REFINEMENT OF MICROPROPAGATION PROTOCOL AND RAPD ASSAY FOR SEX DETERMINATION IN KODAMPULI
(*Garcinia gummi-gutta var.gummigutta*)

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Refinement of micropropagation protocol and RAPD assay for sex determination in kodampuli (*Garcinia gummi-gutta* var. *gummigutta*)"**is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Refinement of micropropagation protocol and RAPD assay for sex determination in kodampuli (*Garcinia gummi-gutta* var. *gummigutta*)"** is a record of research work done independently by **Mr. Premjith Gopinath** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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Premjith Gopinath


Dedicated to My
Parents

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ABBREVIATIONS

A	-	Absorbance
ABA	-	Abscisic acid
b	-	Bases
BA	-	Benzyl Adenine
BAP	-	Benzyl Amino Purine
bp	-	Base pair
cDNA	-	Complementary DNA
cm	-	Centimeter
CO_2	-	Carbon dioxide
CPBMB	-	Centre for Plant Biotechnology and Molecular Biology
CTAB	-	Cetyl trimethyl ammonium bromide
DAI	-	Days after inoculation
DNA	-	Deoxyribo Nucleic Acid
d NTP	-	Deoxy Nucleoside Tri Phosphate
EDTA	-	Ethylene diamine tetra acetic acid
g	-	Gram
gl^{-1}	-	Gram per Litre
IAA	-	Indole Acetic Acid
IBA	-	Indole butyric acid
K	-	Kinetin

KAU	-	Kerala Agricultural University
Kg	-	Kilogram
kb	-	Kilo base
L	-	Litre
m	-	Metre
m^3	-	Metre cube
ml	-	Millilitre
NAA	-	Naphthalene Acetic Acid
ng	-	Nano gram
OD	-	Optical Density
PAGE	-	Polyacrylamide Gel Electrophoresis
PCR	-	Polymerase chain reaction
ppm	-	Parts per million
PVP	-	Polyvinyl Pyrrolidone
RAPD	-	Random Amplified Polymorphic DNA
RNA	-	Ribo Nucleic Acid
RNAase	-	Ribonuclease
rpm	-	Rotation per minute
SDS	-	Sodium dodecyl sulphate
TAE	-	Tris Acetic Acid EDTA
Taq	-	*Thermus aquaticus*
TE	-	Tris EDTA
UV	-	Ultra violet

V	-	Volt
2,4 –D	-	2,4 Dichlorophenoxy acetic acid
µg	-	Microgram
%	-	Percentage
µl	-	Micro litre
µm	-	Micro metre
ºC	-	Degree Centigrade

Introduction

Introduction

Garcinia gummi-gutta var. gummigatta (L.)Rob. (syn.*G. camboge* L.) is known in vernacular as kodampuli, camboge or Malabar Tamarind belongs to the family Clusiaceae under the natural order Guttiferales. The large genus of polygamdioecious evergreen trees or shrubs are distributed in Tropical Asia, Africa and Polynesia. It includes 200 species of which over 20 species are found in India (Mohammed *et al.*, 1994). The economic significance of this allogamous miscellaneous non-timber spice tree has excited the scientific community as the richest natural source of potential anti-obesity phytometabolite (-)Hydroxy citric acid (-HCA) from its fleshy fruit rind.

Obesity due to fatty acid synthesis and lipogenesis has become one of the major causes for lifestyle diseases like Type II diabetes mellitus, hypertension and cardiovascular diseases. The (-) HCA is unique biologically active acids lowers blood lipids such as cholesterol and triglycerides by triggering fatty acid oxidation in the liver via thermogenesis. It inhibits the conversion of carbohydrates to fat without affecting Kreb's cycle through a powerful citrate cleavage enzyme, ATP citrate lyase which mobilizes the human body fat stores and dissolves in the liver for weight management. In Ayurveda and herbal medicines, Kodampuli finds use in the treatment of rheumatism, bowl complaints, rickets and uterine contractions after delivery (Keertikar and Basu,1984).

The extensive cultivation of this polygamdioecious miscellaneous homestead spice tree is crippled due to difficulty in differentiating male and female trees before flowering (after 8 years), low recovery of female productive trees, prolonged seed dormancy of 7-8 months, low percentage of germination (40-60%) and non-availability of sufficient number of orthotrophic shoots of normal growth habit as scion materials for grafting . the perennial trees as such and the perennial tree spices in particular are tacticum lots showing recalcitrant response in *in vitro* studies. *In vitro* germination and micropropagation protocol is the single ideal alternative to overcome these limitations. In addition to that the

totipotency of triploid endosperm tissue through the routes of somatic embryogenesis and direct multiple shoot regeneration is a paradigm of excellence in micropropagation. It provides triple edges of developing seedless plantlets, production of propagules at large scale and also brings easiness in HCA extraction and from fleshy fruit rind. Therefore, the present investigation was undertaken to refine the available micropropagation protocol with the following objectives:

- Refinement of standardized micropropagation protocol (nodal segments) and somatic embryogenesis (immature endosperm).
- Standardization of DNA isolation techniques from male and female trees, in vitro seedling progenies and polyembryonic seedlings in kudampuli.
- RAPD assay using selected oligoneucliotide decamer primers to ascertain the sex at juvenile phase in the species.

Review of Literature

REVIEW OF LITERATURE

The available literature though scanty on various aspects relevant to the present investigation on "Refinement of micropropagation protocol and RAPD assay for sex determination in Kodampuli (*Garcinia gummi-gutta var. gummigutta*)"is reviewed here under.

1. Biochemical and therapeutical significance

Keertikar and Basu, (1984) reported that in Ayurveda and herbal medicines, kodampuli finds its use in the treatment of rheumatism, bowl complaints, rickets and uterine contractions after delivery. *Garcinia sp.* includes 200 species of which over 20 species are found in India (Mohammed *et al.,* 1994). The isolation of anti-obesity phytometabolite (-)hydroxycitric acid [(-)-HCA] from the fleshy fruit rinds of *G. cambogia, G. indica* and *G. atrovirdis* has excited the scientific community as a whole, biochemists and health practitioners in particular. The HCA inhibits the conversion of carbohydrates into fats by inhibition of ATP citrate lyase, an important enzyme in Kreb's cycle (Heymesfield *et al.,* 1998). Obesity has become one of the major concerns in health research and many scientists across the world consider it as the cause of diabetes, hypertension and cardiovascular diseases. The changing life style causes diseases of the heart and diabetes which are the two major consequences of excess body fat (IDF, 2006).

2. Micropropagation

Most of the studies pertaining to *in vitro* culture of genus *Garcinia* have been conducted in *G.mangastana* using seed and leaf explants (Goh *et al.,* 1990; Normah *et al.,* 1992; Huang *et al.,* 2000; Te Chato and Lim, 2000). Limited attention has been given to *G. indica*, which has recently been recognized as an important horticultural and medicinal tree species. Deshpande *et al.* (1999) and Mathew *et al.* (2001) conducted preliminary studies on *in vitro* establishment of kokum apical buds.

Kozai and Kubota (2001) were attempted to develop a photoautotrophic micropropagation system for woody plants such as mangium (A*cacia mangium*), coffee (C*offea arabuta*), eucalyptus (*Eucalyptus camadulensis*), mangosteen (*G. mangastana*), neem (*A. indica*), pulownia (*P. fortunei*) and pine (*Pinus radiata*). They revealed that most chlorophyllous explants *in vitro* including cotyledonary stage somatic embryo have the ability to grow photoautographically (without sugar in the culture medium), and that the low or negative net photosynthesis rate of plants *in vitro* is due not to prior photosynthetic ability, but to the low CO_2 concentration in the air-tight culture vessel during the photoperiod.

The major constraint in popularizing this species as a potential horticultural crop is the dioceious nature of the tree. Differentiation between male and female trees is known only at the flowering stage (7-9 years of age). The tree growth is slow and propagation is usually done by seeds and softwood grafting (George *et al.*, 2002).

Later Kozai *et al.* (2003) continued the similar line of research by using large culture vessels and reported the enhanced rate of growth and development of *in vitro* plants under heterotrophic or photomixotrophic condition as well as the cost reduction by photoautotrophic micropropagation.

Micropropagation is considered to be a tool to develop an efficient and reproducible method for rapid *in vitro* multiplication, conservation and *ex vitro* establishment of species using seeds (Malik *et al.*, 2005).

2.1 Explant source

Chun *et al.* (2000) were noticed for the tissue culture parameters of slow growing species *Garcinia mangostana* by rigorous monitoring of treatments through two or more successive, relatively long passages of two eight week passages were necessary to observe difference in phytohormone effects, photoperiod and temperature effects which are not clearly evident until tissues cultured through three passages. The optimum photoperiod and temperature for shoot proliferation could not be established until after the 5^{th} passage. Continuous culturing in an eight hour photoperiod at $30^{o}C$ resulted in progressively intensified

degeneration of shoots after 3 passages. In contrast, successive passages in a 16 hr. photoperiod/26°C regime enabled sustained regeneration of shoots.

According to Rajendran *et al.* (2005), the grafted plants in pots were nursed in the glasshouse of the Centre for Plant Biotechnology and Molecular Biology (CPBMB) for excising the explants. These plants were given fungicidal spray with 0.1% Bavistin /Fytolan/Companion once in a week so as to reduce the microbial load of the explants.

2.1.1 Pre-Treatment and surface sterilization

Rajendran *et al.* (2005) collected the explants in the conical flask containing 0.1per cent Bavistin solution as pretreatment during the first and second year for about 20 minutes.

These pretreated explants were wiped with 70 per cent alcohol. Thereafter these explants were surface sterilized for 3-5 minutes with 0.1 per cent $HgCl_2$. These explants were surface explants were given 3-4 repeated rinsing with sterile distilled water under laminar hood. Administration of 70 per cent alcohol wiping for 1.0 minute as pre-treatment along with 0.1 per cent Bavistin as media additive were tried during the second year. Pretreatment with 0.1 per cent Fytolan/Companion/Bavistin with 0.1 per cent $HgCl_2$ surface sterilization were attempted for inhibiting the inherent latent contamination from active endogenous fungi in the system of the explant. Thirty different MS media supplements with various hormonal combinations were tried.

2.1.2 *In vitro* culturing

Lakshmisita *et al.* (1982) could isolate callus from shoot segment of (0.5 – 0.8 cm) mature sandal trees of 20-25 years. These segments have callused on MS basal medium supplemental with 2,4-Dichlorophenoxy acetic acid (2,4-D). Kulkarni and Deodhar (2001) cultural explant such as leaves, petiole and nodal segments from *in vitro* developed plantlets were also cultured on MS + NAA (5-21μM) and BAP (2.2-13.2 μM).

Mary Mathew *et al.* (2001) got multiple shoot proliferation in both Comboge and kokum could be promoted by culturing on half strength MS medium fortified with 1.0 mgl^{-1} BAP, 0.5 mgl^{-1} GA and 0.5 mgl^{-1} NAA and 0.5 per cent activated charcoal. Best response of 2.1± 0.4 shoots explant was observed for Comboge and 2.6± 0.5 for kokum. Though the leaves were healthy and unfurled within a few week of initial response, premature leaf fall was observed in all the cultures irrespective of various growth regulators used. However, addition of activated charcoal in the media, promoted shoot growth and sustained leaf development *G. indica* showed better response in terms of number of multiple shoots than *G. gummi-gutta var.gummigutta* . The *in vitro* culture of comboge showed severe fungal contamination within 15-20 days of inoculation in 95-100 per cent cultures.

According to Rajendran *et al.*, (2005) 85 per cent of shoot tips and 60 per cent of nodal segments exhibited regeneration and axillary shoot growth free from microbial contamination within 59 days of inoculation in ¼ MS media fortified with 0.5 mgl^{-1} BA +2.5 mgl^{-1} IBA + 0.75 % Agar +2.0% Sucrose in *Garcinia gumi-gutta var.gummigutta*.

