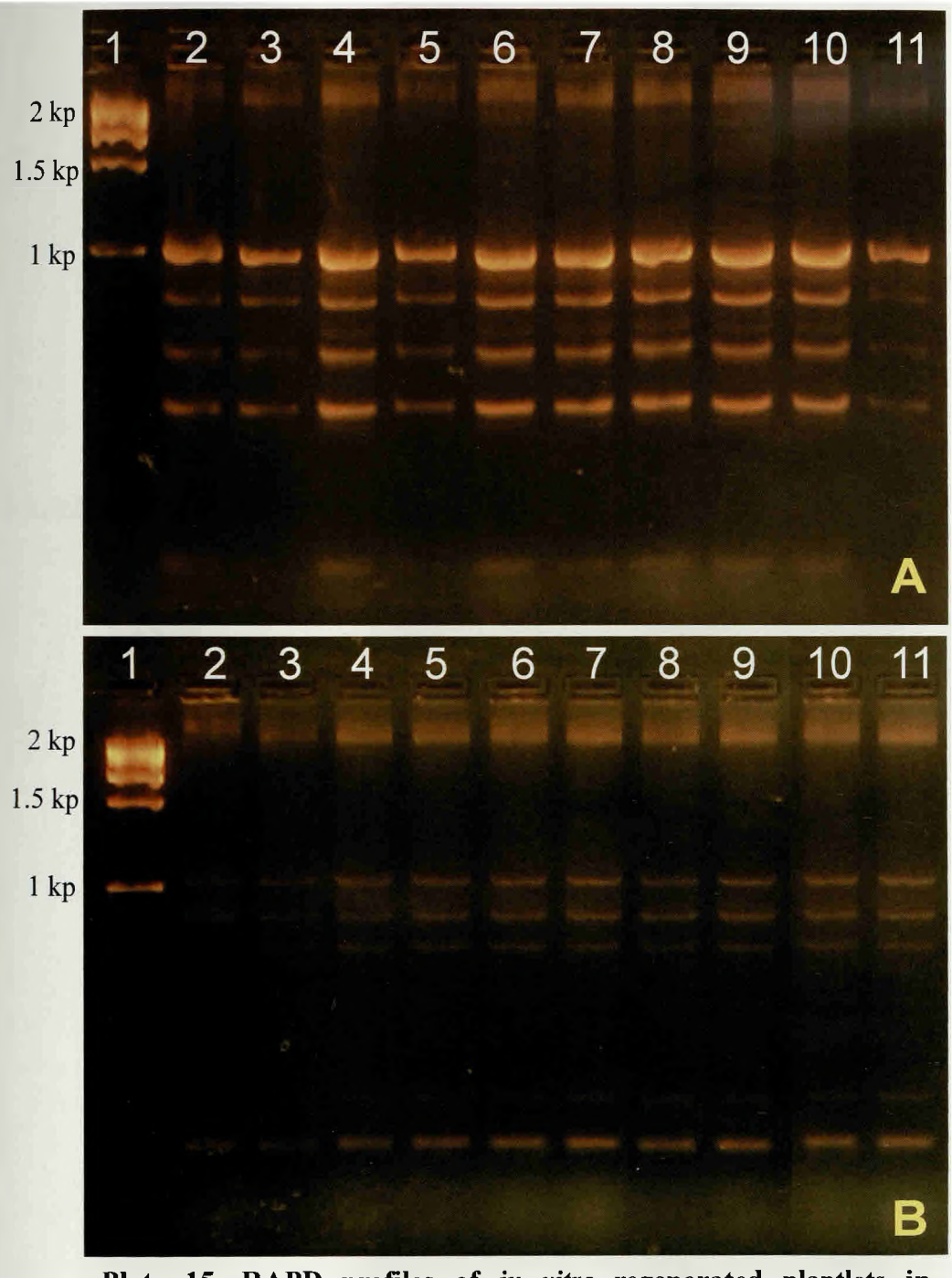


Plate 15. RAPD profiles of *in vitro* regenerated plantlets in comparison with mother plants of *Garcinia indica.* A. Primer OPA 9; B. Primer AO 12; Lane 1, 1kb ladder; Lane 2 & 3, DNA from mother plants; Lane 4-11, DNA from *in vitro* regenerated plants.

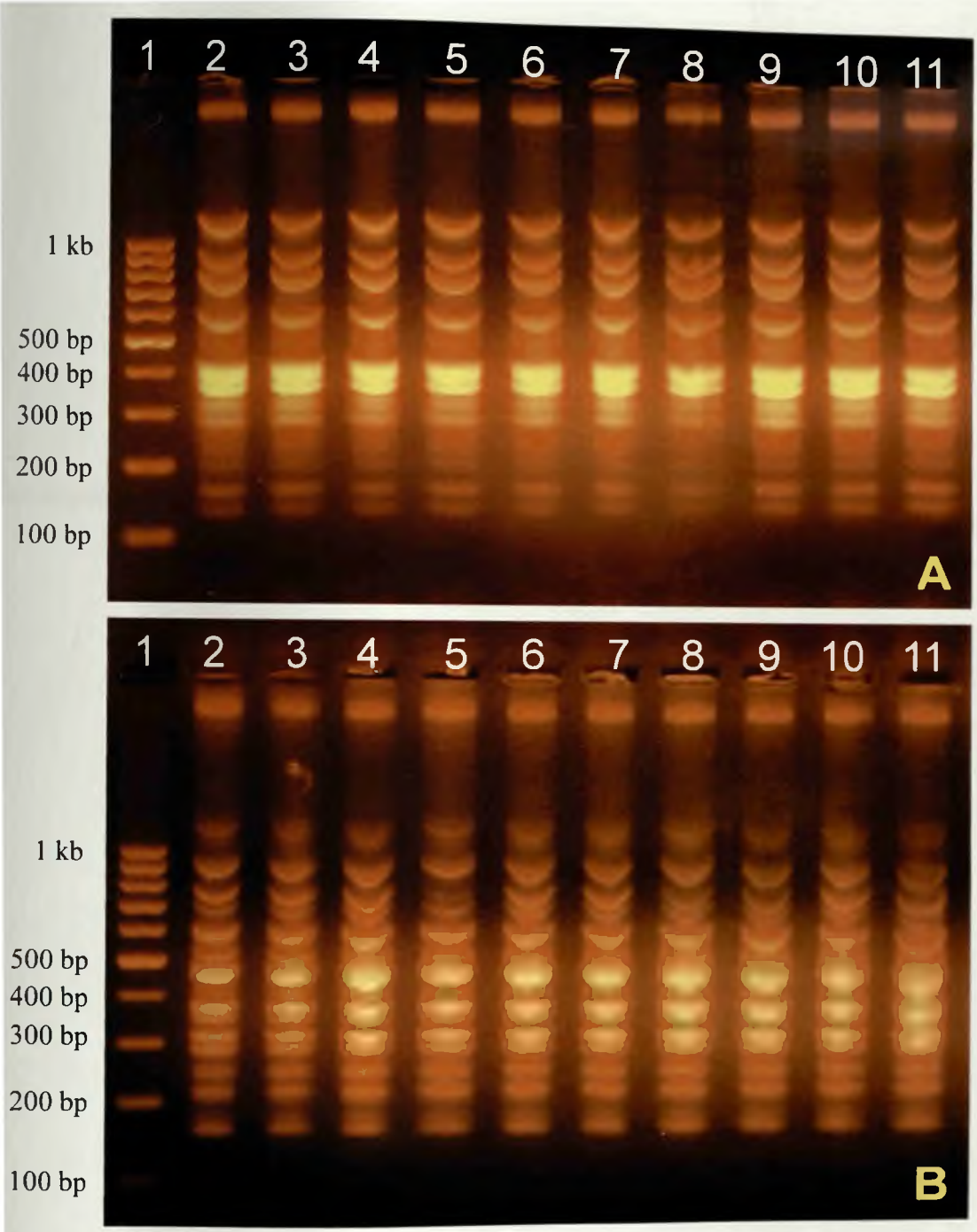


Plate 16. ISSR profiles of *in vitro* regenerated plantlets in comparison with mother plants of *Garcinia indica*. A. Primer IB 1; B. Primer IB 2; Lane 1, 100 bp ladder; Lane 2 & 3, DNA from mother plants; Lane 4-11, DNA from *in vitro* regenerated plants.

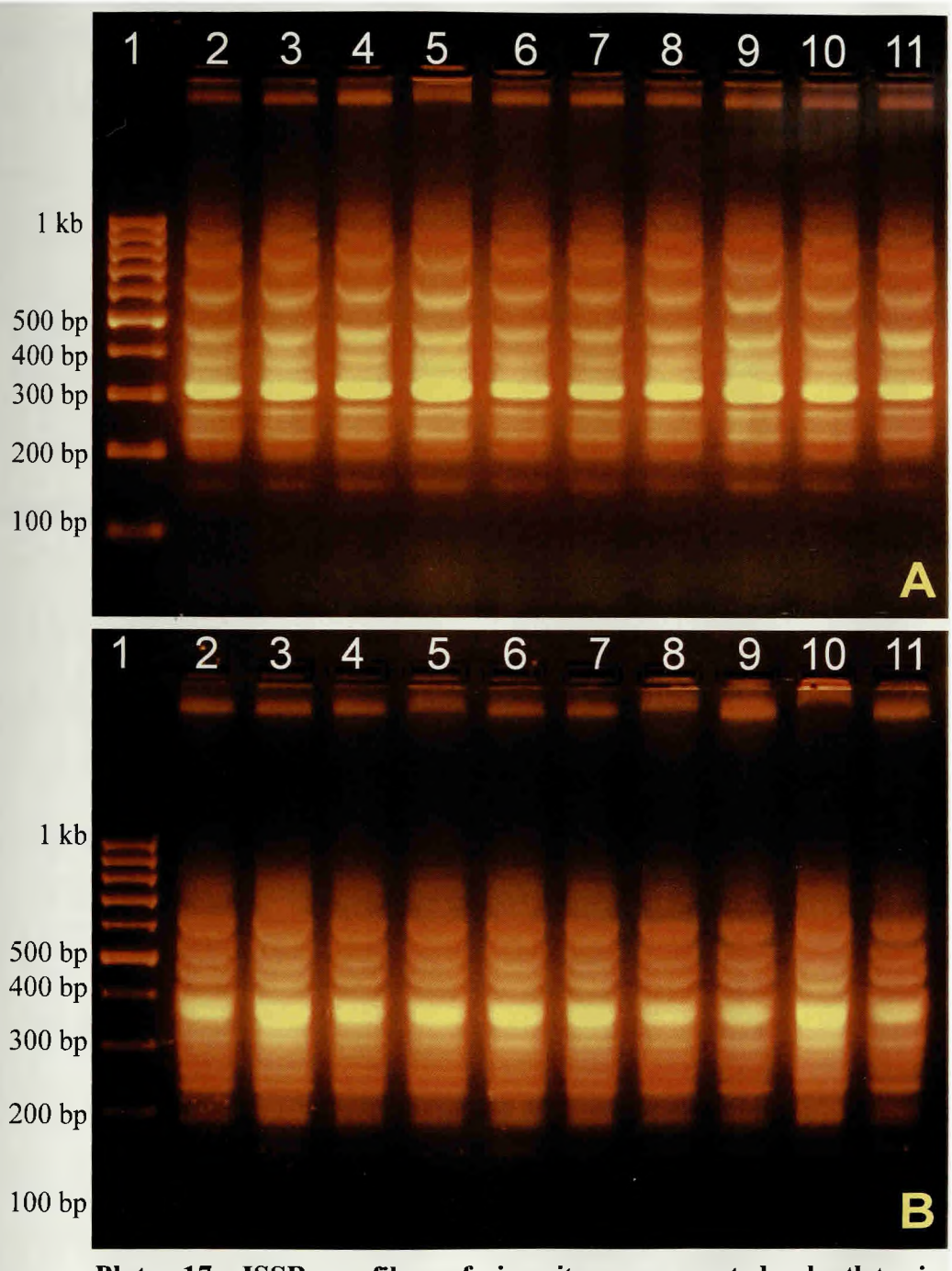


Plate 17. ISSR profiles of *in vitro* regenerated plantlets in comparison with mother plants of *Garcinia indica.* A. Primer IB 4; B. Primer ISSR-808; Lane 1, 100 bp ladder; Lane 2 & 3, DNA from mother plants; Lane 4-11, DNA from *in vitro* regenerated plants.

3, ISSR-ST2 and ISSR-834 did not show any amplification during PCR. The details of results were summarized in the Table 25 .There after seven primers were used for PCR amplification of DNA from regenerated plants as well as mother plants. Primer IB 2 recorded maximum number of bands (15) with clear banding pattern after PCR amplification (Plate 16B). Primers IB 1 and ISSR-812 accounted for 14 and 12 amplified bands, respectively (Plate 16A). Amplified bands (11 and 10) were scored by primer ISSR-810 and IB 4, respectively (Plate 17A). Minimum number of amplified bands (9) was noticed in two primers, namely IB 5 and ISSR-808 (Plate 17B).

Comparison of DNA of regenerated plants with mother plants using RAPD random primers and ISSR random primers, showed no variation in number of bands and banding pattern. Intensity of bands in regenerated plant DNA was however, little less as compared to the mother plant DNA. Moreover molecular characterization of *in vitro* regenerated plants with their corresponding mother plants (donor) using random primers of RAPD and ISSR revalued that genetic stability is maintained in the regenerated plants.

4. 12. Biochemical Analysis of Hydroxycitric acid and Garcinol

Fruits of *Garcinia indica* were collected at different stages of development, from high yielding trees grown in Marcel (Tree No.1) (Plate 18A), Priol (Tree No.2) (Plate 18B) and Ponda (Tree No.3) (Plate 18C). Collected fruits were separated to different developmental stages (6 to 7 stages) depending on the size of the fruits and the same is summarized in Table 26. Fruits collected from Marcel (Tree No.1) and Priol (Tree No.2) were separated to 7 different developmental stages while fruits collected from Ponda (Tree No.3) were separated to 6 different developmental stages.

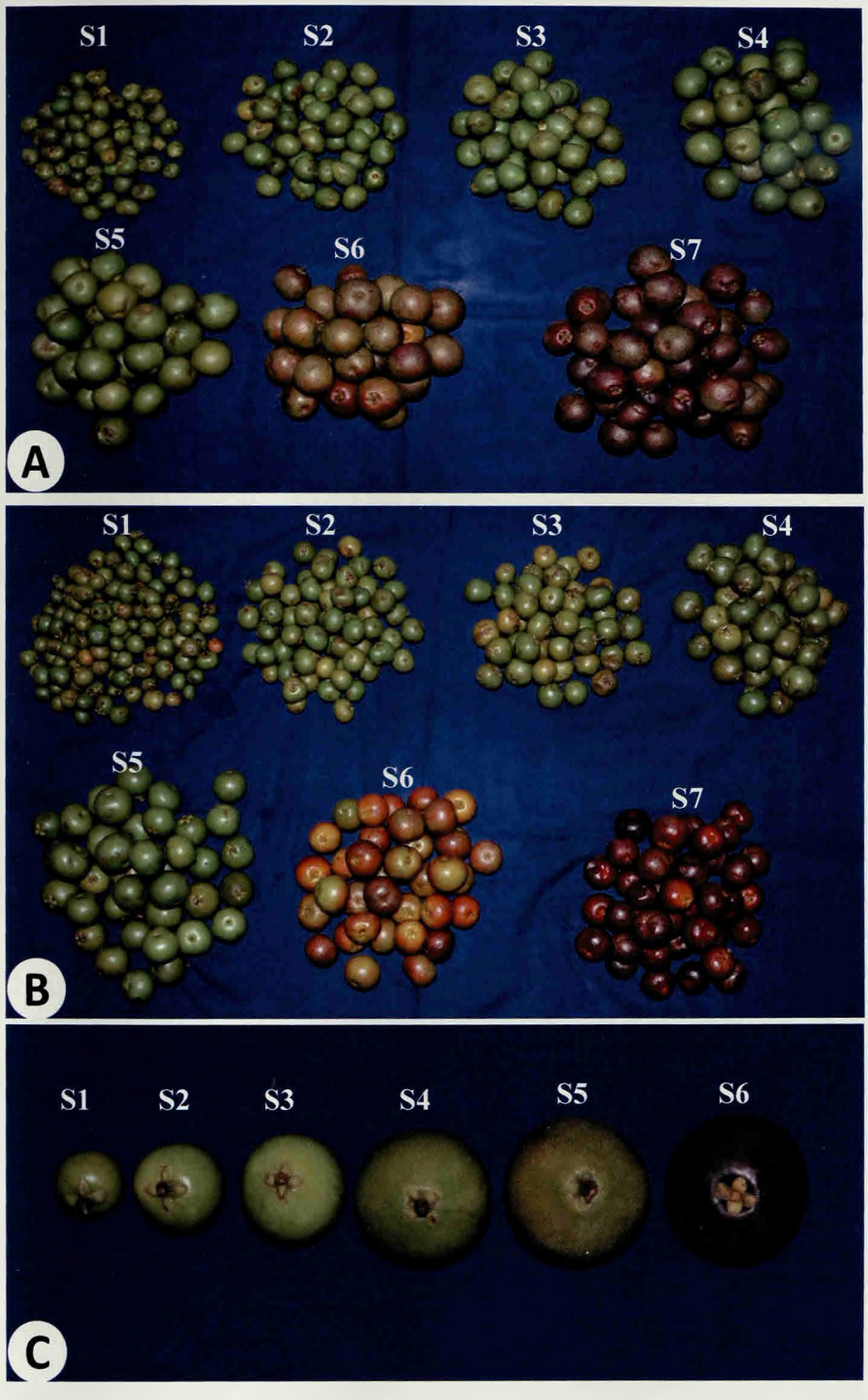


Plate 18. Different stages of development of fruits of *Garcinia indica* used for biochemical analysis collected from different places of Goa. A. Tree No. 1 from Marcel; B. Tree No. 2 from Priol; C. Tree No.3 from Ponda. S1, stage 1; S2, stage 2; S3, stage 3; S4, stage 4; S5, stage 5; S6, stage 6; S7, stage 7.

4. 12. 1. Biochemical Analysis of Hydroxycitric acid

4. 12. 1. 1. Thin Layer Chromatography (TLC) for Confirmation of HCA

TLC aluminum sheets (20×20 cm) were used for confirmation of the presence of HCA in the samples. The samples were dissolved in methanol and 2-3 drops of the same were loaded at a respective loading point with the help of fine capillary tube and kept for 5 minutes at room temperature (RT). Butanol: Formic acid: Water (4:1:5) was used as a solvent system for running the TLC. Sodium metavanadate (1%) was used as a spray reagent (using TLC sprayer) and kept for 10-15 minutes at room temperature. Orange spots indicated the presence of HCA in the samples (Plate 19A, B, C).

4. 12. 1. 2. Determination of HCA by HPLC System

Hydroxycitric acid was resolved as a single peak in the HPLC chromatogram of all samples. HCA peak was determined by comparison of retention time of standard HCA. The retention time of standard HCA was 3.7 min and all twenty samples analyzed for HCA was quantified at similar retention time (3.7 ± 0.1) (Fig. 26-28).

4. 12. 1. 2. 1. HCA content of Tree No. 1 at Marcel

HCA was quantified using HPLC system with standard HCA as reference. HCA content in the samples was calculated by peak area of standard HCA verses peak area of sample. HCA content of seven different developmental stages of fruits collected from the Kokum tree at Marcel area was calculated (Table 26). Amongst the stages, maximum HCA content of 11.45 g/100g fruits was recorded in stage 7 i.e. ripened fruits (85 days after fertilization). In the 6th developmental stage of the fruit (80 days after fertilization), the HCA content was 10.70 g/100g fruits while at stage 5 (75 days after fertilization) the content was 9.59 g/100g fruit. Medium levels of HCA (8.53 g/100g fruits) was measured in 4th developmental stage of fruits (60 days after fertilization), followed by third

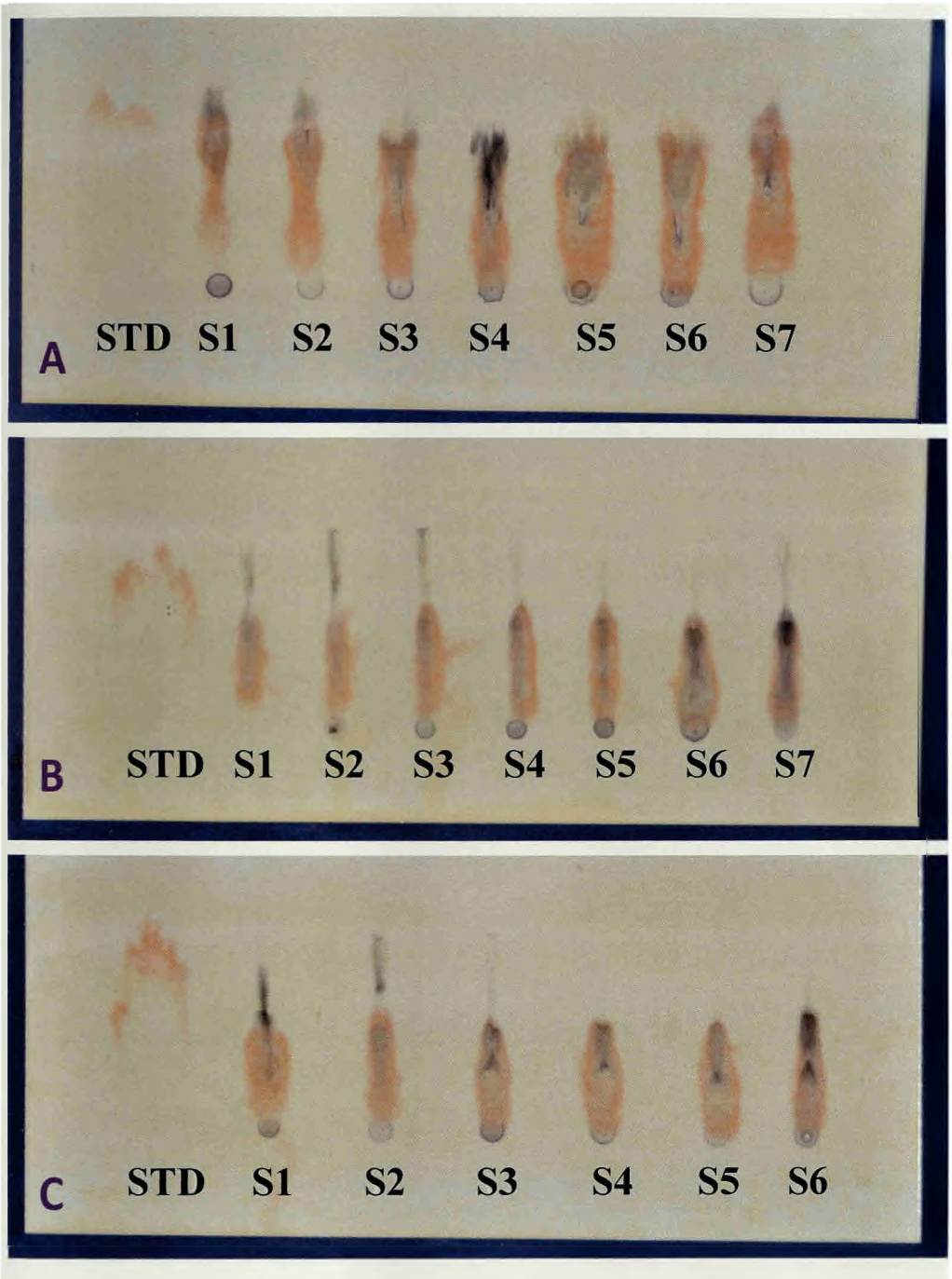


Plate 19. The thin layer chromatography (TLC) of different stages of development of fruits of *Garcinia indica*. A. Fruit extract of Marcel tree; B. Fruit extract of Priol tree; C. Fruit extract of Ponda tree. STD. standard; S1, stage 1; S2, stage 2; S3, stage 3; S4, stage 4; S5, stage 5; S6, stage 6;S7, stage 7.

development stage of fruit (50 days after fertilization) which recorded 8 g HCA/100g fruits. HCA content was 6.30 g/100g fruit in the second developmental stage of fruit (35 days after fertilization). Minimum quantity of HCA (4.86 g/100g fruits) was recorded in the early stages of fruit development (stage 1, about 20 days after fertilization).

4. 12. 1. 2. 2. HCA content of Tree No. 2 at Priol

HCA content of fruits at various stages of development collected from a place called Priol, Goa, is summarized in Table 26. Maximum quantity of HCA (10.56 g/100g fruits) was recorded in fruits of 7th development stage (about 85 days after fertilization) by HPLC system. 8.98 and 8.72 g of HCA/100g fruits was calculated in stage 6 (80 days after fertilization) and stage 5 (75 days after fertilization), respectively. Moderate amount of HCA (7.59 and 7.11 g/100g fruits) was noted in the 4th (60 days after fertilization) and 3rd developmental stage (50 days after fertilization) of fruits, respectively. The 2nd developmental stage (35 days after fertilization) measured HCA content of 5.65 g/100g fruits. Lowest HCA content (4.14 g/100g fruit) was recorded in 1st developmental stage of the fruits.

4. 12. 1. 2. 3. HCA content of Tree No. 3 at Ponda

Fruits at six developmental stages were collected from a tree selected for the study in Ponda (Tree No.3). HCA content of first stage to sixth stage of development of fruits ranged from 6.40-12 g/100g fruits (Table 26). The ripened fruits (stage 6) recorded the maximum HCA content of 12.00 g/100g of fruits. The fruits just before ripening (stage 5) contained 11.18 g/100g of HCA, followed by medium quantity of HCA (8.14 g/100g) in stage 4 (65 days after fertilization). The fruits of developmental stage 3 (50 days after fertilization) showed HCA content of 7.63 g/100g fruits. Lower levels of HCA (6.40 and

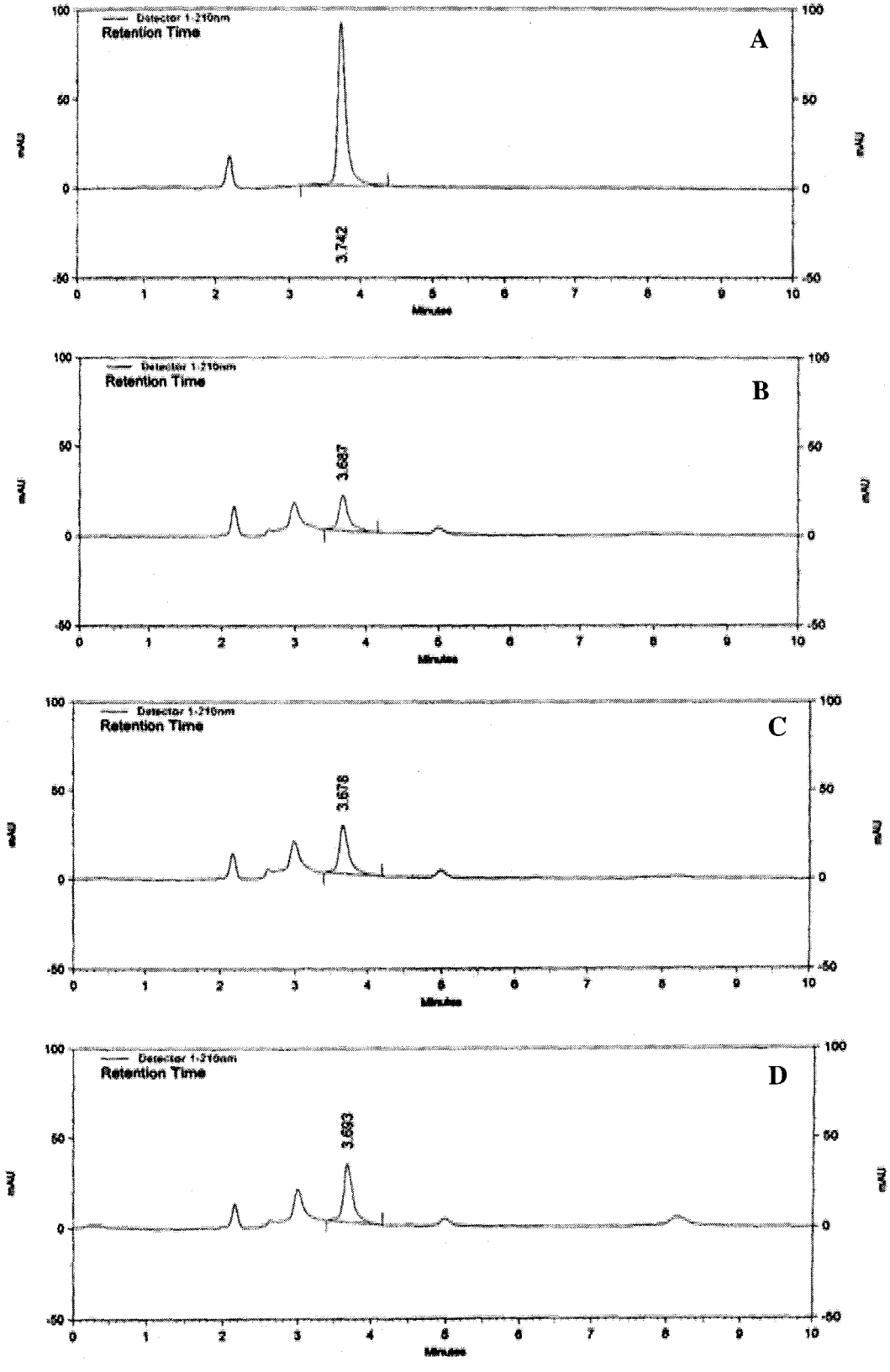


Fig. 26. HPLC chromatogram of HCA of fruit extract of *Garcinia indica* (Tree No. 1, Marcel). A. Standard HCA; B. Stage 1; C. Stage 4; D. Stage 7.

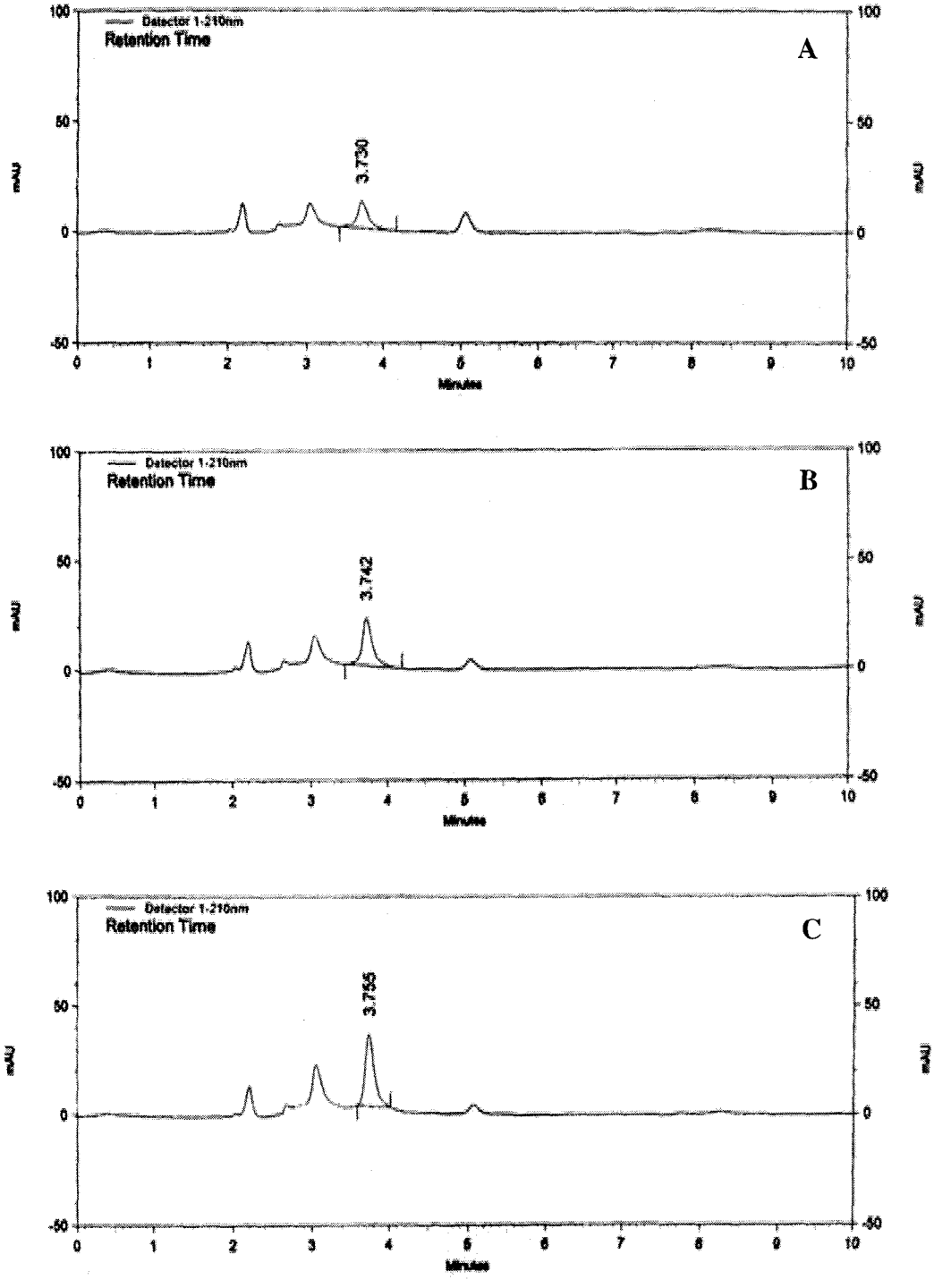


Fig. 27. HPLC chromatogram of HCA of fruit extract of *Garcinia indica* (Tree No. 2, Priol). A. Stage 1; B. Stage 4; C. Stage 7.

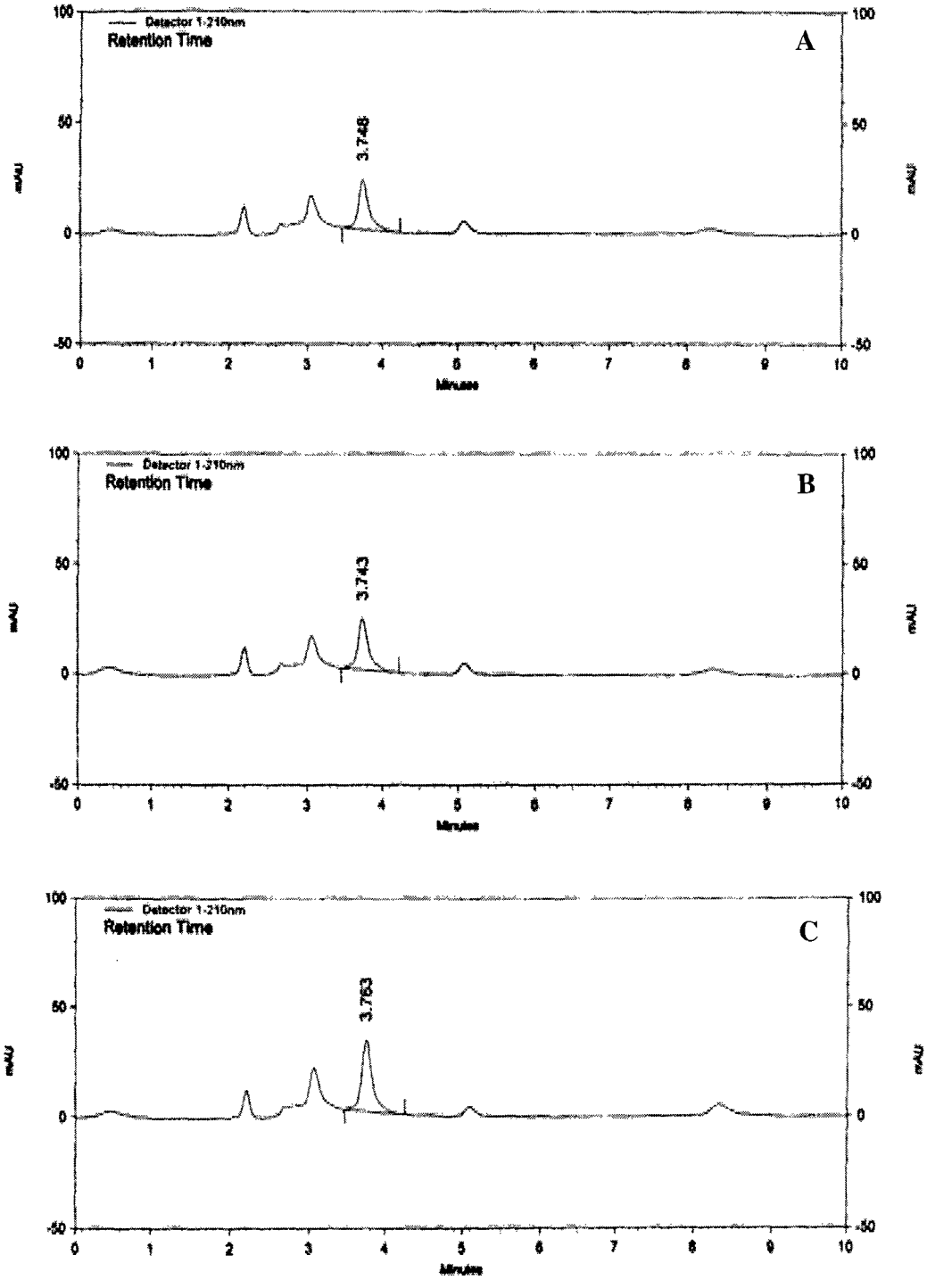


Fig. 28. HPLC chromatogram of HCA of fruit extract of *Garcinia indica* (Tree No. 3, Ponda). A. Stage 1; B. Stage 4; C. Stage 6.

Table 26. HCA content of different developmental stages of fruits of *Garcinia indica* by HPLC analysis.

Trees and Location	Samples	HCA (g/100g)
Tree No. 1 (Marcel)	Stage 1 (20 days after fertilization)	4.86
	Stage 2 (35 days after fertilization)	6.30
	Stage 3 (50 days after fertilization)	8.00
	Stage 4 (60 days after fertilization)	8.53
	Stage 5 (75 days after fertilization)	9.59
	Stage 6 (80 days after fertilization)	10.70
	*Stage 7 (85 days after fertilization)	11.45
Tree No. 2 (Priol)	Stage 1 (20 days after fertilization)	4.14
	Stage 2 (35 days after fertilization)	5.65
	Stage 3 (50 days after fertilization)	7.11
	Stage 4 (60 days after fertilization)	7.59
	Stage 5 (75 days after fertilization)	8.72
	Stage 6 (80 days after fertilization)	8.98
	*Stage 7 (85 days after fertilization)	10.56
Tree No. 3 (Ponda)	Stage 1 (20 days after fertilization)	6.40
	Stage 2 (35 days after fertilization)	6.96
	Stage 3 (50 days after fertilization)	7.63
	Stage 4 (65 days after fertilization)	8.14
	Stage 5 (75 days after fertilization)	11.18
	*Stage 6 (80 days after fertilization)	12.00

*Matured fruits (ripened)

6.96 g/100g fruits) were recorded in the fruits at developmental stage 1 (20 days after fertilization) and stage 2 (35 days after fertilization), respectively.

Hydroxycitric acid was the major organic acid present in the fruit extracts of *Garcinia indica*. Amongst the different developmental stages of fruits collected from three different locations, HCA content ranged from 4.14 to 12 g/100 g of fruits. Early stage of fruit development (20 days after fertilization) recorded the lowest quantity of HCA (4.14 g/100g of fruits) in tree from Priol (Tree No.2), Goa. Highest quantity of HCA (12 g/100g) was recorded in the mature fruits (80-85 days after fertilization) in tree at Ponda, Goa. Mature completely ripened fruits collected from all locations recorded the HCA content from 10.56 to 12 g/100g of fruits (Table 26).

Amongst the three different locations, mature fruits collected from Ponda detected maximum quantity of HCA (12.00 g/100g), followed by Marcel (10.70 g/100g). Least quantity of HCA was detected in fruits collected from Priol area (10.56 g/100g). Amongst the various different stages of development of fruits, early stage of development of fruit (stage1) collected from three different locations (Marcel, Priol, Ponda) recorded lower content of HCA. The lowest HCA of 4.14/100g was detected in the early stage of development of fruits (stage1) collected from Priol (Table 26).

