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Breaking seed dormancy and genderwise dimorphic differentiation in *Garcinia gummi-gutta* var. *gummigutta* (L.) *Rob.*

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Garcinia gummi-gutta var. *gummigutta* (L) Rob. is a polygamodioecious tree indigenous to India remained neglected even though they are highly valued in South Indian cuisine and in traditional systems of medicine. Poor seed germination and breaking the prolonged seed dormancy could achieve within 7 days by *in vitro* inoculation of ¾ mature seed on to ½ MS basal medium. Genetic basis for sex specificities in sexually dimorphic plant species could identify by isozyme analysis of esterase using PAGE and DNA finger prints using RAPD-PCR techniques. RAPD screening using 35 random primers has identified the female specific KIT-C 14 and KIT-C 15 oligonucleotide decamer primers. Enhanced germination per cent with breaking seed dormancy and gender dimorphism at juvenile stage would bring about a revolutionary gallop for the extensive cultivation of Malabar tamarind or kodampuli under the homestead cropping system and massive afforestation programme.

Key words : Malabar tamarind, Sex determination, Seed dormancy, *In vitro* germination, Gel electrophoresis, Isozyme banding, RAPD analysis, *Garcinia gummi-gutta* var. *gummigutta* (L.)Rob.

INTRODUCTION

Garcinia gummi-gutta var. *gummigutta* (L) Rob. known in vernacular as Kudampuli or Malabar Tamarind is a handsome evergreen tree species confined to backyards. This perennial under exploited tree belonging to the family Clusiaceae (Lewis and Neelakandan, 1965) thrives best in the evergreen forests of Konkan, coastal and southern parts of Kerala and Western Ghats upto 180m in the Nilgiris (Varghese, 1996). It is commonly grown as miscellaneous stray spice tree in the homesteads of coastal saline belt of Kerala, Karnataka and Sri Lanka. Though the tree is much adapted to hill tops and plains its best performance is shown in river banks, valleys and coastal saline belts. The vast stretches of coastal saline belts of Indian sub-continent and the entire South East countries may be utilized for its commercial cultivation in future so as to support the indigenous pharmaceutical industry. The fleshy fruit rind of the tree has excited the scientific community as the richest natural source of potential anti-obesity plant metabolite (-) hydroxyCitric acid (HCA). It has the property to lower the blood lipid such as cholesterol and triglycerides. HCA inhibits the conversion of carbohydrates to fat without affecting Kreb's cycle through an enzyme, ATP citrate lyase (Watson *et al.*, 1965). In Ayurveda, it finds use in the treatment of rheumatism, bowel complaints, rickets and uterine contraction after delivery. High economic value of this therapeutically important perennial spices tree has hindered the extensive cultivation due to prolonged seed dormancy, low germination percentage and polygamodioecious nature of the crop. The sprouted seedling yield 50-60 per cent males after 7-8 months of dormancy. The remaining 40 per cent of productive female trees may be inefficient proportion to revolutionise its cultivation in extensive scale due to want of its own seedlings for conventional soft-wood grafting. In the case of dioecious species, sex identification at the juvenile plants is of considerable importance to cultivation practices, since female plants are usually valued for commercial production of fruits and seeds. Growers never have a control over the manipulation of sex ratio of saplings to eliminate unproductive male trees at juvenile stage, thus causing a substantial lose of resources and productivity.

The last decade has witnessed an increasing number of research efforts directed at identifying and characterizing molecular markers and genes involved in plant dioecy (Paranis *et al.*, 2000; Kafkas *et al.*, 2001; Rajendran *et al.*, 2004; Rajendran *et al.*, 2005; Xu *et al.*, 2004; and Yakubov *et al.*, 2005). Modern DNA technology provides a variety of techniques to produce molecular markers. The RAPD assay is rapid, inexpensive, simple and used for identification of genderwise dimorphic differentiation. Of the estimated 25.0 lakhs angiosperm species, only about 6% (14,620 species) are dioecious (Kumar and Singh, 2008). Hence, the present study initiated for developing biotechnological tools to enhance

the percentage of germination and early detection of gender dimorphism in this under-exploited multipurpose spice tree.

MATERIALS AND METHODS

In vitro germination, pre-treatment and surface sterilization:

Around 1750 immature fruits of ¾ maturity were given half an hour 0.1 per cent Bavistin pre-treatment for half an hour with few drops of detergent extra at the time of explant collection from the field grown trees. These pre-treated immature fruits were washed thoroughly with distilled water to remove fungicide residue and then wiped with 90 per cent alcohol.