2.1.3 Sub culturing

Proliferation of multiple shoot from nodal segments was recorded upto 15 shoots per culture and had excited upto 1200 shoots within eight months of cultural duration (Rajendran *et al.*, 2005). The interval between the two subculture cycles was 30 days while culturing on ¼ MS +2.0 mgl^{-1} IBA + 2.0 mg l^{-1} BA .

2.1.4 Elongation of shoot buds

Kulkarni and Deodhar (2001) observed elongation of shoot in NAA concentration media with MS was most effective.

2.1.5 Shoot proliferation

Mary Mathew *et al.* (2001) reported that shoot proliferation in *Garcinia indica* to some extent in *Garcinia gummiguta* .In a medium containing ½ MS with 1.0 mg l^{-1} BAP + GA at 0.5 mg l^{-1} + NAA at 0.5 mg l^{-1} with 0.5% activated

charcoal. Rajendran *et al.*, (2005) got multiple shoot proliferation upto 15 shoots per culture in ¼ MS + 2.0 mg l^{-1} BA + 5.0 mg l^{-1} IBA, ¼ MS + 2.0 mg l^{-1} IAA + 2.0 mg l^{-1} IBA + 2.0 mg l^{-1} NAA and ¼ MS + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} IBA . But did not get any shoot growth in ½ MS + 0.5 mg l^{-1}BA + 2-5 mg l^{-1}IBA with 2 per cent sucrose + 7.5 per cent agar in all the media combinations.

2.1.6 Rooting of cultures

According to Normah *et al.* (1990) in mangosteen shoots were rooted in MS medium supplemented with 20-30m µM IBA and the plantlets formed were successfully transplanted into a vermiculite and sand mixture. In another experiment, callus formation was most successful in segmented seed culture supplemented with 30 mgl^{-1} 2,4-D and maintained under a 12 hour photoperiod.

Normah *et al.* (1995) later reported rooting in mangosteen was 75 per cent of the plants produced on the MS medium with activated charcoal rooted after 6 weeks in rooting medium. The number of roots formed from each shoot was as high as 6 to 7 per shoot. The average length of time from bud to plantlet formation was approximately 5 months.

These cultures were shown rooting in ¼ MS + 2 mg l^{-1} IAA + 2.0 mg l^{-1} IBA + 2.0mgl^{-1} NAA in 60 days after inoculation Rajendran *et al.* (2005)

2.1.7 Hardening and planting out

The mixture of sand, soil and organic material (3:2:1) gave the best survival of 98 per cent. After six months, the plants were transferred into polythene bags of size 25x45 cm with the same original media composition and after a year all plantlets were transferred into the sand, soil and organic material medium. Data on plantlet height was taken at a monthly interval. Plant in both soil and sand (1:1) or sand, soil and organic material (3:2:1) medium grew at a higher and uniform rate when compared to the other two media. The two year old plants were later transferred to the field. Due to a limited area of planting, only 64 plants were transferred to the field. The survival rate of plants in the field assessed after three months of transfer was 97 per cent.

Lichun *et al.* (2000) in mangosteen reported that shoots rooted at a rate of 85 per cent when pre cultured for 3 days in a medium containing 4921.3µM

indole-3-butyric acid or 10 days at 492.1µM, then cultured for two 8 weeks passage in phytohormone free medium. The 95 per cent of survived rooted shoots acclimatized in the growth chamber by gradual lowering the relative humidity were transferred to green house for hardening.

In a study conducted by Kulkarni and Deodhar (2001) they found that 75 per cent of rooting of the elongated shoots of mangosteen was achieved in ½ MS medium, supplemented with 10.74 µM NAA. However, the root initiation occurred through the intervention of unorganized callus. As a result most of the plantlets failed to survive in soil. When the elongated shoots were dipped in different concentrations of NAA (1342.5, 2685, 5370 and 10740 µM) or IBA(1225, 2450, 4900 and 9800 µM) for 30 seconds and cultured on ½ MS basal medium, 70 percent rooting was achieved in 25 days with 4900 µM of IBA dip. The resulting plantlets were devoid of callus at the root shoot junction and survived well in pots. The rooted plantlets were transferred to the pots containing soil, sand coccopeat in the ratio 1:2:1 such mixture gave 60 per cent survival and the plantlets showed growth in the apical region after about 40 days.

Malik *et al.* (2005) transferred shoots (15-25mm long) to ½ strength MS medium supplemented with 5-25 µM IBA and NAA added singly and 2 per cent sucrose for root initiation. The well formed plantlets, after 4 weeks on rooting medium were washed thoroughly and transferred to wide mouth bottles containing quarter–strength liquid MS medium with 1.0 per cent sucrose and absorbent cotton as a support for hardening. The hardened plants were established in pots containing a mixture of soil, vermiculate and farmyard manure (FYM) in 1:1:1 ratio after 7-8 weeks of transfer, the *in vitro* raised plantlets were observed for leaf shape and growth pattern.

2.2. *In vitro* endosperm and regeneration culture

There have been many workers undertaken in different tree crops including Garcinia with the use of immature seeds as a mode of mass multiplication explants. Lumpe and Mills (1933) are credited for the pioneering attempts in endosperm culture of maize. Later, La Rue *et al.* (1949) cultured immature

endosperm of maize and succeeded to exploit the property of unlimited endosperm growth *in vitro*. The first successful tissue culture in forest trees was established by Rangaswamy and Rao (1963) from mature endosperm of sandalwood.

Browning of explants caused by polyphenol oxidation is a major problem in *in vitro* clove cultures (Stevenson and Harris, 1980 and Mathew and Rajendran, 1996). Activated charcoal has the ability to absorb toxic metabolites released into the culture medium (Fridborg and Erikson, 1975). Non-endospermous immature seeds of apple (Mu *et al.*, 1977) and citrus (Wang and Chang, 1978) were attempted and found that the free nucellar endosperm did not survive in culture. They further noticed that the endosperm from immature seeds did not exhibit dependence on the embryo. Broome and Zimmerman (1978) formulated a method to remove phenolic compounds, in which the explants are transferred to fresh medium (containing 0.5 % PVP without any hormones) twice a day for 2-3 days and the tubes are then kept under continuous darkness.

Addition of activated charcoal or PVP in the culture medium prevented accumulation of polyphenols in the culture medium and browning of explants (Bajaj, 1978; Stevenon and Harris, 1980; Zaid and Tisserat, 1983). Since the harmful phenolic oxidation products are formed under illumination, reduction of light intensity at the initial period may be beneficial (Hu and Wang, 1983). Sealing the cut end of the explants with paraffin wax for preventing the polyphenol oxidation was reported in clove and banana (Bhat and chandel, 1991; Mathew and rajendran, 1996).

Normah *et al.* (1992) got multiple shoot production from mature mangosteen seeds (which are apomictic) were sterilized segmented and cultured on MS or modify MS (marcoelements of half strength) medium supplements with 1.0 or 2.5 mμM NAA and 0, 30, 40 or 50 μM BA. Seeds cut into 6 pieces produced more shoot, than seeds cut into 3 pieces. Cultures under an 8-hour photoperiod produced more shoots than those under a 12-h photoperiod. Combination of BA at 40 or 50 μM and 0 or 2-5 μM NAA were best for multiple shoot productions. Normah *et al.* (1995) also reported that in apomictically formed mangosteen seeds

were developed adventive embryos from the integument cells of ovule. They were harvested surface sterilized in 20 per cent Clorox with a few drops of Tween 20 for 30 minutes, followed by rinsing three times in sterile distilled water. These seeds were then cut into six segments (twice lengthwise and once across) before culturing them on MS or WPM media. Both media with or without the addition of activated charcoal (2.0 gl^{-1}) were supplemented with 40 µM BA or a combination of 40 µM BA and 2.5 µM NAA. Control media did not contain any plant growth regulators. All media were supplemented with 20 gl^{-1} sucrose and 7 gl^{-1} Bacto agar prior to autoclave sterilization for 20 minutes at 121°C.

The cultures were incubated at 25°C or 30°C, under an 8 hour photoperiod with a light intensity of 25mmol $m^{-2}s^{-1}$. Each treatment consisted of 10 explants and was repeated three times.

Slow growth, low propagation rate and weak *in vitro* performance of mature explants as compared to juvenile shoots have been reported in jack (Rajmohan and kumaran, 1988) and clove (Mathew and Rajendran, 1996). Growing stock plants under controlled conditions and regularly spraying the plants with systemic and contact fungicides can reduce or avoid contamination problem to certain extent (Malik *et al.,* 1992; Mathew and Rajandran, 1998). Addition of activated charcoal or polyvinyl pyrrolidone (PVP) in the cultured medium prevented accumulation of polyphenols in the culture medium and browning of explants of kudampuli (Rajendran *et al.*,1998).

Rajendran *et al.* (1998) later reported that the immature endosperms excised from 30–40 days old fruits were successfully used for callus induction and proliferation in Kudampuli. They further reported induction of somatic embryos directly on ½ MS medium supplemented with 0.5 mg l^{-1} NAA + 0.2 mg l^{-1} BA and 0.3 mg l^{-1} kinetin. According to them surface sterilization treatment with 0.1 per cent $HgCl_2$ for 3-5 minutes after pre-treatment with 70 per cent alcohol wiping of freshly harvested immature fruits and the treatment of extracted seeds with 0.1 percent bavistin for 20 minutes could effectively check the *in vitro* contamination problem in Malabar Tamarind. They further achieved 92 per cent of germination of seeds by inoculating three fourth mature fruits within 7 days of *in vitro* culture

on ½ MS medium (ICAR Annual report 2003). According to Kulkarni and Doodhar (2001) the immature green fruits were washed with water containing 2-3 drops of Tween 80 and treated with 70 per cent alcohol for 1.0 minute for surface sterilization. The seeds were removed aseptically from the fruit and cut into 2 segments. Explants such as tender leaves, axillary and apical leaf buds, nodal segments and petiole were surface sterilized with 70 per cent alcohol for 1.0 minute followed by 2 percent sodium hypochlorite for 5-7 minutes and washed with sterile distilled water and cultured on MS medium supplemented with 3 per cent (w/v) sucrose, 0.8 per cent (w/v) agar and various combinations of growth regulators, such as 2,4-D (9μM) + K (22.3 μM); NAA (10.74 μM + K (0.93 μM); NAA (2.69 μM) + BAP(22.3 μM); NAA (5-37 μM) + BAP (8-9 μM) + K (0.93 μM); IBA(9-8 μM) + KN(0.93 μM); IBA (9.8 μM) + BAP (0.84 μM); IAA (11.42 μM) + BAP (0.89 μM).

The cultures according to Kulkarni and Deodhar (2001) reported that the cultures were incubated at 25±2°C under illumination 40 μM m^{-2} s^{-1} with 16 hours photoperiod. The explants were subcultured at 30 days intervals. The frequency of explants producing shoots was scored after every 15 days by Analysis of Variance (ANOVA) in *garcinia indica*.

Ferwerda and Licha (2003) reported a simple and efficient protocol for the *in vitro* propagation of mangosteen using seeds aseptically dissected from fruits were cultured in tubes containing 1.8g vermiculate (used as a solid substrate) and 10.2 ml liquid Wood Plant Medium (WPM) for germination. The cultures were maintained under 25 μM , 24-26^{0}C and a 16/8 h photoperiod.

2.3 Somatic embryogenesis

Balaji *et al.* (2000) observed highest frequency of somatic embryo regeneration upon subculturing in media supplemented with TDZ in kokum (*Garcinia indica).*

2.4 Induction of auxillary buds.

Li Chun *et al.* (2000) revealed that no auxin supplementation was necessary for bud primordium differentiation in cotyledon explants of mangosteen or proliferation of regenerated shoots. The optimum N_6-benzyladenine concentration for primordium differentiation was 13.3 µM and for shoot proliferation ranged from 4.4 to 13.3 µM. Continuous culturing in an 8 hour photoperiod at 30°C resulted in progressively intensified regeneration of shoots after three passages.