4. 12. 1. 3. Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis of HCA

The liquid chromatographic peak at RT 0.49-0.91 (Fig. 33A) was analyzed using the mass spectrometry (MS) interface of negative ion mode $[M–H]^-$ recorded the molecular mass as 207 (Fig. 33C). The LC peak of 0.68-0.61 RT (Fig. 33A) was also analyzed using the MS interface of positive ion mode $[M+H]^+$ recorded the molecular mass as 209 (Fig. 33B). When MS analysis carried out using negative ion mode, the

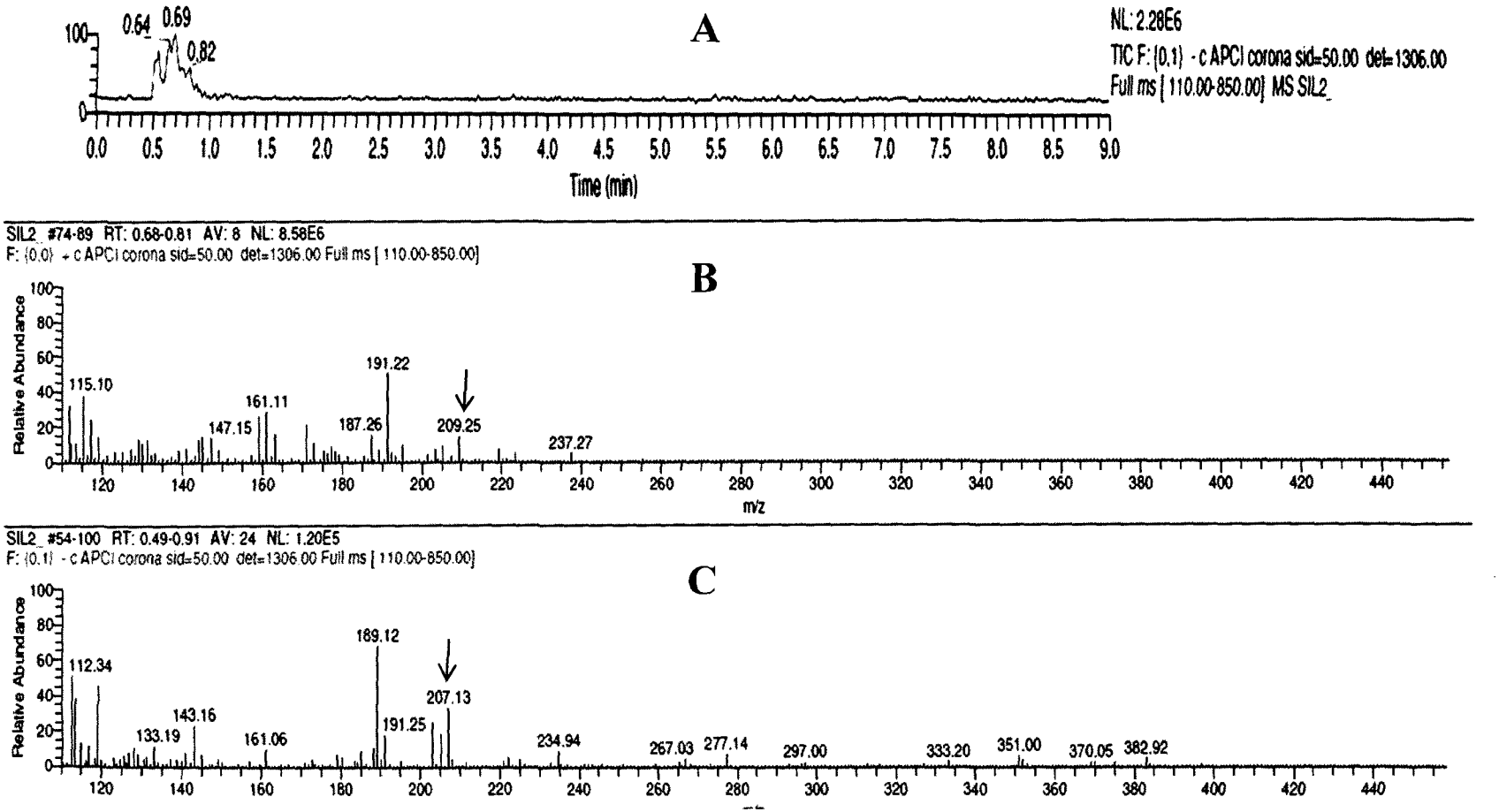


Fig. 33. LC-MS chromatogram of hydroxycitic acid from fruit extract of 50 DAF of *Garcinia indica*. A. LC chromatogram; B. Mass spectrum taken in positive ionization mode, arrow indicate the molecular mass of HCA; C. Mass spectrum taken in negative ionization mode, arrow indicate the molecular mass of HCA.

molecular mass obtained (207) +1 (plus one) indicates the actual molecular mass of HCA 208. Similarly, when MS analysis carried out using positive ion mode, the molecular mass obtained (209) -1 (minus one) indicates the actual molecular mass of HCA 208.

4. 12. 2. Biochemical Analysis of Garcinol

4. 12. 2. 1. Determination of Garcinol by HPLC System

The HPLC analysis of garcinol standard resolved with single peak at retention time (RT) 1.6 (Fig. 34A). However, the samples of various developmental stages of fruits collected from all three locations revealed with two prominent peaks vary close to each other at RT 2 (Fig. 34B, C, D). The resolving of two peaks may due to the fragmentation of garcinol or unstable nature of garcinol (high sensitive to oxygen and existence of large number of isomers). Because of this high sensitivity and presence of numerous isomers in Garcinol, quantitative estimation of garcinol could not be possible and needs further study.

4. 12. 2. 2. Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis of Garcinol

The liquid chromatographic peak resolved at RT 5.77-5.83 was further analyzed using the mass spectrometry (MS) interface negative ionic mode $[M–H]^-$ recorded the molecular mass as 601 (Fig. 35B). Since, the MS analysis carried out using negative ion mode, the molecular mass obtained (601) +1 (plus one) indicates the actual molecular mass of garcinol 602. During this investigation, the presence of garcinol in fruit extract was confirmed using LC-MS (Fig. 35A, B).

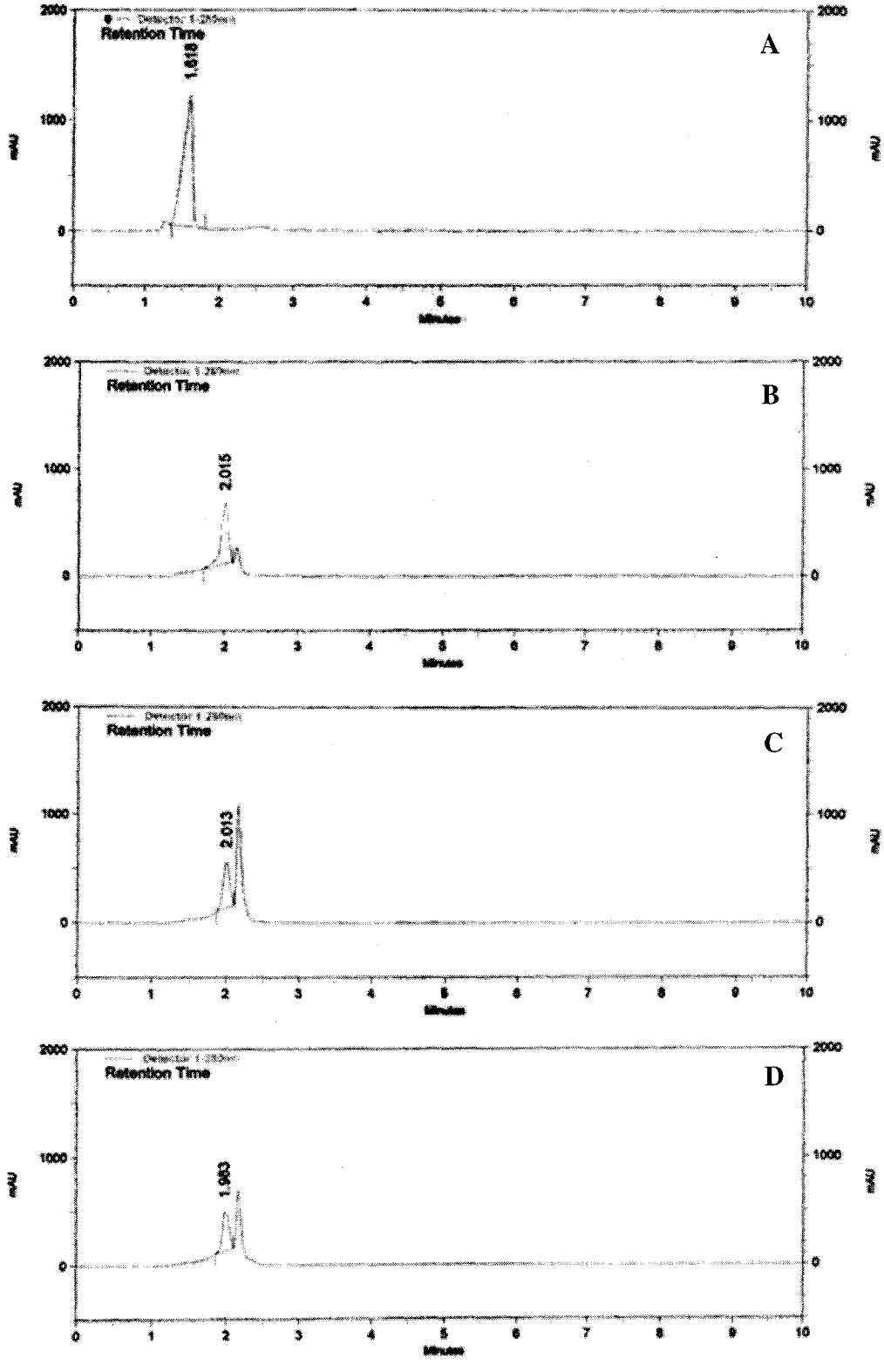


Fig. 34. HPLC chromatogram of garcinol of fruit extract of *Garcinia indica* (Tree No. 1, Marcel). A. Standard HCA; B. Stage 1; C. Stage 4; D. Stage 7.

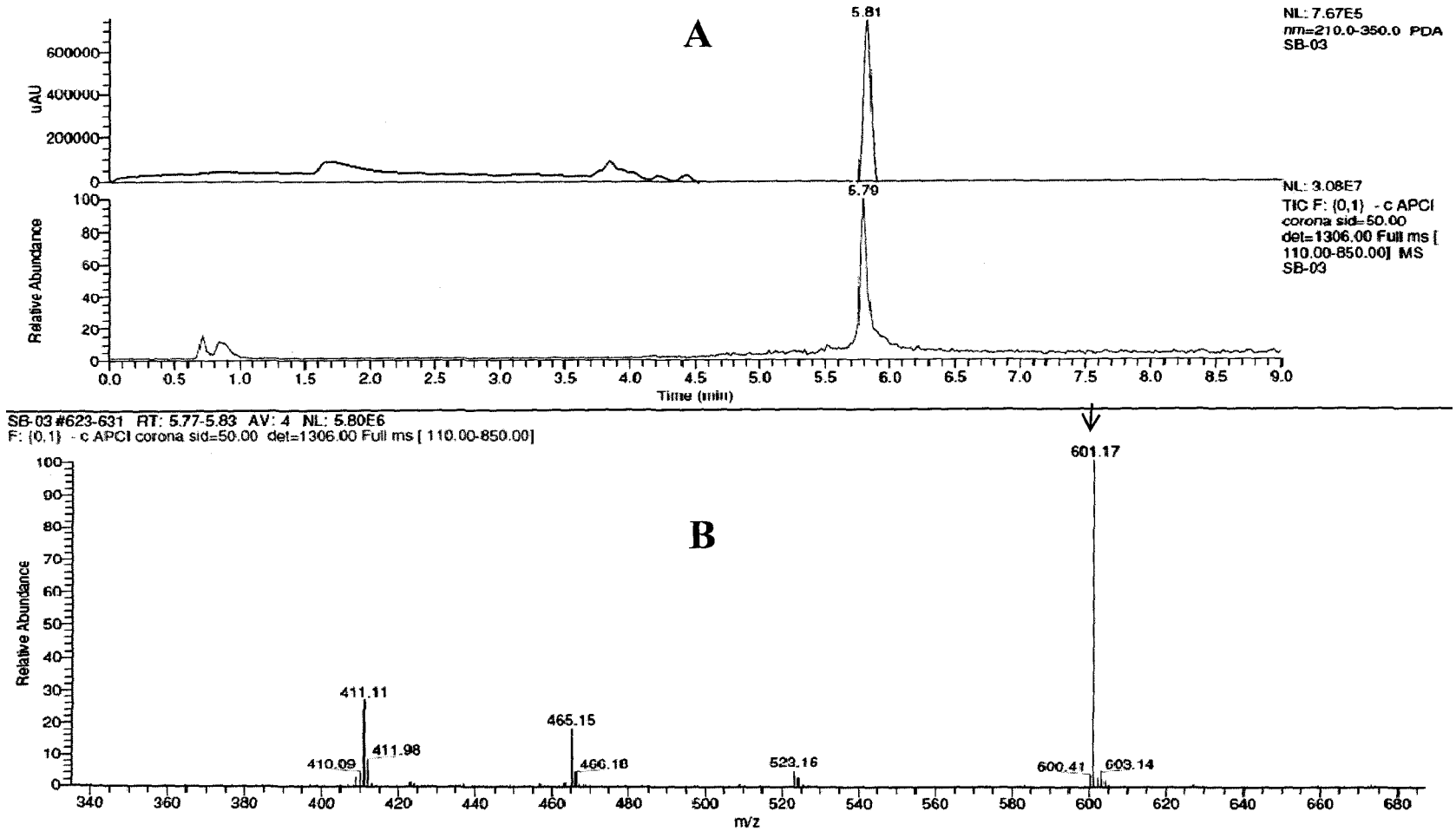


Fig. 35. LC-MS chromatogram of garcinol from fruit extract of 50 DAF of *Garcinia indica*. A. LC chromatogram; B. Mass spectrum taken in negative ionization mode, arrow indicate the molecular mass of garcinol.

4. 13. Developmental and Histochemical Studies

4. 13. 1. Developmental Studies

4. 13. 1. 1. Floral Characteristics

The systematic observation of *Garcinia indica* trees reveal three different types of trees (polygamodioecious nature): (i) Trees with only male flowers; (ii) Trees with only female flowers; (iii) Trees with bisexual flowers and the same tree containing male flowers. Flowering in Kokum starts during the month of November and continues up to February. Fruiting season is usually from April to June. The flowers are axillary or terminal, solitary or clusters. Each flower has four sepals, thick, fleshy, green in colour (Plate 20G, H, I). Petals are four, yellow to purple in colour. Male flower has numerous short filamented anthers (Plate 20A, D, G), two lobed and each lobe has two locules (tetraloculate anthers). It was observed that in bisexual flowers ovary is with short style and 3-4 stigmatic lobes (Plate 20B). Female flower is mostly solitary, ovary with sessile style and 6 stigmatic lobes (Plate 20C). Ovary superior (Plate 20F); ovules are orthotropous, axial placentation, each ovary has 4-8 locules and 4-8 ovules.

Flowering in male trees starts one week earlier than other trees. It was observed that the male flower has long pedicel, female flower has short pedicel and bisexual (hermaphrodite) flowers have intermediate length of pedicel (Plate 20D, E, F). Within bisexual (hermaphrodite) trees, variation was observed with reference to number of flowers, size of the flowers, length of the pedicel (stalk) and number of stamens (9-24). The male flowers contain more number of stamens per flower (36 to 38) in comparison to bisexual flowers.

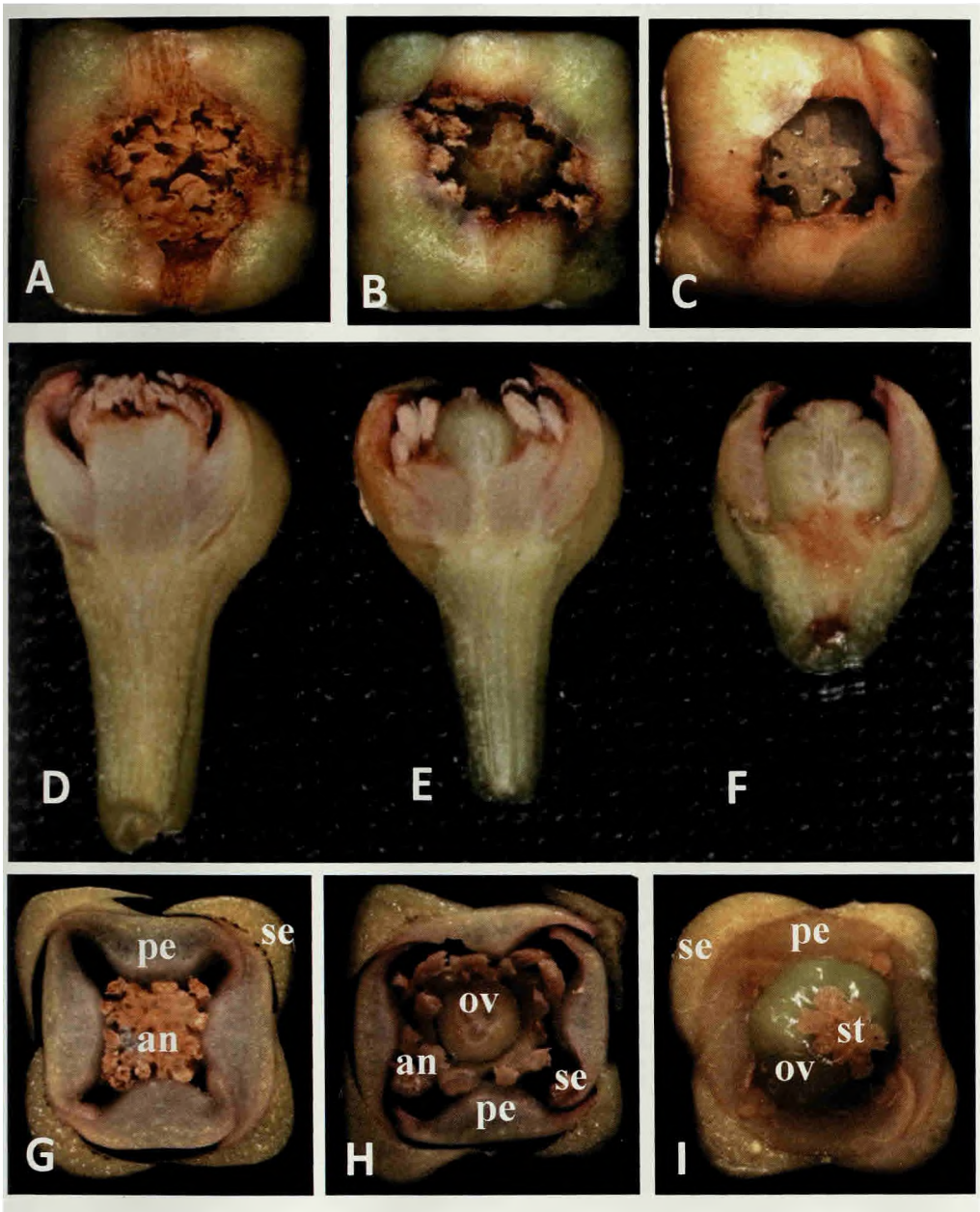


Plate 20. Different types of flowers found in *Garcinia indica*. A, D & G. Male flowers showing numerous anthers, four petals and four sepals; B, E & H. Bisexual flowers. B. Distinct ovary with four lobed stigma surrounded by anthers; E. Showing the superior ovary; H. Ovary surrounded by filamentous anthers, four sepals and four petals; C, F & I. Female flowers. C. Ovary with six lobed stigma; F. Female flower showing the short pedicel and superior ovary; I. Female flower showing ovary with distinct stigmatic lobes. an, anthers; pe, petal; ov, ovary; se, sepal; st, stigma. All X8.

4. 13. 1. 2. Structure and Development of Fruit

At the time of anthesis, the outer most layer of ovary consists of uniseriate epidermis with small rectangular cells. Thin cuticle was found over the epidermis. The few cell layers of ovarian mesophyll are present in between outer and inner epidermis. The ovarian mesophyll cells are found to be fundamental parenchyma, arranged compactly with few intercellular spaces. In between the ovarian mesophyll cells, large number of branched laticifers was observed. The inner epidermis differentiated in to elongated cells of endocarp which at fruit ripening stage forms part of the pulp. During the growth of ovary, the cellular divisions occur in all directions in the entire ovarian mesophyll cells. Immediately after fertilization, the developing fruit does not show any distinct exocarp, mesocarp and endocarp (pulp). They appear as homogenous mass of cells (Plate 21).

About 5 days after fertilization (DAF), the outer epidermis and sub epidermal cells of the ovary develops into 2-3 layers of exocarp. The ovarian mesophyll cells differentiate in to several layers of mesocarp. The mesocarp cells are tangentially elongated. At this stage, laticifers increase in size and secrete the yellow latex. The mesocarp derived from the outer region of ovarian mesophyll cells (many layered) is actually derived from the ventral meristem. Similarly, the endocarp (parenchymatous cells) were also derived from a ventral meristem and developed from inner part of the inner region of ovarian mesophyll (few layer) and the inner epidermis which delimits the locule. Laticifers or secretory ducts were absent in endocarp region (Plate 21). The peripheral region of endocarp shows vascular bundles (Plate 23B). At this stage, the middle and outer region of endocarp contains several developing juice cells (Plate 21).

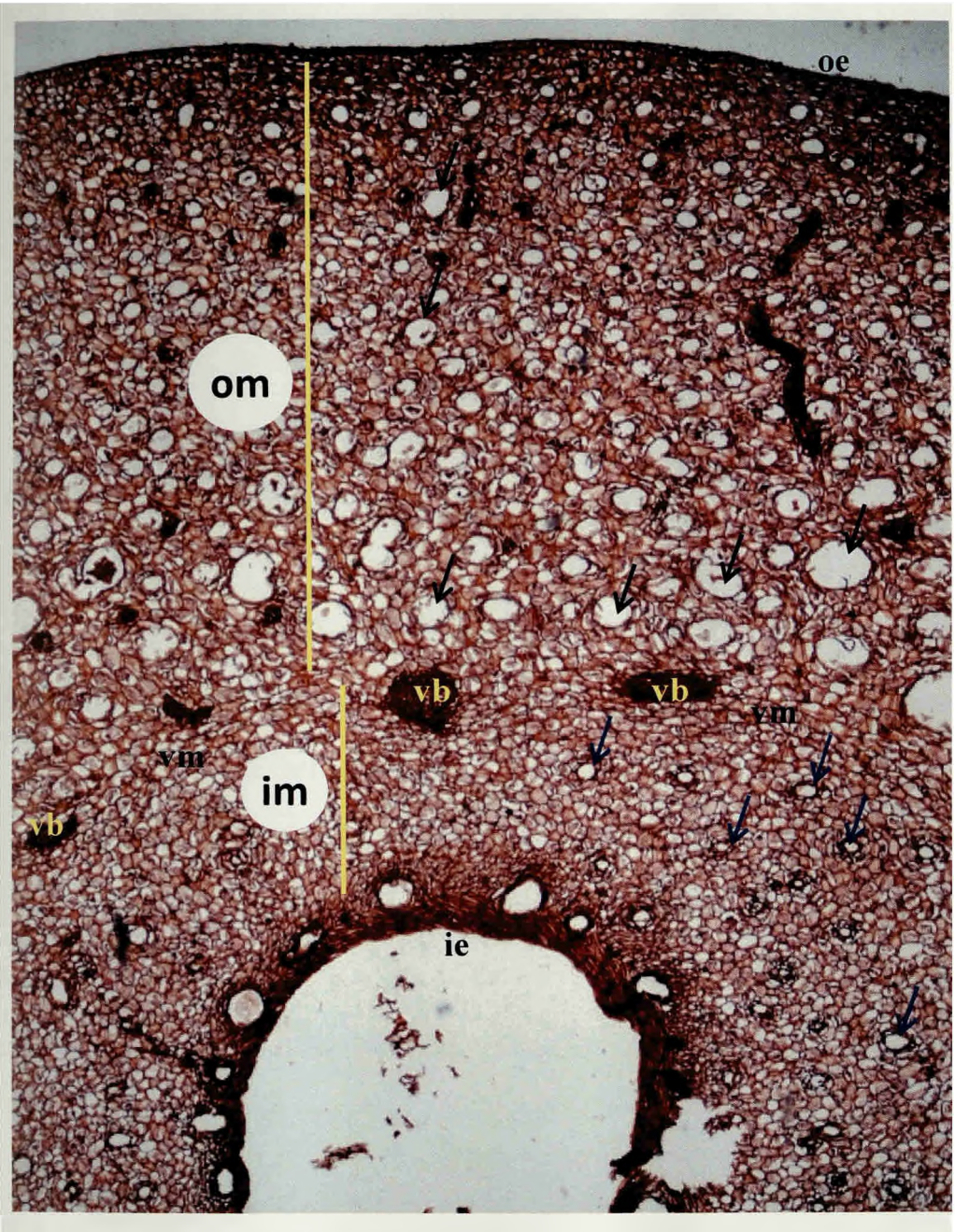


Plate 21. Transverse section of young fruit of 5 DAF of *Garcinia indica* stained with safranin showing different parts of fruit. The outer epidermis (oe), outer region of ovarian mesophyll cells (om), inner region of ovarian mesophyll cells (im), inner epidermis (ie), ventral meristem (vm), vascular bundle (vb), developing juice cells (blue arrows) and laticifer cells (black arrows).

Later, inner cell layers of the endocarp undergo a radial elongation as the seed develops and it firmly attaches to the fibrous testa (Plate 23C). About 15 days after fertilization (DAF) fruit showed the exocarp, mesocarp and endocarp (Plate 23A). Juice cells always occur as group (Plate 23D). Fruit 70 DAF showed the distinct exocarp, mesocarp with non-functional laticifers and endocarp with juice cells (Plate 22, 23E). The matured fruits (ripened) usually have two distinct parts: rind and pulp along with seeds. The rind consists of exocarp and mesocarp, which stores primarily secondary metabolites i.e. hydroxycitric acid, garcinol and others. The rind also contains large quantity of anthocyanin pigments. The endocarp (pulp) is white, edible and rich in sugars (Plate 23F).

4. 13. 1. 3. Laticifers in Fruit

An early developmental stage of fruit has large number of laticifers, which secretes the yellow coloured latex. Longitudinal section of young fruit (5 DAF) was noted with a network of branched tubular laticifers (Plate 24A). The number of laticifers was more near the locular region and the pedicel region than towards the peripheral region of fruit (Plate 24C). It was observed that the size of the laticifers were larger towards the locular region in comparison with the peripherial region of the fruit (Plate 24D, E). Transverse section of fruit after anthesis showed dense laticifers, which are dark stained with safarinn (Plate 24B). In about to ripe fruits, it was observed that laticifers become non-functional and stops growth (Plate 24F).

4. 13. 1. 4. Structure and Development of Seed

At the time of anthesis, the ovule has large amount of nucellar tissue.Successful growth of nucellar tissue confirms seed setting. The nucellar tissue is semi-solid and grows towards the centre of the ovule (Plate 25B). Within the nucellar tissue, embryo sac develops (Plate 25C). After fertilization, zygote divides and the development of seed

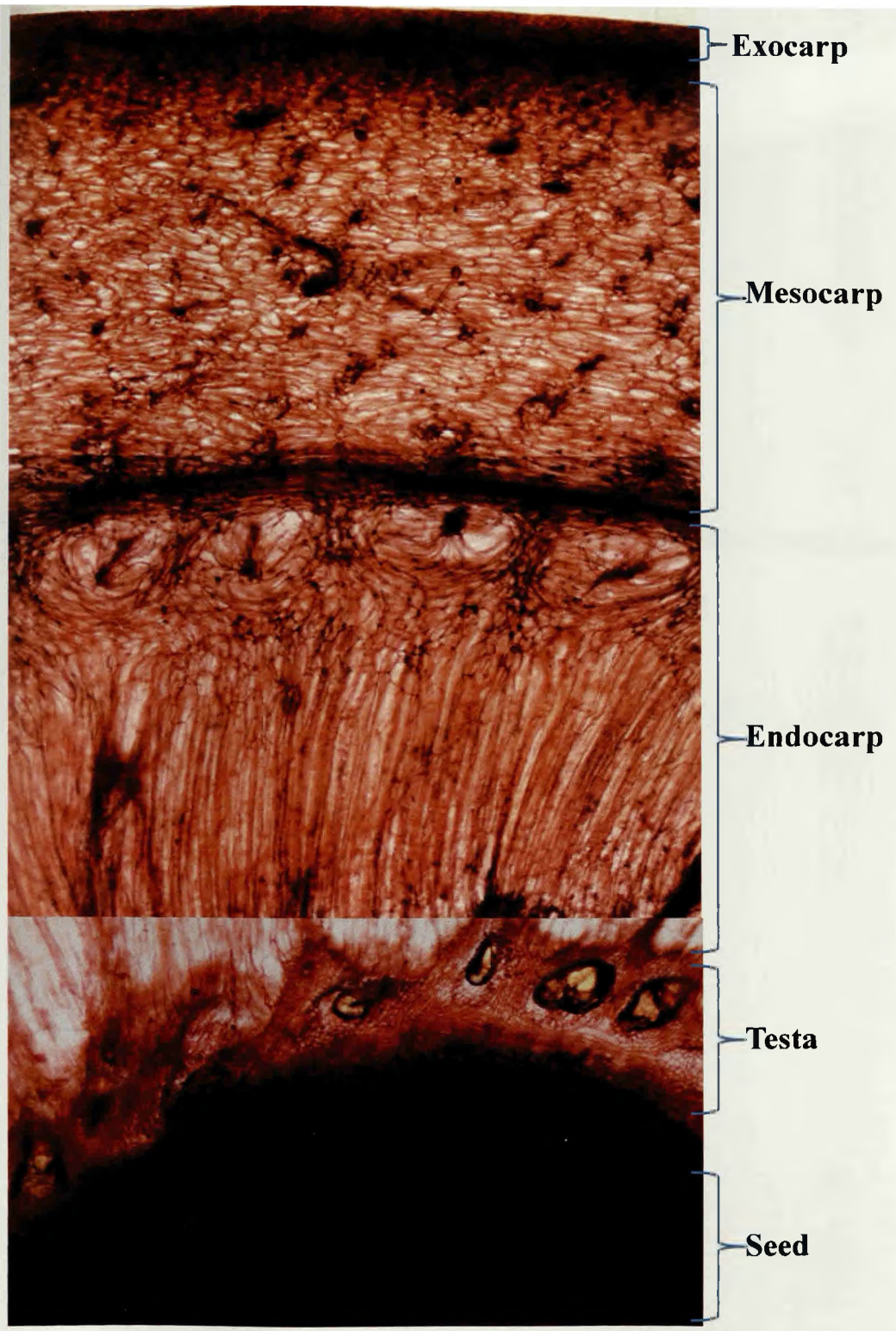


Plate 22. Transverse section of fruit 50 DAF of *Garcinia indica* stained with safranin showing major parts of fruit.

Plate 23. Structure and development of fruits of *Garcinia indica*. Transverse section of fruit stained with safranin.

A. Young fruit of 15 DAF showing early stages of development of exocarp, mesocarp and endocarp. X8

B. Young fruit of 5 DAF revealing the early stage of development of vascular bundle in the mesocarp and exocarp. X20

C. Fruit of 50 DAF showing the juice cells and endocarp. X20

D. Enlarged view of juice cell. X100

E. Section of 70 DAF fruit with distinct exocarp, mesocarp with non-functional laticifers and endocarp with juice cells. X20

F. Ripened fruit of 85 DAF with rind and pulp.

en, endocarp; ex, exocarp; jc, juice cell; l, laticifers; mc, mesocarp; p, pulp; r, rind; vb, vascular bundle.

Plate - 23

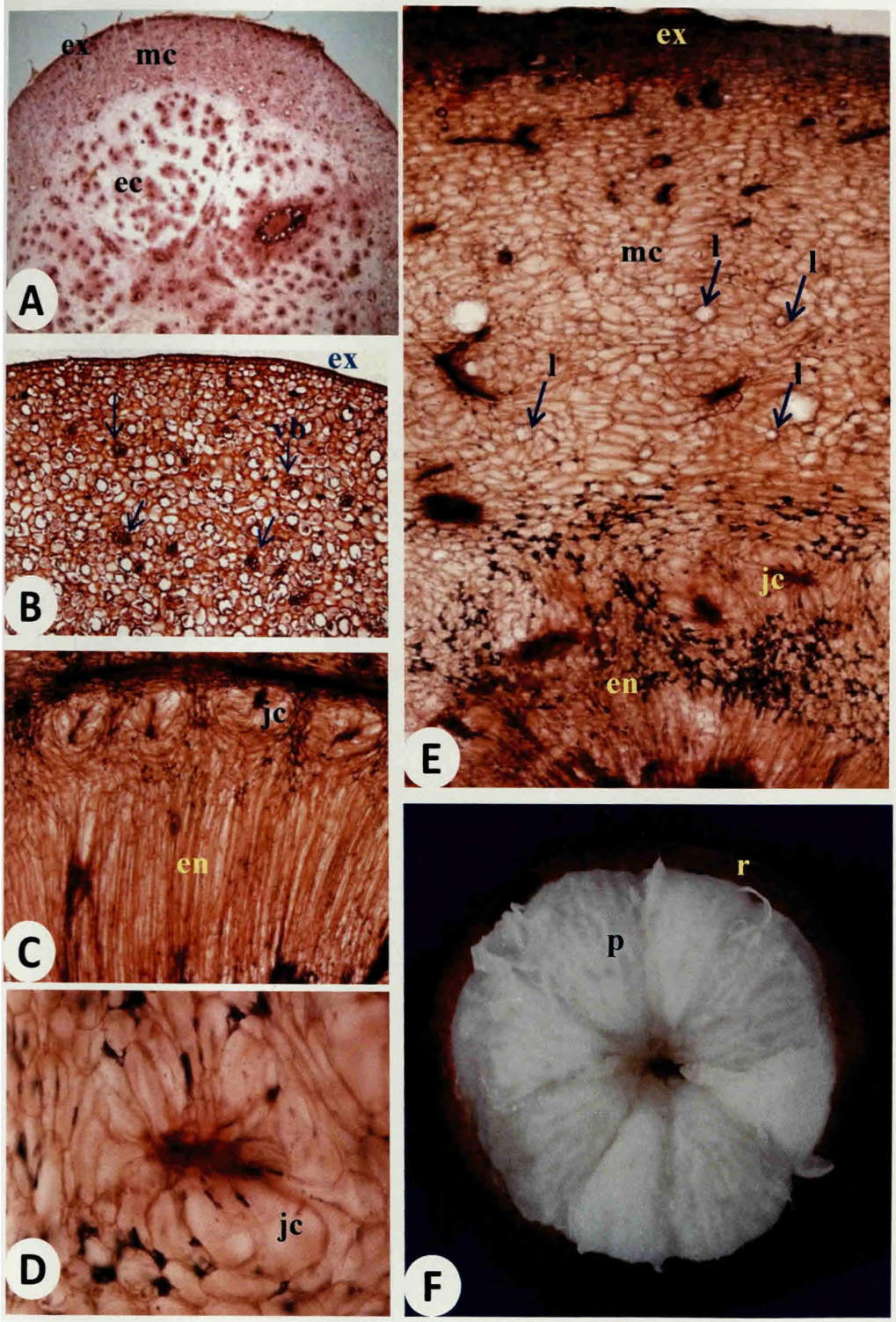


Plate 24. Structure and development of laticifers in the fruits of *Garcinia indica.*

A. Longitudinal section (LS) of early stage of fruit showing laticifers in ovary wall, pedicel and sepal. X8.

B. Transverse section (TS) of fruit of 5 DAF stained with safranin, laticifers stained dark. X20.

C. LS of portion of fruit stained with safranin showing the net work of laticifers. X50.

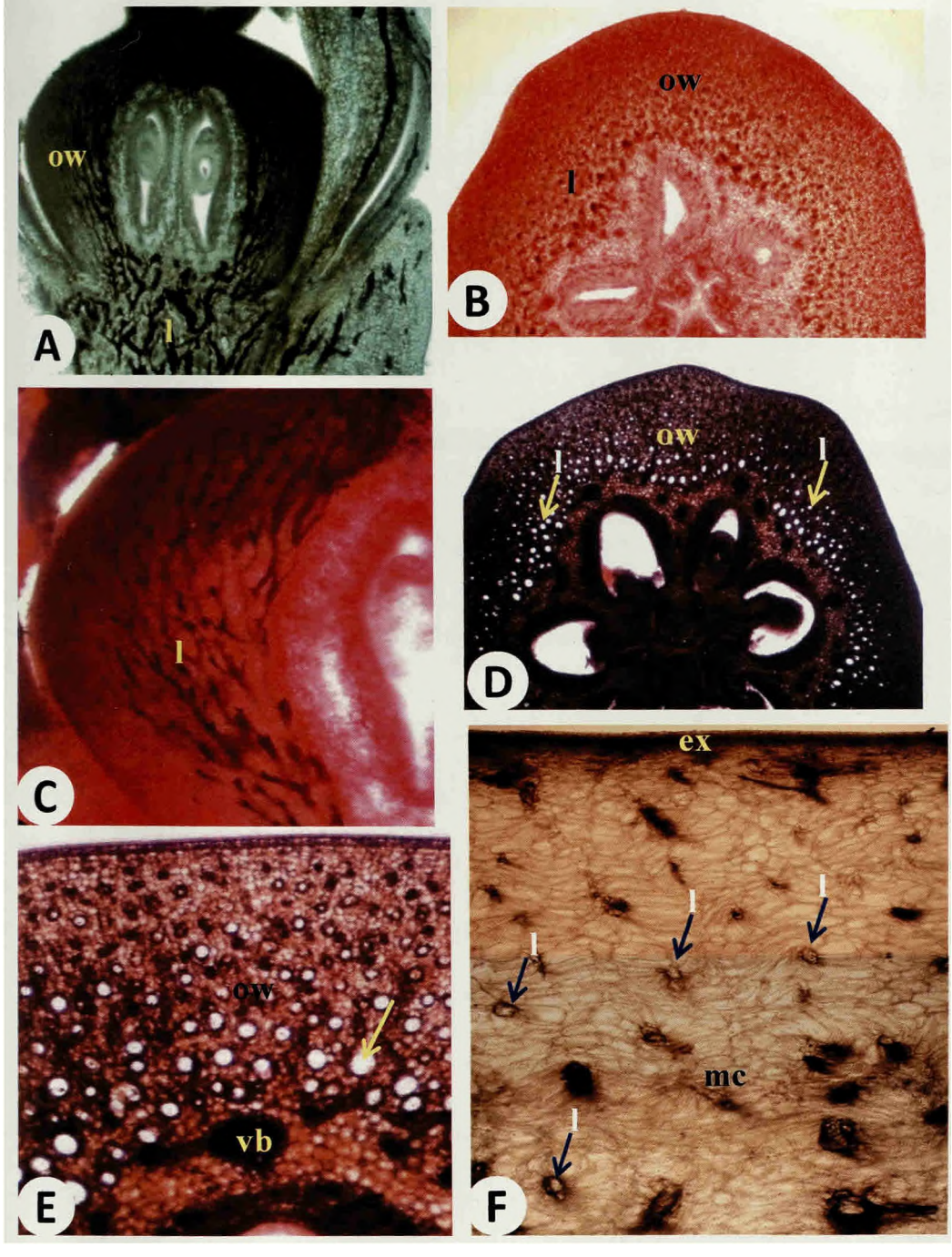
D. TS of fruit of 10 DAF stained with safranin showing large number of empty cells which are actually tubular laticifers in the mesocarp. X20.

E. Enlarged view tubular laticifers in mesocarp region. X50.

F. TS of ripened fruit rind showing non-functional laticifers in the mesocarp (blue arrows). X20.

ex, exocarp; l, laticifers; mc, mesocarp; ow, ovary wall; vb, vascular bundle.

Plate - 24



starts. In a single fruit, 4-8 seeds develop (Plate 25A). Seed maturation occur form periphery towards the centre. About 15 DAF, peripheral regions of seed gets filled with seed storage parenchyma cells and central region appears empty (Plate 25D). Even though the Kokum fruit contains 4-8 seeds, the observation revealed that the filling of seed storage parenchyma cells in kokum was much faster than in other seeds (Plate 25E). At this stage, when single seed was observed closely, the seed coat appeared brown indicating the early deposition of phenolic compounds (Plate 25F).

About 20 DAF, the outer most layers of seed storage parenchyma cells differentiates into distinct single layer of epidermis. At this stage, just below the epidermis and adjacent region of cells get organized into storage parenchyma cells (Plate 25G). The seed storage parenchyma cells get completely filled and no any empty space is observed in seeds, about 35 DAF (Plate 25H).

Transverse section of seed 50 DAF, noted the appearance of vasculature in near central portion of the seed which appears like a small ring like structure which stains darkly (Plate 26A). The vascular structures run almost in the entire length of the seed, usually starting from one side of the top portion of the seeds and ending towards the bottom on other side (Plate 26B). When the seed with vasculature region was stained with Sudan IV, it did not stain the vascular region; however, lipids were localized in the cells present in the inner side and outer side of the vasculature (Plate 26C). The vasculature consists of procambial strand which acts as an embryonic tissue during germination. When seeds were germinated, it was observed that the shoot emerges out from the top portion of seed and radical arise from the bottom portion of the seed. Longitudinal section of mature seed stained with toluidine blue revealed the presence of spiral thickening of tracheary elements with alternate simple pits in the protoxylem elements (Plate 26D). Transverse

Plate 25. Structure and development of fruit and seed of *Garcinia indica.*

A. Transverse section of female flower showing the ovary with seven ovules with axial placentation. Note the yellow latex exudation on ovary surface. X10.

B. Transverse section of portion of ovary showing single ovule with nucellus tissue. X20.

C. Closer view of ovule showing nucellus tissue (brown in colour). X100.

D. Transverse section of fruit 15 days after fertilization (DAF) stained with safranin showing the development of seed and pulp (ovary wall). X4.

E. Transverse section of fruit 15 days after fertilization stained with toluidine blue showing seeds and pulp region. X4.

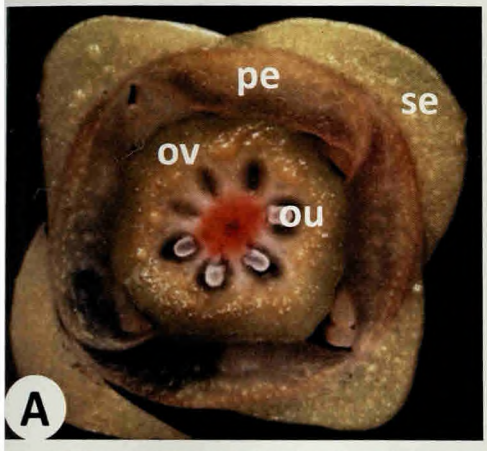
F. Transverse section of seed 15 DAF showing the central air space indicate the seed filling starts form peripheral towards centre and note the seed coat in early stage of development. X20.