These pre-treated seeds were given following treatments for breaking seed dormancy of 7-8 months duration: T_1 – Dipping mature seed in hot water for 1.0 minutes, T_2 – Dipping mature seed in conc. H_2SO_4 for 1.0 minutes, T_3 – Overnight soaking of mature seeds in aqueous mixture containing 1.0% urea + 0.1% Bavistin for one hour, T_4 – Removing seed coats of mature seeds, T_5 – Overnight soaking of decorticated mature seeds in distilled water, T_6 – Soaking decorticated mature seeds in 0.1% Bavistin solution for 45 minutes, T_7 – Soaking decorticated mature seeds in 250 ppm GA_3 for 6 hrs, T_8 – Soaking decorticated mature seeds in 250 ppm GA_3 for 6 hrs + 4 % Mancozeb for 2 hrs, T_9 – Soaking decorticated mature seeds in GA_3 250 ppm for 6 hrs + 4 % Mancozeb for 2 hrs plus incubation of seeds under air tight condition, T_{10} – Immature seeds extracted from three-fourth mature fruits.

The treated mature seeds were washed thoroughly, dried and wiped with absolute alcohol. Thereafter, both the treated, mature and immature seeds of ¾ maturity were given surface sterilization using 0.1 per cent $HgCl_2$ for 3-10 minutes under aseptic condition of laminar hood. Subsequent to surface sterilization, both the lots of seeds were dried over the sterilized filter paper so as to minimize the risk of contamination. Then they were inoculated onto ½ MS basal medium for *in vitro* germination. Response for germination was observed on alternate days.

Sex determination and PAGE gel electrophoresis:

Sex detection of *in vitro* germinated seedlings as compared to adult male trees and grafted female trees grown in the field were subjected to isozyme analysis of Esterase using PAGE. One gram of fully grown, light green coloured juvenile leaves of these three categories of plant material samples were taken and washed thoroughly and thereafter ground in the mortar using 0.5 ml of 0.1M phosphate extraction buffer at pH 7.0. After initial grinding another 0.5 ml of phosphate buffer was added to the mortar and the whole aliquot samples were transferred individually to the centrifuge tubes. These samples were immediately spun in the refrigerated centrifuge at 10,000 RPM for 15 minutes at 4°C. The supernatants of these samples were transferred to the fresh centrifuge tubes and kept in the ice so as to avoid denaturation of enzyme.

PAGE Gel electrophoresis unit was arranged as per instructions given in the Hoeffer model 96 manual. Prepared separating gel at pH 8.8 by polymerizing the monomer acrylamide with cross linking agent N,N'-bis acrylamide in presence of catalyst ammonium persulphate and chain initiator N,N,N',N'-tetra ethylene tetra amide (TEMED). Then the separating gel was allowed to polymerize. The stacking gel of pH at 6.8 was also prepared as in the case of separating gel. Staining solution was swiveled gently and poured over the separating gel and then the comb was kept over the gel. The stacking gel was allowed to polymerize and then the comb was removed. Plant extract of 30µl each from three categories of samples along with 10µl of loading dye were added to the well. Totally 24 samples were analysed at different electric impulses and timings using various stains.

The electrophoresis was done and transferred the gel to a staining tray containing a mixture of 100 ml 0.1M sodium phosphate buffer at pH 7.0, 0.25 mg Naphthyl acetate dissolved in 1.0 ml of 50 per cent acetone and 5.0 mg of fast blue RR salt. The gel was kept in the solution for about 30 minutes at room temperature. Then the gel was fixed in the distilled water and the photographs were taken.