The study conducted by Malik *et al.* (2005) in *G. indica* on the effect of seed maturity on *in vitro* differentiation of adventitious buds, seeds at two maturity stages were used. Creamish white seeds were extracted from greenish-red mature fruits. While dark brown seeds were separated out from fully ripe dark red fruits. The seeds (being too large they were segmented into four pieces (5-6mm x 6-8mm pieces). The segments were labeled in order to study the effect of seed polarity if any, on adventitious bud differentiation. The cut segments were cultured on media supplemented with different concentrations of plant growth regulators for adventitious bud differentiation.

The seed segments were cultured on MS medium supplemented with various concentrations of BAP (5, 12.5, 25, 37.5 and 50µM) Kinetin (12.5µM) and TDZ (0.1, 0.2, 0.5 and 12.5µM) was added in combination with BAP (5-50µM).

2.5 Elongation of shoot buds

The concentration of BA and NAA used in the above study were chosen from a preliminary experiment (Normah and Siti Maisarah, 1990). The activated charcoal has been known to absorb growth regulators (Ebert and Taylor, 1990). The observation of shoot bud regeneration from *in vitro* nodal explants was maximum at NAA (2.69 µM) + BAP (17.6 µM). This medium is comparable with the observation made in *G. mangostana* (Goh *et al.*, 1990) where the optimum level of BAP conducive for shoot bud development was 22.2 µM. The number of shoots obtained in the study was higher when compared to those found by

Normah and Siti Maisarah (1990) and Goh *et al.* (1998).The segmentation of seed and the effect of photoperiod were reported earlier by Normah *et al.* (1992).

Normah *et al.* (1995) reported in mangosteen that MS medium was suitable for shoot proliferation. However, most shoots formed on seed segments cultured on WPM produced roots in culture. Activated charcoal reduced shoot formation in most cases. Multiple shoot buds were observed on seed segments cultured on MS medium supplemented with 40µM BA alone or in combination with 2.5 µM NAA after 2-3 weeks of culture. Buds were formed about a week later on seed segments cultured on WPM. Mature segmented seed cultured on MS medium supplemented with BA and NAA without activated charcoal and kept at 30°C formed the highest mean number of shoots per explant of 16.8±1.2 after 13 weeks of culture. Another experiment was carried out to confirm this result using MS medium supplemented with 40 µM BA and 2-5 µM NAA with or without activated charcoal. It was found that after 6 months with frequent subculture and rooting of shoots formed on seed segments, from one seed cultured on medium with the addition of activated charcoal was 13 and for those cultured on medium without activated charcoal the maximum number of shoots obtained was 88.

Kulkarni and Deodhar (2001) had registered elongation of *in vitro* shoots in *G.indica* by keeping NAA concentration constant at 0.54 µM. Among the various concentrations of BAP (0-0.89µM) and Kinetin (0-1.86µM) tested, ½ MS medium containing NAA (0.517µM) + BAP (0.44µM) + Kinetin (0.93µM) was most effective.

For immature seed explants of *G.indica* Kulkarni and Deodhar (2001) reported NAA 5-37 µM + BAP 13.2µM + Kinetin 0.93µM was most conducive for callus initiation. *In vitro* leaf explants showed bulging at the petiole and, which subsequently developed unorganized proliferation to give friable callus in medium containing NAA 16.11 µM + BAP 2.2 µM + Kinetin 0.93µM. Callus initiation also occurred from root explants in the same medium. The callus initiated from all the three explants grew well in subsequent transfer to fresh media containing MS + NAA 10.74 µM + BAP 13.2 µM + Kinetin 0.93 µM supplemented with (w/v) sucrose 3 per cent ,(w/v) agar 0.8 per cent at pH of the medium at 5.7.

In another experiment conducted by Ferwerda and Licha (2003), the seedlings were cut in two segments, one containing the apical bud and a portion of the stem, and the other containing the remaining stem and the root system. The cut surface of the shoot section was soaked for 10 seconds in BAP, NAA or 2, 4-D solution at 30 g per kg. Both segments were subsequently transferred to ¼ strength liquid WPM supplemented with 7 per cent sucrose and vermiculite as the substrate. The cultures were maintained as previously described seeds. Cultured in the liquid medium generated after 3 weeks. Plantlets were obtained after 4 weeks in ¼ strength WPM liquid medium. Shoot and root induction were observed at 3-4 weeks after transplanting of the 114 plantlets cut into two sections and soaked in BAP, 113 top sections resulted in new plants (28 %). For the 37 plants cut into two and soaked in 2, 4-D, no new plantlets were obtained, but some callus tissues were evident in the area exposed rapid clonal propagation at times when seeds are scarce or unavailable.

Malik *et al.* (2005) reported in *Garcinia indica* that seed segments with induced adventitious buds (of about 1-7mm length) after 5 weeks of culture on hormone supplemented medium, were further segmented into smaller parts of approximately 3mmx4mm before transferring to MS basal medium supplemented with 0.2 per cent activated charcoal for shoot elongation. The elongated shoots after attaining a height of about 15-25mm were excised and transferred to either rooting or multiplication medium, while the segments with smaller buds were cultured again on basal medium for allowing their further elongation. The induction and elongation media were supplemented with 5 percent sucrose and solidified with 0.8 percent agar.

3.1 Direct and indirect organogenesis

In horticulture crops, tissue culture plantlets from immature endosperm of C*ucumis sativus* were established by Nakajima (1962). The endosperm proliferates only if it is excised 7-10 days after pollination. Lakshmisita *et al.* (1982) produced triploids by inducing embryogenesis from endosperm callus. Although the triploid plants are seed sterile and consequently undesirable, where

seeds are of commercial importance, there are instances where seedlessness caused by triploids is of no serious consequence.

Sealing the cut end of the explants with paraffin wax for preventing the polyphenol oxidation was reported in clove by Mathew and Rajendran (1996). Rajendran *et al.* (1998) also observed that the immature endosperm excised from 30-40 day old fruits could be successfully used for callus induction and proliferation in *G. gummi-gutta var.gummigutta*. They further noticed callus initiation from immature endosperm of Malabar tamarind after 24 days of inoculation when kept initially in dark for two weeks followed by light incubation for one week.

3.2 Caulogenesis and rhizogenesis

Kulkarni and Deodhar (2001) have reported root initiation through the intervention of unorganized callus in *Garcinia indica*. According to Sarin *et al*. (1986) in *cocos nucifera*, differentiation of shoots and roots were achieved by transferring callus to B5 medium supplemented with IAA or NAA. In this study complete plantlets were obtained when media was supplemented with NAA (0.5 mgl^{-1}), BAP (2.0 mgl^{-1}) and PVP (1.0 gl^{-1}).

4 RAPD analysis and Sex Determination

Sex determination using RAPD is done exploiting the polymorphic nature of DNA sequence and its different banding patterns. This may prove to be an effective tool to analyze and determine male and female tree crops at the nursery or early stages. Hormaza *et al.* (1994) reported a specific female 945bp RAPD marker of *P. vera* identified by an Operon decamer primer, OPO-08. The sex linkage of this marker was maintained in every individual of two crosses and in 14 cultivars unrelated to these crosses.

Kafkas *et al.* (2001) conducted studies to develop sex-associated RAPD markers in wild *Pistacia sp.* They said that early diagnosis of seedling sex type would assist breeding and nursery management in these dioecious species. They searched for RAPD markers linked to sex in *P. atlantica, P. terebinthus* and *P.*

eurycarpa, the main wild species in Turkey that were used as rootstocks for *P.vera*. They mixed DNA of ten male and ten female individuals to screen for sex associated RAPD bands. A total of 472 primers were screened and from these two bands, amplified by primers BC156 and BC360, appeared to be sex associated in *P. eurycarpa*.

Wen-Jie Xu *et al.* (2004) conducted work in *Eucommia ulmoides* Oliv., which is a dioecious perennial tree native to China. The pistillate plants are economically more useful than the staminate plants. The random amplified polymorphic DNA (RAPD) technique was used to screen markers of sex determination in this species. A 569bp RAPD marker linked to sex determination in *E. ulmoides* (MSDE) was found in all the pistillate but not in the staminate plants, its exclusiveness to pistillate plants was confirmed by Southern Blotting .

The work conducted by Rajendran and Al-Mssallem (2005) reported the sex specific variations in the genomic DNA of male and female date palms as revealed from an extensive study conducted with palms representing different date palm growing regions of the Kingdom of Saudi Arabia. The genomic DNA content in female palms was less than 60 per cent compared to those of the male genotypes. Polymerase chain reactions carried out to amplify different segments of the alcohol dehydrogenase (AdhA) gene from the date palm genomic DNA yielded two DNA fragments each in female genotypes whereas the same primers amplified a single fragment in male plants.

In another study conducted by Yakubov *et al.* (2005) on *Pistacia vera* L. Sequence Characterized Amplified Region (SCAR) primers designed on the basis of the RAPD female molecular marker, amplified a 905bp female and a 909bp male fragment. Sequencing these fragments revealed high homology, with several point mutations, namely four deletions in female and one in the male sequence. A second internal set of SCAR primers designed on the basis of a polymorphic locus, in combination with Touchdown-PCR technique, amplified a specific female 297bp product. The diagnostic reliability of the new female specific marker was verified on 54 different genotypes.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled 'Refinement of micropropagation protocol and RAPD assay for sex determination in kudampuli (*Garcinia gummi-gutta* var. *gummigutta*) was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2004-2006. The materials used and the methodologies adopted in this study are described below.

3.1 MATERIALS

3.1.1 Plant Materials

Plant materials from the field of Department Of Olericulture, College of Horticulture, Vellanikkara were used for the present study. The juvenile nodal segments at the fag end of parrot green colour and immature endosperm excised from 30-45 day old seeds were used as explants for refining micropropagation protocol. The embryo portions excised from immature seeds with a bit of endosperm were also used as explant source for studies on breaking seed dormancy and sex determination.

3.1.2 Glasswares

Borosilicate glasswares from Borosil and Corning were used for the experiments. They were initially soaked in hot water at 100^0 C for half an hour. On cooling, they were washed with detergent solution, rinsed with potassium dichromate solution in sulphuric acid, washed free of detergent using tap water and finally rinsed with distilled water. Washed glass wares were dried in hot air oven at 60^0C and were stored away from dust and contaminants.

3.1.3 Media composition for *in vitro* multiple shoot induction from nodal segments.

MS medium (Murashige and Skoog, 1962) and Woody Plant Medium (Lloyd and McCown, 1980) for plant tissue culture was used as the nutrient

medium in the present study. Composition of MS and WPM medium is given in Table 1 and 2**.** Half strength MS and full strength WPM was also tried for nodal segments along with growth regulators viz., Indole butyric acid (IBA), Benzyl adenine (BA), Naphthalene acetic acid (NAA) and Kinetin (K). Separate stocks were prepared for macronutrients, micronutrients, Fe-EDTA, vitamins and amino acids. For making one litre full strength MS and WPM, 20:20:10:10:10 ml was taken from five stocks respectively. Separate stocks were prepared for each of the growth regulators used. Sucrose (Sisco Research Laboratories and Sigma, USA) was used as the carbon source at the concentration of $30gl^{-1}$ in the experiments. Activated charcoal at a concentration of 0.5 gl^{-1} was used in the media to reduce the effect of phenolic exudation from the nodal segments. Myo- inositol at the concentration 0.1 gl^{-1} was used as an osmoticum. The pH of the medium was adjusted 5.6 - 5.7 before adding agar. For solidifying the culture medium, agar (Sisco Research Laboratories and Sigma, USA) was used at the concentration of 7.5 gl^{-1}. Aquaguard (Eureka Forbes,USA) water was used for the preparation of stocks and media. Sterilization of media was done by subjecting them to temperature of 121^0C at a pressure of 15psi for 20 minutes. After sterilization, the media were allowed to cool to room temperature and stored in cool dry place.