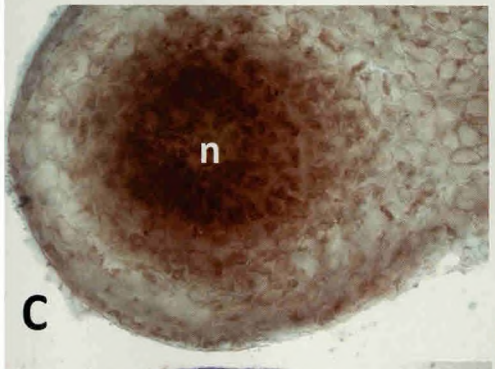
G. Single seed showing the storage parenchyma cells except in the central region and prominent seed coat. X20.

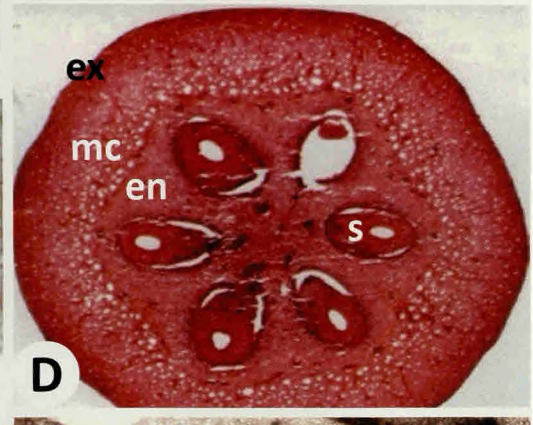
H. A portion of transverse section of fruit 35 DAF showing the storage parenchyma which completely filled seed. Note the exocarp, mesocarp and endocarp of fruit. X8.

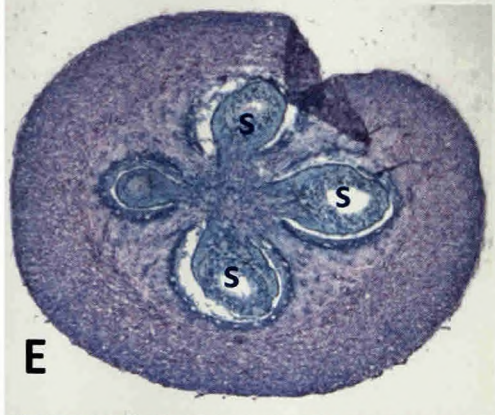
en, endocarp; ex, exocarp; ms, mesocarp; n, nucellus; ou, ovule; ov, ovary; pe, petal; s, seed; sc, seed coat; se, sepal.

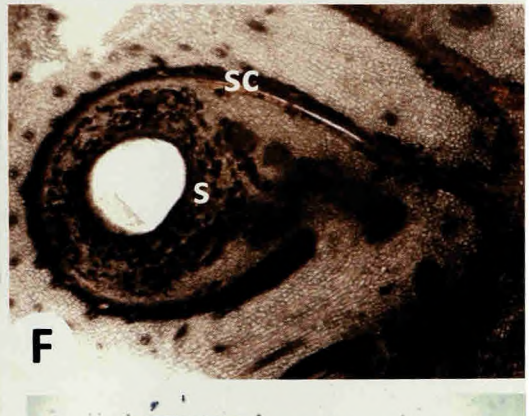
Plate - 25

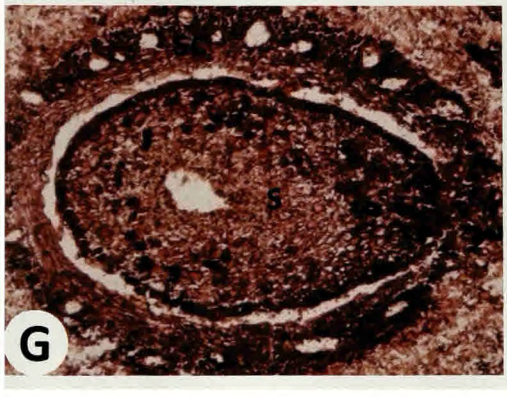












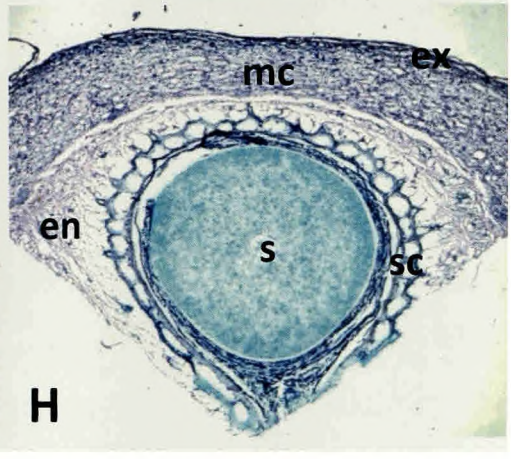


Plate 26. Structure and development of seed of *Garcinia indica.*

A. Transverse section of part of fruit 50 DAF stained with safranin, showing the development of vasculature in the seed (arrow). X4.

B. Overview of seed showing the vasculature structure (macro photograph).

C. Transverse section of seed 50 DAF stained with Sudan IV showing the vasculature, lipids in both inner side and outer side of the vasculature as shown by red staining. X20.

D. Longitudinal section of mature seed stained with toluidine blue showing the presences of annular and spiral thickening of tracheary elements in the vasculature. X50.

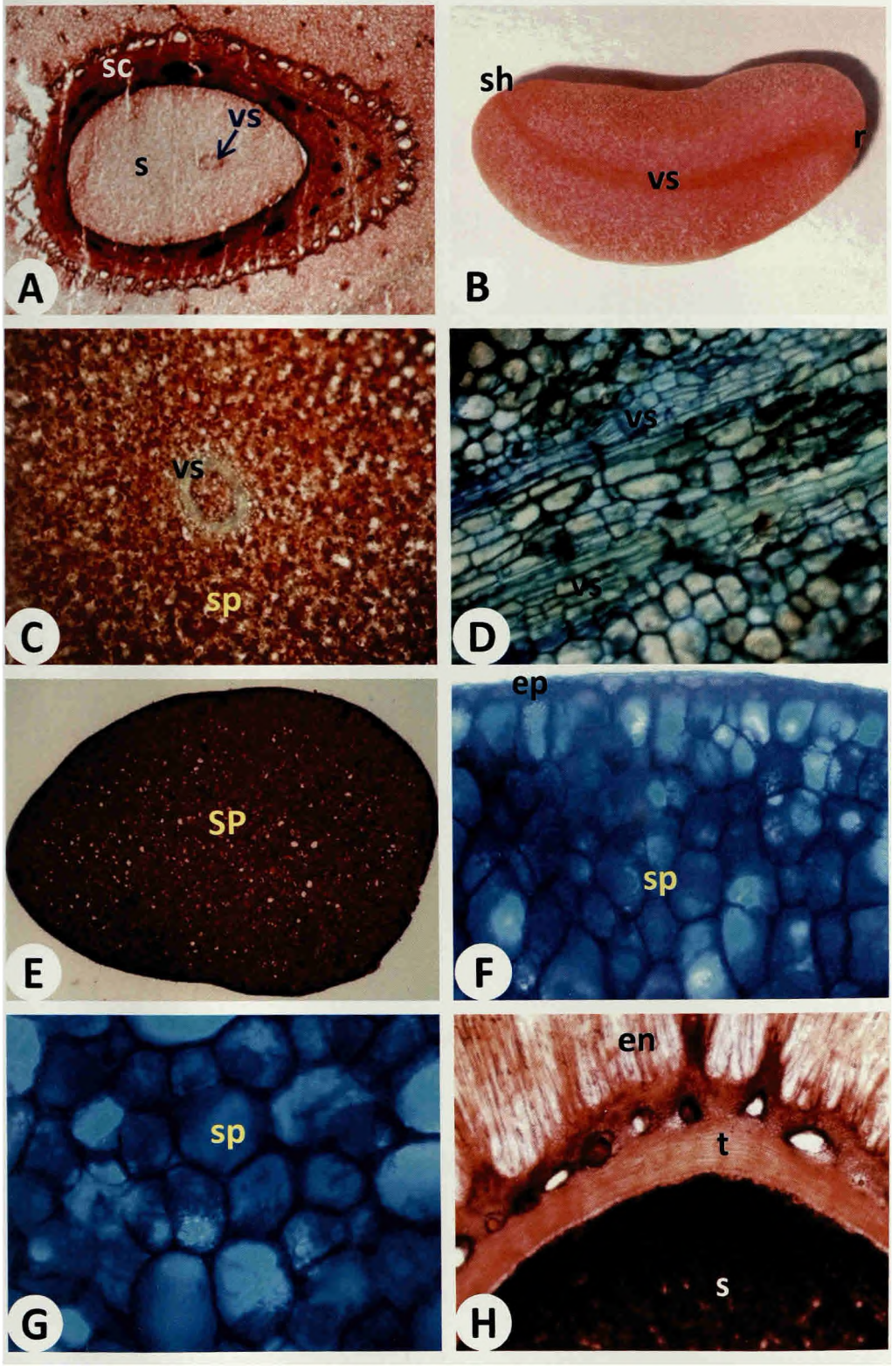
E. Transverse section of entire mature seed stained with safranin showing the storage parenchyma cells. X8.

F & G. Transverse section of portion of mature seed stained with toluidine blue showing single layer epidermis and storage parenchyma cells. F. X100; G. X200.

H. Transverse section of fruit 50 DAF showing seed, testa and endocarp. X20.

en, endocarp; ep, epidermis; r, root emergence; s, seed; sc, seed coat; sh, shoot emergence; sp, storage parenchyma; t, testa; vs, vasculature structure.

Plate - 26



section of mature seed stained with safranin shows the over view of compactly arranged storage parenchyma cells (Plate 26E). A portion of mature seed stained with toluidine blue showed distinct single layer of epidermis followed by several layer of rectangular storage parenchyma cells (Plate 26F). It was observed that the epidermal cells were comparatively much smaller than the storage parenchyma cell as shown in Plate 26F. The closer view of seed storage parenchyma cells shows that they were rectangular in shape and of non-uniform size (Plate 26G).

4. 13. 2. Histochemical studies

4. 13. 2. 1. Localization of Starch

The male, bisexual and female flowers stained with iodine- potassium iodide (I_2KI) revealed the presence of starch as indicated by brown-black deposits (Plate 27A-C). In male flowers, starch was observed in thalamus and petal regions (Plate 27A). In bisexual flowers, starch was localized in petal, base of the anther filament and thalamus regions (Plate 27B). In female flowers, starch was present in sepals, petals and ovary, however, starch deposition was high in the sepal and peripheral regions of the pedicel (Plate 27C).

The young ovary at anthesis stage was detected with minute quantity of starch in the nucellus and locular wall (Plate 27D). The fruit 15 DAF, showed starch deposition in the seed coat, in developing seed and in small quantity in the fruit (Plate 27E). Transverse section of seed about 35 DAF, showed starch all over, including epidermal cells (Plate 27F, G). Matured seed, 85 DAF, showed clear presence of starch deposits in each seed storage parenchyma cells at indicated by I_2KI. The starch was also observed in adjacent regions of the vasculature (inner and outer side of the vasculature), however, actual vasculature region was devoid of starch (Plate 27H, I).

Plate 27. Histochemical localization starch in fruit and seed of *Garcinia indica*. Longitudinal and transverse section of different stages of flower, fruits and seed stained with I_2KI.

A. Longitudinal section of the male flower observed with minute quantity of starch in pedicel, sepal, petal and anthers. X10.

B. Longitudinal section of the bisexual flower showing the presence of starch in sepal, petal and pedicel and no starch was observed in ovary. X10.

C. Longitudinal section of the female flower showing the presence of starch in entire pedicle, petal, sepal and very minute quantity of starch was detected in ovary. X10.

D. Transverse section of fruit of 5 DAF noted starch in the ovary wall and in fertilized ovule. X50.

E. Transverse section of fruit of 15 DAF the starch was observed in over all the fruit, but more concentration was noted in seed and seed coat. X20.

F. Transverse section of seed 20 DAF showing the presence of starch in entire seed, but patches of parenchyma cells detected with intense starch as it appeared black. X10.

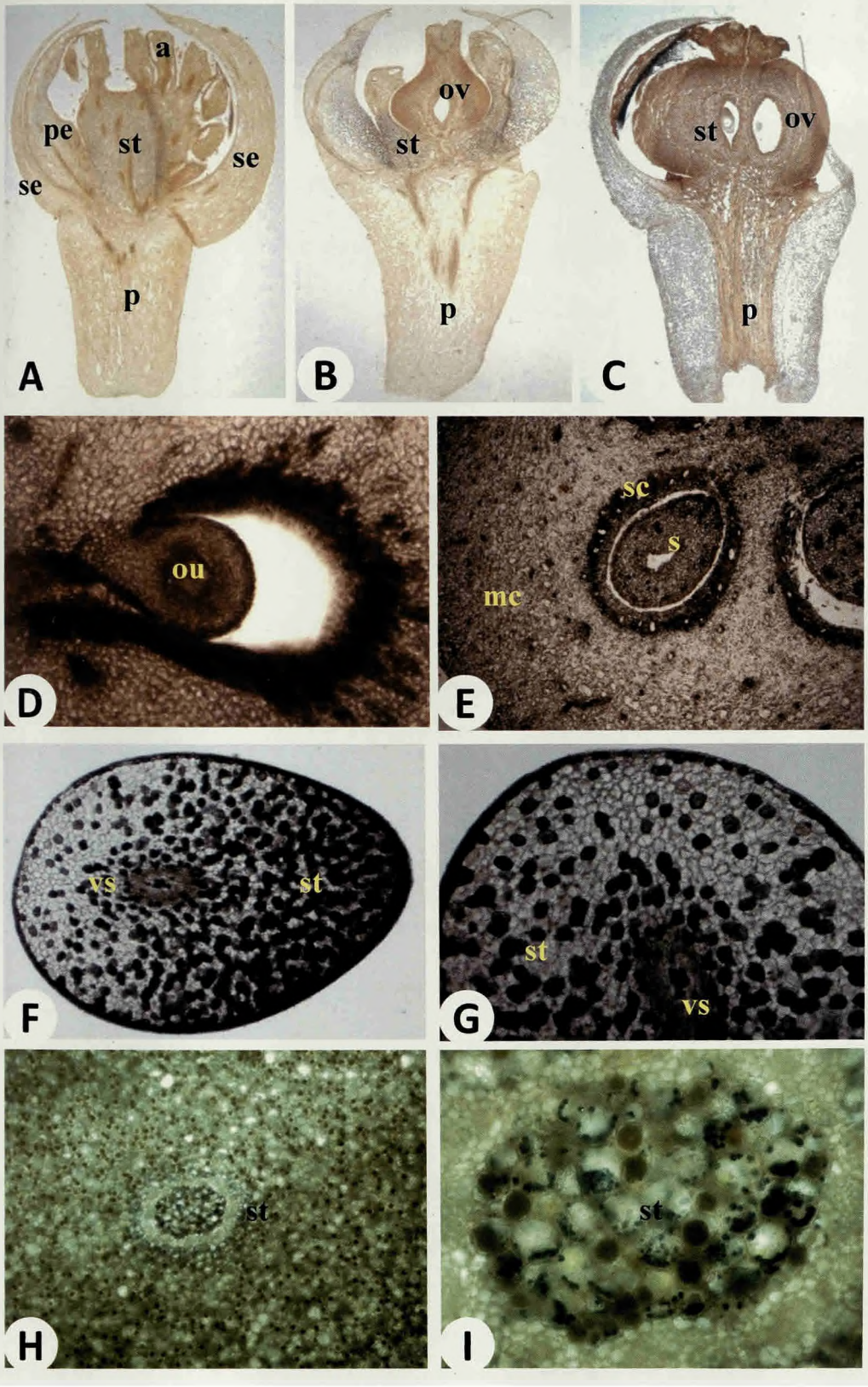
G. Enlarged portion of the F showing the starch accumulation in the rectangular parenchyma cells. X20.

H. Transverse section of mature seed noted with starch all over the seed, it appeared dark purple to black, cells in the inner side of the vasculature also detected with starch. X20.

I. Enlarged view of vasculature showing absence of starch in the xylem tissue, but inner side of the vasculature showed the presence of starch. X50.

a, anther; mc, mesocarp; ou, ovule; ov, ovary; p, pedicel; pe, petal; sc, seed coat; se, sepal; st, starch; vs, vasculature structure.

Plate - 27



4. 13. 2. 2. Localization of Proteins

Proteins were detected in seeds of Kokum as indicated by a fluorescent dye, 8-anilino-1-naphthalenesulfonic acid (ANS). In the influence of this dye, the proteins fluoresces whitish blue, under UV (330-380 nm) excitation filter, in fluorescent microscope. At early stage of development of seed no significant quantity of proteins was detected. The seeds about 20 DAF showed very small quantity of proteins as indicated by mild fluorescence (Plate 28A). Transverse section of seed 35 DAF, observed higher accumulation of proteins in comparison with early stages (Plate 28B). Transverse section of seed 50 DAF, revealed the presence of high concentration of proteins as indicated by bright whitish blue fluorescence (Plate 28C). Transverse section of seed 70 DAF (Plate 28D), 82 DAF (Plate 28E) and 85 DAF (Plate 28F) observed higher concentrations of proteins in comparison with early stages. The protein concentration increases as the seed matures. It was also noted that protein bodies are prominent and larger in size as the seed attains maturity.

4. 13. 2. 3. Localization of Lipids

The deposition of lipids in seed starts little earlier than proteins. Lipids were localized in seeds using a fluorescent dye Nile blue. Lipids fluoresces yellow under blue excitation filter (450-490 nm). The early stages of development of seed (15 DAF) detected low concentration of lipids as indicated by faint yellow fluorescence (Plate 29A). Transverse section of seed 50 DAF, showed higher fluorescence in comparison with early stages, however lipids bodies were not prominent as compared to later stages (Plate 29B). The seeds 70 DAF revealed higher accumulation of lipids as shown by intense fluorescence. It was noted that at this stage, the seed storage parenchyma cells got well organized and the lipid bodies were comparatively larger than early stages (Plate 29C). Transverse section of seed 85 DAF was localized with the highest quantity of lipids. It

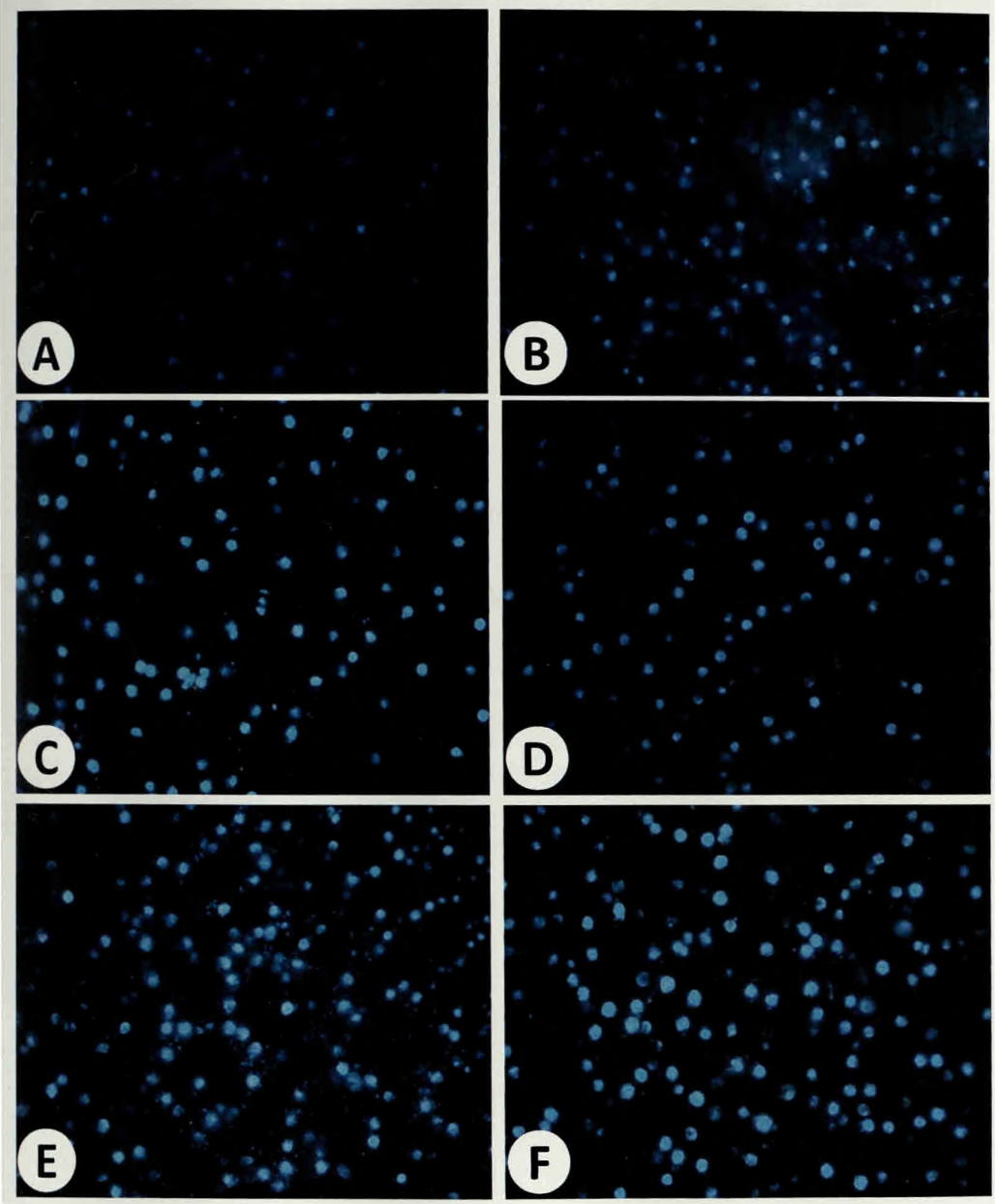


Plate 28. Histochemical localization of protein in the seed of Garcinia indica during various developmental stages. All sections were stained with 8-anilino-1-naphthalinesulphonic acid (ANS). A. Transverse section of seed 20 DAF showing the initiation of protein deposition as indicated by faint whitish blue fluorescence; B. Transverse section of seed 35 DAF slight increase protein concentration; C. Transverse section of seed 50 DAF; D. 70 DAF; E. 82 DAF; F. 85 DAF. Note the increased protein concentration during maturity of seed as compared to early stages, as shown by increased distinct fluoresces in the mature seed. All X50.

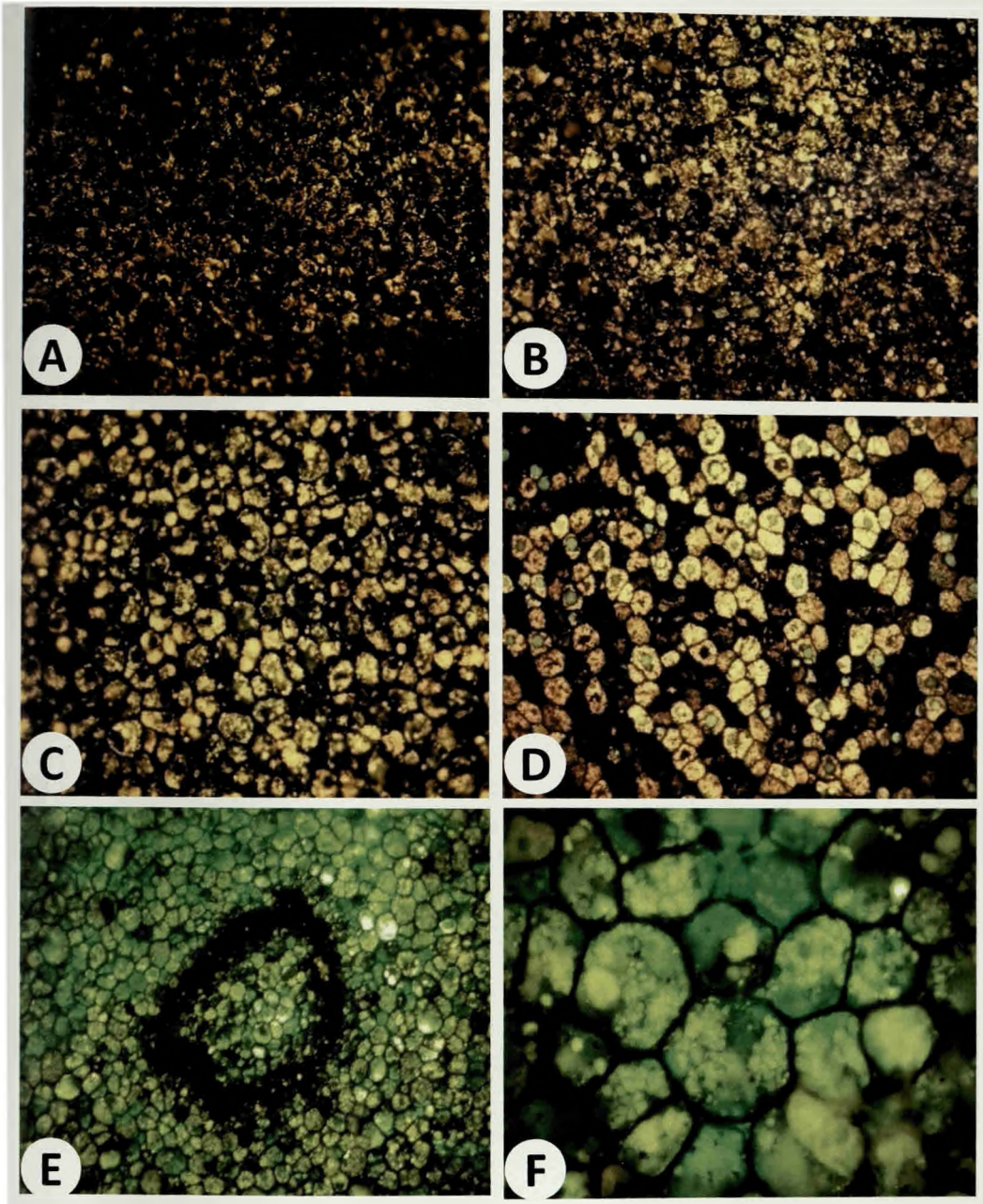


Plate 29. Histochemical localization of lipids in the seed of various developmental stages of *Garcinia indica*. All sections were stained with Nile blue under blue excitation filter (A-D) and green excitation filter (E & F). A. Transverse section of seed 20 DAF showing presences of less concentration of lipids as indicated by yellow fluoresces; B. Transverse section of seed 50 DAF for lipids; C. Transverse section of seed 70 DAF, lipids stored inside the rectangular storage parenchyma cells; D. Transverse section of seed 85 DAF, intense yellow fluorescence indicates the high concentration of lipids; E. Transverse section of seed 70 DAF showing the presence of lipids in the inner and outer side of the vasculature; F. Enlarged view of seed 70 DAF showing seed storage parenchyma cells with distinct lipid bodies. A-E. X50; F. X200.

was observed that early stages of development of seed contain very low concentration of lipids. As the seed matures and the seed storage parenchyma cells get well developed, the lipid concentration reaches to the maximum as shown by the fluorescent dye (Plate 29D). The matured seeds (70 DAF) show the single prominent vasculature structure. Although the vasculature itself does not store any lipids, lipids were detected in both inner and outer side of the vasculature tissue (Plate 29E). The enlarged view of seed storage parenchyma cells stained with Nile blue reveals the distinct range of lipid bodies from very small size to very large bodies (Plate 29F).

4. 13. 2. 4. Localization of Lignin and Tannins

Lignin was detected in seeds as well as in fruits of Kokum. Lignin was localized in mature seeds (85 DAF), in the vasculature structure, where xylem vessels are present. In the vasculature, the xylem vessels were found as a patch, as revealed by histochemical staining reaction with phloroglucinol which stains the lignin red. (Plate 30A, B). In fruits, lignin was found in laticifers structures and vascular tissues (Plate 30C, D). Tannins were localized only in the testa or seed coat. It was observed that the tannin deposition starts 20 DAF, as revealed by ferric chloride staining reaction (Plate 30 F). As the seed matures and seed coat development completes, the tannin concentration continuously increases and reaches a maximum level at maturity. The fruit 70 DAF without any staining reaction showed clear presence of tannins, as by black deposits in a macro-photograph (Plate 30 E).

4. 13. 2. 5. Localization of Hydroxycitric acid (HCA)

HCA was localized during various stages of Kokum fruit development, using a biochemical reaction of 1% sodium metavanadate. The thick sections of fruits were reacted with sodium metavanadate solution. Sodium metavanadate reacted with HCA and formed orange-red colour. This reaction was confirmed using standard HCA (Plate 31 E).

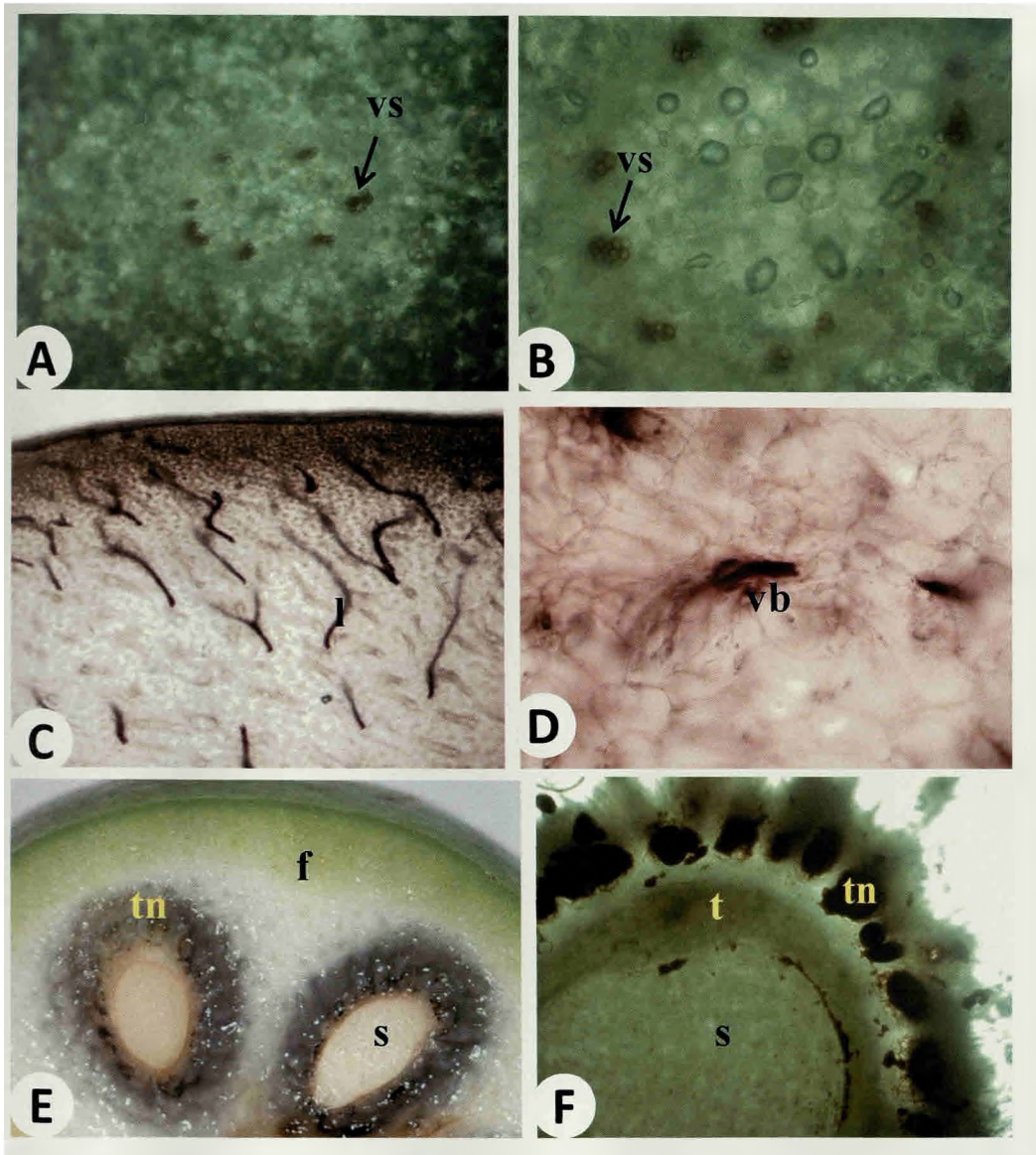


Plate 30. Histochemical localization of the lignin and tannins in fruits and seeds of *Garcinia indica*. A. Transverse section of mature seed stained with Phloroglucinol for lignin. Lignin in vasculature appeared red; B. Closer view of vasculature showing the presence lignin in xylem element; C. Transverse section of fruit 35 DAF detected with lignin in laticifer cells; D. Transverse section of fruit 50 DAF presence of lignin in ; E. Transverse section of fruit 70 DAF (unstained), tannins detected in the seed coat; F. Closer view of seed coat of 20 DAF showing the presence of tannins as indicated by ferric chloride reaction (dark-brown deposits). A, B and CX20; B and D X50. f, fruit; l, laticifers; s, seed; t, testa; tn, tannins; vb, vascular bundle; vs, vasculature structure.

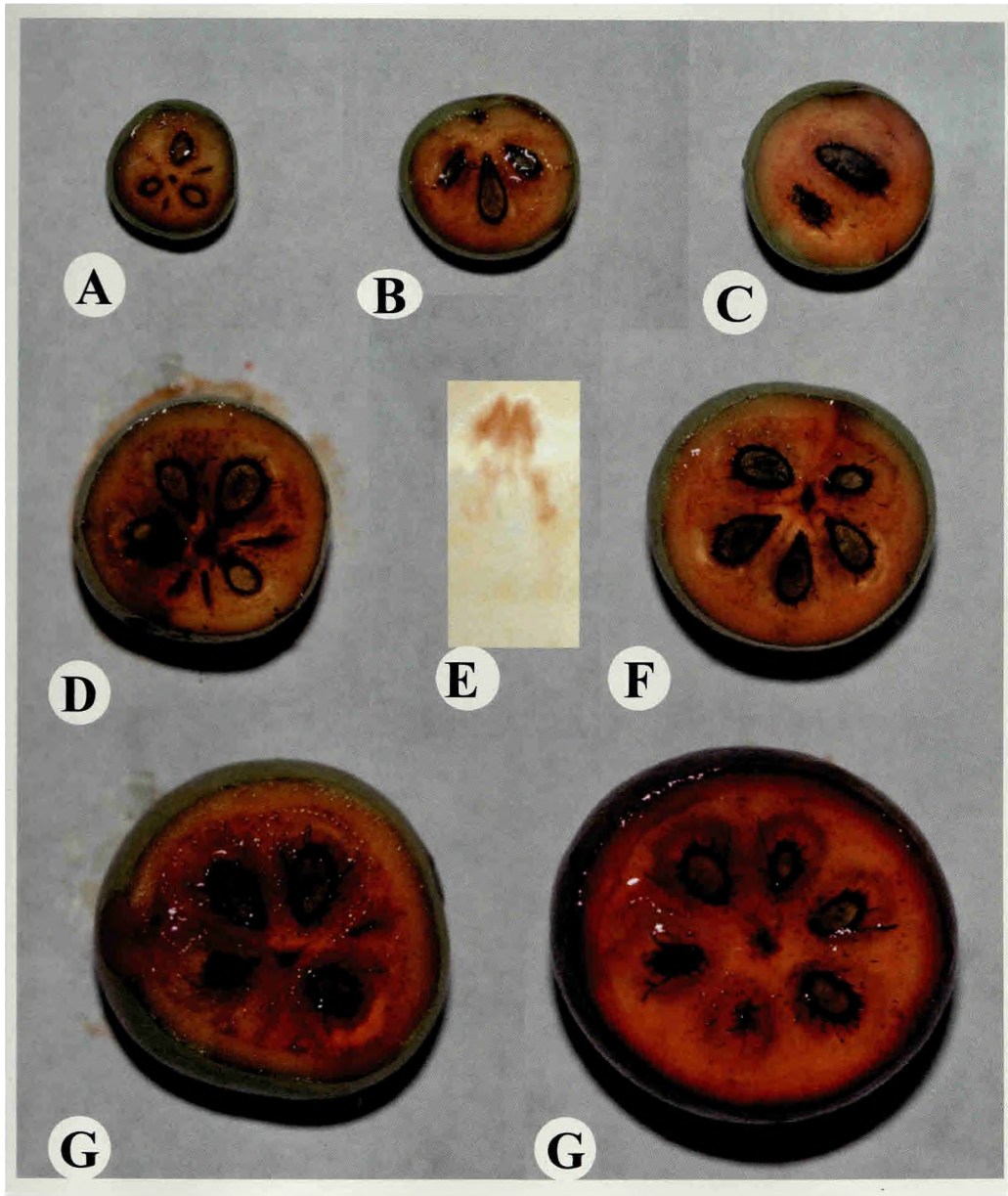


Plate 31. Histochmical localization of the hydroxcitric acid (HCA) during various stages of development of fruits of *Garcinia indica* by sodium metavanadate reaction. A. Thick transverse section of fruit of 15 DAF detected with less concentration of HCA as indicated by orange red colour; B. Transverse section of fruit 20 DAF; C. Transverse section of fruit 35 DAF; D. Transverse section of fruit 50 DAF; E. Transverse section of fruit 70 DAF; F. Transverse section of fruit 82 DAF; G. Transverse section of fruit 85 DAF. Note the gradual increase of HCA content from early stage of development of fruit to maturity.

Early stages of fruit (15 DAF) revealed the presence of low concentration of HCA as indicated by biochemical reaction (faint orange red colour) (Plate 31 A). The fruit 20 DAF depicted a higher concentration of HCA in comparison with early stages (Plate 31 B). The continuous increase in HCA content was noted in 35 DAF (Plate 31 C), 50 DAF (Plate 31 D), 70 DAF (Plate 31 F) and 82 DAF (Plate 31 G). The highest concentration of HCA was detected in matured fruit (85 DAF) as revealed by high intensity of orange-red staining reaction (Plate 31 H). It was observed that at early stage of fruit development, the HCA concentration was lower. As the fruit matures, HCA concentration gradually increases and reaches to the maximum level at maturity.

DISCUSSION

5. DISCUSSION

5. 1. Micropropagation

5. 1. 1. Response of Explants

In the present study, developed and standardized the protocol for *in vitro* regeneration and mass multiplication of plants from mature seed segments of *Garcinia indica*. Tembe and Deodhar (2011) established a protocol for *in vitro* propagation of Kokum using root-sucker explants. Malik *et al.* (2010) developed method for regeneration of plantlets via adventitious bud differentiation on leaf explants from *in vitro* raised seeding as well as mature trees. Deodhar *et al.* (2008) reported the *de novo* shoot formation of *Garcinia indica* using the *in vitro* developed roots as explants. Thengane *et al.* (2006a, b) studied plant regeneration through repetitive somatic embryogenesis and via direct somatic embryogenesis using immature seed explants. Chabukswar and Deodhar (2006) established method for restoration of rooting competence through *in vitro* serial shoot tip grafting for shoots developed from nodal explants of *Garcinia indica*. Malik *et al.* (2005a) developed protocol for regeneration of plantlets via adventitious bud differentiation in *Garcinia indica* from mature seed explants. Kulkarni and Deodhar (2002) developed protocol for multiple shoots and callus development using immature seeds.

5. 1. 2. Culture Medium

MS medium and WPM were supplemented with various concentrations and combinations of plant growth regulators. All the types of explants mentioned above were cultured on MS medium and WPM. While culturing, WPM was found more efficient in terms of culture regeneration and multiple shoot bud formation as compared to MS

medium. Since the present study mainly aimed to develop an efficient protocol for regeneration and mass multiplication, the more efficient medium of WPM was selected for the *in vitro* regeneration and mass multiplication. It was noted that various research workers used different culture media during their studies. For tissue culture of *Garcinia indica*, many of researchers used WPM supplemented with various concentration and combination of plant growth regulators (Thengane *et al.*, 2006a, b; Chabukswar and Deodhar, 2006; Deodhar *et al.*, 2008; Tembe and Deodhar, 2011) and MS supplemented with various concentrations and combinations of plant growth regulators (Kulkarni and Deodhar, 2002; Malik *et al.*, 2005a, 2010).

Matured seed segments of *Garcinia indica* were inoculated on WPM supplemented with various concentrations and combinations of plant growth regulators. After one week of culture seed segments showed swelling. Initiation of shoot bud primordial structures was noticed within 2-4 weeks of culture (Plate 2A). Malik *et al.* (2005a) observed similar response of swelling of mature seed explants along with shoot bud primordia within 2-3 week of culture on MS medium supplemented with plant growth regulators in *Garcinia indica*. The immature seeds of *Garcinia indica* showed swelling after one week and initiation of small protuberances was observed after 2-3 weeks of incubation on WPM supplemented with different combinations of cytokinins and auxins (Thengane *et al.*, 2006a, b). Direct shoot initiation was noticed on root sucker explants when cultured on WPM medium containing cytokinin after 4 weeks of inoculation (Tembe and Deodhar, 2011). Malik *et al.* (2010) noticed multiple shoot primordial structures within 2-3 weeks from leaf explants of *in vitro* raised seedlings of *Garcinia indica* when cultured on MS medium supplemented with various concentration of thidiazuron (TDZ). Chabukswar and Deodhar (2006) reported the development of axillary buds from nodal explants within 25

days of culture on WPM supplemented with cytokinins. Sirchi *et al.* (2008b) observed swelling and shoots emergence in seed explants of *Garcinia mangostana* after 2 weeks of culture on MS medium supplemented with BAP alone or in combination with NAA. Huang *et al.* (2000) observed the shoot formation from cotyledon explants of *Garcinia mangostana* within 4-6 weeks on MS medium supplemented with cytokinins and auxins.