Sex Determination and RAPD banding pattern:

The newly emerged parrot green colour leaves were excised from the field grown 15-20 years old male and 25 female trees. Cut ends of the leaves dipped in liquid nitrogen, immediately wrapped in aluminium foils and stored at -80°C. The genomic DNA was isolated from samples using a modified CTAB method with necessary modifications. The leaf samples (0.5 g) were surface sterilized with 70% ethanol, after throwing on ice, cut into small pieces and ground into fine powder in a mortar using liquid nitrogen. Then they were transferred to microcentrifuge tubes containing freshly prepared equal volume of extracton buffer (100 mmol/l, Tris buffer pH 8.0, 20 mmol/l Na_2 EDTA, 1.4mol/l $NaCl_2$, 2% CTAB, 1% polyvinyl pyrrolidone). The suspension was gently mixed and incubated at 60°C for 60 min. with occasional mixing. The suspension was then cooled to room

temperature and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The mixture was centrifuged at 13000 rpm/min for 10 min. The clear upper aqueous phase was then transferred to a new tube containing 0.5 ml ice-cooled isopropanol and incubated at -20^0 C for 30 min. The resulting pellet was washed twice with 70% ethanol containing 10 mmol/l ammonium acetate. The pellet was air-dried under a sterile laminar hood and the nucleic acid was dissolved in TE (10 mmol/l Tris buffer pH 8.0, 1 mmol/l Na_2EDTA) at 4^0 C. The contaminating RNA was eliminated by treating the sample with RNase A (20 µg/µl) for 30 min at 37^0 C. DNA concentration and purity were determined by measuring the absence of diluted DNA solution at 260nm and 280 nm.

A total of 35 decamer primers were used for performing RAPD analysis of the bulked 5 samples of DNA from each of the sex types (Table 1). PCR amplification of purified genomic DNA were performed in a master cycler (Eppendorf, Germany.). The programme included 1 cycle of 2 min. at 94^0 C, followed by 35 cycles of 45 sec. at 94^0 C, 1 min at 36^0 C and 2 min. at 72^{0}C, followed by a final incubation for 5 min. at 72^0 C. Amplification products were analyzed by gel electrophoresis in 1.8% agarose, 1 × TBE buffer (54.0g Tris-pase 27.5g boric acid, 0.5 m EDTA pH 8.0 in 1000ml volume), stamed with ethidium bromide (0.5 g/ml) and photographed under transilluminator. The amplification was repeated 2-3 times to ensure that the amplification obtained with the primers is reproducible and consistent. The 2kb λ DNA (Genei, Bangalore) was used as molecular size marker.

Table 1 : Code and sequence details of the random decamer primers

Primer code	Primer sequence	Name of firm
OPE-3	CCAGATGCAC	Operon Technologies, Inc. California
OPE-5	GCAGGGAGGT	Operon Technologies, Inc. California
OPE-6	AAGACCCCTC	Operon Technologies, Inc. California
OPE-7	AGATGCAGCC	Operon Technologies, Inc. California
OPE-11	GAGTCTCAGG	Operon Technologies, Inc. California
OPE-12	TTATCGCCCC	Operon Technologies, Inc. California
OPF-1	ACGGATCCTG	Operon Technologies, Inc. California
OPF-2	GAGGATCCCT	Operon Technologies, Inc. California
OPF-3	CCTGATCACC	Operon Technologies, Inc. California
OPF-5	CCGAATTCCC	Operon Technologies, Inc. California
OPF-6	GGGAATTCGG	Operon Technologies, Inc. California
OPF-7	CCGATATCCC	Operon Technologies, Inc. California
OPF-8	GGGATATCGG	Operon Technologies, Inc. California
OPF-9	CCAAGCTTCC	Operon Technologies, Inc. California
OPF-10	GGAAGCTTGG	Operon Technologies, Inc. California
KIT-C-1	TTCGAGCCAG	IDT, USA.
KIT-C-2	GTGAGGCGTC	IDT, USA.
KIT-C-3	GGGGGTCTTT	IDT, USA.
KIT-C-4	CCGCATCTAC	IDT, USA.
KIT-C-5	GATGACCGCC	IDT, USA.
KIT-C-6	GAACGGACTC	IDT, USA.
KIT-C-7	GTCCCGACGA	IDT, USA.
KIT-C-8	TGGACCGGTG	IDT, USA.
KIT-C-9	CTCACCGTCC	IDT, USA.
KIT-C-10	TGTCTGGGTG	IDT, USA.
KIT-C-11	AAAGCTGCGG	IDT, USA.
KIT-C-12	TGTCATCCCC	IDT, USA.
KIT-C-13	AAGCCTCGTC	IDT, USA.
KIT-C-14	TGCGTGCTTG	IDT, USA.
KIT-C-15	GACGGATCAG	IDT, USA.
KIT-C-16	CACACTCCAG	IDT, USA.
KIT-C-17	TTCCCCCCAG	IDT, USA.
KIT-C-18	TGAGTGGGTG	IDT, USA.
KIT-C-19	GTTGCCAGCC	IDT, USA.
KIT-C-20	ACTTCGCCAC	IDT, USA.