Table 1. Composition of Murashige and Skoog basal medium

Stock	Composition	Concentration (mgl^{-1})	Stock strength	Quantity(l^{-1})
I	**Macronutrients**		50X	20 ml
	NH_4NO_3	1650.00		
	KNO_3	1900.00		
	$CaCl_2.2H_2O$	440.00		
	KH_2PO_4	170.00		
	$MgSO_4.7H_2O$	370.00		
II	**Micronutrients**		50X	20 ml
	H_3BO_3	6.20		
	$MnSO_4.4H_2O$	22.30		
	KI	0.83		
	$ZnSO_4.7H_2O$	8.60		
	$Na_2MoO_4.2H_20$	0.25		
	$CuSO_4.5H_2O$	0.025		
	$CoCl_2.6H_2O$	0.025		
III	**Fe stock**		100X	10 ml
	Na_2EDTA	37.30		
	$FeSO_4.7H_2O$	27.80		
IV	**Vitamins**		100X	10 ml
	Thiamine HCl	0.10		
	Nicotinic acid	0.50		
	Pyridoxine HCl	0.50		
V	**Amino acid**		100X	10 ml
	Glycine	2.00		

Table 2. Composition of Woody Plant Medium basal medium

Stock	Composition	Concentration(mgl^{-1})	Stock strength	Quantity(l^{-1})
I	**Macronutrients**			
	K_2SO_4	990.0	50X	20 ml
	NH_4NO_3	400.0		
	KH_2PO_4	170.00		
	$MgSO_4.7H_2O$	370.00		
II	**Micronutrients**			
	$CaNO_3.4H_2O$	556.0	50X	20 ml
III	**Micronutrients**			
	$CaCl_2.2H_2O$	96.0	50X	10 ml
IV	**Fe stock**			
	Na_2EDTA	37.3	100X	10 ml
	$FeSO_4.7H_2O$	27.8		
V	**Micronutrients**			
	H_3BO_3	6.2		
	$MnSO_4.4H_2O$	22.3		
	$ZnSO_4.7H_2O$	8.6		
	$Na_2MoO_4.2H_20$	0.25		
	$CuSO_4.5H_2O$	0.25	100X	10 ml
VI	**Vitamins**			
	Thiamine HCl	1.00		
	Nicotinic acid	0.50	100X	10 ml
	Pyridoxine HCl	0.50		
	Amino acid			
	Glycine	2.00		

3.1.4 Media compositions for immature endosperm.

Half strength MS and full strength WPM was also tried for 30-45 day old immature endosperm along with growth regulators viz., Benzyl amino purine (BAP) and Naphthalene acetic acid (NAA) along with an antibiotic ampicillin. Separate stocks were prepared for macronutrients, micronutrients, Fe-EDTA, vitamins and amino acids. For making one litre full strength MS and WPM , 20:20:10:10:10 ml was taken from five stocks respectively. Sucrose (Sisco Research Laboratories and Sigma, USA) was used as the carbon-source at the concentration of 30 gl^{-1}in the experiments. Myo-inositol at the concentration 0.1 gl^{-1} was used as an osmoticum. The pH of the medium was adjusted 5.7-5.8 before adding agar. For solidifying the culture medium, agar (Sisco Research Laboratories and Sigma, USA) was used at the concentration of 7.5gl^{-1}. Distilled water from Aquaguard (Eureka Forbes, USA) was used for the preparation of stocks and media and thereafter sterilized the media as above. After sterilization, the media were allowed to cool to room temperature and stored.

3.1.5 Media composition for *in vitro* germination

Half strength MS basal medium was used for the initial establishment and germination of the immature embryos of the seeds. Later it was transferred to specialized quarter and half strength MS medium with plant growth hormones IBA, NAA, BA at different concentrations. Carbon source sucrose and solidifying agent agar was added at the same concentration as above and sterilized.

3.2 METHODS

3.2.1 Production of multiple shoots from nodal segments.

3.2.1.1 Procedure

The nodal segments from immature green shoots were collected in fungicide solution in the early morning hours. The fungicides, Fytolan/Bavistin/Indofil at 0.1 per cent concentration along with Teepol were used for 45 minutes pre-treatment of nodal segments. These explants were washed thoroughly with tap water, allowed to dry and then wiped out with 70 per cent alcohol. These explants were taken to the laminar air flow for surface treatment and inoculation.

3.2.1.2 Culturing in Laminar air flow chamber

In the laminar air flow chamber the explants were surface sterilized with $HgCl_2$ for 1-3 minutes and later rinsed thrice with autoclaved distilled water. This was dried on autoclaved blotting paper and it was inoculated in half strength MS and full strength WPM media fortified with plant growth regulators at different concentrations and activated charcoal (Table 3). These cultures were kept under incubation under dark conditions for fortnight so as to reduce the effect of phenolic exudations. After a fortnight incubation it was transferred to light all under culture conditions and controlled temperature regime of $28\pm2^{o}C$.

Table 3. Induction media composition for auxillary bud initiation for nodal Segments

Treat-ment	Basal medium	Growth regulators (mgl^{-1})	Sucrose(gl^{-1})	Agar(gl^{-1})
1	½ MS	5.0 IBA +1.0 BA +0.5% AC	20	7.5
2	MS	3.0 NAA +1.0 BA +1.0 Kinetin	20	7.5
3	WPM	1.0 BA + 1.0Thiourea	30	7.5

3.2.1.3 Subculturing

The explants were subcultured at monthly interval or whenever showing contamination .It was transferred to half MS basal medium in between these subculture regimes. The explants were cultured in different MS medium with hormonal concentrations (Table 4).

Table 4. Media composition for elongation of multiple shoots from nodal segments

Treat-ment	Basal medium	Growth regulators (mgl^{-1})	Sucrose(gl^{-1})	Agar(gl^{-1})
1	½ MS	1.0 NAA +3.0 BA	30	7.5
2	½ MS	3.0 NAA +1.0 BA + 1.0 Kinetin	30	7.5

3.2.2 Production of triploid plants from immature endosperms

3.2.2.1 Procedure

Immature fruits (30-45 day old) from elite trees in the field of the Department of Olericulture were harvested and brought to the tissue culture laboratory in fungicide solution (0.1per cent). These fruits were excised inside laminar air flow chamber and the seeds were sterilized in $HgCl_2$ solution of 0.1per cent for 1-3 minutes. They were rinsed with autoclaved distilled water thrice before drying on autoclaved blotting paper. On drying, the two ends of seeds were cut and only the endosperm part was inoculated into jam bottles containing half strength MS medium fortified with various concentrations of plant growth hormones (Table 5). These cultures were kept in dark cultural conditions in the culture room for a fortnight to reduce phenolic exudation and thereafter transferred to light conditions in the culture room. The light and temperature regime were controlled in the culture room with light and temperature $28 \pm 2^{o}C$. Subculturing was carried out at monthly intervals or when the media shows phenolic exudation by the cut ends of the endosperm.

Table 5. Media composition for somatic embryogenesis from immature endosperm

Treat-ment	Basal medium	Growth regulators (mgl^{-1})	Sucrose(gl^{-1})	Agar(gl^{-1})
1	½ MS	2.0 BAP	20	7.5
2	½ MS	5.0 BAP	30	7.5
3	½ MS	1.5 BA+0.1 NAA+ 1ppm Amp	30	7.5
4	WPM	5.0 BAP	30	7.5

3.2.2.2 Regeneration

The sub cultured immature endosperms were transferred to half strength MS media containing growth hormones and other additives (Table 6) to initiate shoot and root growth in the explants .

Table 6. Media composition for regeneration of immature endosperm

Treat-ment	Basal medium	Growth regulators (mgl^{-1})	Sucrose(gl^{-1})	Agar(gl^{-1})
1	½ MS	2.0 BAP	20	7.5
2	½ MS	2.0 IBA+2.0 BA +1.0 GA_3	20	7.5
3	½ MS	1.5 BA+0.1 NAA+ 1ppm Amp	20	7.5

3.2.3 Production of *in vitro* seedlings from immature seeds

3.2.3.1 Procedure

The excised ends of the immature endosperm containing embryo were inoculated into half strength MS basal. These inoculated embryo portions with a bit of endosperm were initially kept in dark for two weeks and later transferred to light in the culture room. The *in vitro* germinated seeds were transferred to MS basal medium with different hormonal compositions (Table 7).

Table 7. Media composition for *in vitro* germination from immature seeds

Treat-ment	Basal medium	Growth regulators (mgl^{-1})	Sucrose(gl^{-1})	Agar(gl^{-1})
1	½ MS	2.0 IAA +2.0 IBA +2.0 NAA	20	7.5
2	½ MS	1.0 IAA +2.0 BA	20	7.5

3.2.4 Sex determination through molecular technique

3.2.4.1 Isolation of genomic DNA

Parrot green leaves from the field grown male and female trees were collected and the leaves of *in vitro* germinated seedlings were collected for isolation of DNA, and further RAPD analysis to detect distinct sex forms in this polygamodioecious perennial spice taxa. Procedure 1: Doyle and Doyle (1987) method.

Reagents

1. Extraction buffer (4X) – 1.0litre

   Sorbitol – 25.6 g

   Tris – 48 g

   EDTA disodium salt – 7.4 g

2. Lysis buffer-1litre

   1 M Tris pH 8 – 200 ml

   0.25 M EDTA – 200 ml

   CTAB – 20 g

3. Sarcosine -5 %

4. TE buffer

   10 mM Tris (pH 8)

   1 mM EDTA (pH 8)

5. Ice-cold Isopropanol

6.Chloroform-Isoamyl alcohol (24:1 v/v)

7.Ethanol 70 per cent (v/v)

Procedure 1

Leaf samples weighing 1.0 g were freeze powdered in liquid nitrogen and ground with 6 ml of 1X extraction buffer, 50 µl β mercaptoethanol and a pinch of sodium metabisulphate using chilled autoclaved mortar and pestle. The homogenate was transferred to a 50 ml oak ridge tube containing 6.0ml pre-warmed lysis buffer and 2.0 ml sarcosine. The tubes were incubated in a water bath at 65° C for 10 minutes with intermittent shaking. The tubes were removed from the water bath and equal volume of chloroform:isoamyl alcohol mixture (24:1 v/v) was added and mixed by gentle inversion. Centrifugation was carried out at 10,000 rpm for 10 min at 4°C. The clear aqueous phase was transferred to a fresh tube. To this, 0.6 volume ice-cold isopropanol was added and after gentle mixing, kept in - 20 °C deep freezer for 30 minutes for complete precipitation of DNA. DNA was pelleted by centrifuging at 10,000 rpm for 10 min at 4°C. The isopropanol was poured out and the pellet was washed with 70 per cent (v/v) ethanol by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and air-dried the pellet. Dried pellet was dissolved in 100 µl of TE and stored at – 20°C until further use.

In another procedure the complete precipitation of DNA in - 20 °C deep freezer was extended to one hour 30 minutes. The air-dried DNA pellet was dissolved in 100µl Milli Q water stored at - 20 °C until further use in this modified procedure.

Procedure 2: Rogers and Bendich (1994) method

Reagents

1. 2X CTAB extraction buffer

   2% CTAB (w/v)

   100 mM Tris (pH 8)

   20 mM EDTA (pH 8)

1.4 M NaCl

1% PVP

2. 10X CTAB extraction buffer

10% CTAB (w/v)

0.7M NaCl

3. TE buffer
10 mM Tris pH 8
1 mM EDTA pH 8

4. Ice-cold Isopropanol
5. Chloroform-Isoamyl alcohol (24:1 v/v)
6. Ethanol 70 per cent (v/v)
(Chemical compositions of reagents are given in Annexure I)

Procedure 2.1

Leaf sample weighing 1.0g was accurately weighed and ground in a pre-chilled mortar and pestle in the presence of liquid nitrogen. The ground tissue was transferred into a 30ml oak ridge tube containing 5 ml pre-warmed 2X CTAB extraction buffer. The contents were mixed well and incubated at 65°C for 15 minutes. Then equal volume of chloroform:isoamyl alcohol (24:1 v/v) mixture was added, mixed gently by inversion and centrifuged at 10,000 rpm for 15 minutes at 4 °C. The mixture separated into three distinct phases from which the upper aqueous phase containing DNA was pipetted out into a fresh 30ml oak ridge tube. To this, 1/10th volume 10 per cent CTAB was added and mixed gently by inversion. Equal volume of chloroform:isoamyl alcohol mixture (24:1 v/v) was added, mixed gently to form an emulsion and centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was collected in a fresh oakridge tube and 0.6 volume of chilled isopropanol was added and mixed gently to precipitate the DNA. It was incubated at - 20°C for 20 minutes. The contents were then centrifuged at 10,000 rpm for 5 minutes at 4°C to pellet the DNA. The

isopropanol was poured off retaining the DNA pellet. It was washed first with 70 per cent alcohol and then with absolute alcohol. The DNA pellet was air dried to remove the alcohol and then dissolved in 100 µl of TE buffer.