5. 1. 3. Culture Regeneration and Shoot Bud Induction

During the study, increasing the concentration of BAP from 0.5 to 4.5mg/L in the WPM greatly increased the induction of shoot buds as well as regeneration frequency (Fig. 3, 4). Similar results were reported by Deodhar *et al.* (2008), who observed a significant increase in the number of shoots produced in *Garcinia indica* when the concentration of BAP was increased from 0.44 μM to 2.22 μM (Malik *et al.*, 2005a) observed in *Garcinia indica*, that with an increase in concentration of BAP from 5 to 50 μM, an increase in number of shoots occurred. Purohit *et al.* (2003) reported that the increase in concentration of BAP (0.5-5 mg/L) resulted in more shoot proliferation in *Achras sapota*. In *Garcinia mangostana*, Goh *et al.* (1990) reported that the increase in number of shoots per explant depends on the increment of the concentration of BAP while Rostika *et al.* (2008) observed that the higher concentration of BAP in the medium increased the number of shoots per seed segment.

During the study, mature seed segment explants were cultured on WPM supplemented with BAP at 0.5 to 5 mg/L concentration. Amongst the various concentrations BAP at 4.5 mg/L recorded maximum number of shoot buds (27) per explant with 100% regeneration (Table 14; Fig. 3, 4). Similarly, maximum number of shoots was reported on 2.22 μM BAP medium (Deodhar *et al.*, 2008). Higher number of

Fig. 3. Effect of BAP on culture regeneration of *Garcinia indica*.

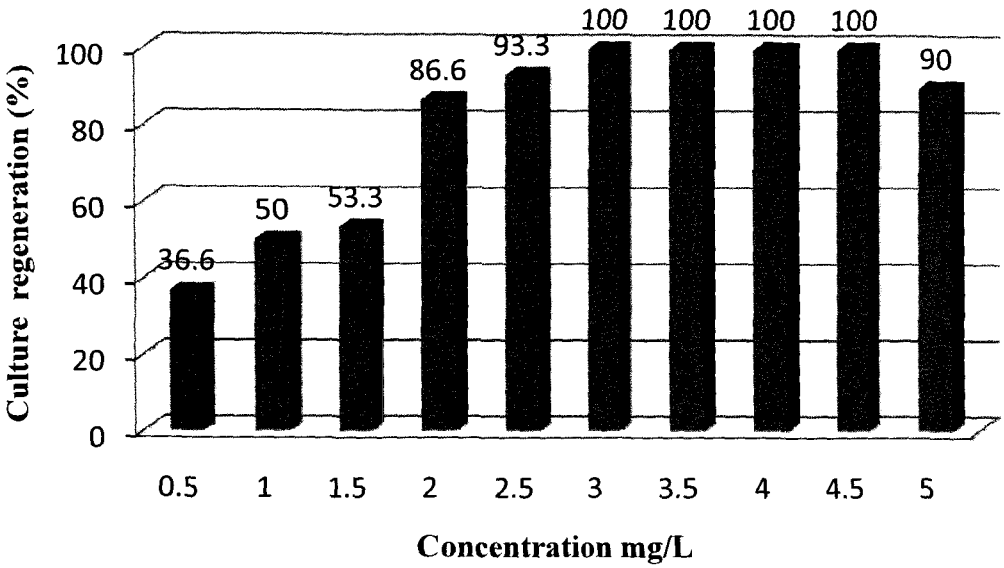
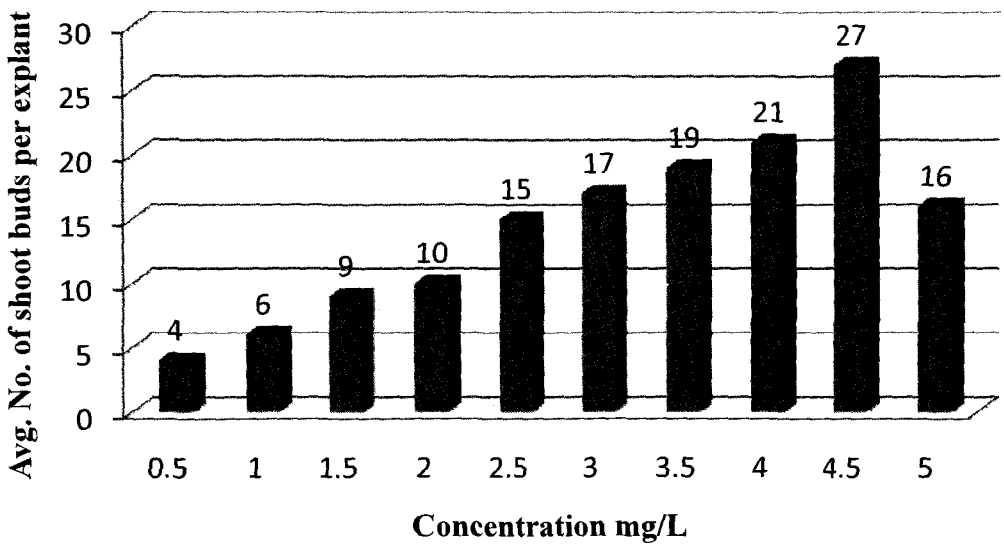


Fig. 4. Effect of BAP on development of shoot buds.



shoots were also exhibited in a medium supplemented with 12.5-50 μM BAP (Malik *et al.*, 2005a). In *Garcinia mangostana*, 5 mg/L BAP in the medium gave maximum number of shoots per explant (Goh *et al.*, 1990; Rostika *et al.*, 2008). In *Garcinia quaesita,* BAP at a concentration of 10 mg/L produced maximum number of shoot buds with higher percentage of culture regeneration (Farzana *et al.*, 2010). In the present study, the increase in BAP concentration beyond the 4.5 mg/L (optimal concentration) did not increase the number of shoot buds per explant. Malik *et al.* (2005a) reported similar observation of no significant increase in number of shoots per explant beyond optimal concentration of BAP (50 μM) in *Garcinia indica* and the same response in *Garcinia mangostana* (Goh *et al.*, 1990; Huang *et al.*, 2000).

During the study, KIN treatments did not give any good response in terms of number of shoot buds per explant, culture regeneration percentage and length of the regenerated shoots (Fig. 6-8) as compared to the other cytokinin (BAP). It was observed that KIN was not suitable for regeneration of *Garcinia indica* by using mature seed segments. Malik *et al.* (2005a) reported that KIN in the medium gave poor regeneration as well as less number of shoot per explant. In present study, BAP was found to be more potential and effective for induction of multiple shoot bud formation than KIN. Similar results were obtained in *Garcinia indica* by Malik *et al.* (2005a), in *Calophyllum apetalum* (Nair and Seeni, 2003) and in *Syzygium cuminii* (Jain and Babbar, 2000; 2003). The increased effects of BAP in inducing multiple shoots were previously reported in *G. mangostana* (Huang *et al.*, 2000; Goh *et al.*, 1990). However when Loc *et al.* (2005) supplied BAP and KIN individually in the medium, both showed similar effect on number of shoot formation in *Curcuma zedoaria*. Zhang *et al.* (2006) stated that KIN had mild stimulating effect on bud initiation of *Pinus massoniana*. In contrast, kinetin was found to

Fig. 5. Effect of BAP on length of shoots.

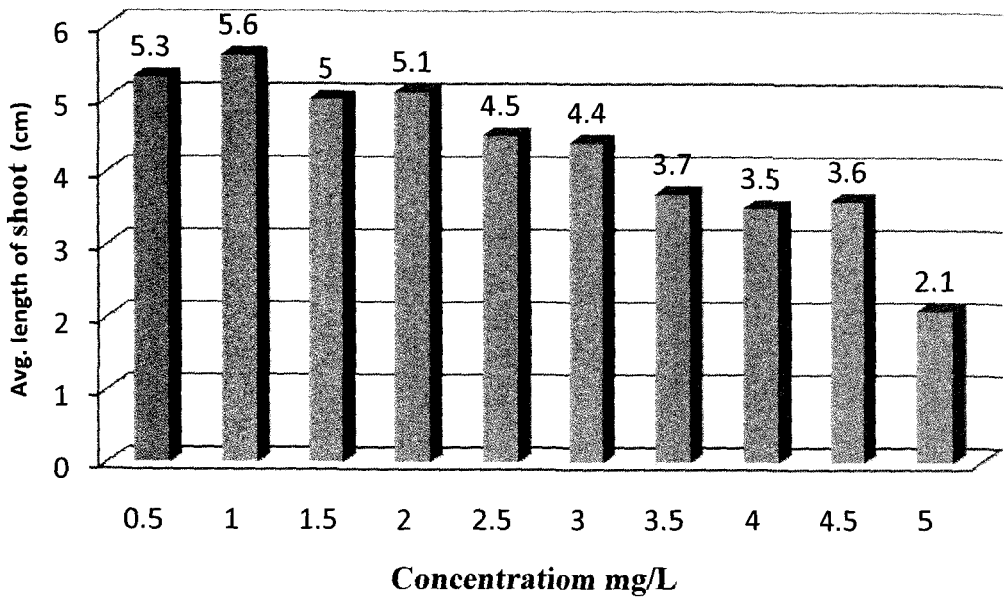


Fig. 6. Effect of KIN on culture regeneration of *Garcinia indica*.

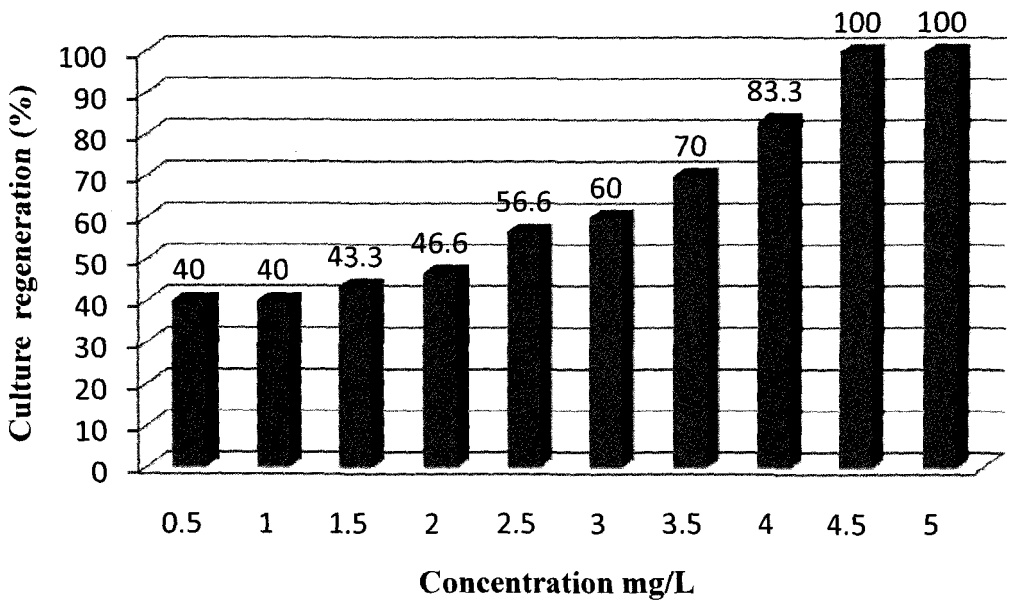


Fig. 7. Effect of KIN on development of shoot buds.

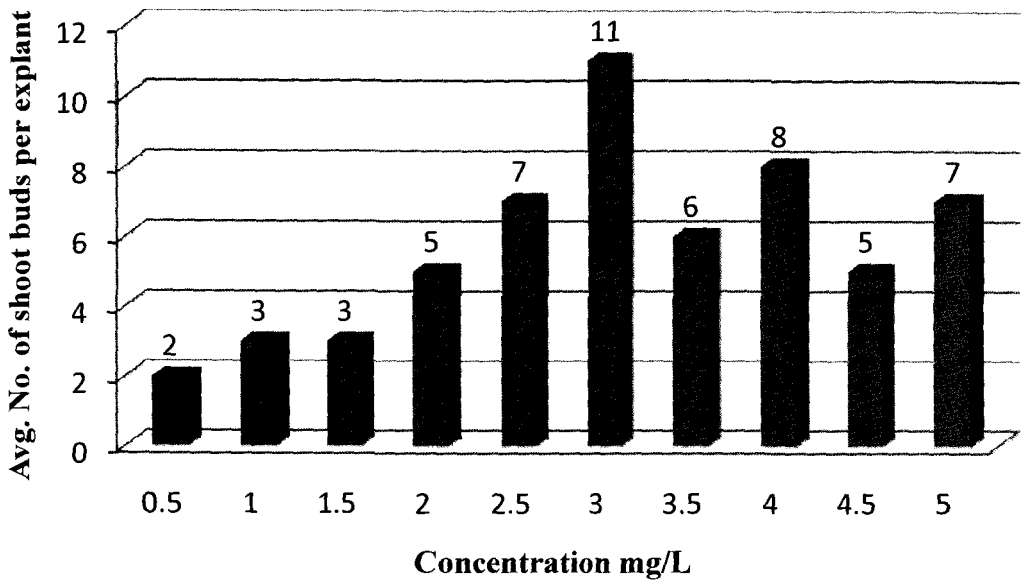
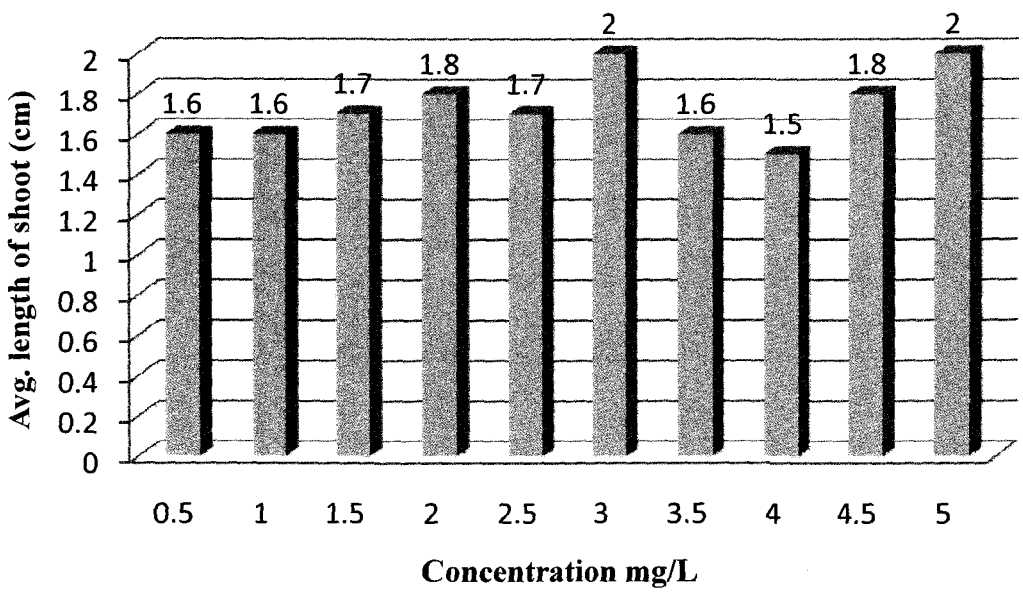


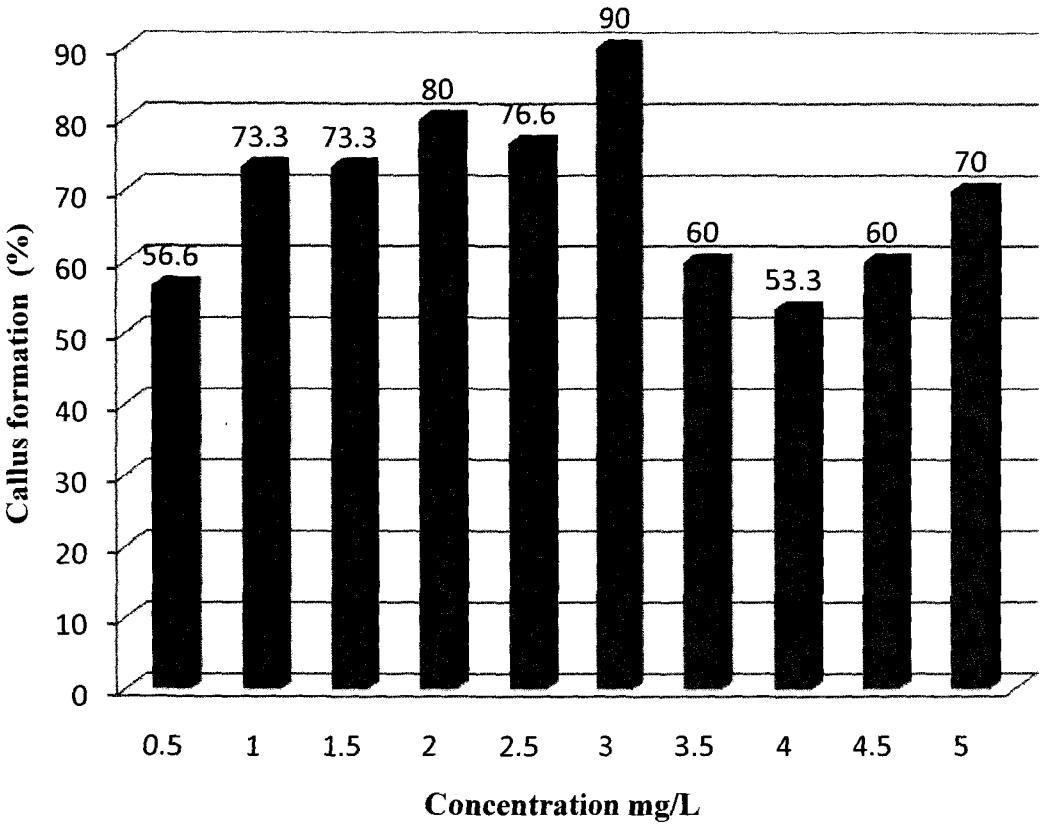
Fig. 8. Effect of KIN on length of shoots.



be most efficient PGR to induce shoot organogenesis in yam petiole explants of *Dioscorea* species (Anike *et al.*, 2012). During this study, the shoot buds regenerated from KIN treatments, showed very small shoots as compared to other treatments. In contrast, KIN enhanced shoot growth in *Garcinia mangostana* (Sirchl *et al.*, 2008).

During this study, seed segments inoculated on WPM containing 2, 4-D at 0.5-5 mg/L showed callus formation after 8-10 days, however, the percentage of callus varied among the concentration (Fig. 9). The callus was not compact, appeared very spongy and whitish yellow. The sub-culturing of callus was carried out at 3-4 weeks interval in fresh same media composition or BAP supplemented media. The callus did not respond further. It neither showed any further growth nor any morphological change (induction of shoot bud or organogenesis). The callus turned black and lost their potency of regeneration. Similarly, in *Carthamus tinctorius,* 2, 4-D medium produced calli which did not support any further regeneration (Walia *et al.*, 2007). Rineksane *et al.* (2012) observed that 2, 4-D in the medium induced callus formation in *Garcinia mangostana* with no further regeneration. In rice, Li *et al.* (2007) reported the development of whitish-yellow calli in MS medium supplemented with 2, 4-D after 12 days of inoculation. Later after 25 days of sub-culture the callus turned brown and died. In contrast of our results, in many plant species, organogenesis was observed in the presence of 2, 4-D medium. Srivastava *et al.* (2009) reported that the induced callus regenerated to shoots in *Azadirachta indica.* Vikrant and Rashid (2003) observed in *Paspalum scrobiculatum* that the callus regenerated into somatic embryos and similar somatic embryogenesis was also noted in chickpea (Kiranghanti *et al.*, 2010).

Fig. 9. Effect of 2, 4-D on callus formation of *Garcinia indica*.



The low concentrations of auxins along with cytokinins were shown to play critical roles in organogenesis of several Crassulaceae species (Brandao and Salema 1977; Dobos *et al.*, 1994; Liu *et al.*, 2006). In the present study, mature seed segments were cultured on WPM supplemented with BAP from 0.5-4.5 mg/L concentration along with a low concentration (1 mg/L) of auxins (IAA, NAA and IBA). The resulting media showed better induction of multiple shoot bud formation as compared to BAP and KIN alone. The synergistic effect of low concentrations of auxins along with cytokinins on shoot formation using seed explants were reported in *Garcinia mangostana* (Sirchl *et al.*, 2008). Huang *et al.* (2000) reported in contrast to the above results that low concentrations of auxins along with varying levels of cytokinins inhibited tissue growth and development in cotyledon segments of *Garcinia mangostana.*

In the present study, amongst the BAP and auxin combinations, BAP (2 mg/L) with IAA (1 mg/L) produced highest number of shoots buds (86.2 per explants) with a frequency of 100% regeneration (Fig. 10, 11) and varying shoot length (Fig. 12). (Baskaran and Krishnan, 2011). In *Emblica officinalis*, maximum number multiple shoots per explant was obtained on MS medium containing 2 mg/L BAP + 0.25 mg/L IAA (*Nayak *et al.*, 2010). Thengane *et al.* (2006a) reported that in *Garcinia indica,* the maturation of somatic embryos was achieved on WPM containing BAP 16.08 μM, kinetin 4.65 μM and IAA 5.71 μM. During this study, when seed segment explants were cultured, WPM supplemented with BAP (2.5 mg/L) and IAA (1 mg/L) resulted in 58 shoot buds per explant, followed by 47.2 shoot buds per explant (3 mg/L BAP + 1 mg/L IAA) and 40 shoot buds per explant (1.5 mg/L BAP + 1 mg/L IAA) (Fig. 11). Parimalan *et al.* (2009) reported that BAP (8.87 μM) and IAA (0.05 μM) combinations produced high number of shoots in *Bixa orellana.*

Fig. 10. Effect of BAP and IAA on culture regeneration of *Garcinia indica*.

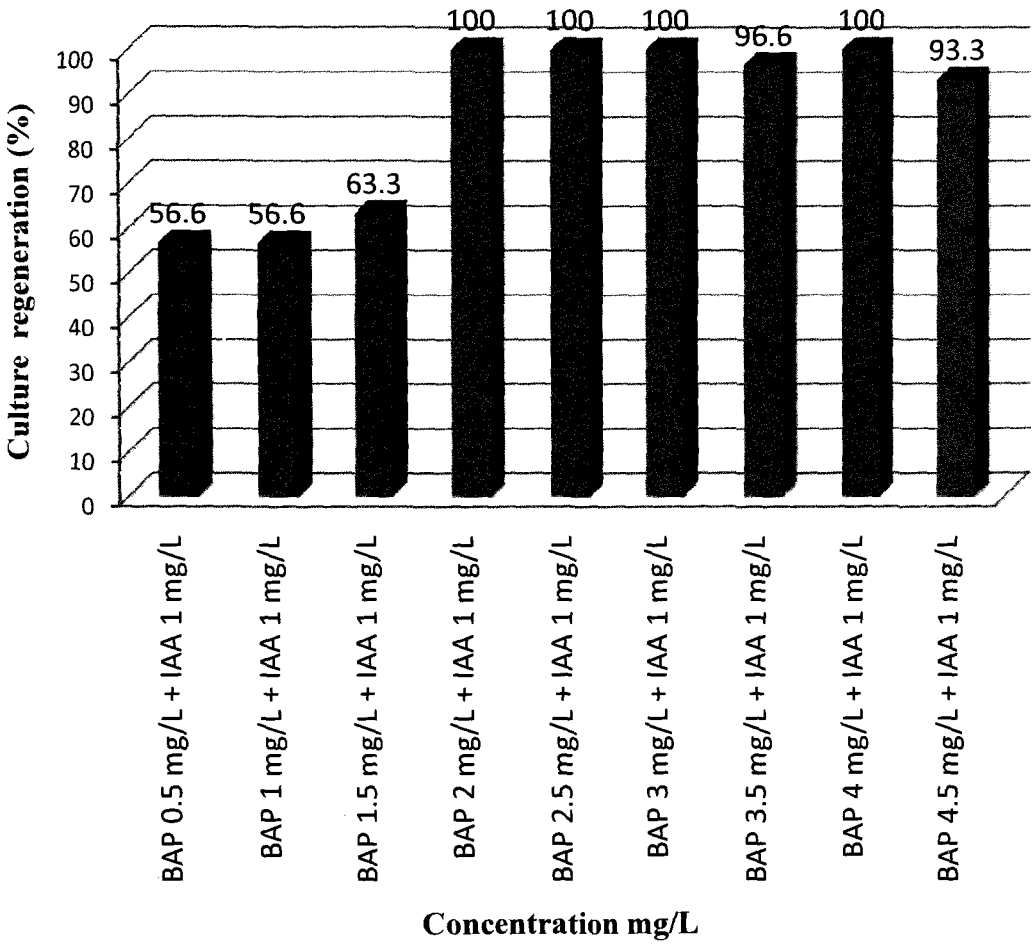


Fig. 11. Effect of BAP and IAA on development of shoot buds.

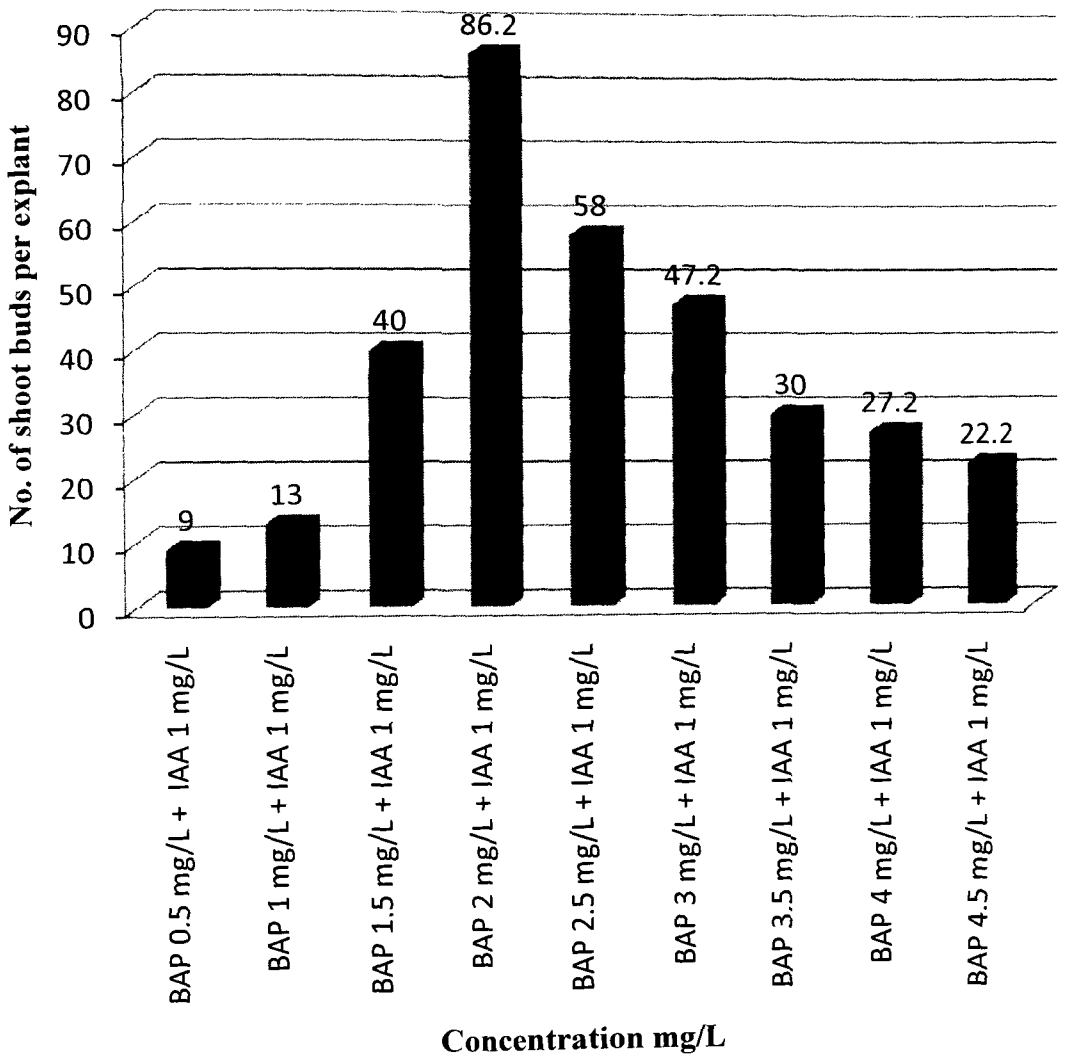
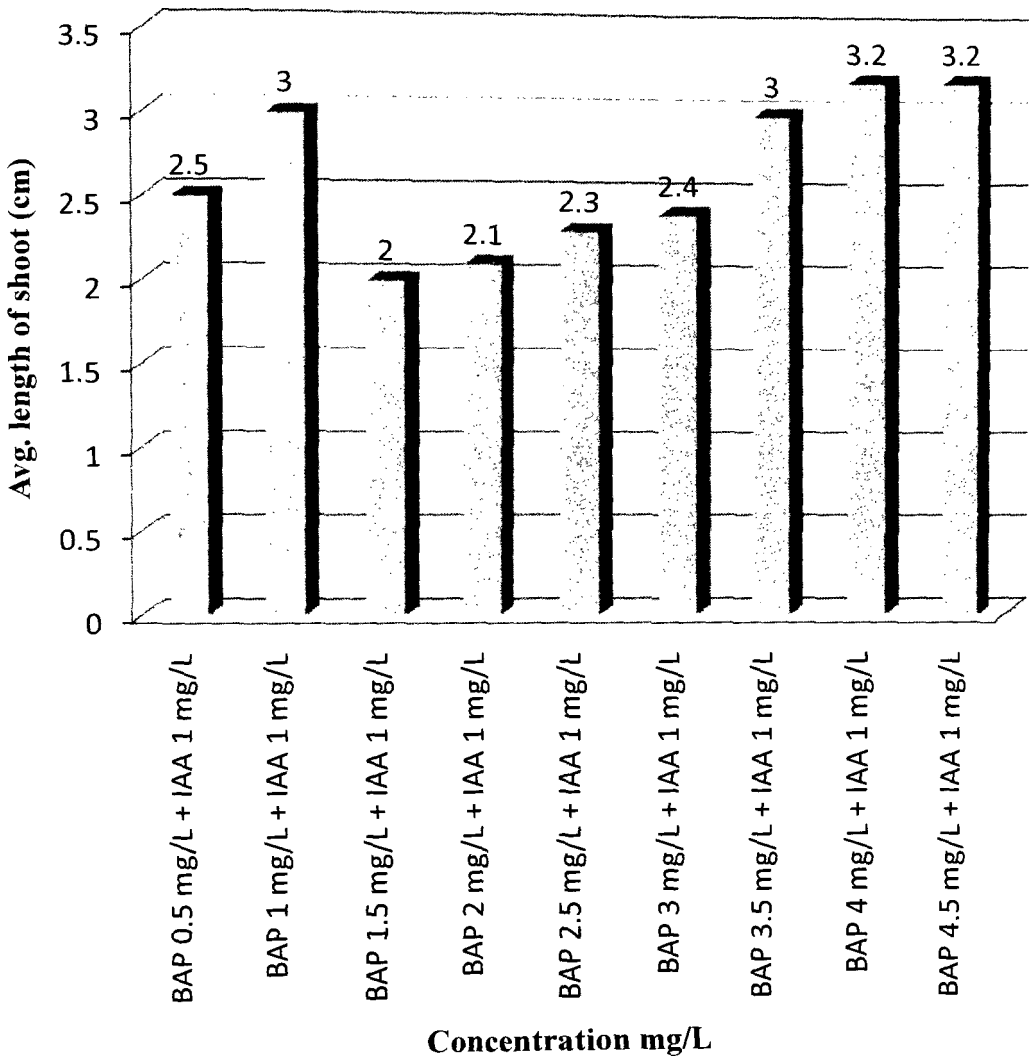


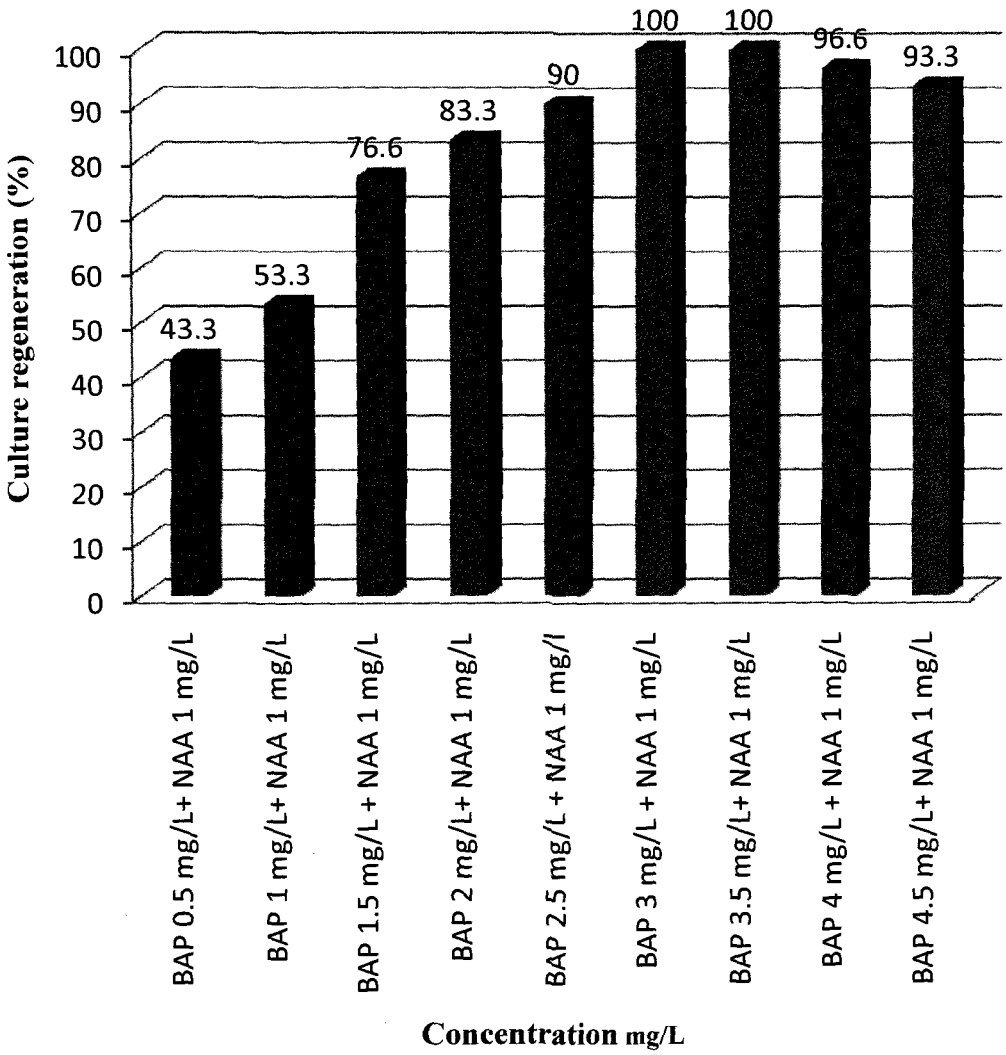
Fig. 12. Effect of BAP and IAA on length of shoots.



The synergistic effect of BAP and NAA on shoot formation using seed explants was also reported in *Garcinia mangostana* (Sirchl *et al.*, 2008). Mature seed segments when cultured during this study on WPM supplemented with BAP (0.5- 4.5 mg/L) and 1 mg/L NAA, multiple shoot buds induction along with callus formation was observed. The maximum number of shoot buds (61.2) was induced with 93.3% culture regeneration on medium supplemented with 4.5 mg/L BAP + 1 mg/L NAA (Fig. 13, 14). The maximum callus formation (60%) was seen in a medium supplemented with 0.5 mg/L BAP+1 mg/L NAA, while minimum callus formation (10%) was recorded in 4.5 mg/L BAP + 1 mg/L NAA supplemented WPM. In *Garcinia indica*, shoot bud induction along with callus formation was observed on MS medium containing BAP and NAA (Malik *et al.*, 2005a). Similar results of shoot formation along with callus induction from seed explants of *Garcinia mangostana* was reported in medium supplemented with BAP and NAA (Normah *et al.*, 1995; Huang *et al.*, 2000; Sirchl *et al.*, 2008). On the contrary, Kulkarni and Deodhar (2002) observed a positive effect of shoot morphogenetic responses in *Garcinia indica* when the media was supplemented with NAA.

In the present study, BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 mg/L) combinations with 1 mg/L IBA also contributed towards culture regeneration and multiple shoot buds formation in *Garcinia indica*. Amongst the concentrations studied, medium containing 4.5 mg/L BAP and 1 mg/L IBA reported maximum average number shoot buds (42.7) with 100% culture regeneration. Minimum number of shoot buds was noted in medium supplemented with 0.5 mg/L BAP and 1 mg/L IBA with 50% of regeneration frequency (Fig. 16, 17). BAP in combination with IBA was less effective in shoot bud induction when compared to BAP + IAA or BAP + NAA treatments. In contrast, Sun *et al.* (2009) reported the formation of the maximum number of shoots per explant (13.72) on WPM

Fig. 13. Effect of BAP and NAA on culture regeneration of *Garcinia indica*.



Concentration mg/L

BAP 0.5 mg/L + NAA 1 mg/L

BAP 1 mg/L + NAA 1 mg/L

BAP 1.5 mg/L + IBA 1 mg/L

BAP 2 mg/L + NAA 1 mg/L

BAP 2.5 mg/L + NAA 1 mg/L

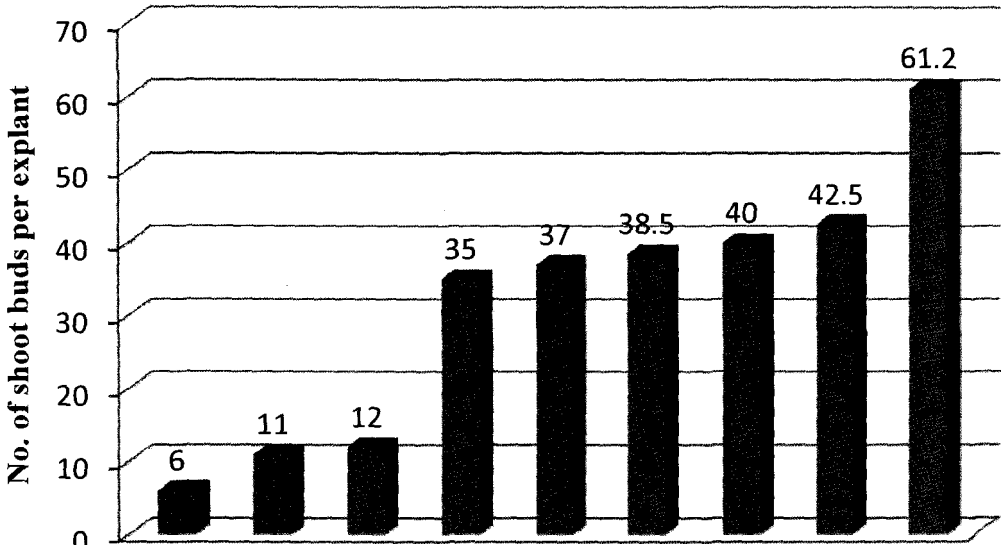
BAP 3 mg/L + NAA 1 mg/L

BAP 3.5 mg/L + NAA 1 mg/L

BAP 4 mg/L + NAA 1 mg/L

BAP 4.5 mg/L + NAA 1 mg/L

Fig. 14. Effect of BAP and NAA on development of shoot buds.



supplemented with BAP and IBA as compared to BAP + NAA and KIN + IBA in *Platanus occidentalis*.

During this study, it was observed that BAP was more effective cytokinin as compared to KIN for morphogenic responses. Shoot bud induction capacity of BAP depended on its concentration in the media. During the present investigation, amongst the cytokinins (BAP and KIN), BAP (4.5 mg/L) supplemented medium showed maximum numbers shoots. Similar result was noted in *Garcinia indica* (Malik *et al.*, 2005a). When BAP was combined with IAA, NAA and IBA, BAP (2 mg/L) + IAA (1 mg/L) recorded maximum number of shoot bud formation. In the present study, seeds were cut into three segments and used for regeneration on WPM supplemented with various concentrations and combinations of BAP, IAA, NAA and IBA. It was found that the regeneration ability of all seed segments were almost similar. They did show a significant difference in terms of percentage regeneration, number of multiple shoot buds per segment and the length of the shoots. Malik *et al.* (2005a) recorded no major differences among the seed segments, in the term of culture regeneration as well as number and length of induced buds of *Garcinia indica*.