RESULTS AND DISCUSSION

In vitro germination:

The prolonged seed dormancy, delayed germination and low percentage of germination could be overcome by extracting seeds from immature fruits of ¾ maturity stage. Among the ten treatments experimented, the ¾ mature seeds with 250ppm GA3 for 6h+4.0 per cent Mancozeb for 2h+ incubation of seeds under air tight condition. The treatment has registered 64.0 per cent germination within 12.5 days. The result of T_9 was in corroboration with the earlier findings of Nazeema *et al*. (1993) having 66.0 per cent germination within 12.0 days of conventional mature seed sowing in *ex vitro* nursery. *In vitro* germination of freshly extracted immature seeds on half MS basal medium surpassed the earlier results with extraneous hormonal treatment. However, the increased germination per cent of 92.0 per cent within 7.0 days of *in vitro* culture as compared to 7-8 months of

germination time is noteworthy (Table 2; Fig.1). Germination of immature seeds would be an accidental gift to augment the large-scale vegetative propagation and to initiate commercial cultivation of this versatile condiment-cum-spice tree of therapeutic significance. After eliminating, the 50-60 per cent male seggregants from the germinated seeds and the remaining 40-50 per cent productive females will not be an inefficient proposition to revolutionize the cultivation of Malabar tamarind or Kodampuli in extensive scale outside the boundary of homestead gardens. The major limitation lies in the fact that the sex determination would be feasible only after eight years of planting in the main field (Sarah and Mathew, 2000).

Sex determination using PAGE gel electrophoresis and RAPD analysis:

The conventional *ex vitro* nursery requires 7-8 months for germinating fully mature seeds. Though the germination per cent of mature seeds could be hastened by specialized seed treatment to the range of 64-66 per cent, the sprouted lots yield upto 50-60 per cent of male seedlings. It requires 7-8 years to identify the productive females in this polygamodioecious condiment-cum-spice tree species. The isozyme analysis of esterase using PAGE and the RAPD analysis using 35 random decamer primers with the three categories of plants could help to detect the gender dimorphism at juvenile seedling phase within 7.0 days after germination (Fig. 2). The sex detection of other dioecious perennial species like nutmeg and papaya could not be standardized using isozyme banding technique (Beena and Kurian, 1994 b). Therefore, higher percentage of germination and breaking seed dormancy using three-fourth matured seeds within 7.0 days of *in vitro* culture and *in vitro* sex determination through molecular techniques like isozyme and RAPD analyses may be able to avoid strenuous top working and *in situ* budding of this under-exploited tree.

The sex of many plant species cannot be detected from external morphology before flowering. Obtaining a marker linked to a gene/genomic region through RAPD analysis depends to a large extent on chance. In the present investigation, 35 decamer primers were tested with the DNA bulks of the dimorphic sex types and a total of 6 bands were amplified. On an average, each primer amplified 2 and 4 bands from DNAs of male and female plants, respectively.

Optimization of PCR conditions for the RAPD markers showed that the concentration of primer and magnesium in the reaction were important. Primer concentration of 7.8µm was optimal for KIT-C1; higher

Table 2 : *In vitro* germination from immature seeds

Treat. No.	No. of seed explants treated										No. of seeds germinated										Percentage of seed germination										Days taken for seed germination									
Duration	3	4	5	6	7	8	9	10	3	4	5	6	7	8	9	10	3	4	5	6	7	8	9	10	AV	3	4	5	6	7	8	9	10	AV
T_1	20	20	20	20	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T_2	20	20	20	20	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T_3	20	20	20	20	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T_4	20	20	20	20	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T_5	20	20	20	20	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T_6	20	20	20	20	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T_7	20	20	20	20	20	20	20	20	6	5	5	5	4	3	2	2	30	25	25	25	20	15	10	10	20	58	61	66	68	69	72	74	81	68.5
T_8	20	20	20	20	20	20	20	20	10	11	9	8	7	7	7	6	50	55	45	40	40	35	35	30	41.25	22	18	21	22	21	23	23	24	21.75
T_9	20	20	20	20	20	20	20	20	13	14	14	14	13	13	12	10	55	65	70	70	65	65	60	50	64	12	11.5	12.4	13.2	13.0	12.5	13.1	13.2	12.5
T_{10}	50	50	50	50	50	50	50	50	46	48	48	46	45	44	42	43	90	96	95	90	92	90	89	84	92	7	6.5	7	7.5	7	6.5	8	7.5	7.12