A modified DNA isolation protocol by increasing incubation at - 20 ^{o}C for 1 hour 30 minutes and thereafter dissolved the dried pellet in 100 µl MilliQ water.

3.2.4.2 Purification of DNA

The DNA isolated contains RNA and protein as contaminants. The DNA sample was hence treated with RNase and Proteinase K.

3.2.4.3 Preparation of RNase and Proteinase K

Ribonuclease A (Genei,Bangalore) was dissolved at a concentration of 10mgl^{-1} in 0.01 M sodium acetate (pH 5.2). The solution was heated at 100^{o}C for 15 minutes and then cooled to room temperature. The pH was finally adjusted by adding 100µl Tris base (pH 7.4) and stored at - 20^{o}C.

Proteinase K (Genei, Bangalore) was prepared at a concentration of 20 mgl^{-1} in distilled water and stored at - 20^{o}C.

3.2.4.4 RNase and Proteinase treatment

Hundred microlitre of DNA suspended in TE buffer was treated with 2µl of RNase solution and incubated at 37^{o}C for one hour. Thereafter 2µl of Proteinase K solution was added and again incubated at 45^{o}C for one hour. The content was made into total volume of 500µl with distilled water and equal volume of phenol:chloroform isoamyl alcohol mixture (1:1) was added. It was centrifuged at 10000 rpm for 10 minutes at 4^{o}C. The top layer was transferred to a fresh eppendorf tube and equal volume of chloroform :isoamyl alcohol mixture was added. The upper layer was saved and this step was repeated twice. The final aqueous phase was collected into a fresh eppendorf tube and 0.6 volume of chilled isopropanol was added, mixed gently and incubated at - 20^{o}C for 30 minutes to precipitate the DNA. It was centrifuged at 10000 rpm for 10 minutes at 4^{o}C. The DNA pellet was retained and washed first with 70 per cent alcohol

and then with absolute alcohol. It was then air dried and dissolved in 25µl TE buffer.

3.2.6 Quantification of DNA

The quality of isolated DNA samples were evaluated through 0.8 percent agarose gel electrophoresis.

3.2.6.1 Materials Required

1. Agarose
2. 50X TAE buffer (pH 8)
3. Gel casting tray, comb, electrophoresis unit and power pack.
4. Ethidium bromide solution (stock 10mg/ml; working concentration, 0.5µg/ml)
5. 6X loading dye
6. Double digest marker
7. UV transilluminator
8. Gel documentation and Analysis system

(Chemical compositions of the buffer and dyes are given in Annexure II)

3.2.6.2 Procedure for casting the gel and electrophoresis.

1X TAE buffer was prepared from 50 X TAE stock buffer. Agarose {0.8 per cent (w/v) for genomic DNA and 1.2 percent (w/v) for RAPD} was weighed and added to the volume of buffer required in the casting tray. It was then dissolved by boiling. The open ends of the gel-casting tray were sealed properly with cellotape and arranged on a horizontal level platform. The comb was placed at an open end such that it is 0.5-1.0mm from the base. After the agarose dissolved completely it was cooled to lukewarm temperature and ethidium bromide was added to a final concentration of 0.5µg/ml. It was poured into the gel mould and allowed to solidify for about 30-45 minutes at room temperature. The comb and cellotape were removed carefully and the gel was placed in the electrophoresis unit with the well side directed towards the cathode. 1X TAE

buffer was added to the buffer tank so as to cover the gel to a depth of 1mm. DNA sample of 5µl was mixed with 1µl tracking dye and carefully loaded into the wells using a micropipette. The λDNA/EcoRI HindIII Double Digest (Bangalore Genei) was used as molecular weight marker. The cathode and the anode of the electrophoresis unit were connected to the power pack (Biotech) and the gel was run at constant voltage of 5 V/cm. The power was turned off when three-fourth running was completed which could be identified from the position of the tracking dye.

3.2.6.3 RAPD markers

Random amplified polymorphic DNA (RAPD) analysis was carried out after isolation of good quality DNA from all the collected samples. This technique allows DNA amplification using random primers. The differences in the patterns of bands amplified from genetically distinct individuals behave as genetic markers. Primers were used to generate the amplification patterns and many loci can be analysed as each primer anneals to a different region of the DNA which can be viewed on an agarose gel.

3.2.6.4 Composition of RAPD reaction mixture

A total volume of 25µl mixture was prepared for each reaction. The RAPD reaction mixture consists of 10X assay buffer for Taq DNA polymerase, $MgCl_2$, dNTPs, template DNA, random primer and Taq polymerase enzyme which was subjected to cycling among three temperatures for denaturing, annealing and extension. The composition of reaction mixture is as follows :

10X Assay buffer for Taq DNA polymerase (15mM $MgCl_2$)	- 2.5 µl
$MgCl_2$	- 1.0 µl
dNTP mix (100µM each of dATP, dCTP, dGTP, dTTP)	- 1.0 µl
DNA template (20 – 50 ng)	- 2.0 µl
Random decamer primer	- 2.0 µl
Taq DNA polymerase (0.6 U)	- 2.0 µl
Milli Q water	- 14.5µl
Total volume	**- 25 µl**

3.2.7 Screening of random primers for RAPD

Primer screening was carried out to identify best primers for RAPD analysis. Random decamer primer kits obtained from Operon Technologies, USA and Kit C (Integrated Technologies) series were used for screening. Six primers under OPE series and nine primers under OPF series were tried. The details of the primers are given in the Table 8.

The template DNA was kept the same throughout the screening procedure. A master mix was prepared for the required number of reactions by adding all the constituents except the primer. Aliquots of the master mix were pipetted out into each of the 0.2ml PCR tubes and then the primer was added into each tube separately. The primers that gave good amplification with four or more distinct and reproducible bands were selected and used for screening of the field grown trees and *in vitro* germinated seedlings for sex determination.

3.2.8 RAPD analysis

Reaction mixture (25 µl) containing 2 µl genomic DNA (20-50 ng), 2.5 µl 10X assay buffer (Bangalore Genei), 1 µl Mg Cl_2 (Bangalore Genei), 1 µl dNTPs (100 µ M each of dATP, dGTP, dCTP, dTTP), 2 µl of primer (Table 8), 2 µl of Taq polymerase enzyme (Bangalore Genei) and 14.5 µl Milli Q water was prepared in 30 µl PCR tubes . Genomic DNA was replaced by equal volume of MilliQ water keeping all other constituents same in one PCR tube which was taken as control. Polymerase Chain Reaction was carried out in the thermal cycler of model PTC 200 of MJ Research, USA programmed for an initial denaturing period of 94^{o}C for 3 minutes followed by 40 cycles of one minute denaturation at 92 ^{o}C, 1 minute primer annealing at 37 ^{o}C and 2 minutes polymerisation at 72 ^{o}C. After completion of amplification, the reaction was held at 4 ^{o}C for 10 minutes.

The amplified products were resolved on a 1.2 percent w/v agarose gel with ethidium bromide as intercalating agent for visualisation. The agarose gel was prepared in 1X TAE buffer and run in a horizontal electrophoresis (Bangalore Genei) at 100 V supplied from a power pack (Biotech). Double digest marker λDNA/EcoRI HindIII (Bangalore Genei) was used as molecular weight marker

and visualised under UV light in a transilluminator (Herolab) and documented with the help of an alpha imager (Alpha Innotech, USA).

3.2.9 Gel Documentation

The gel was taken from the electrophoresis unit and the separated DNA bands were viewed under UV light in a transilluminator. The DNA fluoresces under UV light on account of intercalating ethidium bromide dye. The image was stored in the gel documentation system (Alpha Imager).

Table. 8. List of primers used for screening in RAPD analysis

Sl. No.	Primer code	Primer sequence
1	OPE-3	CCAGATGCAC
2	OPE-5	GCAGGGAGGT
3	OPE-6	AAGACCCCTC
4	OPE-7	AGATGCAGCC
5	OPE-11	GAGTCTCAGG
6	OPE-12	TTATCGCCCC
7	OPF-1	ACGGATCCTG
8	OPF-2	GAGGATCCCT
9	OPF-3	CCTGATCACC
10	OPF-5	CCGAATTCCC
11	OPF-6	GGGAATTCGG
12	OPF-7	CCGATATCCC
13	OPF-8	GGGATATCGG
14	OPF-9	CCAAGCTTCC
15	OPF-10	GGAAGCTTGG
16	KIT-C-1	TTCGAGCCAG
17	KIT-C-2	GTGAGGCGTC
18	KIT-C-3	GGGGGTCTTT
19	KIT-C-4	CCGCATCTAC
20	KIT-C-5	GATGACCGCC
21	KIT-C-6	GAACGGACTC
22	KIT-C-7	GTCCCGACGA
23	KIT-C-8	TGGACCGGTG
24	KIT-C-9	CTCACCGTCC
25	KIT-C-10	TGTCTGGGTG
26	KIT-C-11	AAAGCTGCGG
27	KIT-C-12	TGTCATCCCC
28	KIT-C-13	AAGCCTCGTC
29	KIT-C-14	TGCGTGCTTG
30	KIT-C-15	GACGGATCAG
31	KIT-C-16	CACACTCCAG
32	KIT-C-17	TTCCCCCCAG
33	KIT-C-18	TGAGTGGGTG
34	KIT-C-19	GTTGCCAGCC
35	KIT-C-20	ACTTCGCCAC

Results

4. RESULTS

The results of the study on "Refinement of micropropagation protocol and RAPD assay for sex determination in Kodampuli (*Garcinia gummi-gutta var. gummigutta*)" are given below.

4.1 Effects of media composition for multiple shoot induction from nodal segments

Among the media composition the treatment T_1 with ½ MS media fortified with 5.0 IBA, 1.0 BA and 0.5% activated charcoal gave 25 per cent response with an average of three multiple shoots per explant as compared to the treatments T_2 and T_3 (Table 9) (Plate1a). Due to lack of response in T_2 and T_3, the cultures were discarded and not used for further inoculation.

4.1.2. Effect of media compositions for shoot elongation

Elongation of nodal segments were studied and results are represented in Table 10 (Plate1e). The explants subcultured to the T_1 and T_2 were shown signs of contamination within 15DAI. These contaminations were of fungal in nature and due to which all the inoculated explants were lost. Thus, there were no cultures which survived more than 15 DAI in these two treatments (Plate1c & d).

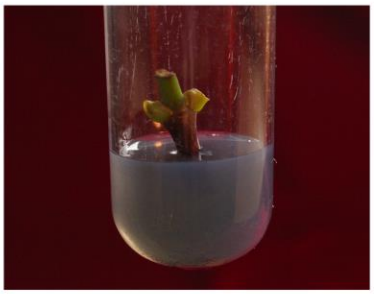
4.2 Effects of media composition for triploid plantlets production from immature endosperm

The treatment T_1 showed good response with 78 per cent of the inoculated survival with shoot growth at 15DAI, whereas the other treatments T_2, T_3, T_4, T_5 and T_6 were not shown any response. The immature embryo in T_1 has showed multiple shoot initiation at 30 DAI and 45 DAI (Plate2 & 3). These cultures were sub-cultured at 15 days interval. The survival rate at each subculture cycle had sequentially reduced due to fungal contamination. At 30 DAI, the survival per cent was reduced to 53 per cent (Table 11) and at 45 DAI none of them were survived.