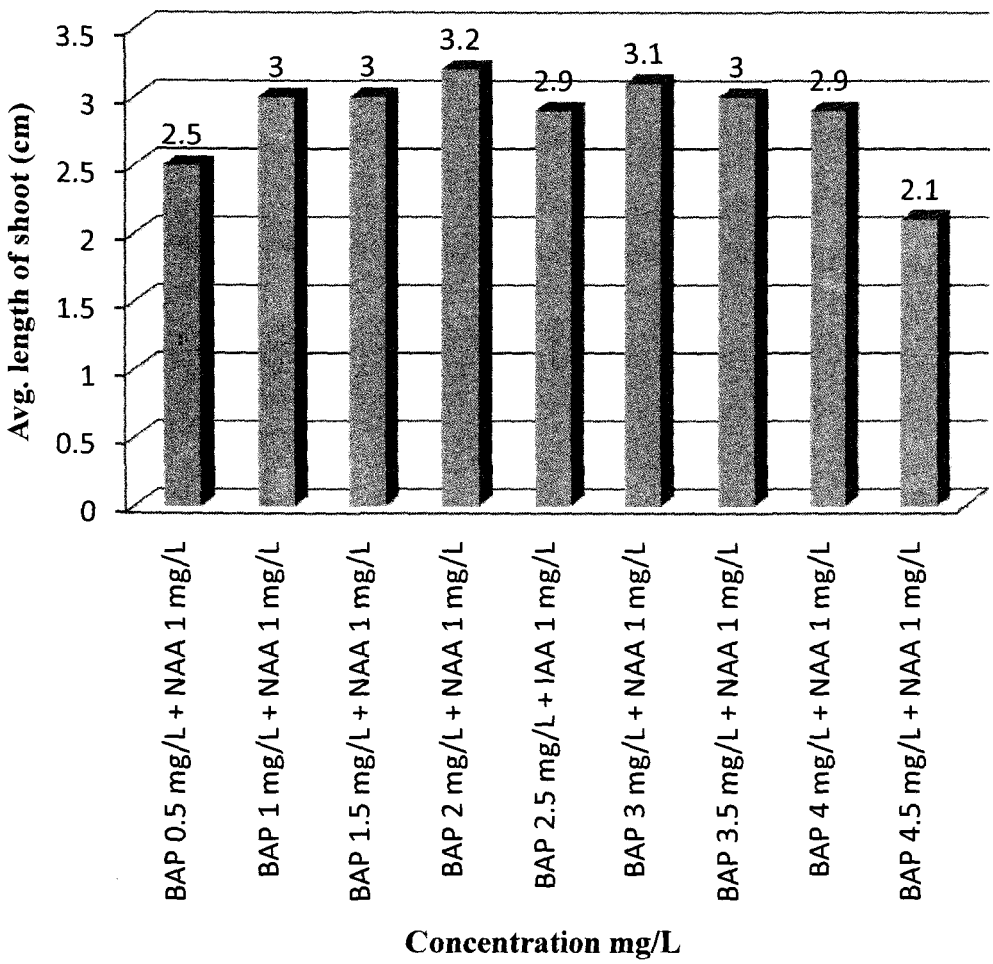
During this study, the maximum shoot length (5.6 mm) was recorded in WPM supplemented with 1mg/L BAP after 42 day of culture (after first sub-culture on same medium). Similar results of maximum length of shoots with lower concentration of BAP were reported by Malik *et al.* (2005a). Reduction in length of shoots was noticed when the concentration of BAP was increased from 2 to 5 mg/L (Table 14; Fig. 5). Similar responses of decrease in shoot length were reported while increasing concentration of BAP by Malik *et al.* (2005a). Stunted growth of shoots was also reported in *Garcinia*

mangostana in the presence of high concentration of BAP (Goh *et al.*, 1990). The present study revealed that amongst the six different treatments, BAP at 1 mg/L recorded maximum length of shoots (5.6 mm) (Fig. 5). Minimum length of shoots (1.6 mm) was reported on medium supplemented with 4 mg/L KIN after 42 days of culture (after first sub-culture on same medium) (Fig. 6). Shoot lengths of BAP and IAA treatments ranged from 2 to 3.2 mm (Table 17; Fig. 12). In case of BAP and NAA treatments, shoot length ranged from 2.1 to 3.2 mm (Table 18; Fig. 15). In BAP and IBA treatments, shoots measured from 2.2 to 3.5 mm after 42 day of culture (after first sub-culture on same medium) (Table 19; Fig. 18).

5. 1. 4. Elongation of Shoots

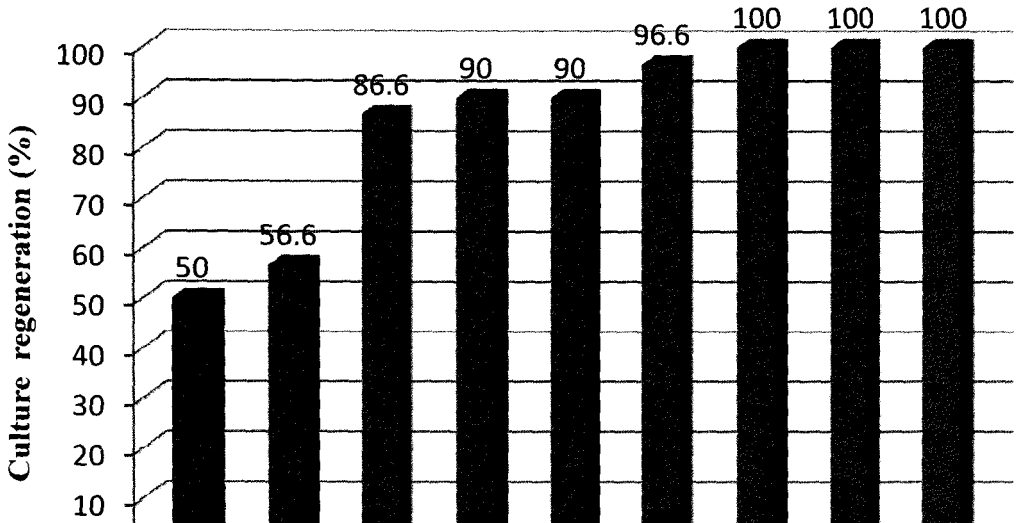
Induced shoots (after 42 days of culture) were cultured on WPM containing 1 mg/L BAP with (0.1%) and without activated charcoal, for further shoot bud growth and elongation of shoots. Amongst the above, best shoot bud growth and elongation was observed in the elongation medium without activated charcoal. Similar results of shoot elongation in *Garcinia indica* was achieved on half-strength WPM containing 0.4 μM BAP (Chabukswar *et al.*, 2006), MS basal medium supplemented with 0.2% activated charcoal (Malik *et al.*, 2010, 2005a) and WPM containing 22.19 μM BAP + 4.65μM KIN + 5.71μM IAA (Deodher *et al.*, 2008). In *Garcinia mangostana*, shoot elongation was achieved on medium supplemented with 2 mg/L BAP (Sirchl *et al.*, 2008a). Rostika *et al.* (2008) stated that low concentration BAP in the medium supported the elongation of shoots of *Garcinia mangostana*. In *Celastrus paniculatus,* Rao and Purohit (2006), achieved shoot elongation on MS medium containing low concentration of BAP (2.22 μM BAP). The present study revealed that all shoot bud induction treatments supported shoot elongation, except KIN treatment. Shoot buds induced using KIN treatment did not

Fig. 15. Effect of BAP and NAA on length of shoots.



0
BAP 0.5 mg/L + IBA 1 mg/L
BAP 1 mg/L + IBA 1 mg/L
BAP 1.5 mg/L + IBA 1 mg/L
BAP 2 mg/L + IBA 1 mg/L
BAP 2.5 mg/L + IBA 1 mg/L
BAP 3 mg/L + IBA 1 mg/L
BAP 3.5 mg/L + IBA 1 mg/L
BAP 4 mg/L + IBA 1 mg/L
BAP 4.5 mg/L + IBA 1 mg/L
Concentration mg/L

Fig. 16. Effect of BAP and IBA on culture regeneration of *Garcinia indica*.



Concentration mg/L

BAP 0.5 mg/L + IBA 1 mg/L

BAP 1 mg/L + IBA 1 mg/L

BAP 1.5 mg/L + IBA 1 mg/L

BAP 2 mg/L + IBA 1 mg/L

BAP 2.5 mg/L + IBA 1 mg/L

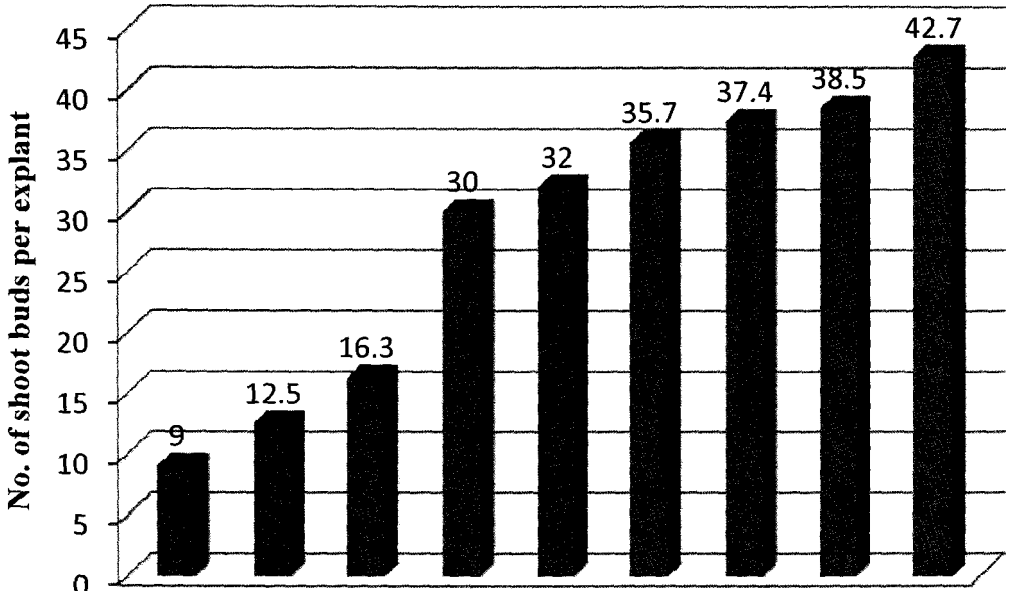
BAP 3 mg/L + IBA 1 mg/L

BAP 3.5 mg/L + IBA 1 mg/L

BAP 4 mg/L + IBA 1 mg/L

BAP 4.5 mg/L + IBA 1 mg/L

Fig. 17. Effect of BAP and IBA on development of shoot buds.



Concentration mg/L

BAP 0.5 mg/L + IBA 1 mg/L

BAP 1 mg/L + IBA 1 mg/L

BAP 1.5 mg/L + IBA 1 mg/L

BAP 2 mg/L + IBA 1 mg/L

BAP 2.5 mg/L + IBA 1 mg/L

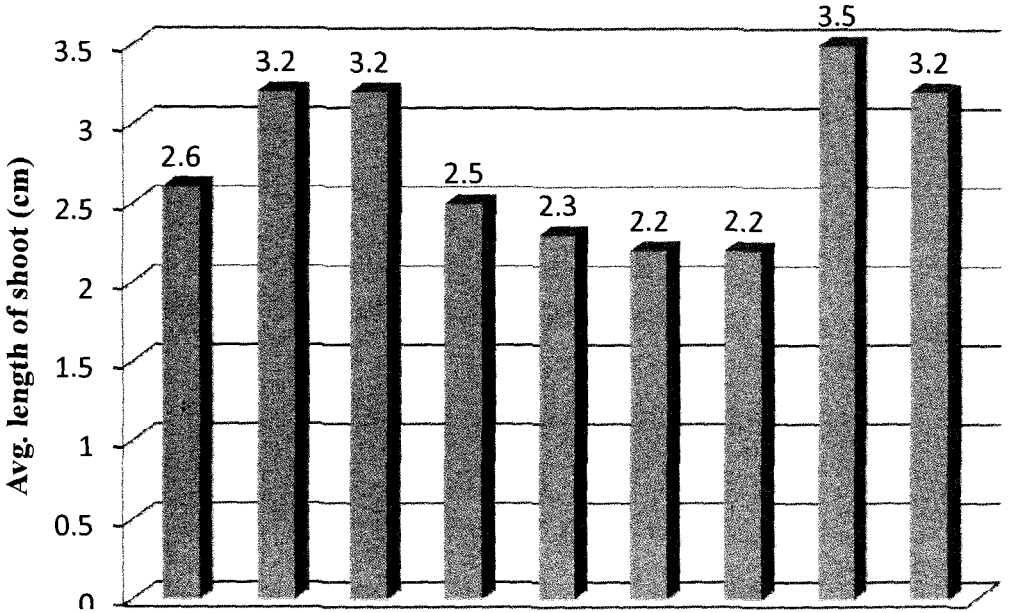
BAP 3 mg/L + IBA 1 mg/L

BAP 3.5 mg/L + IBA 1 mg/L

BAP 4 mg/L + IBA 1 mg/L

BAP 4.5 mg/L + IBA 1 mg/L

Fig. 18. Effect of BAP and IBA on length of shoots.



support better elongation after 2-3 sub-culture on elongation medium (1 mg/L BAP). In contrast, Sirchl *et al.* (2008b) stated that KIN plays a role in rejuvenating the cell activity and shoot elongation in *Garcinia mangostana.*

5. 1. 5. Root induction

In the present study, *in vitro* regenerated shoots were cultured on half-strength WPM supplemented with auxins for root induction. The percentage of root induction varied in IBA and NAA concentrations (Fig. 19, 22). The number of roots per shoot ranged from 1 to 2.8 in IBA (Fig. 20). Increased number of roots (2-5) was observed in NAA (Fig. 23). Hundred percent root induction was seen in IBA at 3 and 4 mg/L concentration while in NAA the percentage of root induction ranged from 60-90. Amongst the IBA and NAA treatments, the best response was noticed in IBA at 3 mg /L, which showed maximum number (2.8) (Fig. 21) and increased length (3.65 cm) (Fig. 24) of root . Similar root induction by IBA and NAA was reported in *Garcinia indica* (Malik *et al.*, 2005a; Thengane *et al.*, 2006b; Deodhar *et al.*, 2008). During several studies, it was proved that IBA promotes better root induction responses than other auxins, in plants such as *Terminalia bellerica* (Rathore *et al.*, 2008), *Artemisia judaica* (Liu *et al.*, 2003), *Bixa orellana* (Paiya Neto *et al.*, 2003) *Dioscorea zingiberensis* (Chen *et al.*, 2003) and *Garcinia mangostana* (Rostika *et al.*, 2008). In contrast to the above, it was reported that the half-strength WPM with 2.5-245 μM IBA did not induce any rooting in *Garcinia indica* (Chabukswar *et al.*, 2006).

In present study, all roots were formed without any callus formation. Roots induced on IBA medium was supported for acclimatization process and showed high rate of plant survival as compared to NAA. Even though the number of roots formed was high

Fig. 19. Effect of IBA on percentage of rooting of *Garcinia indica.*

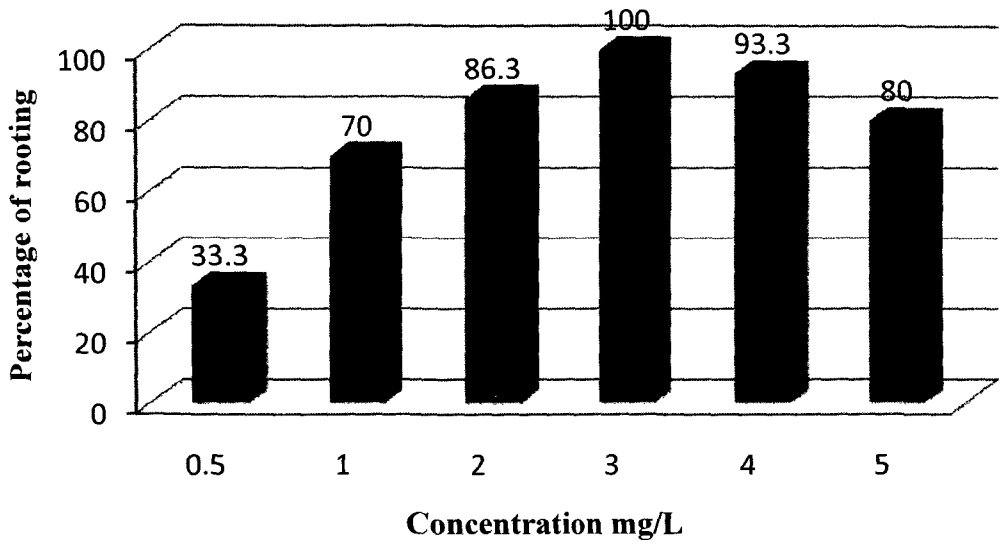


Fig. 20. Effect of IBA on number of roots in regenerated shoots of *Garcinia indica.*

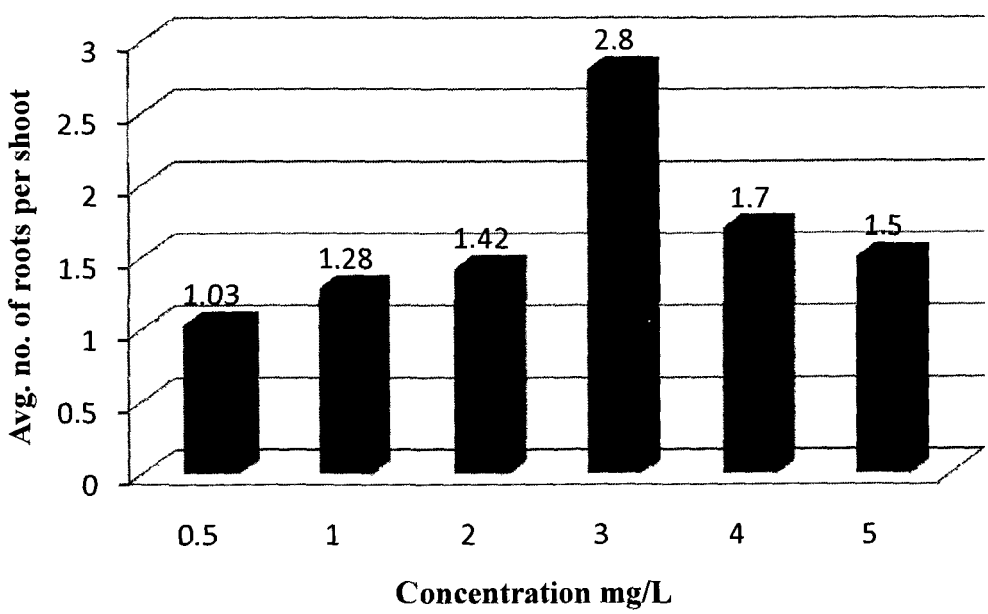


Fig. 21. Effect of IBA on length of roots of Garcinia indica.

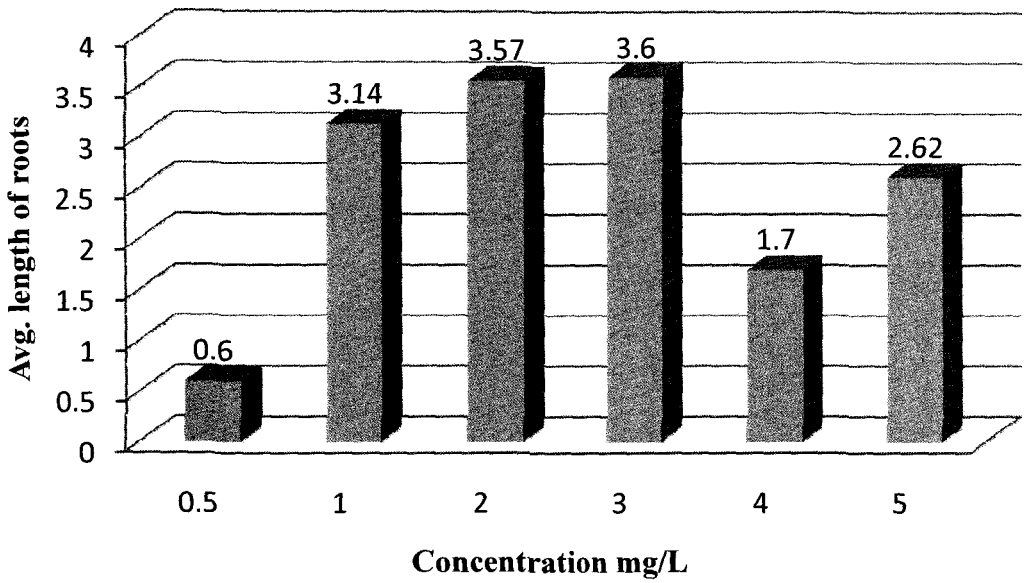


Fig. 22. Effect of NAA on percentage of rooting in *Garcinia indica.*

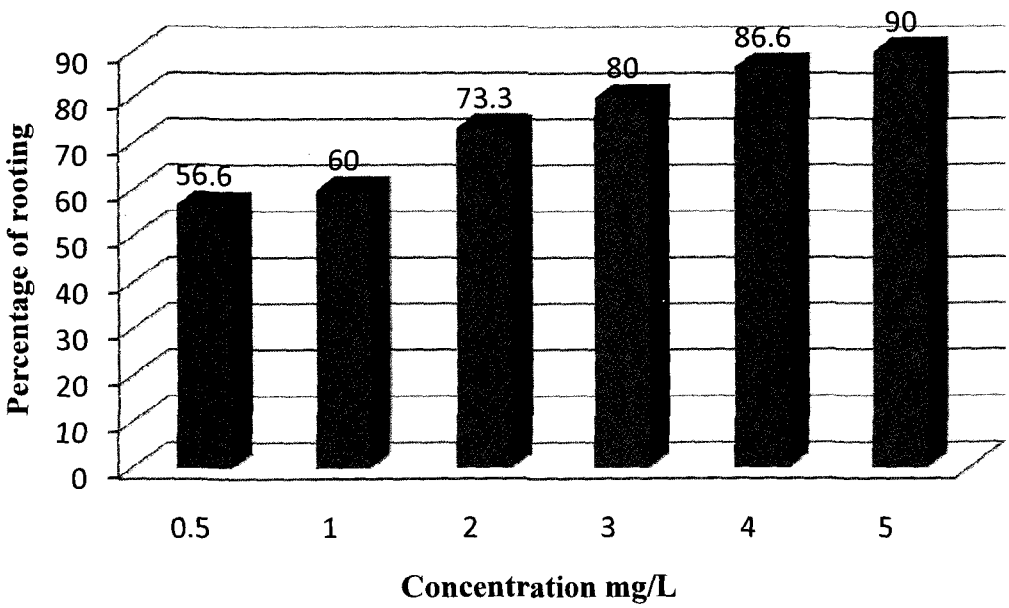


Fig. 23. Effect of NAA on number of roots in regenerated shoots of *Garcinia indica*.

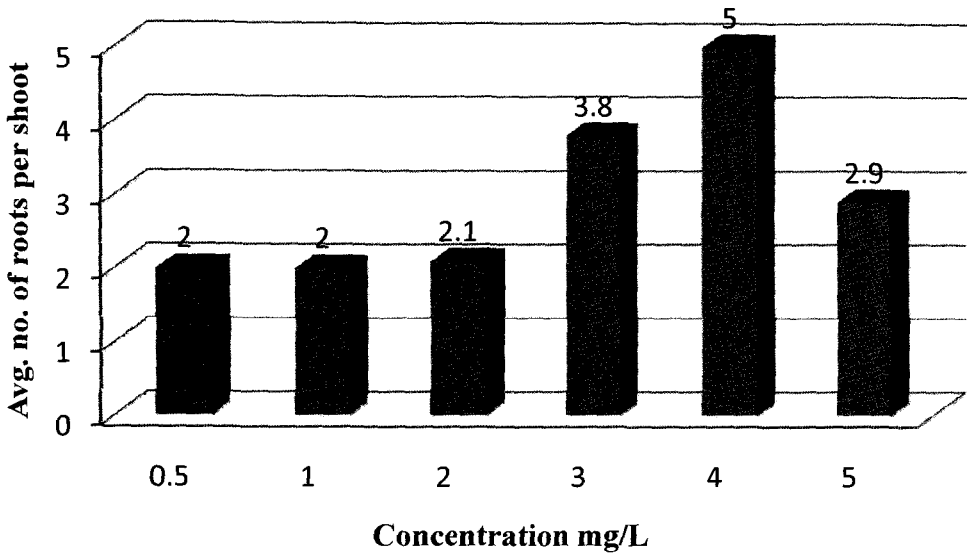
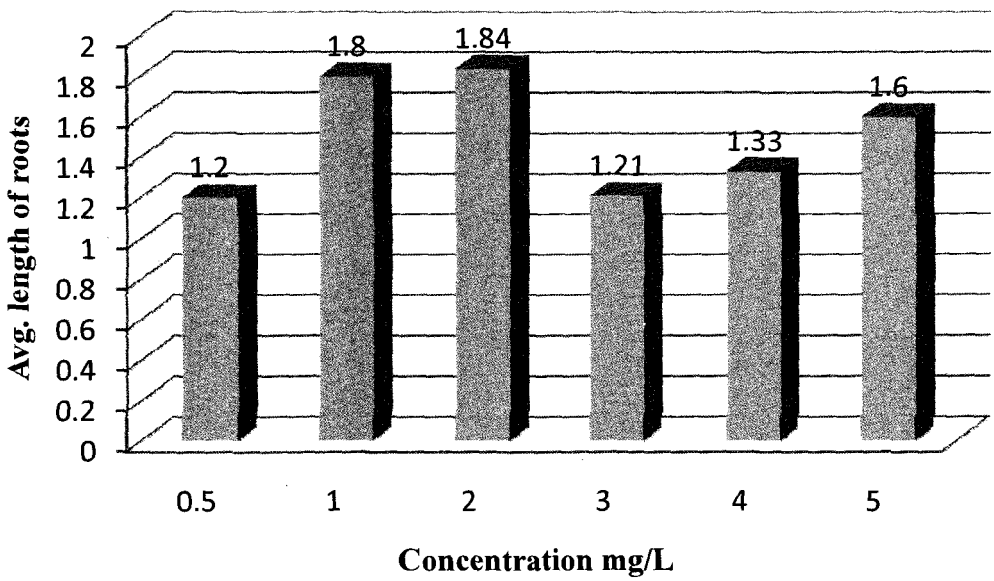


Fig. 24. Effect of NAA on length of roots of *Garcinia indica*.



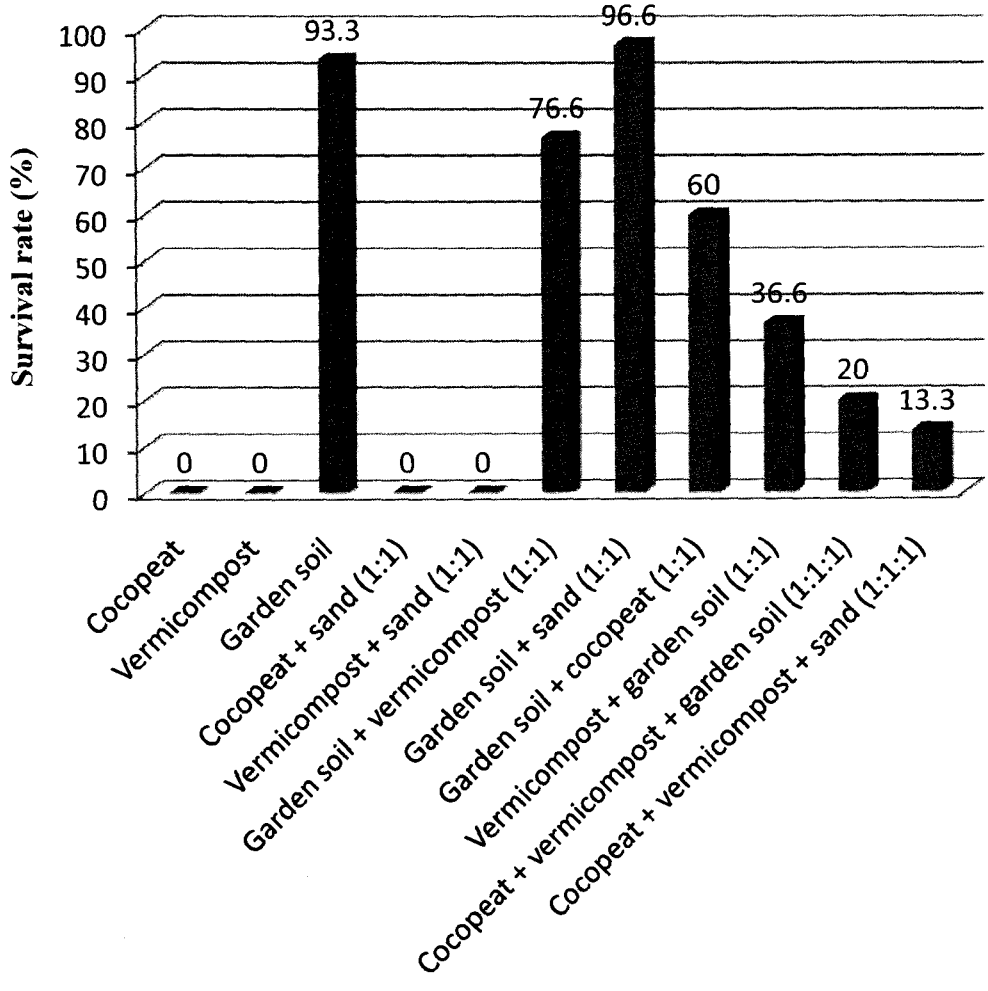
(5) in 4 mg/L NAA as compared to IBA, the roots induced from NAA were not stronger and did not support the acclimatization process. It was proved that in *Garcinia indica*, the IBA treatments induced longer and thinner roots in large number of explants as compared to thick and stunted roots produced by NAA supplemented medium (Malik *et al.*, 2005a). It was reported that in *Garcinia mangostana,* IBA induced longer and thinner roots in higher number of explants as compared to thick stunted roots produced on NAA supplemented medium (Goh *et al.*, 1988; Goh *et al.*, 1994; Normah *et al.*, 1995; Huang *et al.*, 2000). In contrast to the above, Kulkarni and Deodhar (2002) observed intervention of callus on rooting of shoots of *Garcinia indica* in NAA supplemented medium.

5. 1. 6. Acclimatization

Rooted plantlets were transferred to culture tubes containing quarter-strength liquid WPM without sucrose, for root hardening. Malik *et al.* (2005a) reported the use of quarter-strength liquid MS medium with 1% sucrose for hardening in *Garcinia indica*. In the present study, root hardened, healthy and actively growing plantlets of about 2-3 roots and 6-8 leaves were transplanted to seed tray filled with different potting mixture (Table 22).

The survival rate of transferred plants ranged from 13.3 to 96.6%. Maximum survival rate of 96.6% was recorded in the combinations of garden soil + sand (1:1), followed by garden soil (93.3%), garden soil + vermicompost (76.6%), cocopeat + garden soil (60%), vermicompost + garden soil (36.6%) and 20% survival was noted in both cocopeat + vermicompost + garden soil and vermicompost + sand. Minimum survival rate was observed in cocopeat + vermicompost + sand (13.3%) (Fig. 25). Different potting media such as soil, vermiculite and farm yard manure (1:1:1) showed 90% survival rate (Malik *et al.*, 2005a; 2010). In sand and soil (1:1) mixture, 92% survival rate (Thengane *et*

Fig. 25. Survival rate of regenerated plants in different potting mixtures.



al., 2006b) and 90% survival rate (Deodher *et al.*, 2008) were observed. In cocopeat, 76.2% survival rate (Chabukswar and Deodher *et al.*, 2005) and 75% survival rate (Tembe and Deodhar, 2011) were reported for acclimatization of regenerated plantlets of *Garcinia indica* by different workers. In *Garcinia mangostana*, soil + compost (1:1) proved to be better for acclimatization (Rostika *et al.*, 2008) while, soil + sand + organic matter + vermiculite (2:2:1:1) showed 80% survival rate (Sirchl *et al.*, 2008a, b). In *Emblica officinalis*, 80% survival rate was recorded in garden soil (*Nayak *et al.*, 2010).

In the present study, protocol was developed for regeneration and mass multiplication of Kokum using mature seed segments. The maximum numbers of shoot buds per explant (86.2) was significantly higher than other studies reported. An average number of 57.67 shoot buds per mature seed segment (Malik *et al.*, 2005a), 30 shoots per root sucker explant (Tembe and Deodhar, 2011), 29.3 shoots per root explants (Deodhar *et al.*, 2008), 16.7 shoots per immature seed explant (Kulkarni and Deodhar, 2002), 2.6 shoot buds per leaf explant from *in vitro* raised plant (Malik *et al.*, 2010), 2.6 shoots per nodal explant (Chabukswar and Deodher *et al.*, 2005) and 48.53 somatic embryos per immature seed explant (Thengane *et al.*, 2006b) were reported previously in *Garcinia indica.*

When seed segments were cultured on WPM supplemented with BAP alone, the induction of shoot buds took 4-5 weeks duration, conversion of shoot buds to shoots and elongation of shoots took around 7 weeks, root initiation and root elongation took about 6 weeks, root hardening about 2 weeks (10-12 days) and acclimatization of regenerated rooted plantlets in suitable potting mixture about 2 weeks. A total of 22 weeks were taken to regenerate complete plants from the mature seed segment explants. Malik *et al.* (2005a) reported in *Garcinia indica,* that in mature seed segments on MS medium containing BAP

alone, the initiation of regeneration to transplanting to soil was completed in 15-17 weeks. The study by Thengane *et al.* (2006a) showed that the complete processes of plant regeneration via direct somatic embryogenesis using the immature seeds took 28-30 weeks period.

During this study, when mature seed segment was cultured on WPM supplemented with BAP, in combination with IAA, NAA and IBA, initially the induction of shoot buds took the period of 5-6 weeks, conversion of shoot buds to shoot and elongation of shoots took around 9 weeks, root initiation and elongation took 6 weeks, root hardening took about 2 weeks (10-12 days) and acclimatization of regenerated and rooted plantlets in suitable potting mixture took about 2 weeks. So, a total of about 25 weeks were taken to complete the entire protocol of regeneration to transfer of the plant to the poly-house.

5. 1. 7. Histological Studies of Development of Shoot Buds

Histological studies were carried out to determine the origin and development of shoot bud primordial structures and shoot buds. Transverse section of seed segments after one week of culture showed the presence of large number of meristematic zones mostly in the central region (dark stained) (Plate 11A, B). Similar observation of formation of meristematic zones on cortical region of root was reported by Deodhar and Thengane *et al.* (2008). After three weeks of culture, the meristematic zones developed in to shoot primordium (Plate 11C, D) and then the shoot primordium developed in to shoot buds after four weeks of culture (Plate 11E, F). The study also revealed the initiation of leaves from the shoot bud primordium. Deodhar and Thengane *et al.* (2008) noted the formation of shoots from meristemetic zones in *Garcinia indica*. In contrast, meristematic pockets were observed in periphery of immature seed explants and the same developed into

somatic embryos (Thengane *et al.*, 2006a, b). In *Garcinia mangostana*, somatic embryos developed from the meristematic regions of seed (Elviana *et al.*, 2011).

5. 1. 8. Molecular Characterization of Regenerated Plants

During this investigation, DNA was isolated from the regenerated plants as well as corresponding mother plants (donor). The polymerase chain reaction (PCR) amplification were carried out using RAPD and ISSR primers to check genetic stability and variation, if any, between the regenerated plant and the corresponding mother plant (donor). To understand the genetic stability within micropropagated plants, various DNA marker techniques were employed (Saker *et al.*, 2006; Feyissa *et al.*, 2007; Xing *et al.*, 2010). Molecular markers have been the most desirable tool for establishing genetic uniformity in micropropagated plantlets. PCR based markers such as RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) were used for the characterization of genomic instability within the micropropagated plants in comparison with mother plant (Borchetia *et al.*, 2009; Mohan *et al.*, 1012).

5. 1. 9. RAPD Banding Pattern of PCR Products

Eighteen RAPD primers were used for PCR amplification of DNA from regenerated plants as well as mother plants. Maximum number of bands (9) was obtained using the primer OPC 2 and least number of bands (2) by primer OPF 10. RAPD primers recorded 2 to 9 bands during the RAPD analysis of regenerated plants as well as corresponding mother plants (Table 24). Xing *et al.* (2010) used a total of 42 RAPD primers for the analysis of 40 randomly selected regenerated plants. Out of 42 primers, 16 produced clear identifiable bands and yielded 91 countable bands with an average of 5.7 bands per primer. The results indicated that almost no somaclonal variation existed in the

regenerated eggplant. Rai *et al.* (2012) reported, in spine gourd (*Momordica dioica*), that sixteen RAPD random primers produced reproducible bands. The number of amplified bands produced by RAPD primer ranged from 3 to 9. The results of this molecular study revealed, that the micropropagated plants were genetically identical to mother plant and that no genetic variation was induced during clonal propagation.

5. 1. 10. ISSR Banding Pattern of PCR Products

Out of ten ISSR primers screened, seven primers produced clear banding pattern of amplified DNA and three primers such as IB 3, ISSR-ST2 and ISSR-834 did not amplify during PCR amplification. Primer IB 2 recorded maximum number of bands (15) with clear banding pattern after PCR amplification. Minimum number of amplified bands (9) was noticed in two primers namely, IB 5 and ISSR-808 (Table 25). Many researchers have used ISSR primer for assessment of genetic fidelity of micropropagated plants with their corresponding mother plants. Joshi and Dhawan (2007) assessed the genetic fidelity of micropropagated plant *Swertia chirayita* by inter simple sequence repeat (ISSR) marker. Gangopadhyay *et al.* (2010) showed that the ISSR profiles of micropropagated plants produced monomorphic bands indicating the genetic uniformity in tissue culture derived plants. Huang *et al.* (2009) used ISSR molecular markers for the assessment of genetic stability of long-term micropropagated plantlets of London plane tree (*Platanus acerifolia*).

In the present study, the molecular characterization of regenerated plants in comparison to their mother plants, using both RAPD and ISSR random primers, showed no variation in banding patterns indicating that the genetic stability was maintained in the regenerated plants. Many researchers used both RAPD and ISSR random primers to

revalue the genetic stability of regenerated plants and confirm the genetic homogeneity: Kumar *et al.* (2010) in *Chlorophytum borivilianum*, Pati *et al.* (2008) in *Aegle marmelos*, Kumar *et al.* (2010) in date palm *Phoenix dactylifera,* Mohanty *et al.* (2011) in *Zingiber rubens* and Mohan *et al.* (2012) in *Garcinia gummigutta, Garcinia indica* and *Garcinia tinctoria.*

Many researchers have reported that the *Garcinia indica* seeds were formed apomictically (agamospermy). They were not true seeds because, the seeds do not have any definite endosperm/cotyledons and embryonic axis, but a vascular strand was noticed in the seed (Mohan *et al.*, 2012; Malik *et al.* 2005b). Although the seeds were formed apomictically, from the present study, the molecular characterization of regenerated plants, using molecular markers (RAPD and ISSR), showed that no any genetic variability exists, indicating that the regenerated plants were clones of mother plants and true to type. So, all regenerated plants (clones) were identically same as mother plant.

5. 2. Biochemical Study

5. 2. 1. Sample extraction

Six and seven developmental stages of fruits of *Garcinia indica* were collected from high yielding trees grown in three different places of Goa such as Marcel (Tree No.1), Priol (Tree No.2) and Ponda (Tree No.3). The fruits collected from Marcel (Tree No.1) and Priol (Tree No.2) were separated into 7 different developmental stages. The fruits collected from Ponda (Tree No.3) were separated into 6 different developmental stages. 100 g of fruits of each developmental stage was used for extraction with 1.3 L of methanol using Soxhlet apparatus. Hot methanol extraction was carried out for the duration of 8 hours to have a complete extraction. Several researchers have used different

solvents and various methods of extraction of HCA from *Garcinia* species. Jayaprakasha and Sakariah (2002) used water for the extraction of HCA from leaves and rind samples of *Garcinia indica* by autoclaving at 15 lbs/inch^2 for 20 min. Jayaprakasha and Sakariah (1998) reported the extraction of HCA from dried rind (obtained from local market) of *Garcinia combogia* using water and autoclaved at 15 lbs/inch^2 for 20 min. Jayaprakasha *et al.* (2003) carried out extraction of HCA from rind, pulp, fruits and leaf of *Garcinia pendunculata* using water at 15 psi for 30 min. Jena *et al.* (2002b) reported the extraction of HCA from dried rind of *Garcinia cowa* using water and autoclaved at 15 lbs/inch^2 for 30 min. Vinh *et al.* (2011) used water as solvent for microwave extraction of HCA from rind of *Garcinia oblongifolia.*

5. 2. 2. Determination HCA by HPLC Method

100 mg solid sample extracts of each developmental stage of fruits was dissolved in 100 ml of 0.1% perchloric acid in water. Then 10μl of sample was used for HPLC analysis. The mobile phase was 0.1% perchloric acid in water, and the flow rate was set to 0.8 ml/min. The peak area was obtained at wavelength of 210 nm. Jayaprakasha and Sakariah (2002) used 8 mM sulfuric acid as mobile phase, a flow rate of 1ml/min and detection at 210 nm, for HPLC quantification of HCA of *Garcinia indica.* Jayaprakasha and Sakariah (1998) used 10 mM sulfuric acid as mobile phase, flow rate of 0.7ml/min and detection at 210 nm, for HPLC quantification of HCA from *Garcinia combogia.* Jayaprakasha *et al.* (2003) worked with 6 mM sulfuric acid as mobile phase with flow rate of 1ml/min, for LC method for determination of HCA from *Garcinia pendunculata.* Jena *et al.* (2002b) used phosphoric acid (0.01 M) as mobile phase with flow rate of 0.7ml/min and detection at 210 nm, for HPLC quantification of HCA from *Garcinia cowa.* Vinh *et*

al. (2011) used 0.1% phosphoric acid as mobile phase with a flow rate 1ml/min, to analyse the HCA content in the rind of *Garcinia oblongifolia,* through HPLC.