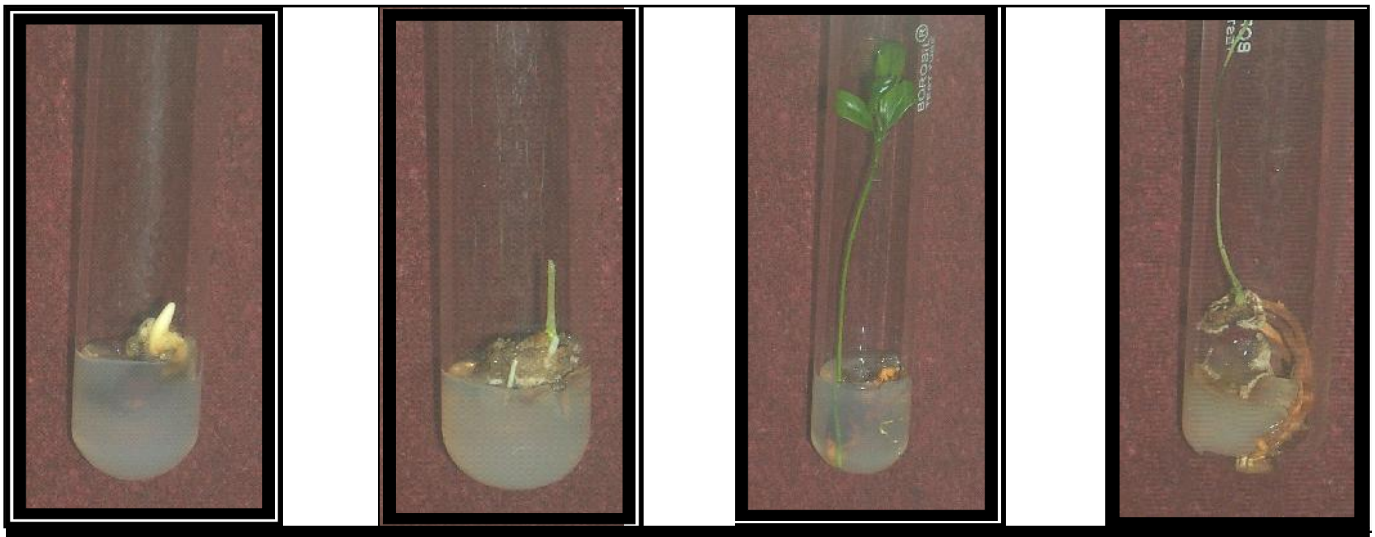


Fig: 1 : *In vitro* germination from immature seeds

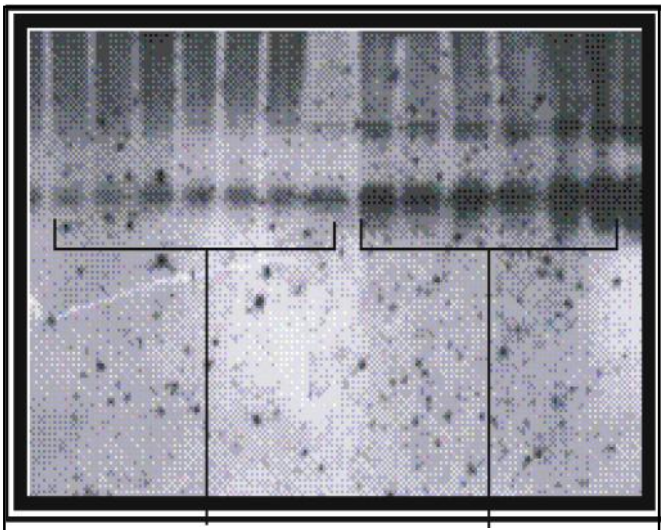


Fig. 2 : Isozyme banding of field grown plants (8% gel)