Table9. Effect of media composition on multiple shoot induction

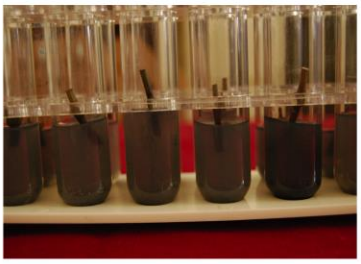
Treatment	Basal medium	Growth regulators (mgl^{-1})	No. of explants inoculated	No. of explants survived 15DAI	No. of explants shown response	Nature of response	No. of multiple shoot per explant	(%) Cultures responded	Nature of loss
T_1	½ MS	5.0 IBA+1.0 BA +0.5% AC	1139	285	285	Multiple shoot	3	25%	FC
T_2	MS	3.0 NAA+1.0 BA + 1.0 K	442	0	0	-	-	-	FC
T_3	WPM	1.0 BA +1.0 Thiourea	450	0	0	-	-	-	FC

a. Explant collected at parrot green stage



b. Inoculated nodal segment

c. Fungal contamination



d. Fungal contamination

e. Nodal segment at 15DAI

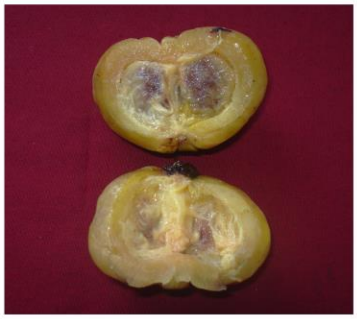
f. Nodal segment at 21 DAI

Plate 1. Stages and responses of nodal explants

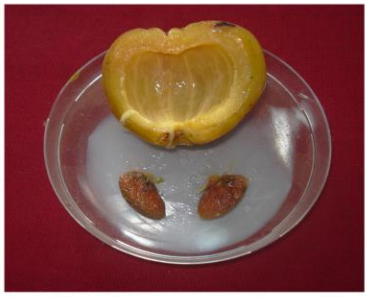
Table10. Effect of media composition on elongation of multiple shoot

Treatment	Basal medium	Growth regulators (mgl^{-1})	No. of explants inoculated	Nature of response	No. of explants showing contamination	(%) Cultures survived
T_1	½ MS	1.0 NAA +3.0 BA	140	contamination	140	0
T_2	½ MS	3.0 NAA + 1.0 BA +1.0 K	140	contamination	140	0

a. 30-45 day old fruits



b. Fruit excised into two halves



c. Seed used for endosperm and *in vitro* seedling cutlure

Plate 2. Explant source for endosperm and *in vitro* seedling culture

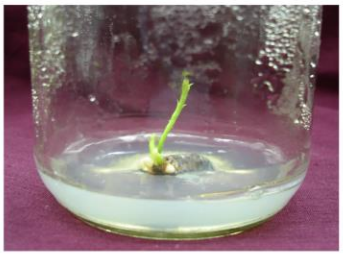
Table 11. Effect of media compositions on triploid plantlet production from immature endosperm

Treatment	Basal medium	Growth regulators (mgl^{-1})	No.of explant inoculated	No. of explants survived			No. of explants shown response	Nature of response	No.of multiple shoots per explants	(%)Cultures responded			Nature of loss
				15 DAI	30 DAI	45 DAI				15 DAI	30 DAI	45 DAI	
T_1	½ MS	2.0 BAP	306	245	162	0	245	Shoot initiation	4	78	53	0	FC
T_2	½ MS	2.0 IBA+2.0 BA +1.0 GA_3	85	76	0	0	0	-	0	0	0	0	FC
T_3	½ MS	1.5 BA+0.1 NAA+ 1ppm Amp	113	86	0	0	0	-	0	0	0	0	FC
T_4	½ WPM	Basal	93	72	0	0	0	-	0	0	0	0	FC
T_5	¼ MS	2.5 BA+0.5 GA_3 +0.5 K	38	-	0	0	0	-	0	0	0	0	FC
T_6	½ MS	Basal	39	-	0	0	0	-	0	0	0	0	FC

a. Endosperm culture

b. Endosperm culture

c. Shoot growth in Media



d. Multiple shoot initiation

e. Multiple shoot growth

f. Root growth in media

Plate 3. Endosperm culture and multiple shoot growth

Table12. Effect of different media composition for *in vitro* germinated seeds

Treatment	Basal medium	Growth regulators(mgl^{-1})	No. of explants taken to dark incubation	No. of explants survived 21 DAI	Nature of response	(%) Cultures survived
T_1	½ MS	2.0 IAA+ 2.0IBA +2.0 NAA	289	252	Shoot initiation	87%
T_2	½ MS	1.0 IAA+ 2.0 BA	289	210	Shoot initiation	73%

4.3 Effects of *in vitro* seedlings from immature seeds

The excised embryo ends from immature endosperm inoculated onto half strength MS basal media were germinated within 7 days of transfer to the light condition of the culture room, after the fortnight dark incubation. The subcultured *in vitro* germinated seedlings were elongated and produced two leaves at 21 DAI (Plate 4 & 5) (Table 12).

4.4 Sex determination through RAPD assay

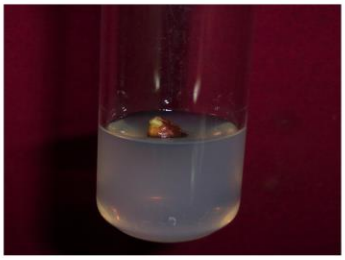
4.4.1 Isolation of DNA

DNA isolation and purification by CTAB method from field grown male, female and *in vitro* germinated seedlings were produced good quality DNA having OD value of 1.848 and 1.930 at absorbance range of A_{260} - A_{280} (Table13) The presence of intact discrete band indicated non-degraded DNA (Plate 7 a & b).

Table 13. Quality and quantity of DNA extracted from field grown male, female trees and *in vitro* germinated seedlings

Sl No.	Plant samples	Optical Density Values		Quantity of DNA (µg/ml)	Ratio OD_{260}/OD_{280}	Remarks on quality
		OD_{260}	OD_{280}			
1.	Female trees	0.0851	0.0463	425.5	1.848	Good
2.	Female trees	0.0726	0.0375	363.0	1.930	Good
3	Male trees	0.0467	0.0249	233.5	1.875	Good
4	Male trees	0.0494	0.0256	247.0	1.926	Good
5	*In vitro* germinated seedlings	0.0724	0.0373	362.0	1.941	Good
6	*In vitro* germinated seedlings	0.0469	0.0251	232.8	1.869	Good
7	*In vitro* germinated seedlings	0.0851	0.0460	423.8	1.850	Good
8	*In vitro* germinated seedlings	0.0465	0.0249	233.7	1.867	Good

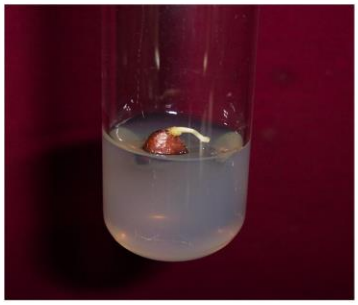
a. Immature embryo inocutated



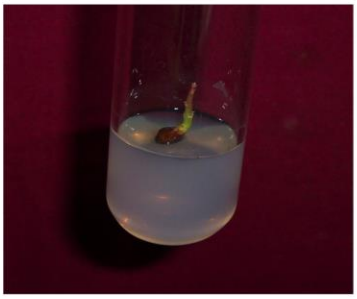
b. Immature embryo15 DAI

c. Immature embryo 21 DAI

d. Immature embryo 30 DAI



e. Immature embryo 45 DAI



f. Immature embryo 60 DAI

Plate 4. Stages of *in vitro* seedling culture

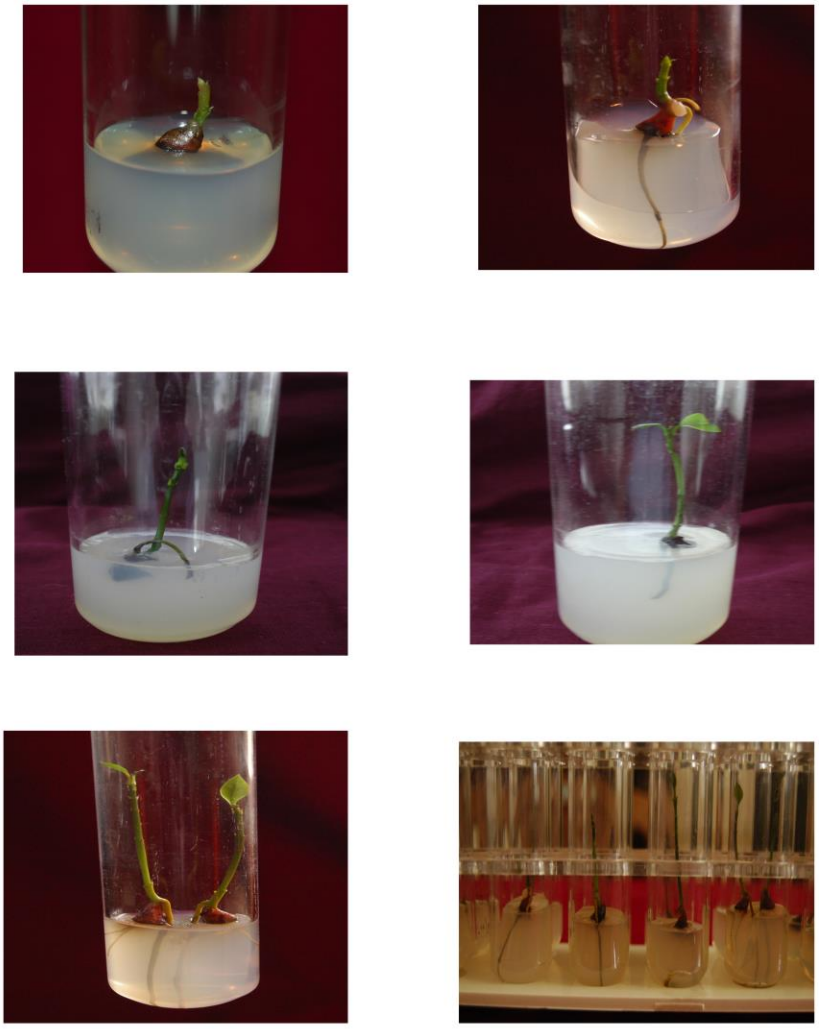


Plate 5. Stages of shoot and root growth of *in vitro* seedlings

4.4.2 Screening of random primers for RAPD

Out of thirty five random primers were screened with the selected PCR reaction mixture and thermal settings. The screening with OPE series 3, 5, 6, 7, 11 and 12 were shown no amplification in the RAPD cycle and thus these primers were not used for further study in sex determination of Kudampuli(Table 14). The screening with OPF decamer primers were also not recorded any amplification. Thus, these primer series were also discarded from future RAPD assay studies (Table 15). The primers KIT-C 1-10 (Plate 7 d and e) were registered amplification and hence among these primers KIT-C 8 and 9 were bestowed with good amplification pattern. The primer sets KIT-C 1-10 were selected for further screening of the *in vitro* germinated seedlings. Though they have recorded maximum number of amplicons (KIT-C 8 & 9), they were not shown any difference between male and female trees (Table 16). No amplifications were exhibited by the decamer primers KIT-C 11-20 (Table 17).

Table 14. Amplification pattern of genomic DNA with different decamer primers under OPE series

Primer code	Primer sequence	No.of amplicons
OPE-3	CCAGATGCAC	Nil
OPE-5	GCAGGGAGGT	Nil
OPE-6	AAGACCCCTC	Nil
OPE-7	AGATGCAGCC	Nil
OPE-11	GAGTCTCAGG	Nil
OPE-12	TTATCGCCCC	Nil

Good =>5, Average= 4-5 and poor= <4 amplicons

.