Hydroxycitric acid was resolved as a single peak in HPLC chromatogram. HCA peak was determined by comparison with the retention time of standard HCA. The retention time of standard HCA was 3.7 and all twenty samples analyzed for HCA was quantified at the similar retention time (3.7 ± 0.1) (Fig. 26-28). HCA was the major organic acid present in the fruit extracts of all seven stages of fruit development with the content varying from 4.14 to 12 g/100 g fruits. Jayaprakasha and Sakariah (2002) reported in *Garcinia indica*, that the HCA content of the rinds ranged from 10.27-12.74 g/100 g in samples collected from coastal Karnataka. Jayaprakasha and Sakariah (1998) found varying HCA content (16-18 g/100 g) in three different rinds of *Garcinia combogia*, obtained from local market in Karnataka. Jayaprakasha *et al.* (2003) reported that the HCA content varied from 2.45-20.11 g/100 g amongst the samples (leaf, fruit, pulp and rind) of *Garcinia pendunculata* using liquid chromatography (LC). Jena *et al.* (2002b) noted a varying the HCA concentration, from 1.67-12.69 g/100 g, amongst the different plant parts (leaf, fruit and rind) of *Garcinia cowa.*

In the present investigation, the highest content of HCA (12 g/100 g) was recorded in the mature fruit rinds (80 days after fertilization) collected from Tree No. 3 (Ponda). Similarly, Jayaprakasha and Sakariah (2002) reported maximum HCA content (12.74 g/100g) in rind-III of *Garcinia indica* collected from coastal Karnataka. Jayaprakasha and Sakariah (1998) recorded maximum HCA content (18 g/100g) in rind III of *Garcinia combogia,* obtained from local market in Karnataka. Jayaprakasha *et al.* (2003) found the maximum HCA content to be 20.11g/100g of rind of *Garcinia pendunculata,* using LC.

Vinh *et al.* (2011) recorded the maximum amount of HCA to be 10.14 g/100 g of rind of *Garcinia oblongifolia,* using microwave extraction system by HPLC. Jena *et al.* (2002b) accounted the maximum quantity of HCA to be 12.69 g/100 g of rind of *Garcinia cowa,* using HPLC.

In the present study, amongst the matured fruits (85 days after fertilization), minimum quantity of HCA of 10.56/100 g was recorded in the rind collected from Tree No. 2 (Priol). Jayaprakasha and Sakariah (2002) reported minimum HCA content to be 10.27 g/100 g in rind-I of *Garcinia indica* collected from coastal Karnataka. Jayaprakasha and Sakariah (1998) recorded minimum concentration of HCA to be 16 g/100 g in rind I of *Garcinia combogia*, obtained from local market in Karnataka. Jayaprakasha *et al.* (2003) recorded minimum HCA content in pulp (0.821 g/100 g) of *Garcinia pendunculata.* Jena *et al.* (2002b) observed the minimum HCA content of 1.97 g/100 g leaves and 2.85 g/100 g fruits of *Garcinia cowa.*

Amongst the three locations studied, the HCA content in Tree No.1, Marcel, ranged from 4.86 - 11.45 g/100 g of fruits (Fig. 29); in Tree No. 2, Priol, the HCA content varied from 4.14 - 10.56 g/100 g of fruits (Fig. 30) and in Tree No. 3, Ponda, the HCA content ranged from 6.40 - 12.00 g/100 g of fruits (Fig. 31). The HPLC analysis revealed that, in the early stages of development the HCA content in the fruits was low and as the fruit matured the contents gradually increased. Comparison amongst the matured fruit rinds collected from three different locations, revealed that Tree No. 3, Ponda area, contained maximum quantity of HCA (12.00 g/100 g), followed by Tree No. 1, Marcel (10.70 g/100 g) and Tree No. 2, Priol area (10.56 g/100 g). Minimum HCA content was recorded in early stage of fruits (20 days after fertilization) in Tree No. 2, Priol (4.14 g/100

Fig. 29. HCA content of different developmental stages of fruits of *Garcinia indica* (Tree No.1, Marcel).

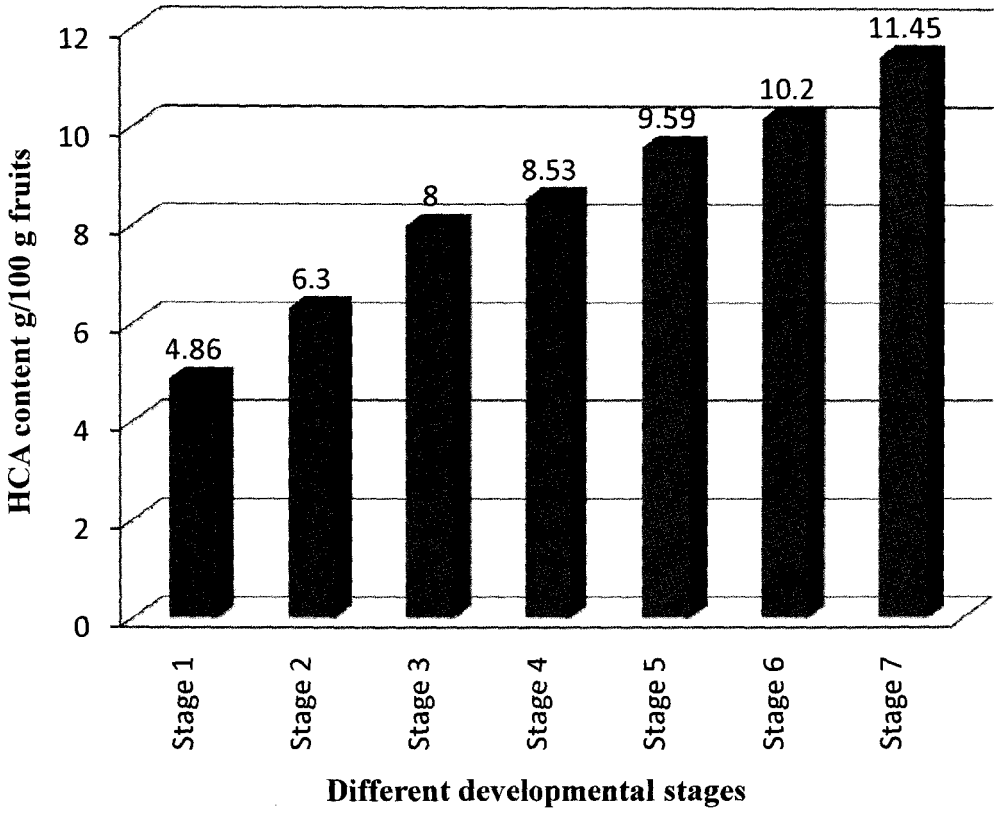


Fig. 30. HCA content of different developmental stages of fruits of *Garcinia indica* (Tree No.2, Priol).

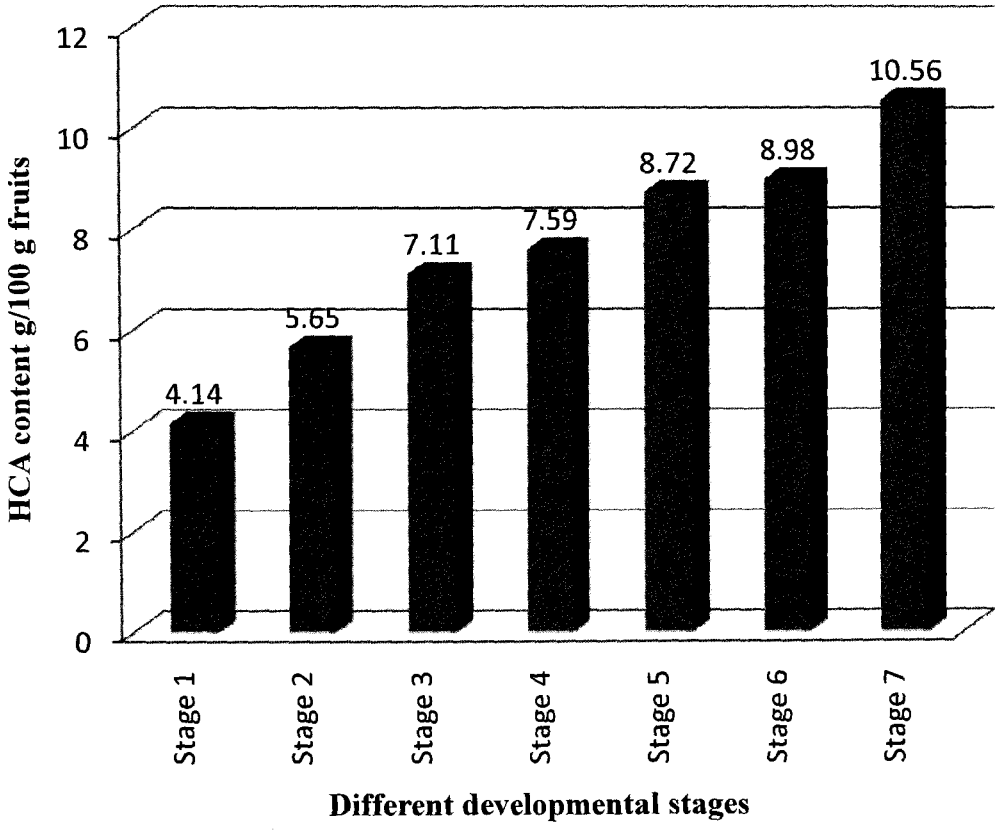


Fig. 31. HCA content of different developmental stages of fruits of *Garcinia indica* (Tree No.3, Ponda).

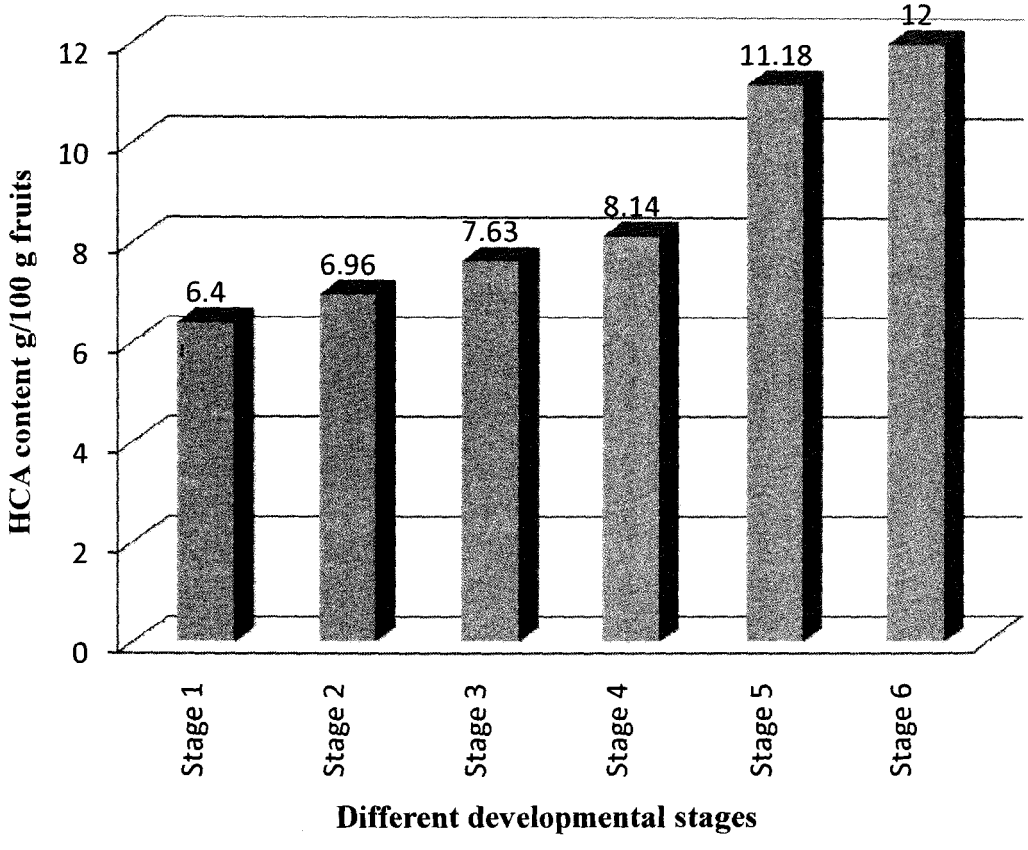
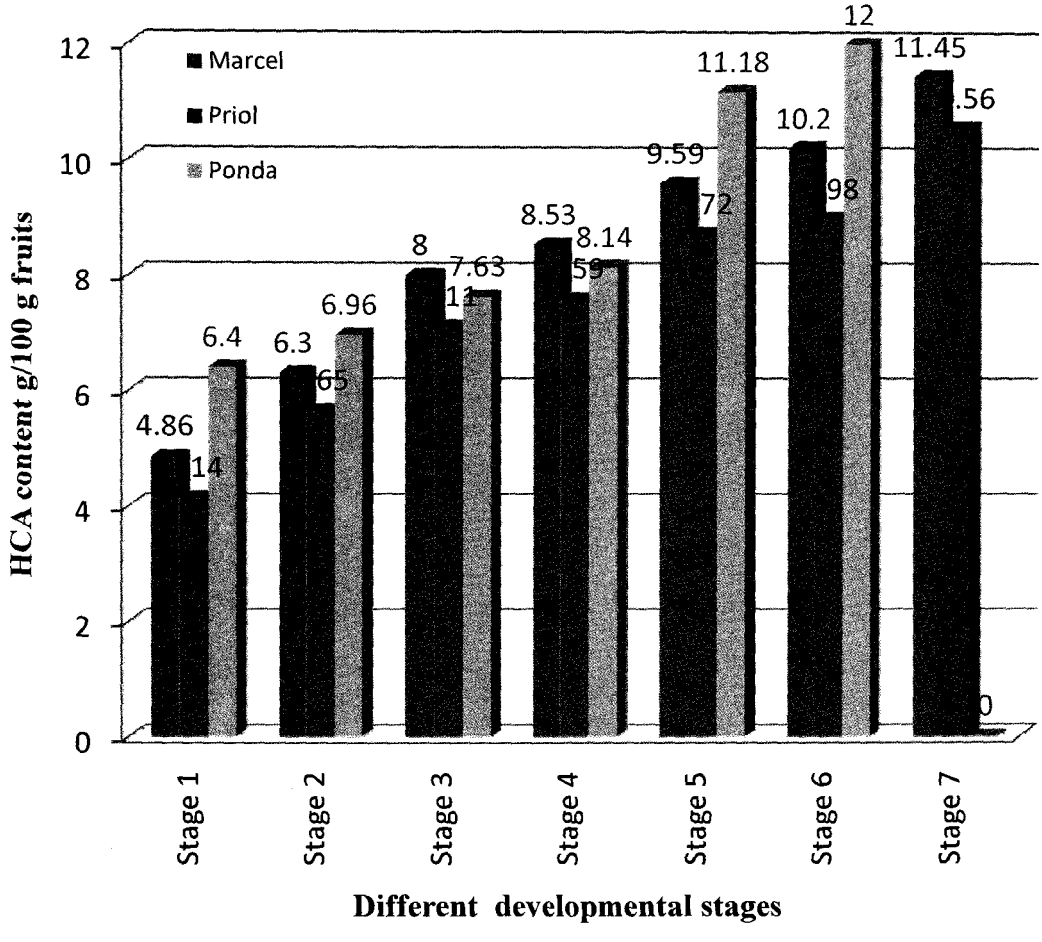


Fig. 32. Comparative account of HCA content of different developmental stages of fruits collected from three different locations.



g), Tree No. 1, Marcel (4.86 g/100 g) and Tree No. 3, Ponda (6.40 g/100 g) (Fig. 32). The variation in HCA content in different locations may be due to the age of the tree in which the fruits were collected or genotype characters of the tree or any specific environmental condition which influences the HCA content, which needs further study.

5. 2. 3. Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis of HCA

The LC-MS analysis of fruit extract using interface of negative ion mode $[M-H]^-$ recorded the molecular mass as 207 (Fig. 33C) which corresponding to HCA molecular mass 208. Similarly, Hida *et al.* (2005) confirmed HCA molecular mass using the LC-MS system in negative ion mode $[M–H]^-$ and recorded the molecular mass as 207. Also, in the present study analyzed using the MS interface of positive ion mode $[M+H]^+$ recorded the molecular mass of HCA as 209 (Fig. 33B) which indicates the molecular mass of HCA 208.

5. 2. 4. Biochemical Analysis of Garcinol

5. 2. 5. Determination of Garcinol by HPLC System

The HPLC analysis of standard garcinol resolved with single peak (Fig. 34A), however, during the analysis of fruit samples of various developmental stages, two peaks were resolved close to each other, this may be due to the fragmentation of garcinol (Fig. 34B-D). Marti *et al.* (2010) also observed fragmentation of garcinol during the analysis of root and bark extract of *Symphonia globulifera*. Padhye *et al.* (2009) and Chattopadhyay *et al.* (2007) have reported the existence of large number of isomeric nature of polyisoprenylated benzophenones derivatives. Due the above nature of garcinol, quantitative estimation of garcinol during this study could not be possible and needs further investigation.

5. 2. 6. Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis of Garcinol

The analysis of fruit sample using LC and mass spectrometry (MS) interface of negative ionic mode $[M–H]^-$ and recorded the molecular mass of garcinol as 601 (Fig. 35B) and indicates the actual molecular mass of garcinol 602. Similarly, Kaur *et al.* (2012) reported the molecular mass garcinol as 601 in negative ionic mode $[M–H]^-$ by LC-MS.

5. 3. Developmental and Histochemical Studies

5. 3. 1. Floral Characteristics

The systematic observation of *Garcinia indica* trees revealed the polygamodioecious nature. Based on the floral characteristics, three different types of trees were noted: trees with only male flowers; trees with only female flowers; trees with bisexual flowers and the same tree containing male flowers. Researchers have reported the polygamodioceous nature of *Garcinia indica* (Thengane *et al.*, 2006a; Deodhar *et al.*, 2008; Tembe and Deodhar, 2011). Flowering in Kokum starts during the month of November and continues up to February. Fruiting season is usually from April to June (Korikanthimath and Desai, 2012).

The flowers of *Garcinia indica* were axillary or terminal, solitary or in clusters. Each flower was noted with four sepals, thick, fleshy, green in colour (Plate 20G, H). Petals were four, yellow to purple in colour. Male flowers consisted of numerous short filamented anthers (Plate 20A, B), two lobed and each lobe with two locules (tetraloculate anthers). It was observed that in bisexual flowers ovary was with short style and 3-4 stigmatic lobes (Plate 20B). Female flowers were mostly solitary, ovary with sessile style and 6-8 stigmatic lobes (Plate 20C, I). Similar observation was reported in *Garcinia indica*

(Himabindu *et al.*, 2010). *Garcinia indica* flowers had superior ovary, orthotropous type of ovule, axial placentation, with 4-8 locules and 4-8 ovules (Plate 25A). The other Clusiaceae members, such as, *Garcinia gardneriana* are reported to have superior trilocular ovary and each locule with one ovule (Asinelli *et al.*, 2011), *Vigmia guianensis* has superior ovary and pentalocular ovules with axile placentation (Mourao and Beltrati, 2001) and *Mammea americana* is reported to have superior ovary, two ovules with axile placentation (Mourao and Beltrati, 2000).

In the present study, it was observed that the flowering in male trees starts one week earlier than other trees. Male flowers had long pedicel, female flowers had short pedicel while bisexual (hermaphrodite) flowers had intermediate length of pedicel (Plate 20D-F). Similar observation of varying pedicle characters was reported in *Garcinia combogia* (Abraham *et al.*, 2010). It was noted during the study that the male flowers contain more number of stamens per flower in comparison to the bisexual flowers (Plate 20A-E).

5. 3. 2. Structure and Development of Fruit

During this study, it was observed in *Garcinia indica* that the ovary at anthesis stage contain uniseriate epidermis with small rectangular cells (Plate 21). Thin cuticle was found over the epidermis. The other members of Clusiaceae was reported to have outer uniseriate epidermis with cubic cells and thick cuticle over the epidermis in the fruits of *Mammea americana* (Mourao and Beltrati, 2000), *Garcinia gardneriana* (Asinelli *et al.*, 2011) and *Vigmia guianensis* (Mourao and Beltrati, 2001).

In the present study, transverse section of the fruit revealed the presence of few cell layers of ovarian mesophyll in between outer and inner epidermis of ovary wall. The

ovarian mesophyll cells are found to have fundamental parenchyma, arranged compactly with few intercellular spaces. In between the ovarian mesophyll cells, large number of branched laticifers was present (Plate 21). Similarly, Mourao and Beltrati (2001) reported in *Vigmia guianensis*, that the ovarian mesophyll is composed of fundamental parenchyma, with few intercellular spaces, where a number of branched secretory ducts occur. In *Mammea americana,* Mourao and Beltrati (2000) stated that the ovarian mesophyll is composed of parenchyma cells of varied sizes with a number of ramified schizogenous secretory ducts. *Garcinia gardneriana* ovary was also composed of mesophyll parenchyma cells with numerous secretory ducts (Asinelli *et al.*, 2011).

It was found during the study that about 5 DAF the ovarian mesophyll cells differentiated in to several layers of mesocarp. The mesocarp cells were tangentially elongated. At this stage, laticifers (which secrete the yellow latex) increased in size. The mesocarp derived from the outer region of ovarian mesophyll cells (many layered) was actually derived from the ventral meristem (Plate 21). Mourao and Beltrati (2001) observed in *Vigmia guianensis* that, the mesocarp derived from the ovarian mesophyll cells remained parenchymatic and many secretory ducts increased in diameter and branched.

The present study revealed that the endocarp of *Garcinia indica* was composed of parenchymatic cells which were derived from a ventral meristem. The endocarp differentiates from the inner few layers of ovarian mesophyll and also partially from inner sub-epidermal cell layers. It was also found that the inner epidermis delimits the locule (Plate 21). Laticifers or secretory ducts were absent in endocarp region or in the peripheral region of endocarp where vascular bundles were located (Plate 23B). At this stage, middle

and outer region of endocarp consisted of several developing juice cells (Plate 23C). Later as the seed developed, it was noted that the inner cell layers of endocarp underwent a radial elongation and the radially elongated cells got firmly attached to the fibrous testa (Plate 22). In other members of Clusiaceae like *Mammea americana,* Mourao and Beltrati (2000) reported that the endocarp contains parenchymatic cells derived from a ventral meristem which later differentiates from an inner part of the ovarian mesophyll cells and also from the inner epidermis. These layers devoid of secretory ducts, later undergo a radial elongation and at the end of seed development, these radially elongated cells, get tightly attached to the fibrous testa. In *Vigmia guianensis*, endocarp was derived partially from the inner epidermis and partially from the inner sub-epidermal layers of the ovary. Both, inner epidermis and inner sub-epidermal layers were formed originally from ventral meristem (Mourao and Beltrati, 2001).

The present study described the structure of matured fruits in *Garcinia indica*. The ripened fruit was observed with two distinct parts: rind and pulp along with seeds (Plate 23F). The rind consisted of exocarp and mesocarp, which stored primarily secondary metabolites like, hydroxycitric acid, garcinol and others. The rind also contained large quantity of anthocyanin pigments. The endocarp (pulp) was white in colour and was the edible part (Plate 23F), which rich in sugars. Asinelli *et al.* (2011) noted in *Garcinia gardneriana* that the pulp was white, edible with a sweet flavor and mainly derived from the endocarp, although it includes part of the spongy mesocarp in which the ducts have lost their functionality. In *Mammea americana,* Mourao and Beltrati (2000) reported that the mature fruit rind to consist of exocarp with periderm and mesocarp with branched secretory ducts and vascular bundles. The pulp was yellowish and edible, rich in sugars.

5. 3. 4. Laticifers in Fruit

An early developmental stage of fruit was observed with large number of laticifers, which secretes yellow latex. Longitudinal section of young fruit (5 DAF) was noted to contain a network of branched tubular laticifers (Plate 24A, C). It was observed that during the development of fruit, laticifers slowly lost their secretory nature and became non-functional when the fruit ripened (Plate 24F). Similarly, it was reported in *Garcinia gardneriana* that the secretory ducts are initially present in mesocarp and performed their function at early stage of development. They lost their function in the ripened fruits (Asinelli *et al.*, 2011). Similar characteristics of laticifers were noted in *Mammea americana* (Mourao and Beltrati, 2000) and *Vigmia guianensis* (Mourao and Beltrati, 2001).

5. 3. 5. Structure and Development of Seed

After fertilization, the zygote divided and formed the seed. Each fruit contained 4-8 seeds (Plate 25A). The maturation of seed occurred from the periphery towards the centre. Mature seeds were filled with seed storage parenchyma cells which stores major storage compounds (Plate 26E-G). The mature seed was creamish, ellipsoid in shape, elongated, angular and broad at one side (top portion) and narrow towards the bottom. It was reported in *Vigmia guianensis* that the mature seeds were red, ellipsoid in shape, elongated and more or less angular (Mourao and Beltrati, 2001). Ellipsoidal shape of mature seed was reported in *Garcinia gardneriana* (Asinelli *et al.*, 2011). In *Mammea americana,* the mature seed was ellipsoid and slightly curved (Mourao and Beltrati, 2000).

In *Garcinia indica*, the vascular structures ran almost in the entire length of the seed, usually starting from one side on top portion of the seed and ending towards the

bottom on the other side (Plate 26B). The vasculature was found to be primary in growth, since no well defined embryo was present in the mature seed. The procambial strand present in the entire length of the seed, probably acted as an embryonic tissue, later giving rise to the plumule and the radical during germination. *Garcinia combogia* seeds are reported to have a vasculature similar to *G. indica* (Malik *et al.*, 2005b). *Garcinia gardneriana* was found to have a procambial strand that runs through the entire seed from down to top (Asinelli *et al.*, 2011). In contrast, *Garcinia xanthochymus* was not observed to have any such vasculature (Malil *et al.*, 2005b). Mourao and Beltrati (2001) noted a whitish-yellow embryo in the seed with two plano-convex cotyledons in *Vigmia guianensis*. Asinelli *et al.* (2011) reported in *Garcinia gardneriana,* many secretory ducts in the central procambial cylinder of the seed. In *Mammea americana,* many branched secretory ducts were found distributed longitudinally in the cotyledonary parenchyma cells of the seed (Mourao and Beltrati, 2000). In the present investigation, however, no laticifers or secretory ducts were found distributed in the entire seed. Mourao and Beltrati (2001) reported no secretory ducts in the seeds of *Vigmia guianensis*.

5. 3. 6. Histochemical Studies of Fruit and Seed

Starch: In male flowers, starch was observed in the thalamus and petal regions (Plate 27A). In bisexual flowers, starch was localized in petal, base of the anther filament and the thalamus region (Plate 27B). In female flowers, starch was present in sepals, petals and ovary, with starch deposition higher in the sepal as well as in peripheral regions of the pedicel (Plate 27C). The young ovary at anthesis stage was detected with minute quantity of starch in the nucellus and lacuolar wall (Plate 27D). Krishnan *et al.* (2001) observed the presence of starch in the nucellus and pericarp of pre-anthesis rice ovary.

The Kokum fruit about 10 DAF was detected with small quantity of starch in the mesocarp. In seeds, starch was localized in the testa as well as in the entire developing seed (Plate 27E). In rice, it was reported that starch begins to accumulate within the endosperm in about 5 DAF (Krishnan *et al.* 2001). In transverse section of Kokum seed about 35 DAF, starch was found all over the seed including in the epidermal cells (Plate 27F, G). In rice, 14 DAF, starch completely disappears in the pericarp (Krishnan *et al.* 2001). In Kokum matured seed, 85 DAF, showed presence of starch deposits in each seed storage parenchyma cells. The starch was also observed in adjacent regions of the vasculature (inner and outer side of the vasculature), however, in actual vasculature region no starch was detected (Plate 27H, I). In matured rice grain (30 DAF), the endosperm cells were completely filled with starch and embryo showed comparatively less quantity of starch (Krishnan *et al.* 2001).

Proteins: Kokum fruit contains about 1% of proteins (Nayak *et al.*, 2010b), however, in the present study, no significant amount of protein was detected in the fruit by histochemical staining reaction. The Kokum seed about 20 DAF, revealed the presence of proteins as indicated by mild fluorescence (Plate 28A, B). Transverse section of seed 35 DAF, observed high accumulation of proteins in comparison to the early stages (Plate 28A). Similarly in rice, in early stages of development of caryopsis, no significant amount of protein was reported, however, during active grain-filling stages (about 15 DAF) protein deposition increased in the endosperm cells (Krishnan *et al.* 2001). Transverse section of seed 85 DAF, observed high concentration of proteins in comparison with early stages. The protein concentration increased as the seed matures (Plate 28A-F). It was also noted that protein bodies were prominent and larger in size as the seed attained maturity (Plate 28F). In mature rice caryopsis, about 80% of the proteins were found distributed in

the sub-aleurone region of endosperm cells, in cultivated variety as well as in the wild species of rice (Krishnan *et al.* 2001).

Lipids: The Kokum seed oil (fat) known as 'Kokum butter' finds several applications (Kshiragar *et al.*, 2003; Maheshwari and Yella Reddy, 2005). In early stages of development of Kokum seed (15 DAF), low concentration of lipids were detected, as indicated by faint yellow fluorescence (Plate 29A). In rice, about 10 DAF, lipids were observed in aleurone as well as in developing embryo (Krishnan and Dayanandan, 2003). Transverse section of Kokum seed 50 DAF showed higher fluorescence (Plate 29B) in comparison with early stages, indicating the high quantity of lipids. However, the lipids bodies in the early stages were not as prominent as compared to later stages (Plate 29A-D). Transverse section of seed 85 DAF (matured Kokum seed), observed maximum quantity of lipids (Plate 29D). At this stage, it was observed that no lipids were present in the actual vasculature, however, lipids were found in the interior cells of the vasculature (Plate 29E). In matured Kokum seeds, distinct lipid bodies were present (Plate 29F). Krishnan *et al.* (2001) and Krishnan and Dayanandan (2003) reported matured caryopsis of rice to contain prominent lipid bodies in the aleurone, the pigment strand region and the embryo.

The histochemical localization of starch, proteins and lipids in Kokum fruit and seed revealed that the early stage of development of fruit and seed contained very low concentration of these storage compounds. As the fruit and seed matured and seed storage parenchyma cells developed, the starch, proteins and lipids reached to its maximum level. Similarly, in rice, an early stage of development contains very low concentration starch, lipids and protein as compared to the matured grain (Krishnan and Dayanandan, 2003;

Krishnan *et al.*, 2001). Krishnan *et al.* (2011) reported the pattern of distribution of starch, proteins and lipids in the cotyledons of *Anacardium occidentale*.

Lignin and Tannins: Lignin was detected in seeds as well as in fruits of Kokum. Lignin was found localized in mature seeds (85 DAF) in the vasculature where xylem vessels were present (Plate 30A). In the vasculature, the xylem vessels were found as patches as indicated by histochemical staining reaction (Plate 30B). In Kiwi fruits, lignin was reported to occur in the xylem vessels and seed as revealed by phloroglucinol staining reaction (Banos and Long, 2003). Tannins in Kokum were localized in the testa or seed coat. It was observed that the tannin deposition starts from 20 DAF, as revealed by ferric chloride staining reaction (Plate 30F). As the seed matures and seed coat development completes, the tannin concentration increases and reaches to the maximum level at maturity. The fruit 75 DAF, without any staining reaction also detected clear presence of tannins, as indicated by black deposits in a macro-photograph (Plate 30E). Tannins in the pseudocarp of *Anacardium occidentale* were localized using ferric chloride staining reaction (Krishnan *et al.*, 2011).

Hydroxycitric acid (HCA): In the present investigation, biochemical reaction was developed for the localization of HCA, during various stages of development of fruit of *Garcinia indica*. When thick sections of fruits were reacted with 1% sodium metavanadate, it formed orange-red colour. This biochemical reaction was confirmed using the standard HCA (Plate 31E). Early stage of development of fruit (20 DAF) showed the presence of low concentration of HCA as indicated by faint orange red (Plate 31A). The gradual increase in HCA content was noted 35 DAF (Plate 31B), 50 DAF (Plate 31C), 60 DAF (Plate 31D), 75 DAF (Plate 31F) and 80 DAF (Plate 31G). HCA level reached to

the maximum in matured fruit (85 DAF) as shown by high intensity of orange-red biochemical reaction (Plate 31H). The increased level of HCA in matured fruits, as observed in the biochemical reaction, was confirmed through detailed HPLC analysis carried out during this investigation (Section 4. 2 & 5. 2). Both biochemical reaction and HPLC analysis revealed that the early stage of fruits contained low quantity of HCA and the HCA content gradually increased and reached to the maximum level at maturity (Table 26; Fig. 32).

SUMMARY

SUMMARY

Garcinia indica Choisy is commonly known as Kokum belongs to the family Clusiaceae (Guttiferae). The genus *Garcinia* comprising over 200 species in the world, of which about 30 different species are known to occur in India, 7 spp. are endemic to Western Ghats, 6 spp. known to grow in Andaman and Nicobar islands and 4 spp. are in North East India. *Garcinia indica* is an endemic tree species found distributed in tropical rain forest of Western Ghats of India.

1. Micropropagation

Mature seed segments cultured on WPM supplemented with 0.5-5 mg/L BAP showed swelling of seed segment after a week of inoculation. Shoot buds were initiated on the outer portion of cut regions. Maximum number of shoot buds (27) was recorded in 4.5 mg/L BAP with 100% regeneration after 31-35 days of culture. Minimum number (4) of shoot buds was noted on medium containing 0.5 mg/L BAP. The length of shoots varied with varying concentrations, and the length shoots ranged from 2.1-5.1 cm. In kinetin treatment (0.5 to 5 mg/L), after 28 days of inoculation, the explants showed shoot bud primodium formation. Maximum number of shoot buds per explant (10.8) and 60% culture regeneration was recorded on medium containing 3 mg/L KIN. Minimum culture regeneration of 40% and minimum number shoot buds per explant (2) with 0.5 mg/L of KIN. The length of regenerated shoots ranged from 0.5-1 cm. The highest length (1 cm) of shoots was obtained on medium fortified with 3 and 5 mg/L KIN. While lowest length (0.5 cm) was observed with 4 mg/L KIN.

Seed segments inoculated on WPM containing 2, 4-D at 0.5-5 mg/L showed callus formation after 7-10 days of culture. Callus development was prominently seen at the cut portion of the explants at all the concentrations. The maximum of 90% callus formation was recorded in 2, 4-D at 3 mg/L. Minimum (53.3%) callus formation was observed at 4

mg/L. The callus was not compact, looked very spongy and was whitish brown. The callus did not respond further, turned black and lost their potential for regeneration.

Mature seed segments cultured on WPM supplemented with BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 mg/L) and addition of 1 mg/L IAA, initiated shoot bud primordium at peripheral (cut region and surrounded areas) of explants, later it was seen all over the explants. Maximum of 86.2 shoot buds per seed segment was recorded in the medium supplemented with combination of 2 mg/L BAP and 1 mg/L IAA. Minimum number of shoot buds (9) was observed in medium supplemented with 0.5 mg/L BAP + 1 mg/L IAA. Maximum length (3.2cm) of shoots was recorded on both 4 and 4.5 mg/L BAP. In combinations of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 mg/L) and NAA at 1 mg/L showed whitish green callus along with the formation of shoot bud primordium. The maximum number (61.2) of shoot buds was induced on medium supplemented with combination of 4.5 mg/L BAP and 1 mg/L NAA. Minimum number (6) of shoot buds per segment was induced on medium supplemented with 0.5 mg/L BAP and 1 mg/L NAA. Highest length of shoots (3.2 cm) was recorded on medium supplemented with 2 mg/L BAP and 1 mg/L NAA while lowest length of shoots (2.1 cm) was noted on medium supplemented with 4.5 mg/L BAP and 1 mg/L NAA.

When explants cultured on WPM supplemented with combination of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5 mg/L) and IBA at 1 mg/L, the maximum number shoot buds (42.7) initiated on 4.5 mg/L BAP and 1 mg/L IBA with 100% of culture regeneration. Minimum number of shoot buds (9.5) with 50% of culture regeneration was observed on medium supplemented with 0.5 mg/L BAP and 1 mg/L IBA. Highest length of shoots (3.5 cm) was measured on BAP (4 mg/L) and IBA (1 mg/L) and lowest (2.2 cm) was noted on 3 and 3.5 mg/L BAP + IBA (1 mg/L).

The elongation of shoots achieved on the WPM containing 1 mg/L BAP. Elongated shoots of 3-5 cm height were cultured on half strength WPM medium with

different concentrations (0.5-5 mg/L) of IBA and NAA for root initiation. Highest frequency of rooting (100 and 93.3%) was recorded on 3 and 4 mg/L IBA, respectively. Maximum number of roots (2.8) as well as maximum length of roots (3.65 cm) was achieved at 3 mg/L IBA. In NAA treatment, maximum percentage (90%) of rooting was noticed at 5 mg/L NAA.

Root hardening was carried out on quarter strength WPM without sucrose. Root hardened plants were transplanted into seed trays filled with different potting mixtures. Survival rate of transferred plantlets ranged from 13.3 to 96.6%. Maximum survival rate of 96.6% was recorded in the combinations of garden soil + sand (1:1). Among the potting mixture, garden soil and sand (1:1) showed the maximum survival rate, hence, garden soil and sand (1:1) was selected for further acclimatization of all regenerated plants during the entire study. After 20 days of growth in seed trays with garden soil and sand (1:1), the plantlets were transplanted into small plastic pots containing garden soil and vermicompost (3:1). After 6 months of growth, the regenerated plants, grown to the height of about 1 feet were transferred to large black polythene bags containing garden soil: manure: sand (2:1:1) for further growth.

Histological studies were carried out to determine the origin and development of shoot bud primordium and shoot buds. Transverse section of seed segments after one week culture showed the presence of large number of meristematic zones, mostly present in central region. After two weeks of inoculation, the explants showed more number of meristematic zones toward the peripheral region. In three week cultures, the meristematic zones further developed into shoot primordium. Later, the shoot primordium developed in to shoot buds after four weeks of culture.

Anatomical characterization of regenerated plants and mother plants revealed no any significant difference in the structure of leaf, stem and root. Similarly, comparison of DNA of regenerated plants with mother plants using RAPD random primers and ISSR

random primers showed no variation in number of bands indicating no genetic variation and genetic stability is maintained in the regenerated plants.

2. Biochemical Analysis of Hydroxycitric acid and Garcinol

Fruits of six and seven different developmental stages were collected from high yielding trees located at three different locations such as Marcel (Tree No.1), Priol (Tree No.2) and Ponda (Tree No.3), Goa. Amongst the three locations, the HCA content in Tree No.1, Marcel, ranged from 4.86 - 11.45 g/100 g of fruits; in Tree No. 2, Priol, HCA content varied from 4.14 - 10.56 g/100 g of fruits and the HCA content of Tree No. 3, Ponda ranged from 6.40 - 12.00 g/100 g of fruits. The HPLC analysis revealed that, in the early stages of development the HCA content in the fruits was low and as the fruit matures the content gradually increases. Liquid chromatography and mass spectrometry (LC-MS) analysis of HCA confirmed the molecular mass of HCA as 208.

The HPLC analysis of garcinol standard resolved with single peak at retention time (RT) 1.6. However, the samples of various developmental stages revealed with two prominent peaks vary close to each other at RT 2 may be due to the fragmentation of garcinol or unstable nature of garcinol (high sensitive to oxygen and existence of large number of isomers). Hence, quantitative estimation of garcinol could not be possible and needs further study. However, liquid chromatography and mass spectrometry (LC-MS) analysis confirmed the presence of garcinol in samples indicating the molecular mass of garcinol as 602.

3. Developmental and Histochemical Studies

Garcinia indica trees revealed with polygamodioecious nature. Three different types of trees were noted, i.e. trees with only male flowers, trees with only female flowers and trees with bisexual flowers and the same tree containing male flowers. Flowering in Kokum starts during the month of November and continues up to February. Fruiting season is usually from April to June.

Structure and Development of fruit and Seed: At the time of anthesis, the outer most layer of ovary consists of uniseriate epidermis with small rectangular cells. The few cell layers of ovarian mesophyll are present in between outer and inner epidermis. In between the ovarian mesophyll cells, large number of branched laticifers was observed. About 5 days after fertilization (DAF), the outer epidermis and sub epidermal cells of the ovary develops into 2-3 layers of exocarp. The ovarian mesophyll cells differentiate in to several layers of mesocarp. At this stage, laticifers increase in size and secrete the yellow latex. The mesocarp derived from the outer region of ovarian mesophyll cells (many layered) is actually derived from the ventral meristem. Similarly, the endocarp (parenchymatous cells) were also derived from a ventral meristem and developed from inner part of the inner region of ovarian mesophyll (few layer) and the inner epidermis. Laticifers were absent in endocarp region. The peripheral region of endocarp shows vascular bundles. At this stage, the middle and outer region of endocarp contains several developing juice cells. Later, inner cell layers of the endocarp undergo a radial elongation as the seed develops and it firmly attaches to the fibrous testa. The ripened fruits have two distinct parts: rind and pulp along with seeds. The rind consists of exocarp and mesocarp. The endocarp (pulp) is white, edible and rich in sugars.

Structure and Development of Seed: At the time of anthesis, the ovule has large amount of nucellar tissue. The nucellar tissue is semi-solid and grows towards the centre of the ovule. Within the nucellus embryo sac develops. After fertilization, zygote divides and the development of seed starts. In a single fruit, 4-8 seeds develop. Seed maturation occur form periphery towards the centre. About 15 DAF, peripheral regions of seed filled with seed storage parenchyma cells and central region appears empty. About 20 DAF, the seed storage parenchyma cells get completely filled in the seeds. Transverse section of seed 35 DAF, noted the appearance of vasculature in near central portion of the seed which appears like a small ring like structure which stains darkly. The vascular structures run

almost in the entire length of the seed, usually starting from one side of the top portion of the seeds and ending towards the bottom on other side. Transverse section of mature seed revealed the presence of distinct rectangular storage parenchyma cells.