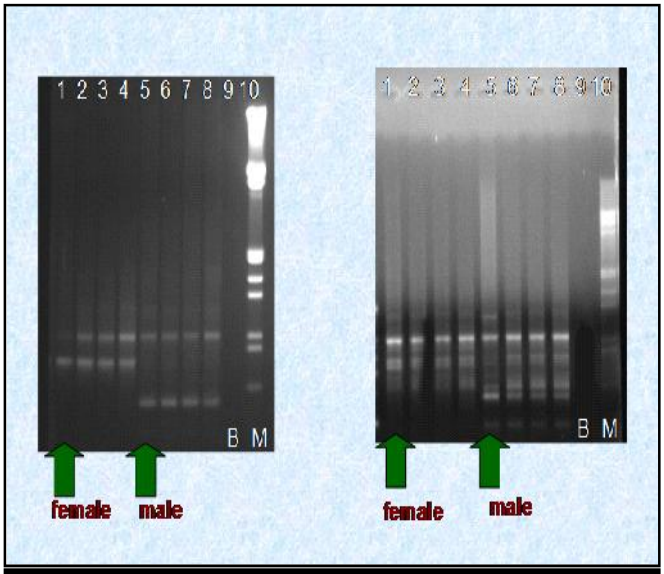
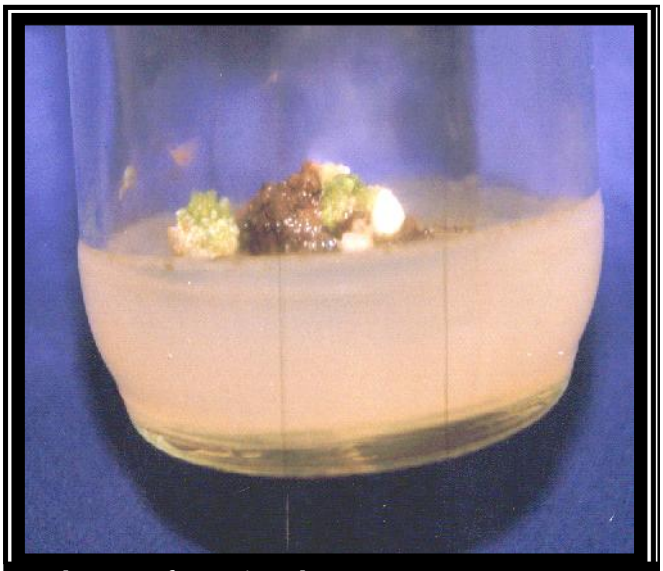


Fig. 3 : RAPD assay for sex determination

concentration reduced its amplification drastically. Primer concentration of 7.8µm was optimal for KIT-C9 magnesium in the range of 20-25mM was optimal for both markers. PCR amplifications of KIT-C1 and KIT-C9 were achieved in the annealing temperature of 37^0 C.

Bulked segregant analysis (BSA) of RAPD-PCR products of male and female DNA pools with 35 decamer primers led to the selection of two female specific RAPD markers. Subsequent tests on two products of 831bp from primer KIT-C1 and 1904bp primer KIT-C9 (IDT,USA) were found to be female specific (Fig. 3). Parrish *et al.* (2004) advocated the use of BSA instead of examining amplification products from individual plant DNAs for the detection of consistent polymorphic bands associated with the trait for which DNAs are bulked. Therefore, it was concluded that the female specific markers KIT-$C1_{831}$ and KIT-$C9_{1904}$ are fruitfully used for an early determination of sexual dimorphism in *G. gummi-gutta* var.*gummigutta*, well in advance the plants attain the reproductive maturity.

In Kerala, there are long stretches of costal saline belts. The main occupations of weaker sections in these areas are coir based industries and fishing. The coir and coconut based industries are facing challenges from dreaded root (wilt) disease and serious infestations of Eryophid mites on coconut. The fishermen communities living in the coastal belts are under utter poverty and starvation during monsoon season as the men folks are