Table 15. Amplification pattern of genomic DNA with different decamer primers under OPF series

Primer code	Primer sequence	No.of amplicons
OPF-1	ACGGATCCTG	Nil
OPF-2	GAGGATCCCT	Nil
OPF-3	CCTGATCACC	Nil
OPF-5	CCGAATTCCC	Nil
OPF-6	GGGAATTCGG	Nil
OPF-7	CCGATATCCC	Nil
OPF-8	GGGATATCGG	Nil
OPF-9	CCAAGCTTCC	Nil
OPF-10	GGAAGCTTGG	Nil

Good = >5, Average= 4-5 and poor= <4 amplicons

Table 16. Amplification pattern of genomic DNA with different decamer primers under KIT-C 1-10 series

Primer code	Primer sequence	No.of amplicons		Quality of amplification
		female	male	
KIT-C-1	TTCGAGCCAG	3	4	Average
KIT-C-2	GTGAGGCGTC	0	4	Average
KIT-C-3	GGGGGTCTTT	5	5	Average
KIT-C-4	CCGCATCTAC	4	3	Average
KIT-C-5	GATGACCGCC	0	0	Poor
KIT-C-6	GAACGGACTC	4	4	Average
KIT-C-7	GTCCCGACGA	0	5	Average
KIT-C-8	TGGACCGGTG	7	7	Good
KIT-C-9	CTCACCGTCC	8	8	Good
KIT-C-10	TGTCTGGGTG	5	5	Average

Good = > 5, Average= 4-5 and poor= <4 amplicons

Table 17. Amplification pattern of genomic DNA with different decamer primers under KIT-C 11-20 series

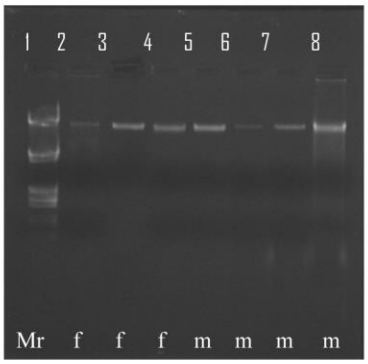
Primer code	Primer sequence	No.of amplicons	
		female	male
KIT-C-11	AAAGCTGCGG	0	0
KIT-C-12	TGTCATCCCC	0	0
KIT-C-13	AAGCCTCGTC	0	0
KIT-C-14	TGCGTGCTTG	0	0
KIT-C-15	GACGGATCAG	0	0
KIT-C-16	CACACTCCAG	0	0
KIT-C-17	TTCCCCCCAG	0	0
KIT-C-18	TGAGTGGGTG	0	0
KIT-C-19	GTTGCCAGCC	0	0
KIT-C-20	ACTTCGCCAC	0	0

Good = > 5, Average= 4-5 and poor =< 4 amplicons

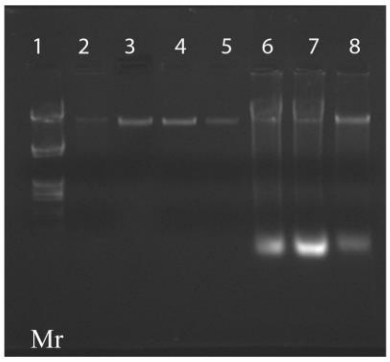
4.4.2 Screening of *in vitro* germinated seedlings

The primer KIT-C 1 was selected for further studies of RAPD analysis with the known male and female field grown trees as well as unknown *in vitro* germinated seedlings . The details of amplification products obtained and the number of polymorphic and monomorphic bands are given in (Table18) (Plate7c).

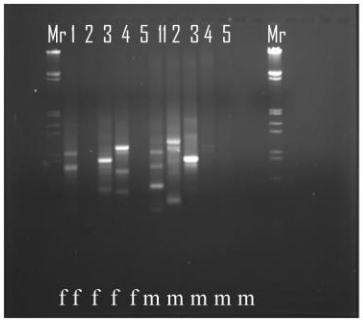
Certain bands in the samples of field grown female trees and some of the *in vitro* seedling were absent when amplified with KIT-C1. The RAPD banding pattern of some of the *in vitro* seedlings were of the copyright of field grown male trees.



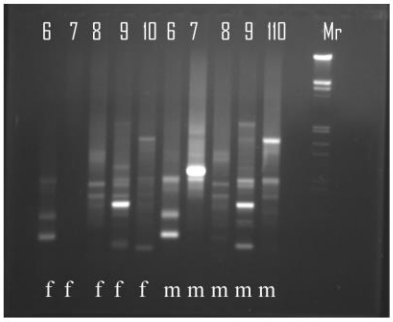
a. DNA isolated from field grown male and female tree



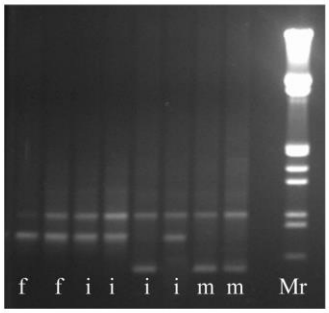
b. DNA isolated from i*n vitro* seedlings



c. Banding pattern of KIT-C-1 to 5 primers lane 1-5 female, 6-10 male



d. Banding pattern of KIT-C-6 to 10 primers lane 1-5 female, 6-10 male



e. Banding pattern of KIT-C-1 primer

F-Female M-Male I- *in vitro* seedlings Mr - Marker

Plate 6. DNA isolation and RAPD analysis for sex determination

Table 18. No. of polymorphic and monomorphic bands obtained for each sample after PCR using selected primer.

Bands	Samples selected for PCR reaction							
	female	female	*In vitro* seedling	*In vitro* seedling	*In vitro* seedling	*In vitro* seedling	male	male
pb	1	1	1	1	2	1	2	2
mb	2	2	2	2	2	2	2	2

Pb-polymorphic band; Mb-monomorphic band

Discussion

5. DISCUSSION

Kodampuli (*Garcinia gummi-gutta var. gummigutta* (L.) Rob.) is one of the under exploited miscellaneous spice tree taxon which is grown as an intercrop in backyard of homesteads in Kerala. This backyard non-timber polygamodioecious spice tree has excited the scientific community as the richest natural source of potential anti-obesity phytometabolite. (-)hydroxycitric acid [(-)-HCA] from the fleshy fruit rinds The present study gains importance when we consider the fact that the kodampuli tree is polygamodioecious in nature and the seed dormancy extends to 7-8 months.

Major aspects of the investigation are as follows:

1. Micropropagation of nodal segments and immature endosperm
2. *In vitro* germination of immature embryos
3. DNA isolation
4. Sex determination using RAPD analysis

5.1 Micropropagation of nodal segments and immature endosperm

5.1.1 Micropropagation of nodal segments

Clonal multiplication of economically and pharmaceutically important species like *Garcinia gummi-gutta var. gummigutta* can be achieved either by softwood grafting or by micropropagation. Softwood grafting however, is a season dependant process requiring large space, trained manpower and large number of rootstocks. The non-availability of orthotropic shoots as scion material has limited the scope of softwood grafting in this pharmaceutically significant tree taxon. Seed dormancy and low germination per cent of seeds were also cause dearth of rootstock for grafting in *Garcinia gummi-gutta var. gummigutta.* Similar opinion was shared by George *et al.* (2002) in *G. indica*.ver However, in most the tropical tree species *in vitro* establishment and multiplication is difficult (Drew, 2000).

The media compositions tried ½ MS + 5.0 mgl^{-1} IBA + 1.0 mgl^{-1} BA +0.5 per cent AC gave 25 per cent response and gave on an average three multiple

shoots per explant. The other two treatments T_1 and T_2 were not registered the similar results of Rajendran *et al.*(2004). Mary Mathew *et al.* (2005) had reported 2.1± 0.4 shoots for Comboge and 2.6± 0.5 for kokum. Goh *et al.* (1990) reported similar finding in shoot bud regeneration from *in vitro* nodal explants inoculated in MS + NAA (2.69 µM) + BAP (17.6 µM). This medium is comparable with the observation made in *G. mangostana* . All the cultures were observed fungal contamination within 45 DAI irrespective of pre-treatment and surface sterilization. The results were on par with the finding of Mary Mathew *et al.* (2001) *in vitro* cultures of camboge where severe fungal contamination within 15-20 days of inoculation in 95-100 per cent cultures due to *Rhizoctonia* and *Fusarium spp*.

These results were contrary to the findings of 69 per cent survival and regeneration of axillary shoot growth free from microbial contamination within 59 DAI in ¼ MS media fortified with 0.5 mg l^{-1} BA + 2.5 mg l^{-1} IBA and 0.75 per cent agar + 2.0 per cent sucrose reported by Rajendran *et al.* (2005).

The dosage of cytokinin in the culture medium is known to be critical for shoot organogenesis. In the present study, BAP was found to be more potent compared to other cytokinins. Zaerr and Mapes (1982) reported that the superiority of BAP for shoot induction may be attributed to the ability of BAP to induce production of natural hormones suc as zeatin within the tissue. The similar promotory effect of BAP in inducing multiple shoots has been previously reported in *G. mangostana* by Goh *et al.* (1990), Huang *et al.* (2000) and Malik *et al.* (2005). Incorporation of NAA in the medium had a deleterious effect on shoot bud formation and the similar effect of NAA was earlier noticed in *G . mangostana* (Normah *et al.*,1992; Huang *et al.*, 2000) and in G. indica (Malik *et al.*, 2005)

5.1.2 Immature endosperm for triploid plantlet production

The 30-45 day old seeds when excised and endosperm cultured in ½ MS + 2 BAP media they showed survival with shoot growth at 15 DAI. Similar results were reported by Rajendran *et al.* (1998) callus induction and proliferation was

obtained along with direct organogenesis when cultured in ½ MS medium supplemented with 0.5 mg l^{-1} NAA + 0.2 mg l^{-1} BA and 0.3 mg l^{-1} K. According to them surface sterilization treatment with 0.1 per cent $HgCl_2$ for 3-5 minutes after pre-treatment with 70 per cent alcohol wiping of freshly harvested immature fruits and the treatment of extracted seeds with 0.1 percent bavistin for 20 minutes could effectively check the *in vitro* contamination problem in Kodampuli.Lakshmisita *et al*. (1982) were induced somatic embryogenesis from Sandalwood endosperm opined that the seed sterility and seedlessness are of no serious consequence where seeds have no commercial importance. They further postulated that the triploid endosperm tissue explants could be exploited for mass producing identical copies of superior trees by millions. Triploid endorsed through endosperm culture will have the unique advantage of seedlessness in perennial fruity spice crop condiment-cum-spice crop like kodampuli (rajendran *et al*., 2004).

Rajendran *et al.* (1998) were observed that the immature endosperm excised from 30-40 days old fruits could induce profuse callusing with embryonic mode of response in the media composition of ¼ MS + 8.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} BA within 55 days. They were produced somatic embryos through the subsequent sub-culture cycles on ½ MS + 0.5 mg l^{-1} NAA + 0.2 mg l^{-1} BA + 0.3 mg l^{-1} K. On the contrary, the multiple shoot regeneration and embryogenic mode of response obtained from the present study were lost due to systemic fungal contamination at 45 DAI. So, it needs further investigation to refine protocol the micropropagation protocol of this perennial tree spices.

5.2 *In vitro* germination of immature embryos

The embryo from immature endosperm was inoculated onto ½ MS basal media which showed germination within seven days after the fortnight dark incubation. They were sub cultured to elongation media and produced two leaves. Li Chun *et al*. (2000) had reported similar finding that no auxin supplementation was necessary for bud primordium differentiation in cotyledon explants of mangosteen or proliferation of regenerated shoots. The optimum N_6-

benzyladenine concentration for primordium differentiation was 13.3 µM and for shoot proliferation ranged from 4.4 to 13.3 µM. Continuous culturing in an 8 hour photoperiod at 30°C resulted in progressively intensified degeneration of shoots after three passages. The findings of Rajendran *et al.* (2004) the *in vitro* germinated embryos with roots were ready to transfer to the polythene bags within one month period could be able to stereotype from the present investigation. Similarly Malik *et al.*(2005) reported in *Garcinia indica* that seed segments with induced adventitious buds (of about 1-7mm length)after 5 weeks of culture on hormone supplemented medium, were further segmented into smaller parts of approximately 3mm x 4mm before transferring to MS basal medium supplemented with 0.2 percent activated charcoal for shoot elongation.