Histochemical Studies

Starch: The young ovary at anthesis starch was detected with minute quantity in the nucellus and locular wall. The fruit 15 DAF showed starch deposition in the seed coat, developing seed and in the fruit. Transverse section of seed about 35 DAF, showed starch all over, including epidermal cells. Matured seed, 85 DAF, showed clear presence of starch deposits in each seed storage parenchyma cells. The starch was also observed in adjacent regions of the vasculature and no starch was detected in actual vasculature region. **Proteins:** An early stage of development of seed no significant quantity of proteins was detected. The seeds about 20 DAF showed very small quantity of proteins as indicated by mild fluorescence. Seed 35 DAF, observed higher accumulation of proteins in comparison with early stages. Seeds of 85 DAF observed with higher concentrations of proteins. The protein concentration increases as the seed matures. It was also noted that protein bodies are prominent and larger in size as the seed attains maturity. **Lipids:** Early stages of development of seed (15 DAF) detected low concentration of lipids. The seed 85 DAF was localized with the highest quantity of lipids. It was observed that early stages of development of seed contain very low concentration of lipids. In matured seeds, lipid concentration reaches to the maximum level, distinct storage parenchyma cells and various sizes of lipid bodies were detected. No any lipids present in vasculature, but lipids were localized in both inner and outer side of the vasculature tissue. **Lignin and tannins:** Lignin was detected in mature seeds (85 DAF), in the vasculature and in xylem vessels. In fruits, lignin was found in laticifers cells and vascular tissues. Tannins were localized only in the testa or seed coat.

Hydroxycitric acid (HCA) was localized during various stages of fruit development using 1% sodium metavanadate. Early stages of fruit (15 DAF) revealed the

presence of low concentration of HCA as indicated by biochemical reaction (faint orange red colour). The fruit 20 DAF depicted a higher concentration of HCA in comparison with early stages. The continuous increase in HCA content was noted in 35 DA, 50 DAF, 70 DAF and 82 DAF. The highest concentration of HCA was detected in matured fruit (85 DAF) as revealed by high intensity of orange-red staining reaction. It was observed that at early stage of fruit development, the HCA concentration was lower. As the fruit matures, HCA concentration gradually increases and reaches to the maximum level at maturity.

In the present investigation, protocol was developed and standardized for *in vitro* regeneration, mass multiplication and production of elite clones from mature seed segments. Histological studies revealed the origin and development of multiple shoots from mature seed segments. The regenerated plants and corresponding mother plants showed anatomically similar structures and molecular characterization revealed no any variation and genetic stability is maintained. The maximum HCA content of 12.00 g/100 g of fruits was recorded in Tree No. 3 (Ponda). Liquid chromatography and mass spectrometry (LC-MS) analysis confirmed the molecular mass of HCA as 208 and molecular mass of garcinol as 602. The developmental studies of Kokum fruits revealed various cells, tissue types including laticifers and cells and tissues responsible for the formation of exocarp, mesocarp and endocarp. Similarly, developmental studies of seed revealed the development of vasculature structure near the central portion of seed which run in the entire length of the seed. Mature seeds revealed the presence of distinct rectangular storage parenchyma cells. Histochemical studies revealed the time-course deposition and distribution of starch, protein, lipids, lignin, tannins and HCA during various developmental stages of fruits and seeds.

CONCLUSIONS

CONCLUSIONS

The cultivation of *Garcinia indica* in Goa and Maharashtra occupies an area of about 1000 hectares and annual production of fruits is about 4500 metric tonnes. There is a continuous increase in demand as evident by the market trends and export scenario. Further, the propagation by seeds is becoming rare due to recalcitrant nature of seed and less viability period (about a month). Vegetative propagation and air-layering is not successful, because of the yellow resin exudates from the stem that interferes with the root formation. Softwood grafting is reported to be ideal, however, it dependent on season and rootstock (seedling). The fruit rind contains bioactive compounds such as hydroxycitric acid (HCA) and garcinol, which are pharmaceutical importance. Considering the above, in the present study, developed and standardized an efficient protocol for plant regeneration and mass multiplication of elite clones from mature seed segments of *Garcinia indica.* This protocol could be used for the production of elite clones throughout the year for large scale cultivation and genetic manipulation to enhance the bioactive compounds in future.

The quantitative estimation of HCA during various developmental stages of fruits revealed the HCA content ranging from 4.14 to 12 g/100 g fruits. The HCA content of trees from three different locations, Tree No.1 (Marcel) ranged from 4.86 - 11.45 g/100 g of fruits; in Tree No. 2 (Priol) ranged from 4.14 - 10.56 g/100 g of fruits and in Tree No. 3 (Ponda) ranged from 6.40 - 12.00 g/100 g of fruits. The histochemical localization of HCA also confirmed the presence of high concentration of HCA in the matured fruits. The results indicated that the concentration of HCA found to be low in the early stages of development of fruit and increases to the maximum level in the matured ripened fruits. Hence, any company/organization/groups targeting for the extraction of HCA may harvest the Kokum fruits at completely ripened stage.

The developmental studies fruit and seed revealed various cells and tissue types including laticifers cells. Similarly, histochemical study revealed the deposition of starch, protein, lipids, lignin, tannins and HCA during various developmental stages of fruits and seeds. The developmental studies of fruit and seed help in overall understanding the cells and tissues involved in the development of fruit (exocarp, mesocarp and endocarp) and seed. The histochemical studies revealed the time-course deposition and distribution of major storage and other compounds including HCA during various developmental stages of fruit and seed. The understanding of structure, development and time-course deposition and distribution of major storage and other compounds may help in further improvement of Kokum.

REFERENCES

REFERENCES

Abraham, Z. and Senthilkumar, R. 2007. Kokum, Malabar tamarind and Mysore gamboges. *In*: Peter, K. V. and Abraham, Z. (eds.), *Biodiversity in Horticultural crops*, **1**: 309-319. Daya Publishing House, Delhi, India.

Anike, F. N., Konan, K., Olivier, K. and Dodo, H. 2012. Efficient shoot organogenesis in petioles of yam (*Dioscorea* spp.). *Plant Cell, Tissue and Organ Culture*, 111(3): 303-313.

Anonymous. 1956. The Wealth of India-Raw materials, **4**: 99-107. Council of Scientific and Industerial Research, New Delhi, India.

Asinelli, M. E. C., De Souza, M. C. and Mourao, K. S. M. 2011. Fruit ontogeny of *Garcinia gardneriana* (Planch. and Triana) Zappi (Clusiaceae). *Acta Botanica Brasilica*, **25**(1): 43-52.

Balasubramanyam, K., Altaf, M., Varier, R. A., Swaminathan, V., Ravindran, A., Sadhale, P. P. and Kundu, T. K. 2004. Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *The Journal of Biological Chemistry*, **279**: 33716-33726.

Banerjee, H., Pai, R. A. and Sharma, R. P. 1999. Restriction fragment length polymorphism and random amplified polymorphic DNA analysis of chickpea accessions. *Biologia Plantarum*, **42** (2): 197-208.

Banos, B. S. and Long, P. G. 2003. Influence of temperature on anatomical and histochemical changes of inoculated Kiwi fruit stem scar with *Botrytis cinerea* Per. Fr. *Revista mexicana Fitopatologia*, **21**(1): 33-39.

Baskaran, M and Krishnan, S. 2011. High frequency plant regeneration from the mature seeds of *Garcinia indica*. *Biologia Plantarum*, **55**(3): 554-558.

Boll, P. M., Else, S. and Erik, B. 1969. Naturally occurring lactones and lactames III. The absolute configuration of the hydroxycitric acid lactones, Hibiscus acid and Garcinia acid. *Acta Chemica Scandinavica*, **23**: 286-293.

Borchetia, S., Das, S. C., Handique, P. J. and Das, S. 2009. High multiplication frequency and genetic stability for commercialization of the three varieties of micropropagated tea plants (*Camellia* spp.). *Scientia Horticulturae*, **120**: 544-550.

Braganza, M. A. and Rodrigues J. C. 2012. Wine of waste tropical fruit. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum,* 88-90. Western Ghats Kokum Foundation, Goa, India.

Brandao, I. and Salema, R. 1977. Callus and plantlets development from cultured leaf explants of *Sedum telephium* L. *Zeitschrift Fur Pflanzenphysiologie*, **85**: 1-8.

Chabukswar, M. M. and Deodhar, M. A. 2006. Restoration of rooting competence in a mature plant of *Garcinia indica* through serial shoot tip grafting *in vitro*. *Scientia Horticulturae*, **108**: 194-199.

Chabukswar, M. M. and Deodhar, M. A. 2005. Rooting and hardening of *in vitro* plantlets of *Garcinia indica* Chois. *Indian Journal of Biotechnology*, **4**: 409-413.

Chattopadhyay, S. K. and Satyanshu Kumar, S. 2007. Liquid chromatography-tandem mass spectrometry method for identification and quantification of two biologically active polyisoprenylated benzophenones, isoxanthochymol and camboginol, in *Garcinia* species. *Biomedical Chromatography*, **21**: 1159-1165.

Chen, Y., Fan, J., Yi, F., Luo, Z. and Fu, Y. 2003. Rapid clonal propagation of *Dioscorea zingiberensis*. *Plant Cell, Tissue and Organ Culture*, **73**: 75-80.

CIMMYT. 2005. Quantification and quality control of DNA. *In*: Laboratory Protocols (3rd Edition), 11-12. CIMMYT Applied Molecular Genetics Laboratory, Mexico.

Ciochina, R. and Grossman, R. B. 2006. Polycyclic polyprenylated acylphloroglucinols. *Chemical Reviews*, **106**: 3963-3986.

Corner, E. J. H. 1988. Wayside trees of Malaya. 1. Malayan Nature Society, Kuala Lumpur, Malaysia.

Dalvi, N. V., Salvi, B. R. and Chavan, S. A. 2010. Value added products of *Garcinia indica* Choisy. *In:* Vasudeva, R., Janagouder, B. S., Reddy, B. M. C., Sthapit, B. and Singh H. P. (eds.), *Garcinia genetic resources*, 162-164. College of Forestry, Sirsi, India.

Das, D., Bandyopadhyay, D., Bhattacharjee, M. and Banerjee, R. K. 1998. Hydroxyl radical is the major causative factor in stress-induced gastric ulceration. *Free Radical Biology and Medicine*, **23**: 8-18.

Decruse, W. S., Gangaprasad, A., Seeni, S. and Menon, S. V. 2003. Micropropagation and ecorestoration of *Vanda spathulatha*, an exquisite orchid. *Plant Cell, Tissue and Organ Culture*, **72**: 199-202.

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

Dobos, E., Danos, B. and Bencsik, L. 1994. Callus induction and shoot regeneration in *Sempervivum tectorum*. *Plant Cell, Tissue and Organ Culture*, **36**: 141-143.

Elviana, M., Rohani, E. R., Ismanizan I. and Normah, M. N. 2011. Morphological and histological changes during the somatic embryogenesis of mangosteen. *Biologia Plantarum*, **55**(4): 731-736.

Faisal, M. and Anis, M. 2003. Rapid mass propagation of *Tylophora indica* via leaf callus culture. *Plant Cell Tissue and Organ Culture*, **75**(2): 125-129.

Faisal, M., Ahmad, N. and Anis, M. 2007. An efficient micropropagation system for *Tylophora indica*: an endangered medicinally important plant. *Plant Biotechnology Reports*, **1**(3): 155-161.

Farzana, A. F. R., Bandara, R. M. I. E. K., Aluwihare, P. C. and Eeswara, J. P. 2010. *In vitro* regeneration of shoots from *Garcinia quaesita* leaf explants. *Journal of the National Science Foundation of Sri Lanka*, **38**(3): 157-162.

Ferrandiz, C., Pelaz, S. and Yanofsky, M. F. 1999. Control of carpel and fruit development in *Arabidopsis*. *Annual Review Biochemistry*, **68**: 321-354.

Feyissa, T., Welander, M. and Negash, L. 2007. Genetic stability, *ex vitro* rooting and gene expression studies in *Hagenia abyssinica*. *Biologia plantarum*, **51**(1): 15-21.

Gangopadhyay, M., Chakraborty, D., Dewanjee, S. and Bhattacharya, S. 2010. Clonal propagation of *Zephyranthes grandiflora* using bulbs as explants. *Biologia Plantarum*, **54**(4): 793-797.

Gawankar, M. S., Shingre, D. V. Dalvi, M. B. and Jamadani. 2012. Variability for morpho-physical properties of fruit in Kokum. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum*, 37-40. Western Ghats Kokum Foundation, Goa, India.

George, G. L., Lakshmanan, R., Mathew, K. M. and Rao, Y. S. 2002. Review of research on kokum, camboge and related species. *Journal of Spices and Aromatic Crops*, **11**(1): 1-12.

Glusker, J. P., Minkin, J. A. and Casciato, C. A. 1971. The structure and absolute configuration of the calcium salt of garcinia acid, the lactone of (-)-hydroxycitric acid. *Acta Crystallographica*, **27**: 1284-1293.

Glusker, J. P., Minkin, J. A., Casciato, C. A. and Soule, F. B. 1969. Absolute configuration of the naturally occurring hydroxycitric acids. *Archives Biochemistry and Biophysics*, **132**: 573-577.

Goh, C. J., Ng, S. K., Lakshmanan, P. and Loh, C. S. 1997. The role of ethylene on direct shoot bud regeneration from Mangosteen (*Garcinia mangostana* L.) leaves cultured in vitro. *Plant Science*, **124**: 193-202.

Goh, C. J., Prakash, L. and Loh, C. S. 1994. High frequency direct shoot bud regeneration from excised leaves of Mangosteen (*Garcinia mangostana* L.). *Plant Science*, **101**: 173-180.

Goh, H. K. L., Rao, A. N. and Loh, C. S. 1988. *In vitro* plantlet formation in Mangosteen *Garcinia mangostana* L. trees. *Annals of Botany*, **61**(1): 87-94.

Goh, H. K. L., Rao, A. N. and Loh, C. S. 1990. Direct shoot bud formation from leaf explant of seedlings and mature Mangosteen (*Garcinia mangostana* L.) trees. *Plant Science*, **68**: 113-121.

Goyal, P., Kachhwaha. S. and Kothari, S. L. 2012. Micropropagation of *Pithecellobium dulce* (Roxb.) Benth - a multipurpose leguminous tree and assessment of genetic fidelity of micropropagated plants using molecular markers. *Physiology and Molecular Biology of Plants*, **18**(2): 169-176.

Gunaga, S., Harshakumar, V. C., Manjunath A. V., Damle, P., Asha, A., Vasudeva and Ganeshaiah, K. N. 2010. Mapping diversity, density and distribution of *Garcinia* species in the Western Ghats, India. *In:* Vasudeva, R., Janagouder, B. S., Reddy, B. M. C., Sthapit, B. and Singh H. P. (eds.), *Garcinia genetic resources*, 1-7. College of Forestry, Sirsi, India.

Haldankar, P. M., Pawar, C. D., Kshirsagar, P. J. and Kulkarni, M. M. 2012. Present status and future thrust areas in production technology of Kokum. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum*, 44-53. Western Ghats Kokum Foundation, Goa, India.

Hemshekhar, M., Sunitha, K., Santhosh, M. S., Devaraja, S., Kemparaju, K., Vishwanath, B. S., Niranjana, S. R. and Girishn, K. S. 2011. Overview on genus *Garcinia*: phytochemical and therapeutical aspects. *Phytochemistry Review*, **10**: 325-351.

Heymsfield, S. B., Allison, D. B., Vasselli, J. R., Pietobelli, D. G. and Nunthe, C. 1998. *Garcinia cambogia* (Hydroxycitric acid) as a potential antiobesity agent a randomized control trial. *Journal of the American Medical Association*, **280**: 1596-1600.

Hida, H., Yamada, T. and Yamada, Y. 2005. Production of hydroxycitric acid by microorganisms. *Bioscience, Biotechnology, and Biochemistry*, **69**(8): 1555-1561.

Hima Bindu, K., Suryanarayana, M. A. and Kumar, T. V. 2010. Evalution of *Garcinia indica* Choisy for floral and yield traits. *In:* Vasudeva, R., Janagouder, B. S., Reddy, B. M. C., Sthapit, B. and Singh H. P. (eds.), *Garcinia genetic resources*, 87-90. College of Forestry, Sirsi, India.

Hong, J., Sang, S., Park, H. J., Kwon, S. J., Suh, N., Huang, M. T., Ho, C. T. and Yang, C. S. 2006. Modulation of arachidonic acid metabolism and nitric oxide synthesis by garcinol and its derivatives. *Carcinogenesis*, **27**(2): 278-286.

Huang, L. C., Huang, B. L., Wang, C. H., Kuo, C. I. and Murashige T. 2000. Developing an improved *in vitro* propagation system for slow-growing species using *Garcinia mangostana* L. (mangosteen). *In Vitro Cellular and Development Biology*, **36**(6): 501-504.

Huang, W. J., Ning, G. G., Liu, G. F. and Bao, M. Z. 2009. Determination of genetic stability of long-term micropropagated plantlets of *Platanus acerifolia* using ISSR markers. *Biologia Plantarum*, **53**(1): 159-163.

Jain, N. and Babbar S. B. 2003. Regeneration of juvenile plants of black plum, *Syzygium cuminii* Skeels, from nodal exp of mature trees. *Plant Cell, Tissue and Organ Culture*, **73**(3): 257-263.

Jain, N. and Babbar, S. B. 2000. Recurrent production of plants of black plum, *Syzygium cuminii* (L.) Skeels, a myrtaceous fruit tree, from *in vitro* cultured seedling explants. *Plant Cell Reports*, **19**: 519-524.

Jayaprakasha, G. K. and Sakariah, K. K. 1998. Determination of organic acids in *Garcinia cambogia* (Desr.) by HPLC. *Journal of Chromatography A*, **806**: 337-339.

Jayaprakasha, G. K. and Sakariah, K. K. 2000. Determination of (-)- hydroxycitric acid in commercial samples of *Garcinia cambogia* extracts by liquid chromatography using ultraviolet detection. *Journal of Liquid Chromatography and Related Technologies*, **23**: 915-923.

Jayaprakasha, G. K. and Sakariah, K. K. 2002. Determination of organic acids in leaves and rinds of *Garcinia indica* (Desr.) by LC. *Journal of Pharmaceutical and Biomedical Analysis*, **28**: 379-384.

Jayaprakasha, G. K., Jena, B. S. and Sakariah, K. K. 2003. Improved liquid chromatographic method for determination of organic acids in leaves, pulp, fruits, and rinds of *Garcinia*. *Journal of AOAC International*, **86**(5): 1063-1068.

Jena, B. S., Jayaprakasha, G. K., Singh, R. P. and Sakariah, K. K. 2002a. Chemistry and Biochemistry of (-)-Hydroxycitric acid from *Garcinia*. *Journal of Agriculture and Food Science*, **50**: 10-22.

Jena, B. S., Jayaprakasha, G. K., Singh, R. P. and Sakariah, K. K. 2002b.Organic acids from leaves, fruits and rinds of *Garcinia cowa*. *Journal of Agriculture and Food Science*, **50**: 3431-3434.

Joshi, G. D. 2012. Traditional method of kokum fruit processing. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum,* 71-76. Western Ghats Kokum Foundation, Goa, India.

Joshi, P. and Dhawan, V. 2007. Analysis of genetic diversity among *Swertia chirayita* genotypes. *Biologia Plantarum*, **51**(4): 764-768.

Kaur, S., Chattopadhyay, S. K., Tandon, S. and Sharma, S. 2012. Large scale extraction of the fruits of *Garcinia indica* for the isolation of new and known polyisoprenylated benzophenone derivatives. *Industrial Crops and Products*, **37**: 420-426.

Kiranghanti, S. S. K., Sujata, K. G., Rao, M. S. and Kishor, P. B. K. 2010. Direct somatic embryogenesis and plant regeneration from immature explants of chickpea. *Biologia Plantarum*, **54**(1): 121-125.

Koeberle, A., Northoff, H. and Werz, O. 2009. Identification of 5-lipoxygenase and microsomal prostaglandin E2 synthase-1 as functional targets of the anti-inflammatory and anti-carcinogenic garcinol. *Biochemical Pharmacology*, 77: 1513-1521.

Korikanthimath, V. S. and Desai, A. R. 2006. Status of Kokum (*Garcinia indica* Choisy) in Goa. *In*: Bhat, D. J., Kamat, N. and Shirodkar, A. (eds.), *Compendium on Kokum* (*Garcinia indica*), 17-27. Western Ghats Kokum Foundation, Goa, India.

Korikanthimath, V. S. and Desai, A. R. 2012. Status of Kokum in Goa. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum*, 21-26. Western Ghats Kokum Foundation, Goa, India.

Krishnamoorthy, B., Rema, J. and Mathew, P. A. 2007. Tree spices. *In*: Peter, K. V. and Abraham, Z. (eds.), *Biodiversity in Horticultural crops*, **1**: 256-277. Daya Publishing House, Delhi, India.

Krishnamurthy, K. V. 1988. Methods in plant histochemistry. S. Viswanathan Printers and Publishes Pvt., Ltd., Chennai.

Krishnamurthy, N., Lewis, Y. S. and Ravindranath, B. 1982. Chemical constituents of *Kokum* fruit rind. *Journal of Food Science and Technology*, **19**(3): 97-100.

Krishnan, S. and Dayanandan, P. 2003. Structural and histochemical studies on grain-filling in the caryopsis of rice (*Oryza sativa* L.). *Journal of Biosciences*, **28**(4): 455-469.

Krishnan, S., Datta, K., Baisakh, N., Vasconcelos, M. D. and Datta, S. K. 2003. Tissue-specific localization of β-carotene and iron in transgenic indica rice (*Oryza sativa* L.). *Current Science*, **84**(9): 1232-1234.

Krishnan, S., Ebenezer, G. A. I. and Dayanandan, P. 2001. Histochemical localization of storage components in caryopsis of rice (*Oryza sativa* L.). *Current Science*, **80**(4): 567-571.

Krishnan, S. Datta, K. Parkhi, V. and Datta. S. K. 2009. Rice caryopsis structure in relation to distribution of micronutrients (iron, zinc, β-carotene) of rice cultivars including transgenic indica rice. *Plant Sciences*, **177**(6): 557-562.

Krishnan, S., Freda C. B. and Supriya, D. 2011. Histochemical and physiological studies of seed (nut) and pseudocarp (apple) of *Anacardium occidentale* L. *Journal of Economic and Taxonomic Botany*, **35**(1): 181-188.

Kshiragar, P. J., Gawankar, M. S., Patil, B. P. and Deshpande, S. B. 2003. Kokum - a unique food spice and medicinal fruit tree of Konkan, 38-41. Spices Production Technology. Proceedings of National Seminar on Spices held at I.C.A.R. Research Complex, Goa, India.

Kulkarni, M. D. and Deodhar, M. A. 2002. *In vitro* regeneration and hydroxycitric acid production in tissue cultures of *Garcinia indica* Chois. *Indian Journal of Biotechnology*, **1**: 301-304.

Kumar, N., Modi, A. R., Singh, A. S., Gajera, B. B., Patel, A.R., Patel, M. P and Subhash, N. 2010. Assessment of genetic fidelity of micropropagated date palm (*Phoenix dactylifera* L.) plants by RAPD and ISSR markers assay. *Physiology and Molecular Biology of Plants*, **16**(2): 1-7.

Kumar, S., Chattopadhyay, S. K., Darokar, M. P., Garg, A. and Khanuja, S. P. 2007. Cytotoxic activities of xanthochymol and isoxanthochymol substantiated by LC-MS/MS. *Planta Medica*, **73**: 1452-1456.

Lakshmanan, P., Ng, S. K., Loh, C. S. and Goh, C. J. 1977. Auxin, cytokinin and ethylene differentially regulate specific development state associated with shoot bud morphogenesis in leaf tissue of mangosteen (*Garcinia mangostana* L.) cultured *in vitro*. *Plant Cell Physiology*, **38**: 59-64.

Lewis, Y. S. 1969. Isolation and properties of hydroxycitric acid. *In*: Colowick, S. P. and Kaplan, N. O. (eds.), *Methods in Enzymology*, **13**: 613-619. Academic Press, New York.

Lewis, Y. S. and Neelakantan, S. 1965. (-)-Hydroxycitric acids - The principal acid in the fruits of *Garcinia cambogia*. *Phytochemistry*, **4**: 619-625.

Li, Z., Duan, S., Kong, J., Li, S., Li, Y. and Zhu, Y. 2007. A single genetic locus in chromosome 1 controls conditional browning during the induction of calli from mature seeds of *Oryza sativa* ssp. Indica. *Plant Cell, Tissue and Organ Culture*, **89**: 237-245.

Liao, C. H., Ho, C.T. and Lin J. K. 2005. Effects of garcinol on free radical generation and NO production in embryonic rat cortical neurons and astrocytes. *Biochemical and Biophysical Research Communications*, **329**: 1306-1314.

Liao, C. H., Sang, S., Liang, Y. C., Ho, C. T. and Lin, J. K. 2004. Suppression of inducible nitric oxide synthase and cyclooxygenase-2 in downregulating nuclear factor-kappa B pathway by Garcinol. *Molecular Carcinogenesis*, **41**: 140-149.

Little, S. A., Stockey, R. A. and Penner, B. 2009. Anatomy and development of fruits of Lauraceae from the middle Eocene princeton chert. *American Journal of Botany*, **96**(3): 637-651.

Liu, C.Z., Murch, S.J., Demerdash, M. E. L. and Saxena, P. K. 2003. Regeneration of the Egyptian medicinal plant *Artemisia judaica* L. *Plant Cell Reports*, **21**: 525-530.

Liu, H., Xu, Y., Liu, Y. and Liu, C. 2006. Plant regeneration from leaf explants of *Rhodiola fastigiata*. *In Vitro Cellular and Developmental Biology-Plant*, **42**: 345-347.

Lloy, G. and McCown, B. 1981. Commercially feasible micropropagation of Mountain laurel, *Kalmia latfolia*, by use of shoot tip culture, *Combined Proceedings of International Plant propagators Society*, **30**: 421-426.

Loc, N. H., Duc, D. T., Kwon, T. H. and Yang, M. S. 2005. Micropropagation of zedoary (*Curcuma zedoaria* Roscoe) a valuable medicinal plant. *Plant Cell, Tissue and Organ Culture*, **81**(1): 119-122.

Lowenstein, J. M. and Bruneugraber, H. 1981. Hydroxycitrate. *In*: Lowenstein, J. M. (ed.), *Methods in Enzymology*, 486-497. Academic Press, New York.

Maheshwari, B. and Reddy, S. Y. 2005. Application of Kokum (*Garcinia indica*) fat as cocoa butter improver in chocolate. *Journal of the Science of Food and Agriculture*, **85**: 135-140.

Malik, S. K., Chaudhury, R. and Kalia, R. K. 2005a. Rapid *in vitro* multiplication and conservation of *Garcinia indica*: a tropical medicinal tree species. *Scientia Horticulturae*, **106**(4): 539-553.

Malik, S. K., Chaudhury, R. and Abraham, Z. 2005b. Seed morphology and germination characteristics in three *Garcinia* species. *Seed science and Technology*, **33**: 595-604.

Malik, S. K., Kalia, R. K. and Chaudhury, R. 2010. *In vitro* regeneration of *Garcinia indica* using leaf explants. *Indian Journal Plant Physiology*, **15**(3): 262-266.

Marti, G., Eparvier, V., Moretti, C., Prado, S., Grellier, P., Hue, N., Thoison, O., Delpech, b., Gueritte, F. and Litaudon, M. 2010. Antiplasmodial benzophenone derivatives from the root barks of *Symphonia globulifera* (Clusiaceae). *Phytochemistry*, **71**: 964-974.

Martin, K. P. and Pradeep, A. K. 2003. Simple strategy for the *in vitro* conservation of *Ipsea malabarica* an endemic and endangered orchid of the Western Ghats of Kerala, India. *Plant Cell, Tissue and Organ Culture*, **74**: 197-200.

Martius, C. and Maue, R. 1941. Preparation, physiological behavior, and importance of hydroxycitric acid and its isomers. *Zeitschrift fur Physiological Chemistry*, **269**: 10-33.

Matsumoto, K., Akao, Y., Kobayashi, E., Ito, T., Ohguchi, K., Tanaka, T., Iinuma, M. and Nozawa, Y. 2003. Cytotoxic benzophenone derivatives from *Garcinia* species display a strong apoptosis-inducing effect against human leukemia cell lines. *Biological and Pharmaceutical Bulletin*, **26**: 569-571.

McGregor, C. E., Lambert, C. A., Greyling, M. M., Louw, J. H. and Warnich, L. 2000 comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm. *Euphytica*, **113**: 135-144.

Mishra, A., Bapat, M. M., Tilak, J. C. and Devasagayam, T. P. A. 2006. Antioxidant activity of *Garcinia indica* (kokam) and its syrup. *Current Science*, **91**: 90-93.

Moffett, S. A., Bhandari, A. K., Ravindranath, B. and Balasubramanvam, K. 1996. Hydroxycitric acid concentrate and food products prepared there from. U. S. Patent, 5656316.

Mohan, S., Parthasarathy, U., Asish, G. R. and Nirmal babu, K. 2012. Evaluation of genetic stability of micropropagated plants of three species of *Garcinia* using random amplifield polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. *Indian Journal of Biotechnology*, **11**: 341-343.

Mohanty, S., Panda, M. K., Sahoo, S. and Nayak, S. 2011. Micropropagation of *Zingiber rubens* and assessment of genetic stability through RAPD and ISSR markers. *Biologia Plantarum*, **55**(1): 16-20.

Mourao, K. S. M. and Beltrati, C. M. 2000. Morphology and anatomy of developing fruits and seeds of *Mammea americana* L. (Clusiaceae). *Revista Brasileira de Biologia*, **60**(4): 701-711.

Mourao, K. S. M. and Beltrati, C. M. 2001. Morphology and anatomy of developing fruits and seeds of *Vismia guianensis* (Aubl.) Choisy (Clusiaceae). *Revista Brasileira de Biologia*, **61**(1): 147-158.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**: 473-497.

Naithani, H.B., Sahni, K.C. and Bennet, S.S.R. 1997. Forest flora of Goa. International Book Distributors, Dehra Dun, India.

Nayak, C. A. and Rastogi, N. K. 2010a. Forward osmosis for the concentration of anthocyanin from *Garcinia indica* Choisy. *Separation and Purification Technology*, **71**: 144-151.

Nayak, C. A. and Rastogi, N. K. 2010b. Comparison of osmotic membrane distillation and forwardosmosis membrane processes for concentration of anthocyanin. *Desalination and Water Treatment*, **16**: 134-145.

Nayak, C.A., and Rastogi, N. K. 2010c. Effect of selected additives on microencapsulation of anthocyanin by spray drying. *Drying Technology*, **28**: 1396-1404.

Nayak, C. A., Srinivas, P. and Rastogi, N. K. 2010a. Characterisation of anthocyanins from *Garcinia indica* Choisy. *Food Chemistry*, **118**: 719-724.

Nayak, C. A., Rastogi, N. K and Raghavarao K. S. M. S. 2010b. Bioactive constituents present in *Garcinia indica* Choisy and its potential food applications: A Review. *International Journal of Food Properties*, **13**: 441-453.

*Nayak, P., Behera P. R., Thirunavoukkarasu, M. and Chand P. K. 2010. High frequency plant regeneration through adventitious multiple shoots organogenesis in epicotyl explants of Indian gooseberry (*Emblica officinalis* Gaertn). *Scientia Horticulturae*, **123**: 473-478.

Nayar, N. M. 2011. Agrobiodiversity in a biodiversity hotspot: Kerala State, India. Its origin and status. *Genetic Resources and Crop Evolution*, **58**: 55-82.

Negi, P. S. and Jayaprakasha, G. K. 2004. Control of foodborne pathogenic and spoilage bacteria by garcinol and *Garcinia indica* extracts, and their antioxidant activity. *Journal of Food Science*, **69**(3): 1-5.

Negi, P. S., Jayaprakasha, G. K. and Jena, B. S. 2008. Antibacterial activity of the extracts from the fruit rinds of *Garcinia cowa* and *Garcinia pedunculata* against food borne pathogens and spoilage bacteria. *Food Science and Technology*, **41**: 857-1861.

Normah, M. N., Nor-Azza, A. B. and Aliudin, R. 1995. Factors affecting *in vitro* shoot bud proliferation and *ex vitro* establishment of Mangosteen. *Plant Cell, Tissue and Organ Culture*, **43**(3): 291-294.

O'Brien, T. P. and McCully, M. E. 1981. The study of plant structure: Principles and selected methods. Termarcarphi Pty., Ltd., Melbourne, Australia.

Pacheco, G., Gagliardi, R. F., Valls, J. F. M. and Mansur, E. 2009. Micropropagation and *in vitro* conservation of wild *Arachis* Species. *Plant Cell, Tissue and Organ Culture*, **99** (3): 239-249.

Padhye, S., Ahmad, A., Oswal, N. and Sarkar, F. H. 2009. Emerging role of garcinol, the antioxidant chalcone from *Garcinia indica* Choisy and its synthetic analogs. *Journal of Hematology and Oncology*, **2**: 1-38.

Paiya Neto, V. B. P., Mota, T. R. and Otoni, W. C. 2003. Direct organogenesis from hypocotyl-derived explants of annatto (*Bixa orellana*). *Plant Cell, Tissue and Organ Culture*, **75**(2): 159-167.

Pan, M. H., Chang, W. L., Lin-Shiau, S. Y., Ho, C. T. and Lin J. K. 2001. Induction of apoptosis by garcinol and curcumin through cytochrome c release and activation of caspases in human leukemia HL-60 cells. *Journal of Agriculture and Food Science*, **49**: 1464-1474.

Parab, G. V. and Krishnan, S. 2008. Assessment of genetic variation among populations of *Rhynchostylis retusa*, an epiphytic orchid from Goa, India using ISSR and RAPD markers. *Indian Journal of Biotechnology*, **7**: 313-319.

Parab, G. V., Krishnan, S., Janarthanam, M. K., Sivaprakash, K. R. and Parida, A. 2008. ISSR and RAPD markers assessed genetic variation of *Aerides maculosum* - an epiphytic orchid from Goa, India. *Journal of Plant Biochemistry and Biotechnology*, **17**(1): 107-109.

Parimalan, R., Giridhar, P., Gururaj, H. B. and Ravishankar, G. A. 2009. Micropropagation of *Bixa orellana* using phytohormones and triacontanol. *Biologia Plantarum*, **53**(2): 347-350.

Pati, R., Chandra, R., Chauhan, U. K., Mishra, M. and Srivastava, N. 2008. *In vitro* clonal propagation of bael (*Aegle marmelos* Corr.) cv. CISHB1 through enhanced axillary branching. *Physiology and Molecular Biology of Plants*, **14**: 1-10.

Patil, B. P., Gawankar, M. S., Sagvekar, V. V. and Jambhale N. D. 2012. Status of existing kokum plantation in Maharashtra. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum*, 19-20. Western Ghats Kokum Foundation, Goa, India.

Patil, B. P., Gawankar, M. S., Sawant, D. S., Pujari, K. H. and Jambhale, N. D. 2006. Cultivation of konkan amruta and search for elite varieties. *In*: Bhat, D. J., Kamat, N. and Shirodkar, A. (eds.), *Compendium on Kokum* (*Garcinia indica*), 42-46. Western Ghats Kokum Foundation, Goa, India.

Pearse, A. G. E. 1972. Histochemistry; Theoretical and Applied **1**, Longman, London.

Pearse, A. G. E. 1988. Histochemistry; Theoretical and Applied **2**, Longman, London.

Ploem, J. S. and Tanke, H. J. 1987. Introduction to fluorescence microscopy. Oxford University Press, Oxford, UK.

Priya Devi, S., Tangam, M., Ashok, V., Ramachandrudu, K. and Korikanthimath, V. S. 2012. Prospects on natural diversity in Kokum with respect to processing and value addition. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum*, 12-18. Western Ghats Kokum Foundation, Goa, India.

Purohit, S. D., Singhvi, A. and Nagori, R. 2003. *In vitro* propagation of *Achras sapota*: optimization of conditions for rooting, hardening and acclimatization. *In*: Kavikishor, P. B. (ed.), *Plant tissue culture and Biotechnology emerging trends*, 134-145. Universities Press (India) Pvt, Ltd, Hyderabad, India.

Rai, M. K., Phulwaria, M., Harish, Gupta, A. K., Shekhawat, N. S. and Jaiswal, U. 2012. Genetic homogeneity of guava plants derived from somatic embryogenesis using SSR and ISSR markers. *Plant Cell, Tissue and Organ Culture*, **111**(3): 259-264.

Rancic, D., Quarrie, S. P. and Pecinar, I. 2010. Anatomy of tomato fruit and fruit pedicel during fruit development. *In*: Mendez-Vilas, A. and Diaz, J. (eds.), *Microscopy*, 851-860. Formatex Research Center, Spain.

Rani, D. and Dantu, P. K. 2012. Direct shoot regeneration from nodal, inter nodal and petiolar segments of *Piper longum* L. and in vitro conservation of indexed plantlets. *Plant Cell, Tissue and Organ Culture*, **109**: 9-17.

Rao, M. S. and Purohit, S. D. 2006. *In vitro* shoot bud differentiation and plantlet regeneration in *Celastrus paniculatus* Willd. *Biologia Plantarum*, **5**(4): 501-506.

Rathore, P., Suthar, R. and Purohit. S. D. 2008. Micropropagation of *Terminalia bellerica* Roxb. from juvenile explants. *Indian Journal of Biotechnology*, 7(2): 246-249.

Rema, J. and Krishnamurthy B. 2000. *Garcinia* species of economic importance, distribution and uses. *Indian Spices*, **37**(1): 20-23.

Rema, J., Krishnamoorthy, B. and Mathew, P. A. 1997. Vegetative propagation of major tree- a review. *Journal of Spices and Aromatic Crops*, **6**(2): 87-105.

Richardson, A. C., Boldingh, H. L., McAtee, P. A., Gunaseelan, K., Luo, Z., Atkinson, R. G., David, K. M., Burdon, J. N. and Schaffer, R. J. 2011. Fruit development of the diploid kiwi fruit, *Actinidia chinensis* 'Hort16A'. *BMC Plant Biology*, **11**: 1-14.

Ridley, N. H. 1967. The Flora of the Malay Peninsula. L. Reeve and Co., Ashford, UK.

Rineksane, I. A., Kadir, M. A., Kadzimin, S. and Zaman, F. Q. 2012. *In vitro* development of embryogenic calli and embryogenic stages in suspension cultures of Mangosteen (*Garcinia mangostana* L.). *Journal of Medicinal Plants Research*, **6**(13): 2549-2559.

Rohani, E. R., Ismanizan, I. and Noor, N. M. 2012. Somatic embryogenesis of Mangosteen. *Plant Cell, Tissue and Organ Culture*, **110**: 251-259.

Rostika, I., Sunarlim, N. and Mariska, I. 2008. Micropropagation of Mangosteen (*Garcinia mangostana*). *Indonesian Journal of Agriculture*, **1**(1): 28-33.

Saker, M. M., Adawy, S. S., Mohamed, A. A. and El-itriby, H. A. 2006. Monitoring of cultivar identity in tissue culture-derived date palms using RAPD and AFLP analysis. *Biologia Plantarum*, **50**(2): 198-204.

Sang, S, Liao, C. H., Pan, M. H., Rosen, R. T., Lin-Shiau, S. Y, Lin, J. K. and Ho, C. T. 2002. Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol with peroxyl radicals and their antitumor activities. *Tetrahedron*, **58**: 10095-10102.

Sang, S., Pan, M. H., Cheng, X., Bai, N., Stark, R. E., Robert, R. T., Lin-Shiau, S. Y., Lin, J. K. and Ho, C. T. 2001. Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol and their antitumor activities. *Tetrahedron*, **57**: 9931-9938.

Seeni, S. and Latha, P. G. 2000. *In vitro* multiplication and ecorehabilitation of the endangered blue *Vanda*. *Plant Cell, Tissue and Organ Culture*, **61**(1): 1-8.

Sharma, S., Rathi, N., Kamal, B., Pundir, D., Kaur, B. and Arya, S. 2010. Conservation of biodiversity of highly important medicinal plants of India through tissue culture technology- a review. *Agriculture and Biology Journal of North America*, **1**(5): 827-833.

Sirchi, M. H. T., Kadir, M. A., Aziz, M. A., Rashid, A. A., Rafat, A. and Javadi, M. B. 2008a. Amelioration of Mangosteen micropropagation through leaf and seed segments (*Garcinia mangostana* L). *African Journal of Biotechnology*, 7(12): 2025-2029.

Sirchi, M. H. T., Kadir, M. A., Aziz, M. A., Rashid, A. A., Rafat A. and Javadi, M. B. 2008b. Plant regeneration as affected by plant growth regulators (PGR) in Mangosteen (*Garcinia mangostana* L.). *African Journal of Biotechnology*, 7(15): 2693-2701.

Sivaprakash, K. R., Krishnan, S., Datta, S. K. and Parida, A. K. 2006. Tissue-specific histochemical localization of iron and ferritin gene expression in transgenic *indica* rice Pusa Basmati (*Oryza sativa* L.). *Journal of Genetics*, **85**(2): 157-160.

Srivastava, P., Singh, M., Mathur, P. and Chaturvedi, R. 2009. *In vitro* organogenesis and plant regeneration from unpollinated ovary cultures of *Azadirachta indica*. *Biologia Plantarum*, **53**(2): 360-364.

Stallings, W., Blount, T. F., Srere, P. A. and Glusker, J. P. 1979. Structural studies of hydroxycitrate and their relevance to certain enzymatic mechanisms. *Archives Biochemistry and Biophysics*, **193**: 431-448.

Sun, Y., Zhao, Y., Wang, X., Qiao, G., Chen, G., Yang, Y., Zhou, J., Jin, L. and Zhuo, R. 2009. Adventitious bud regeneration from leaf explants of *Platanus occidentalis* L. and genetic stability assessment. *Acta Physiologiae Plantarum*, **31**: 33-41.

Tamil Selvi, A., Joseph, G. S. and Jayaprakasha, G. K. 2003. Inhibition of growth and aflatoxin production in *Aspergillus flavus* by *Garcinia indica* extract and its antioxidant activity. *Food Microbiology*, **20**: 455-460.