Development of somatic embroys

Fig. 4 : Embryogenic callus from endosperm

Fig. 5 : Auxiliary shoot buds from endosperm

not able to depend on sea for their livelihood. During the monsoon season, women cannot be able to support the family without proper alternative income generating livelihood. Since the Malabar tamarind or kodampuli fruiting season is coinciding with the monsoon season, it would be worthwhile to switch on processing based industries in such areas for sustainable livelihood through women empowerment. The limited crop diversity in coastal belts is another challenge for commercial exploitation of agricultural crops. The Malabar tamarind or kodampuli is an easy-care heavy yielder which grown as companion crop in the coconut based homestead farming system. The extensive cultivation of this dioecious companion crop is crippled with the non-availability of sufficient quantities propagules. The successful softwood grafting method is now facing death of root stock material due to prolonged seed dormancy and low seed germination. The research results of this investigation may be helpful for extensive cultivation and establish processing unit to support natural soft drinks at par with the kokum fruit (Konkan areas of western coast of India). Establishing one HCA extraction unit and rind drying unit in every village or Grama panchayath may be benefited by selling HCA to pharmaceutical companies, and the rind powder in small plastic bottles (just like asafoetida) as a condiment for the domestic consumption of South Indian fish cuisines. The grass-root level co-ordination of Kudumbasree units (peoples participatory programme) existing in Kerala may be effectively utilized for practical application of this research outcomes. Easy *in vitro* germination protocol

and early gender detection through isozyme screening and RAPD- PCR techniques for productive females would bring about a revolutionary gallop for massive afforestation programme in the tribal settlements also. In future, this crop would become an important economic crop in the rural interior of the coastal saline belts of the humid tropical regions of the third world.

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Abbreviations:

HCA-Hydroxy Citric Acid, H_2SO_4-Sulphuric acid, MS-Murashige and Skoog (1962), $HgCl_2$-Mercuric chloride, PAGE-Polyacrylamide Gel Electrophoresis, RAPD-Random Amplified polymorphic DNA, PCR-Polymerase Chain Reaction, TEMED-N, N, N, N-Tetra ethylene tetra amide, GA_3-Gibberillic acid, mg-milligram, µl-microlitre.

REFERENCES

Beena, S. and Kurian, A. (1994 b). *In situ* budding to assure femaleness in nutmeg. PLACROSYM XI, Calicut (p.58)

Kafkas, S., Centiner, S. and Perl-Treves, R. (2001). Development of sex-associated RAPD marker linked to sex determination in wild *Pistacia specis*. *J. Hort. Sci. and Biotech*., : 242-246.

Lewis, Y.S. and Neelakandan, S. (1965). (-) Hydroxy citric acid-The principle acid in the fruits of *Garcinia cambogia*. Desr. *Phytochem.*, **4** : 619-625

Nazeema, K.K., Laila Mathew, K., Sarah T. George and Krishnan, S. (1993). Softwood grafting- a viable propagation technique for *G. cambogia*. Proceedings of Golden Jubilee Symposium of Horticulture, Bangalore. p.439.

Paranis, A.S., Gupta, V.S., Tamhankar, S.A. and Ranjekar, P.K. (2000). A highly reliable sex diagnostic PCR assay for screening of papaya seedlings. *Mol. Breed.*, **6**: 337-344.

Rajendran, P.C., Srikanth, P.M., Manju, T.G. and Smila, K.H. (2004). Breaking seed dormancy and sex determination through biotechnological approach in *Garcinia gummi-gutta* var. *gummi-gutta* (L.). *National Seminar on Agri-business Concept for Sustainable Livelihood*, 6 November 2004, AIRCT, Thrissur. Pp. 104.

Rajendran, P. and Al-Mssallem, I. (2005). Genetic basis for sex determination in Date palm (*Phoenix dactylifera* L.) *National Symposium on Biotechnological Intervention for Improvement of Horticultural Crops*. 10-12 January 2005. Kerala Agricultural University, Thrissur, p. 291-294.

Sarah, T. George and Mathew, K.L. (2000). Sex determination in Kodampuli (*G. gummigutta*). Proc.87th I*ndian Science Congress*, Pune (pp.310-311)

Varghese, J. (1996). (-) /Hydoxycitrates- linique potent metabolic regulators of obesity. *Indian Spices*, **33** (2): 10-25.

Watson, J.A., Fang, M. and Lovenstein, J.M. (1965). Tricarballylate and hydroxyl citrate substrate and inhibitor of ATP.*Arch.Biochem.Biophys.*, **135** : 209-217

Xu, W., Wang and Cui, K., (2004). RAPD and SCAR markers linked to sex determination in *Eucommia ulmoides* Olive. *Euphytica*, **136** : 233-238.

Yakubov, B., Barazani and Golan-Goldhirish, A. (2005). Combination of SCAR primers and Touchdown-PCR for sex identification in *Pistacia vera* L. *Sci.Hort.*, **103**: 473-478.