5.3 DNA isolation

In the present study, the DNA isolation using the CTAB method and the optical density values of diluted preparation of DNA by UV spectrophotometer at OD_{260}/OD_{280} absorbance was registered in the range of 1.8- to 2.0 which, indicated that DNA was good without much RNA or protein contamination. Since one PCR reaction requires only 25-50 ng template, the DNA recovered was found to be sufficient for further PCR reactions. These isolated DNAs from field grown male, female trees and *in vitro* seedlings of kodampuli expressed the reliable gender-linked polymorphic pattern using RAPD analysis.

5.4 Sex determination using RAPD analysis

During the last decade an increasing trend of research result directed at identifying and characterizing molecular markers and genes involved in plant dioecy by Parasnis *et al.* (2000), Kafkas *et al.* (2001), Khadka *et al.* (2002) and Yakubov *et al.*, (2005) reported that the RAPD is a rapid and inexpensive method for studies on genotypic relationships and selection of traits of interest. Development of a pistillate specific RAPD marker linked to sex determination in *G. gummi-gutta var. gummigutta* may be feasible by converting genetic markers to sequence-characterized amplified regions (SCAR). Paran and Michelmore

(1993) and Xu *et al.* (2004) were also substantiated that by developing longer primers from RAPD sequences significantl improves the reproducibility and reliability of PCR assays in pistilate trees which represent the heterogametic sex.

Among the thirty five primers tested, KIT-C-1 RAPD decamer to distinguish the genders at the early vegetative stage in vitro germinated seedlings in comparison with field grown male and female trees. Distinctive sex-linked amplicons using 569 bp RAPD markers was reported by Wen- Jie *et al*. (2004) in pistillate *Ecommia ulmoides* Oliv., in date palm by Rajendran and Al-Mssallem (2005) and 945 bp RAPD OPO-08 marker in *Pistacia vera* by Yabukov *et al.(*2005).

Future line of work:-

1. Deriving triploid plantlet from immature endosperm
2. Planting out somatically derived plantlets
3. Designing primer specific for sex determination
4. Development of SCAR primers for sex determination

Summary

SUMMARY

The study on "Refinement of micropropagation protocol and RAPD assay for sex determination in kudampuli (*Garcinia gummi-gutta* var. *gummigutta*)" was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2004-2006. The objectives of the study were refinement of standardized micropropagation protocol (nodal segments) and somatic embryogenesis (immature endosperm), standardization of DNA isolation techniques from male and female trees, in vitro seedling progenies and polyembryonic seedlings in kudampuli, RAPD assay using selected oligonucleotide decamer primers to ascertain the sex at juvenile phase in the species. The salient features of the study are summarized below.

1. Surface sterilization of nodal segments with mercuric chloride (0.1%) for 1-3 minutes and inoculation in ½ MS+ 5.0mgl^{-1} IBA + 1.0mgl^{-1} BA + 0.5 % activated charcoal gave 25 per cent response with three multiple shoot per explant .Loss of cultures were due to fungal contamination by *Fusarium sp*.
2. Elongation of nodal segments was tried in two media with ½ MS + 1.0 NAA +3.0mgl^{-1}BA and ½ MS + 3.0mgl^{-1} NAA + 1.0mgl^{-1} BA + 1.0mgl^{-1} K but they showed 95-100 per cent fungal contamination at 15 DAI.
3. Immature endosperm was used to attempt triploid plantlet production which showed direct regeneration (78 %) in ½ MS + 2.0mgl^{-1}BAP media . This on subculturing showed sequential reduction in the survival rate due to fungal contamination.
4. Embryo ends excised from immature endosperm showed 73-87 per cent germination within seven days of transfer to light condition, after a fortnight dark incubation in culture room.
5. DNA isolation was standardized using CTAB method in field grown male, female and *in vitro* germinated seedlings with an extended incubation at – 20^{o}C for 1½ hours.

6. Purity of the DNA was assessed by absorbance study in spectroscope at $A_{260} - A_{280}$. DNA with OD value between 1.8 and 2.0 were selected for RAPD screening.
7. Thirty five decamer primers were selected for RAPD screening of male and female field grown trees. From this one primer (KIT-C 1) which gave conclusive polymorphic bands for male and female sex difference was used in further reaction using *in vitro* germinated seedlings.
8. RAPD analysis with KIT-C 1 in male, female trees and unknown *in vitro* seedlings showed, banding pattern of the copyright of field grown male trees with some *in vitro* seedlings .

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Annexures

ANNEXURE I

The reagents used for DNA isolation

1) Extraction buffer (4X)

Sorbitol - 2.5 g

Tris- HCl - 4.8 g

EDTA - 0.74g

The chemicals were dissolved in 60 ml sterile distilled water. The pH was adjusted to 7.5 and final volume was made up to 100 ml with distilled water and then autoclaved.

2) Lysis buffer

1M Tris-HCl (pH-8.0)	- 20 ml
0.25 M EDTA	- 20 ml
5 M NaCl	- 40 ml
CTAB	- 2 g
Distilled water	- 20 ml

Cetyl Trimethyl Ammonium Bromide (CTAB) was dissolved in 20 ml sterile distilled water. To this solution the required volumes of other stock solutions are added.

3) Tris-HCl 1M (pH-8.0)

Tris-HCl 15.76g was dissolved in 60 ml sterile distilled water. The pH was adjusted to 8.0 and final volume was made up to 100 ml with distilled water and then autoclaved.

4) EDTA 0.25 M

Ethylene Diamine Tetra Acetic acid (EDTA) 9.305 g was dissolved in 100 ml sterile distilled water and autoclaved.

5) NaCl 5M

Sodium chloride 29.22 g was dissolved in 100 ml sterile distilled water and autoclaved.

6) Sarcosine 5 %

Sarcosine 5 g was dissolved in 100 ml sterile distilled water and autoclaved.

7) TE buffer

(Tris HCl -10.0 mM; EDTA -1.0 mM)

Tris-HCl 1.0 M (pH 8.0) - 1.0 ml

EDTA 0.25 M (pH 8.0) - 0.4 ml

Distilled water - 98.6 ml

Autoclaved and stored at room temperature.

8) Ice-cold Isopropanol

9) Chloroform-Isoamyl alcohol (24:1 v/v)

To 24 parts of chloroform, 1 part of isoamyl alcohol was added and mixed properly. The mixture was stored in refrigerator before use.

10) Ethanol 70 per cent.

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

ANNEXURE II

Buffer and dyes used in gel electrophoresis

1) 6x Loading/Tracking dye

Bromophenol blue	- 0.25 %
Xylene cyanol	- 0.25 %
Glycerol	- 30 %

The dye was prepared and kept in fridge at 4^{o}C

2) Ethidium Bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg /ml in water and was stored at room temperature in a dark bottle.

3) 50x TAE buffer (pH 8.0)

Tris base	- 242.0 g
Glacial acetic acid	- 57.1 ml
0.5 M EDTA (pH 8.0)	- 100 ml
Distilled water	- 1000 ml

The solution was prepared and stored at room temperature.

ANNEXURE III

Reagents used for plasmid isolation

1) Solution I (Resuspension buffer)

Glucose	- 50 mM
Tris	- 25 mM
EDTA	- 10mM
pH	- 8.0

2) Solution II (Lysis buffer)

NaOH	- 0.2 M
SDS	- 1 %

3) Solution III (Neutralization buffer)

CH_3COOK	- 5 M
pH	- 5.5

REFINEMENT OF MICROPROPAGATION PROTOCOL AND RAPD ASSAY FOR SEX DETERMINATION IN KODAMPULI (*Garcinia gummi-gutta var.gummigutta*)

By

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ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

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(PLANT BIOTECHNOLOGY)

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ABSTRACT

The study entitled "Refinement of micropropagation protocol and RAPD assay for sex determination in Kodampuli (*Garcinia gummi-gutta var. gummigutta)"* was carried out in the laboratory of Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara to attempt and refine the micropropagation protocol in Kodampuli and to determine the sex of this polygamodioecious tree taxon. The relevance of the study is known by the fact that this polygamodioecious perennial tree spice poses hindrance to large scale multiplication of propagules due to seed dormancy, lack of orthrotropic shoots for grafting, poor germination percentage etc. The diabetes and cardiovascular diseases are the two serious lifestyle diseases, which spread at alarming rate throughout the world. In India, Kerala holds first position in these two obesity related diseases. The fleshy fruit rinds of *Garcinia spp*. are the richest natural source for anti-obesity metabolite, (-)Hydroxy Citric Acid (-HCA).

The major objectives of the study were to 1) Refinement of standardized micropropagation protocol (nodal segments) and somatic embryogenesis (immature endosperm) for large scale multiplication to cope up the anticipated extensive cultivation. 2) Standardization of DNA isolation techniques from male and female trees *in vitro* seedling progenies and polyembryonic seedlings in Kodampuli. 3) RAPD assay using selected oligoneucleotide decamer primers to ascertain the sex at juvenile phase in this polygamodiocious species so as to substantiate the findings of isozyme analysis of esterase using SDS- PAGE .

The nodal segements (immture parrot green shoots) collected in fungicide solution and then with $HgCl_2$ for 1-3 minutes under laminar flow chamber. These explants inoculated in half MS and full strength WPM medium fortified with various concentrations of plant growth regulators and activated charcoal. Best results obtained in ½ MS + 5.0mgl^{-1} IBA + 1.0mgl^{-1} BA + 0.5% AC. They

showed 25% survival in the above media with proliferation of multiple shoots. All cultures transferred to the shoot elongation media were lost due to fungal contamination.

Immature kodampuli fruits (30-45 day old) were collected from the field grown trees of the Department of Olericulture and pretreated with any one of the systemic fungicides like Fytolan/Companion/bavistin (0.1%) for 30 minutes. Seeds from these immature fruits were extracted under the aseptic condition of laminar air flow chamber and thereafter sterilized in 0.1per cent $HgCl_2$ for a period of 1-3 minutes. The two ends of seeds were cut and only the endosperm part was inoculated into jam bottles containing half strength MS medium fortified with different concentrations of plant growth regulators .These were kept for dark incubation in the culture room for a period of 2 weeks to reduce phenolic exudation and later transferred to light condition. Temperature of the culture room was maintained at 26$\pm$ 2^{o}C. The media composition ½ MS + 2.0mgl^{-1} BAP showed good response with 78 per cent of the inoculated immature endosperm survived at 15DAI.

The excised ends of the immature endosperm containing embryo were inoculated into half strength MS basal .These inoculated embryo with a bit of endosperm were initially kept in dark for two week and later transferred to light in the culture room. The *in vitro* seeds on germination were transferred to MS basal medium with different hormonal compositions. These immature seeds took 21 days for germination. These *in vitro* germinated seeds transferred to rooting media and they were initiated roots 60 DAI. These cultures were shown 100% fungal contamination and thus they were discarded.

DNA isolation was standardized by using CTAB method with an extended incubation at – 20^{o}C for a period of 1½ hours. These isolated DNAs from the established male and female trees in the field as well as *in vitro* germination seedlings were subjected to sex determination studies using RAPD analysis.

The RAPD analysis for sex determination was carried out by using 35 decamer primers for screening. Among the OPE (six), OPF (nine) and Kit-C (twenty) primers were tried, and the Kit-C-1 olegonucleotide decamer primer were explicited distinct monomorphic and polymorphic banding pattern. So, this primer can be used for further sex determination studies of Kodampuli in future.