Tanaka, T., Kohno, H., Shimada, R., Kagami, S., Yamaguchi, F., Kataoka, S., Ariga, T., Murakami, A., Koshimizu, K. and Ohigashi, H. 2000. Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats. *Carcinogenesis*, **21**: 1183-1189.

Taylor, G. N. and Soliman, K. M. 1999. Detection of polymorphic DNA and taxonomic relationship among 10 wild perennial soybean species using specific and arbitrary nucleotide primers. *Biologia Plantarum*, **42**(1): 25-37.

Te-chato, S. and Lim, M. 1999. Plant regeneration of mangosteen via nodular callus formation. *Plant Cell, Tissue and Organ Culture*, **59**(2): 89-93.

Te-chato, S. and Lim. M. 2000. Improvement of mangosteen micropropagation through meristematic nodular callus formation form *in vitro* derived leaf explants. *Scientia Horticulturae*, **86**: 291-298.

Tembe, R. P. and Deodhar, M. A. 2011. Clonal propagation and hydroxycitric acid production from in vitro shoot cultures of *Garcinia indica* using root suckers as explants. *In Vitro Cellular and Development Biology-Plant*, **47**: 399-409.

Thakor, N. J., Swami, S. B. and Haldankar, P. M. 2012. Need of standardization and advances in value. *In*: Braganza, M., Shirodkar, A., Bhat, D. J. and Krishnan, S. (eds.), *Resource book on Kokum*, 65-70. Western Ghats Kokum Foundation, Goa, India.

Thengane, S. R., Deodhar, S. R., Bhosle, S. V., Rawal, S. K. 2006a. Repetitive somatic embryogenesis and plant regeneration in *Garcinia indica* Choiss. *In Vitro Cellular and Developmental Biology-Plant*, **42**: 256-261.

Thengane, S. R., Deodhar, S. R., Bhosle, S. V. and Rawal, S. K. 2006b. Direct somatic embryogenesis and plant regeneration in *Garcinia indica* Choisy. *Current Science*, **91**: 1074-1078.

Theodore, C. 1967. The flora of the presidency of Bombay, **1**: 80-83. Botanical Survey of India, Calcutta, India.

Varalakshmi, K. N., Sangeetha, C. G., Shabeena, A. N., Sunitha, S. R. and Vapika, J. 2010. Antimicrobial and cytotoxic effects of *Garcinia indica* fruit rind extract. *American-Eurasian Journal of Agricultural and Environmental Sciences*, **7**(6): 652-656.

Vikrant and Rashid, A. 2003. Somatic embryogenesis or shoot formation following high 2, 4-D pulse treatment of mature embryo of *Paspalum scrobiculatum*. *Biologia Plantarum*, **46**(2): 297-300.

Vinh, D. Q., Cuong, D. H. and Thuong, N. 2011. Extracting (-)-hydroxycitric acid from dried rinds of *Garcinia oblongifolia* Champ. ex Benth by using microwave. *Journal of the Korean Chemical Society*, **55**(6): 983-987.

Vishnu Priya, V., Jainu, M., Mohan, S. K., Saraswathi, P. and Chandra Sada Gopan, V. S. 2010. Antimicrobial activity of pericarp extract of *Garcinia mangostana* L. *International Journal of Pharma Sciences and Research*, **1**(8): 278-281.

Walia, N., Kaur, A. and Babbar, S. B. 2007. Proliferation and differentiation from endosperms of *Carthamus tinctorius*. *Biologia Plantarum*, **51**(4): 749-753.

Whitmore, T. C. 1973. Tree Flora of Malaya: A Manual for Foresters **2**. Longman, Kuala Lumpur, Malaysia.

Xing, Y., Yu, Y., Luo, X., Zhang, J. N., Zhao, B. and Guo, Y. D. 2010. High efficiency organogenesis and analysis of genetic stability of the regenerants in *Solanum melongena*. *Biologia Plantarum*, **54**(2): 231-236.

Yamada, T., Hida, H. and Yamada, Y. 2007. Isolation of hydroxycitric acid producing *Streptomyces* sp. U121 and generation of improved mutants by genome shuffling. *Actinomycetologica*, **21**: 40-45.

Yamaguchi, F., Ariga, T., Yoshimura, Y. and Nakazawa, H. 2000a. Antioxidative and anti-glycation activity of garcinol from *Garcinia indica* fruit rind. *Journal of Agriculture and Food Science*, **48**: 180-185.

Yamaguchi, F., Saito, M., Ariga, T., Yoshimura, Y. and Nakazawa, H. 2000b. Free radical scavenging activity and antiulcer activity of garcinol from *Garcinia indica* fruit rind. *Journal of Agriculture and Food Science*, **48**: 2320-2325.

Zhang, Y., Wei, Z., Xi, M. and Shi, J. 2006. Direct organogenesis and plantlet regeneration from mature zygotic embryos of masson pine (*Pinus massoniana* L.). *Plant Cell, Tissue and Organ Culture*, **84**(1): 119-123.

PUBLICATIONS

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Baskaran, M. and S. Krishnan. **2010**. ***In vitro* regeneration and mass multiplication of medicinal and economically important plant *Garcinia indica* Choisy from Western Ghats of India**. Abstract of the paper published and presented in the National conference on "Biotechnology: Fusion of Advanced Research and Training". 2-4 January 2010. School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu, India.

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BRIEF COMMUNICATION

High frequency plant regeneration from the mature seeds of *Garcinia indica*

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Abstract

A high frequency plant regeneration system was developed for the production of high yielding elite clones of *Garcinia indica via* direct organogenesis. A maximum number of 86.2 shoot buds per explant were induced from the mature seed segments cultured on Woody plant medium (WPM) supplemented with 2.0 mg dm^{-3} N^6-benzyladenine and 1 mg dm^{-3} indole-3-acetic acid. Rooting was achieved on half-strength WPM medium supplemented with 3 mg dm^{-3} indole-3-butyric acid. Rooted plantlets were acclimatized and transferred to greenhouse for further growth. The highest survival rate of 95 % was recorded using a mixture of garden soil and sand. Histological studies clearly revealed multiple shoot formation from mature seed segments.

Additional key words: direct organogenesis, kokum elite clones, mass multiplication, multiple shoots.

Garcinia indica Choisy, an endemic plant to Western Ghats of India, belongs to the family *Clusiaceae* and is popularly known as kokum (Shetty and Kaveriappa 2001). The fruit rind contains bioactive compounds of medicinal importance such as hydroxycitric acid (HCA) and garcinol (Jena *et al.* 2002, Jayaprakasha and Sakariah 2002).

Though *in vitro* shoot regeneration of kokum using apical buds (Mathew *et al.* 2001), immature seeds, young leaves, apical and axillary buds (Kulkarni and Deodhar 2002, Thengane *et al.* 2006), *in vitro* root explants (Deodhar *et al.* 2008) and matured seeds (Malik *et al.* 2005) were reported earlier, the average number of shoots formed ranged from 2 to 58 per explant. In the present study we have developed and standardized an efficient protocol for high frequency plant regeneration from the mature seed segments of *Garcinia indica* for the production of elite clones.

Ripened fruits were collected from the trees with high yielding, large fruit size and high acid taste of *Garcinia indica* Choisy during April - June from the Western Ghats of Goa, India. The fruits were washed thoroughly with clean tap water, followed by quick dip in 70 % ethanol. Seeds were separated and then seed coat was removed. Further steps were carried out under sterile condition in a laminar air-flow chamber. The mature seeds were washed with sterile distilled water, followed by rinsing with 70 % (v/v) ethanol for 2 min. The seeds were then surface sterilized with 0.1 % mercuric chloride for 1 min and rinsed with sterile distilled water 3 - 4 times. Each seed was cut into three pieces (endosperm) and used as explants. The seed segments were cultured on Woody plant medium (WPM; Lloyd and McCown 1980) supplemented with N^6-benzyladenine (BA; 1.0, 2.0, 3.0, 4.0, 4.5 and 5.0 mg dm^{-3}) or in combinations with indole-3-acetic acid (IAA; 1.0 mg dm^{-3}), α-naphthalene acetic acid (NAA; 1.0 mg dm^{-3}) and indole-3-butyric acid (IBA; 1.0 mg dm^{-3}). The medium was fortified with 3 % sucrose and 0.75 % agar. The pH was adjusted to 5.7 ± 0.2 with 0.1 M HCl and 0.1 M NaOH. IAA was filter sterilized and added to autoclaved media. For shoot elongation, multiple shoot buds were cultured on WPM

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Abbreviations: BA - N^6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - α-naphthalene acetic acid; PGR - plant growth regulator; WPM - woody plant medium.

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supplemented with 1.0 mg dm^{-3} BA and 0.1 % activated charcoal. Induction of roots were standardized using half-strength WPM medium containing 1.5 % sucrose and different concentration of IBA and NAA (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg dm^{-3}). The cultures were incubated at 25 ± 2 °C, a relative humidity of 50 - 60 % and 16-h photoperiod (50 μmol m^{-2} s^{-1}; cool white fluorescent tubes). For acclimatization, healthy plantlets were transferred to a mixture of equal volume of garden soil and sand. Plants were grown under high relative humidity in a growth room for two weeks and then they were moved to a greenhouse. The data were analyzed by using *WASP-2.0*. Ten replications were maintained for each treatment and each experiment was repeated three times. Observations were made every week. For histological studies, thin transverse sections of 8 - 10 μm were obtained using cryo-microtome (*Leica CM1800, Leica Instruments*, Nussloch, Germany) at the temperature of -6 ± 1 °C and stained with either 1 % safranin or 0.1 % toluidine blue for 5 min. The sections were mounted in dilute glycerin (5 %) and photographed using *Nikon E800* microscope attached with *Nikon Coolpix4500* digital camera (*Nikon*, Tokyo, Japan).

Seed segments showed prominent swelling after one week of culture in WPM supplemented with various plant growth regulators (Fig. 1*A*, Table 1). Induction of multiple shoot primordia was noticed on all over the surface of explants after 2 - 3 weeks (Fig. 1*B*). After one month of culture the shoot primordia differentiated into shoot buds. Malik *et al.* (2005) observed similar response of swelling of explants along with shoot bud primordia in MS medium supplemented with plant growth regulators. Direct somatic embryogenesis using immature seeds revealed the initiation of small protuberances after 2 - 3 weeks of culture (Thengane *et al.* 2006).

The frequency of shoot buds induction ranged from 20 to 100 % in the media supplemented with BA alone. Maximum number of shoot buds (27 per explant) was observed in 4.5 mg dm^{-3} of BA. Increasing the concentration of BA in the media greatly increased the percentage of shoot bud induction (Table 1). Similar results were also reported in *Garcinia indica* (Malik *et al.* 2005), *Phaseolus vulgaris* (Dang and Wei 2009) and *Citrus volkameriana* (Tavano *et al.* 2009). Maximum shoot bud length of 5.6 mm was observed in presence of 1 mg dm^{-3} BA. Similarly, maximum length of shoot buds with lower concentration of BA was reported by Malik *et al.* (2005). An increase in the concentration of BA beyond the optimum (4.5 mg dm^{-3}) did not affect shoot bud induction significantly, but the buds appeared to be developmentally suppressed and did not grow further. Similar responses of reduction in number of shoots were observed with high concentrations of BA in *Garcinia indica* (Malik *et al.* 2005), *Garcinia mangostana* (Goh *et al.* 1990) and *Holarrhena antidysenterica* (Mallikarjuna and Rajendrudu 2009). Stunted growth of shoot buds was also reported in *Garcinia mangostana* in presence of higher concentration of BA (Goh *et al.* 1990).

Table 1. Influence of plant growth regulators [mg dm^{-3}] on induction of multiple shoot buds in *Garcinia indica*. Regeneration frequency [%], shoot number [$explant^{-1}$] and shoot length [mm] were measured. Means ± SE, n = 10; means followed by the same letter were not significantly different at $P < 0.05$. No buds were induced in basal medium.

BA	IAA	NAA	IBA	Regen.	Shoot number	Shoot length
1.0				20	06.0 ± 0.81^{s}	5.6 ± 0.51^{a}
2.0				80	10.0 ± 0.66^{r}	5.1 ± 0.73^{a}
3.0				100	17.0 ± 0.87^{l}	4.4 ± 0.51^{b}
4.0				100	21.0 ± 0.78^{k}	3.5 ± 0.52^{de}
4.5				100	27.0 ± 0.87^{i}	3.6 ± 0.51^{cd}
5.0				100	16.0 ± 0.66^{m}	2.1 ± 0.56^{i}
1.0	1.0			40	13.0 ± 0.67^{n}	3.0 ± 0.66^{efg}
2.0	1.0			100	86.2 ± 0.78^{a}	2.1 ± 0.59^{i}
3.0	1.0			100	47.2 ± 0.63^{c}	2.4 ± 0.69^{hi}
4.0	1.0			100	27.2 ± 0.63^{i}	3.2 ± 0.63^{def}
4.5	1.0			100	22.2 ± 0.56^{j}	3.2 ± 0.63^{def}
1.0		1.0		50	11.0 ± 0.81^{q}	4.1 ± 0.56^{bc}
2.0		1.0		100	35.0 ± 0.66^{g}	3.2 ± 0.63^{def}
3.0		1.0		100	38.5 ± 0.52^{e}	3.1 ± 0.87^{def}
4.0		1.0		100	42.5 ± 0.52^{d}	2.9 ± 0.87^{fgh}
4.5		1.0		100	61.2 ± 0.78^{b}	2.1 ± 0.56^{i}
1.0			1.0	80	12.5 ± 0.52^{op}	3.4 ± 0.51^{def}
2.0			1.0	100	30.0 ± 0.81^{h}	2.5 ± 0.52^{ghi}
3.0			1.0	100	35.7 ± 0.48^{f}	2.2 ± 0.42^{i}
4.0			1.0	100	38.5 ± 0.70^{e}	3.4 ± 0.51^{def}
4.5			1.0	100	42.7 ± 0.48^{d}	3.2 ± 0.42^{def}

BA in combination with auxins was found to be effective for shoot bud induction; however, it took one week more for the induction of shoot buds in comparison with BA alone. Among the BA and IAA combinations, 2 mg dm^{-3} BA with 1 mg dm^{-3} IAA produced highest number of shoots (86.2 per explant) with 100 % regeneration (Fig. 1*E*). Parimalan *et al.* (2009) reported that BA and IAA combinations produced high number of shoots in *Bixa orellana*. BA (4.5 mg dm^{-3}) in conjugation with NAA (1.0 mg dm^{-3}) also produced 61.2 shoot buds per explant (Fig. 1*C,D*). Dai *et al.* (2009) observed similar response in *Brassica oleracea*. The synergistic effect of BA and NAA on shoot formation using seed explants has been reported in *Garcinia mangostana* (Sirchl *et al.* 2008). BA and NAA combination produced 20 - 40 % callus along with shoot bud formation similarly as Malik *et al.* (2005) reported in *Garcinia indica*. BA in combination with IBA was less effective in shoot bud induction when compared to BA + IAA and BA + NAA (Table 1).

Induced shoot buds were cultured on WPM supplemented with 1.0 mg dm^{-3} BA and 0.1 %

Fig. 1. Plant regeneration from mature seed segments of *Garcinia indica*: *A* - swelling of explants after one week of culture; *B* - initiation of shoot buds after 2 weeks of culture on WPM supplemented with 2 mg dm^{-3} BA and 1 mg dm^{-3} IAA; *C* - early stages of multiple shoot formation on WPM supplemented with 4.5 mg dm^{-3} BA + 1 mg dm^{-3} NAA after 40 d of culture; *D* - multiple shoots developed on WPM 4.5 mg dm^{-3} BA + 1 mg dm^{-3} NAA after 60 d of culture; *E* - multiple shoots developed on WPM 4.5 mg dm^{-3} BA + 1 mg dm^{-3} NAA after 90 d of culture; *F* - rooting in half-strength WPM supplemented with 3 mg dm^{-3} IBA; *G* - hardened plants transferred to seed trays; *H* - after 15 d of growth in seed tray plantlets transplanted into large plastic pots for further growth in a greenhouse; *I* - transverse section of seed segment after two weeks of culture stained with toluidine blue showing early stage of development of shoot meristem (sm) and leaf primordium (lp) (× 20); *J* - transverse section of seed segment after 3 weeks of culture stained with safranin showing well developed shoot meristem and leaf primordium (× 20).

Table 2. Effect of IBA and NAA on rooting of *Garcinia indica*. Means ± SE, n = 10; means followed by the same letter were not significantly different at $P < 0.05$. No rooting in basal medium.

IBA [mg dm^{-3}]	NAA [mg dm^{-3}]	Frequency [%]	Number [shoot^{-1}]	Length [cm]
0.5		30	1.00 ± 0.06^{d}	0.60 ± 0.04^{e}
1.0		70	1.28 ± 0.42^{cd}	3.14 ± 0.31^{b}
2.0		70	1.42 ± 0.51^{bc}	3.57 ± 0.52^{a}
3.0		100	2.80 ± 0.42^{a}	3.65 ± 0.51^{a}
4.0		100	1.70 ± 0.48^{b}	1.75 ± 0.48^{d}
5.0		80	1.50 ± 0.52^{bc}	2.58 ± 0.52^{c}
	0.5	60	2.00 ± 0.66^{d}	1.20 ± 0.12^{d}
	1.0	60	2.00 ± 0.47^{d}	1.81 ± 0.11^{a}
	2.0	70	2.10 ± 0.56^{d}	1.83 ± 0.09^{a}
	3.0	80	3.80 ± 1.13^{b}	1.21 ± 0.15^{d}
	4.0	80	5.00 ± 0.47^{a}	1.33 ± 0.08^{c}
	5.0	90	2.90 ± 0.73^{c}	1.60 ± 0.09^{b}

activated charcoal for better shoot growth and elongation. Two weeks later, the elongated shoots of 3 - 5 cm were cut at basal region and placed on rooting media. The smaller shoots were again cultured on elongation medium. Kalia *et al.* (2007) and Li *et al.* (2009) reported that removal of the elongated shoots at regular intervals was necessary to allow the elongation of smaller shoot buds.

Rooting was achieved in all the combinations of IBA and NAA within a period of 2 - 3 weeks (Table 2). IBA induced the highest frequency of rooting (100 %) at 3 and 4 mg dm^{-3} when compared to NAA. However, highest number of roots (5 per explant) was observed at 4 mg dm^{-3} NAA after 15 d. Among the IBA and NAA concentrations, maximum length of the roots (3.65 cm) was recorded in 3 mg dm^{-3} IBA (Fig. 1*F*). Even though the number of roots produced by NAA was higher, the roots were stunted, swollen, greenish white and did not support the acclimatization process as compared to thick white roots produced by IBA. Shoots cultured on media without IBA and NAA did not develop any roots. Similar responses of root induction by IBA and NAA were reported in *Garcinia indica* (Malik *et al.* 2005, Thengane *et al.* 2006, Deodhar *et al.* 2008). In contrast to the above, half-strength WPM with 2.5 - 245 µM IBA did not induce any rooting in *Garcinia indica* (Chabukswar *et al.* 2006). It was reported that IBA promotes better root induction in plants such as *Azadirachta indica* (Srivastava *et al.* 2009), *Acanthophyllum sordidum* (Meratan *et al.* 2009), *Bixa orellana* (Parimalan *et al.* 2009) and *Plantago algarbiensis* (Goncalves *et al.* 2009).

For hardening, the rooted plantlets were transferred to culture tubes containing quarter-strength liquid WPM without sucrose for 10 - 12 d. The plantlets with healthy roots that were transplanted into a mixture of equal volume of garden soil and sand recorded 95 % survival rate (Fig. 1*G*). Different potting mixtures such as soil, *Vermiculite* and farm yard manure (Malik *et al.* 2005), sand and soil (Thengane *et al.* 2006) and cocopeat (Deodhar *et al.* 2008) were used for acclimatization of regenerated plantlets of *Garcinia indica*. After two weeks growth, hardened plants were transplanted into large pots and moved to a greenhouse (Fig. 1*H*).

Histological studies were carried out to understand the origin and development of shoot buds. Transverse section of seed segments after one week of culture revealed the presence of numerous meristematic zones. After two to three weeks of culture, the meristematic zones developed into shoot and leaf primordium (Fig. 1*I,J*).

In the present study we have developed and standardized an efficient protocol for high frequency plant regeneration from mature seed segments of *Garcinia indica*. This protocol will be useful for the production of elite clones for large scale cultivation and genetic manipulation to enhance the bioactive compounds in future.

References

Chabukswar, M.M, Deodhar, M.A.: Restoration of rooting competence in a mature plant of *Garcinia indica* through serial shoot tip grafting *in vitro*. - Sci. Hort. **108**: 194-199, 2006.

Dai, X.G., Shi, X.P., Fu, Q., Bao, M.Z.: High frequency plant regeneration from cotyledon and hypocotyl explants of ornamental kale. - Biol. Plant. **53**: 769-773, 2009.

Dang, W., Wei, Z.M.: High frequency plant regeneration from the cotyledonary node of common bean. - Biol. Plant. **53**: 312-316, 2009.

Deodhar, S.R., Thengane, R.J., Thengane, S.R.: De novo shoot regeneration from root culture of *Garcinia indica* Choiss. - Indian J. exp. Biol. **46**: 482-486, 2008.

Goh, H.K.L., Roa, A.N, Loh, C.S.: Direct shoot bud formation from leaf explants of seedlings and mature mangosteen (*Garcinia mangostana* L.) trees. - Plant Sci. **68**: 113-121, 1990.

Goncalves, S., Martins, N., Romano, A.: Micropropagation and conservation of endangered species *Plantago algarbiensis* and *P. almogravensis*. - Biol. Plant. **53**: 774-778, 2009.

Jayaprakasha, G.K., Sakariah, K.K.: Determination of organic acids in leaves and rinds of Garcinia indica (Desr.) by LC. - J. pharm. biomed. anal. **28**: 379-384, 2002.

Jena, S., Jayaprakasha, G.K., Sing, P., Sakariah, K.K.: Chemistry and biochemistry of (-) hydroxyl citric acid. - J. agr. Food Chem. **50**: 10-22, 2002.

Kalia, R.K., Arya, S., Kalia, S., Arya, I.D.: Plantlet regeneration form fascicular buds of seedling shoot apices of *Pinus roxburghii* Sarg. - Biol. Plant. **51**: 653-659, 2007.

Kulkarni, M.D., Deodhar, M.A.: *In vitro* regeneration and hydroxycitric acid production in tissue cultures of *Garcinia indica* Chois. - Indian J. Biotechnol. **1**: 301-304, 2002.

Li, J.J., Wu, Y.M., Wang, T., Liu, J.X.: *In vitro* direct organogenesis and regeneration of *Medicago sativa*. - Biol. Plant. **53**: 325-328, 2009.

Lloyd, G.B., McCown, B.H.: Commercially-feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot-tip culture. - Proc. int. Plant Propag. Soc. **30**: 412-427, 1980.

Malik, S.K., Chaudhury, R., Kalia, R.K.: Rapid *in vitro* multiplication and conservation of *Garcini indica*: A tropical medicinal tree species. - Sci. Hort. **106**: 539-553, 2005.

Mallikarjuna, K., Rajendrudu, G.: Rapid *in vitro* propagation of *Holarrhena antidysenterica* using seedling cotyledonary nodes. - Biol. Plant. **53**: 569-572, 2009.

Mathew, K.M., Rao, Y.S., Kuruvilla, K.M., Lakshmanan, R., George, G.L., Madhusoodanan, K.J., Potty, S.N.: Multiple shoot regeneration in kokum and camboge. - J. Spices aromatic Crops. **10**: 151-152, 2001.

Meratan, A.A., Ghaffari, S.M., Niknam, V.: *In vitro* organogenesis and antioxidant enzymes activity in *Acanthophyllum sordidum*. - Biol. Plant. **53**: 5-10, 2009.

Parimalan, R., Giridhar, P., Gururaj, H.B., Ravishankar, G.A.: Micropropagation of *Bixa orellana* using phytohormones and triacontanol. - Biol. Plant. **53**: 347-350, 2009.

Shetty, B.V., Kaveriappa, K.M.: An arboretum of endemic plants of Western Ghats at Mangalore University campus, Karnataka, India. - Zoos Print J. **16**: 431-438, 2001.

Sirchl, M.H.T., Kadir, M.A., Aziz, M.A., Rashid, A.A., Rafat, A., Javadi, B.: Amelioration of mangosteen micro propagation through leaf and seed segments (*Garcinia mangostana* L.). - Afr. J. Biotechnol. **7**: 2025-2029, 2008.

Srivastava, P., Singh, M., Mathur, P., Chaturvedi, R.: *In vitro* organogenesis and plant regeneration from unpollinated ovary cultures of *Azadirachta indica*. - Biol. Plant. **53**: 360-364, 2009.

Tavano, E.C.R., Stipp, L.C.L., Muniz, F.R., Mourao Filho, F.A.A., Mendes, B.M.J.: *In vitro* organogenesis of *Citrus volkameriana* and *Citrus aurantium*. - Biol. Plant. **53**: 395-399, 2009.

Thengane, S.R., Deodhar, S.R., Bhosle, S.V., Rawal, S.K.: Direct somatic embryogenesis and plant regeneration in *Garcinia indica* Choisy. - Curr. Sci. **91**: 1074-1078, 2006.

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Original

In vitro Plant Regeneration of *Garcinia indica* Choisy. from the Western Ghats of Goa, India

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Garcinia indica belongs to the family Clusiaceae. Commonly known as 'Kokum', is an underexploited fruit tree species found in tropical rain forests of the Western Ghats covering Maharashtra, Goa, and Karnataka (Anonymous. 1956). The fruit rind contains bioactive compounds such as Hydroxycitric acid (HCA) and garcinol. Hydroxycitric acid is an important biologically active compound used as an anti-obesity and anti-cholesterol agent (Jena *et al.*, 2002; Leonhardt and Langhans, 2002). Garcinol is a yellow color compound which exerts anticancer, antioxidant, antiulcer and anti-inflammatory activities (Mishra *et al.*, 2006). Anthocyanin red pigments extracted from the ripened fruits is used as natural colorant in wide variety of foodstuffs, beverages, nutraceuticals, pharmaceuticals, toiletries and as a natural anti-oxidant (Bhaskaran *et al.*, 2007). Kokum butter is a nutritive, emollient and demulcent product that is suitable for nutraceutical and pharmaceutical industries. The fruit rind is used as a garnish for curry and in some of the folklore medicine in India (Geroge *et al.*, 2002).

The conventional way of propagation of kokum is by seeds. The natural seedling populations, due to cross pollination and heterozygous nature of the trees, has abundant variation with respect to growth, habit, flowering and fruiting season, fruit yield and morphological and biochemical characteristics of fruits (Korikanthimath and Desai, 2005). Also the early ripening and high yielding genotypes are preferred because harvesting and processing of fruits to be completed before the onset of monsoon (end of May or beginning of June) in the Western India. To overcome the above problems, during this study we have produced high yielding elite clones through *in vitro* plant regeneration using excised seed segments.

Material and Methods

Explants, media and culture conditions

Ripened fruits of *Garcinia indica* were collected from elite trees (high yielding, large fruit size and high acid taste) during the month of May-June from the Western Ghats of Goa, India. The fruits were washed thoroughly with clean tap water, followed by wiping with 70% ethanol. Further steps were carried out under sterile condition in a laminar air-flow chamber. The fruits were dissected out to remove the seeds, and then the seeds were washed with sterile distilled water and followed by rinsing with 70% ethanol (v/v) for 1-2 min, followed by rinsing with sterile distilled water 3-4 times. Seeds were then surface sterilized with 0.1% (w/v) aqueous mercuric chloride for 1 min followed by rinsing with sterile distilled water 3-4 times. Surface sterilized seeds were cut into 3-4 pieces and used as explants. Woody Plant Medium (WPM) (Lloy G. and McCown B. 1981) supplemented with 6-benzylaminopurine (BAP) and Kinetin (Kn) and 2,4-dichlorophenoxyacetic acid (2,4- D) at various concentrations (Table 1). Basal medium without any plant growth regulators was used as control. The cultures were incubated at 25 ± 2°C under illumination of cool-white florescent lamp with a light intensity of 50 μ/mol $m^{-2} s^{-1}$ for 16 hrs photoperiod. Visual observations were made on every 4 days with regular intervals. The effect of plant growth regulators were evaluated on the basis of percentage of cultures showing adventitious shoot bud formation and/ or morphogenic responses.

Results and discussion

Morphogenic responses of matured seed segments of *Garcinia indica* were studied (Table 1). Seed segments showed swelling after 1 week of culture, while shoot buds were initiated after 2-3 weeks. With increase in the BAP concentration from 1 to 4.5 mg/L showed increased number of shoot formation (Table 1). The maximum number of shoots per seed segment was twenty-one in the case of 4 mg/L BAP. The maximum length of shoots was 2.5 cm after 6 weeks of culture. Different concentrations of BAP (1-5 mg/L) used during this study showed 20-80% regeneration frequency. Decrease in the number of shoots per explants was noticed with the increase in concentration of BAP from 4 to 5 mg/L. Kn supplemented in the medium exhibited 40-100% regeneration frequency. Among the Kn concentration, 11 multiple shoots with 1cm of shoot length, were noticed on 3mg/L. Callus induction of 80-90% frequency was observed in 2, 4- D supplemented although those calluses did not respond for shoot regeneration in cytokine medium. The seed segment explants cultured on WPM without any plant growth regulators (basal medium) did not show any regeneration.

The shoots of 3-5 cm length were carefully cut at basal region and transferred to half strength WPM supplemented with IBA (0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l). Maximum rooting (100%) was observed in half strength WPM medium supplemented with 3 and 4 mg /L IBA after two weeks of culture. Maximum number of root (3) was observed in 3 mg /L IBA. Well rooted plantlets were carefully washed in sterile distilled water to remove the traces of agar, and transfered to culture tubes containing quarter-strength liquid WPM without sucrose for 10-12 days. Plantlets were transferred to seed tray filled with mixture of different potting media combinations used for the study. Maximum survival rate (95%) was observed in the combinations of garden soil + sand (1:1).

Table 1. Morphogenic response of mature seed segment explants of *Garcinia indica* cultured on the woody plant medium (WPM) medium supplemented with different plant growth regulators

Plant Hormones Used	Conc. (mg/L)	Cultures regenerated (%)	Cultures with callus (%)	Avg. no. of shoots	Avg. Length of shoots
BAP	1	20	-	06±1.8	2.5±1.2
	2	80	-	08±1.4	1.2±1.8
	3	100	-	18±1.2	1.0±1.6
	4	100	-	21±0.8	1.8±1.0
	5	100	-	10±1.0	2.0±1.8
Kn	1	40	-	03±0.6	0.6±1.0
	2	40	-	05±1.0	0.8±1.4
	3	50	-	11±1.2	1.0±1.4
	4	100	-	08±0.8	0.5±1.8
	5	100	-	07±1.4	1.0±1.2
2,4 D	1	80	40	-	-
	2	80	40	-	-
	3	90	50	-	-
	4	100	30	-	-
	5	70	60	-	-

Reference

Anonymous. (1956). The Wealth of India-Raw materials-IV. Ist edn. Council of Scientific and Industrial Research, New Delhi, India, pp:102-103.

Bhaskaran, Sunil, Mehta and Sevanti. (2007). Stabilized anthocyanin extract from *Garcinia indica*, United States Patent No: 7, 261, 769.

Geroge, G.L, Lakshmanan, R, Mathew, K.M. and Roa, Y.S. (2002). Review of research on Kokum camboge and related species. *J Spices Arom Crops*, 11:1-12.

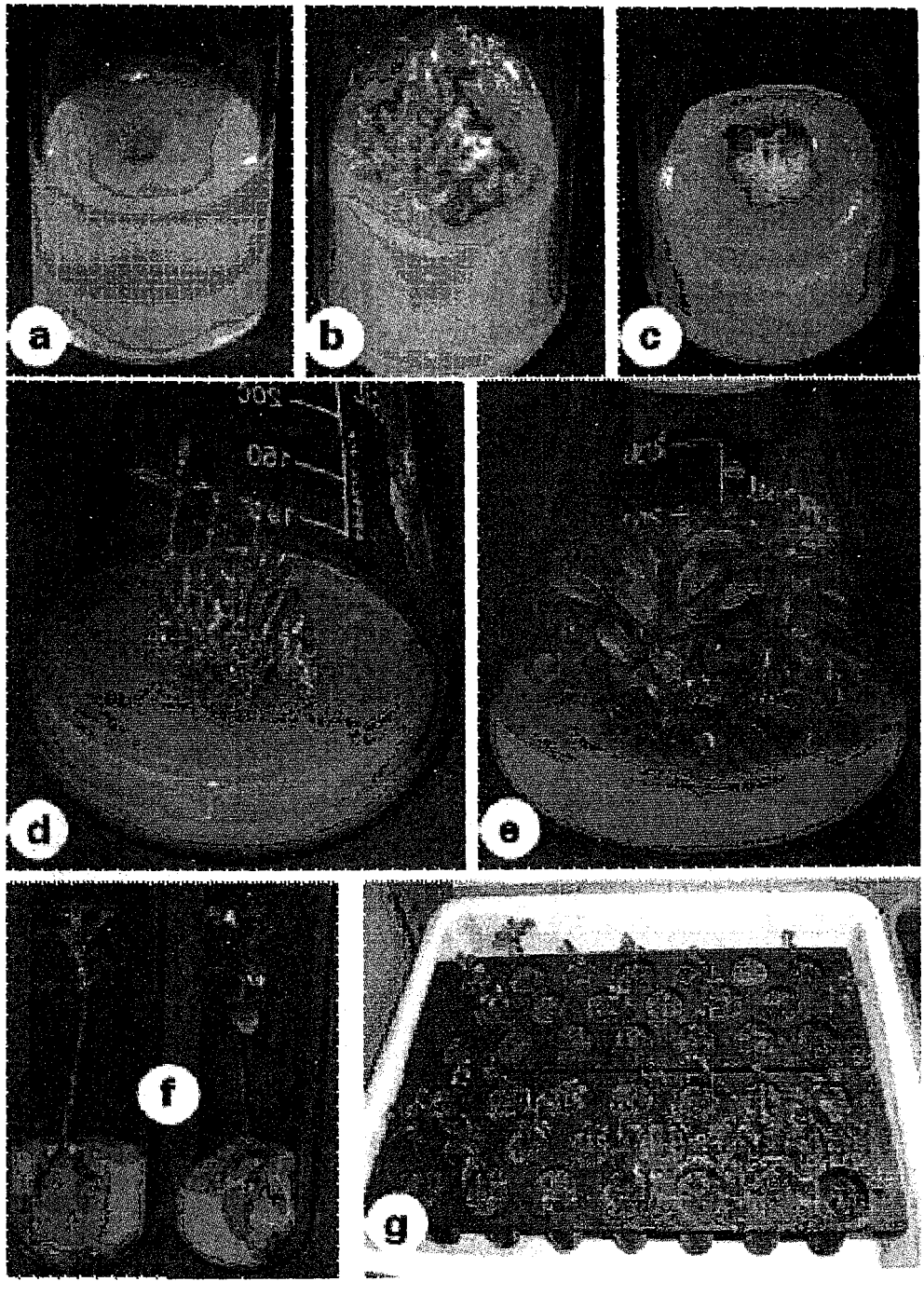

Fig. 1. Plant regeneration from mature seed segments of *Garcinia indica*: a-innoculated explants on WPM medium; b-initiation of shoot buds after 4 weeks of culture 3 mg/L BAP; c-Callus formation on 4 mg/L 2,4-D; d-multiple shoots developed on WPM supplemented with 3 mg/L Kn after 5 weeks of culture; e-multiple shoots developed on WPM supplemented with 4 mg/L BAP; f-rooting in half-strength WPM supplemented with 3 mg/L IBA; g-rooted plants were transplanted in seed tray contains equal mixture of garden soil and sand.

Jena, S, Jayaprakasha, G.K, Sing, P. and Sakariah, K.K. (2002). Chemistry and biochemistry of (-) hydroxyl citric acid. *Agric Food Chem* 50:10-22.

Korikanthimath, V.S. and Desai, A.R. (2005). Status of Kokum (*Garcinia indica* Choisy) in Goa. In: Bhat DJ, Kamat N, Shirodkar A (eds) *Compendium on Kokum (Garcinia indica), Western Ghats Kokum Foundation, Goa, India,* pp:17-21.

Leonhardt, M. and Langhans, W. (2002). Hydroxycitate has long-term effects on feeding behavior, body weight regain and metabolism after body weight loss male Rats. *J Nutr* 132:1977-1982.

Lloy, G. and McCown, B. (1981). Commercially feasible micropropagation of Mountain laurel, *Kalmia latfolia*, by use of shoot tip culture. *Comb Proc Int Plant Prop. Soc.* 30:421-426.

Mishra, A, Bapat, M.M, Tilak, J.C. and Devasagayam, T.P.A. (2006). Antioxidant activity of *Garcinia indica* (kokam) and its syrup. *Curr. Sci* 91:90-93.

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conditions. In the authors' laboratory, the systems of aseptic plants and cell suspensions of *Blumea* and *Opuntia* sps. and hairy roots of *Tagates, Rubia, Brassica* and *Nicotiana* were demonstrated effectively to degrade various harmful textile dyes to non toxic products. Various *in vitro* treatments such as hormonal combinations, precursor feeding and changes in physical status were attempted to enhance the potentiality of these systems for degradation of dyes efficiently. Metabolic pathways, analysis and non toxicity of the resulted products were explicated convincingly. The main thrust focused on isolation of specific enzymes in the metabolic pathways and transfer of genes from other microbial sources to plants is currently underway.

S.8.O.3

EFFICIENT *IN VITRO* REGENERATION, MASS MULTIPLICATION AND ASSESSMENT OF GENETIC STABILITY USING MOLECULAR MARKERS IN *GARCINIA INDICA* CHOISY

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Garcinia indica Choisy is endemic to Western Ghats of India, belongs to the family Clusiaceae. It is popularly known as Kokum. The fruit rind contains bioactive compounds such as hydroxycitric acid (HCA) and garcinol, which are used as an anti-obesity, anti-cholesterol and anti-cancer agent. Anthocyanin pigments extracted from the ripened fruit is used as a natural colorant. Kokum butter is nutritive, emollient and demulcent and finds applications in nutraceutical and pharmaceutical industries. *In vitro* regeneration and mass multiplication was achieved on woody plant medium (WPM) supplemented with 6-benzyladenine (BA) alone or in combinations with naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) using mature seed segments. Maximum number of multiple shoots of 86 was recorded in WPM supplemented with BA (2 mg l^{-1}) + IAA (1mg l^{-1}). Rooting (100%) was observed in half strength WPM medium supplemented with 3 and 4 mg l^{-1} IBA. During acclimatization process, maximum survival rate (96%) was observed in the combinations of garden soil + sand (1:1). Total of twenty random amplified polymorphic DNA (RAPD) and ten inter simple sequence repeat (ISSR) primers were used for initial screening to assess the genetic stability of regenerated plants. Among the primers screened, 18 RAPD and 8 ISSR primers produced reproducible banding patterns (monomorphic). The RAPD primer OPD-20 produced more number of bands (12) followed by primer AB-6 recorded 11 bands and ISSR primer IB-1 produced 8 bands followed by primer IB-4 which produced seven bands. All the regenerated plantlets as well as the mother plant showed similarity in the banding patterns, which indicates that genetic stability is maintained in the regenerated plants.

Key words: *Garcinia indica*, ISSR, mass multiplication, RAPD, woody plant medium.

S.8.O.4

ISOLATION AND CHARACTERIZATION OF *DYAD* ORTHOLOGUES FROM APOSPOROUS AND DIPLOSPOROUS TAXA

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PBT- 7: *In vitro* regeneration and mass multiplication of medicinal and economically important plant *Garcinia indica* Choisy from Western Ghats of India

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Garcinia indica Choisy is endemic to Western Ghats of India, belongs to the family Clusiaceae and is popularly known as Kokum. The fruit rind contains bioactive compounds such as hydroxycitric acid (HCA) and garcinol, which are used as an anti-obesity, anti-cholesterol and anti-cancer agent. Anthocyanin pigments extracted from the ripened fruit is used as a natural colorant. Kokum butter is nutritive, emollient and demulcent and finds applications in nutraceutical and pharmaceutical industries. The conventional way of propagation of kokum is by seeds. These natural seedling populations, due to cross pollination and heterozygous nature of the trees, has abundant variation with respect to growth, habit, flowering and fruiting season, fruit yield and morphological and biochemical characteristics of fruits. To overcome the above problems, during this study developed high yielding elite clones through *in vitro* regeneration and mass multiplication.

The mature seed segments of *Garcinia indica* Choisy were cultured on woody plant medium (WPM) supplemented with 6-benzyladenine (BA) alone or in combinations with naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA). Swelling of the seed segments was observed after one week of inoculation. Initiation of multiple shoot primordia was observed all over the surface of explants after 2-3 weeks of culture. After one month of culture, the multiple shoot primordia differentiated into shoot buds. BA in combination with auxins was found to be effective for multiple shoot formation. Maximum average number of shoots (61.2 /explant) were produced using the combination of BA (4.5 mg l^{-1}) + NAA (1mg l^{-1}). The initiated multiple shoot buds were subcultured on WPM supplemented with 1 mg l^{-1} BA and 0.1% activated charcoal for further growth and elongation. Maximum rooting (100%) was observed in half strength WPM medium supplemented with 3 and 4 mg l^{-1} IBA. During acclimatization process, maximum survival rate (96%) was observed in the combinations of garden soil + sand (1:1). Histological studies clearly revealed the origin and development of multiple shoot formation. The efficient protocols developed for the production of high yielding elite clones of *Garcinia indica* may help the farmers and forest department for large scale cultivation.