STUDY OF REPRODUCTIVE BIOLOGY AND *IN VITRO* PROPAGATION TECHNIQUES IN *KUMIZHU* (*Gmelina arborea* Roxb.)

By

SANI GEORGE

THESIS

submitted in partial fulfilment of the requirement
for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Department of Plant Breeding and Genetics

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2007

DECLARATION

I hereby declare that this thesis entitled **'Study of reproductive biology and *in vitro* propagation techniques in *kumizhu* (*Gmelina arborea* Roxb.)'** is a bonafide record of 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.

Vellanikkara,

Sani George

CERTIFICATE

Certified that this thesis entitled **'Study of reproductive biology and *in vitro* propagation techniques in *kumizhu* (*Gmelina arborea* Roxb.)'** is a record of work done independently by Ms. **Sani George** 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 her.

Vellanikkara,

Dr. Dijee Bastian
(Chairperson, Advisory Committee)
Assistant Professor,
Dept. of Plant Breeding & Genetics,
College of Horticulture,
Vellanikkara.

CERTIFICATE

We, the undersigned members of the Advisory Committee of Ms. **Sani George**, a candidate for the degree of Master of Science in Agriculture with major in Plant Breeding and Genetics agree that the thesis entitled **'Study of reproductive biology and *in vitro* propagation techniques in *kumizhu* (*Gmelina arborea* Roxb.)'** may be submitted by Ms. Sani George in partial fulfilment of the requirements for the degree.

Dr. Dijee Bastian
(Chairperson, Advisory Committee)
Assistant Professor,
Dept. of Plant Breeding & Genetics,
College of Horticulture,
Vellanikkara.

Dr. V.V. Radhakrishnan,
(Member, Advisory Committee)
Associate Professor & Head,
Dept. of Plant Breeding & Genetics,
College of Horticulture,
Vellanikkara.

Dr.C.R.Elsy,
(Member, Advisory Committee)
Assistant Professor,
Dept. of Plant Breeding & Genetics,
College of Horticulture,
Vellanikkara.

Dr. M.Asha Sankar,
(Member, Advisory Committee)
Assistant Professor,
Dept. of Plantation Crops & Spices,
College of Horticulture,
Vellanikkara.

23/12/2006
EXTERNAL EXAMINER

DEDICATED TO MY BELOVED PARENTS

ACKNOWLEDGEMENT

Let me at the outset thank The Almighty who was with me during my course work and blessed me to complete my research work in time.

With deep respect I express my heartfelt gratitude and un forgettable indebtedness to my guide Dr. Dijee Bastian, Assistant Professor, Department of Plant Breeding and Genetics, for her expert guidance, valuable suggestions, constant encouragements and above all painstaking scrutiny of the manuscript, extreme patience and good understanding and affection rendered throughout the course of my study.

I wish to place on record my wholehearted gratitude to Dr.V.V.Radhakrishnan, Associate Professor and Head, Department of Plant Breeding and Genetics and member of my advisory committee for his valuable suggestions in refining the manuscript.

I deeply express my wholehearted gratitude to Dr. C.R. Elsy, Assistant Professor, Department of Plant Breeding and Genetics for her precious suggestions and generous support during my entire study and successful completion of this work.

I am respectfully thankful to Dr. M. Asha Sankar, Assistant Professor, Department of Plantation Crops and Spices, College of Horticulture, for her critical suggestions, kind concern and technical support.

My heartfelt gratitude to Dr. K.T. Pressana Kumari for guidance and detailed discussions in taking observations in connection with reproductive biology and palynology studies.

I also express my thanks to Dr. Achamma Oommen, Retired Professor and Head, Dr. K. Nandini, Associate Professor, Dr. R. Sujatha and Dr. E Srinivasan, Assistant Professors, Department of Plant Breeding and Genetics, College of Horticulture for their kind cooperation and guidance during the entire period of my study.

I especially thank Dr. Arya. K, Assistant Professor, Department of Plant Breeding and Genetics, College of Agriculture, Vellayani for her moral support and suggestions.

I take this opportunity to express my heartfelt gratitude to Scientists of Institute of Forest Genetics and Tree Breeding, Coimbatore for valuable help to get references related to my work.

I am sincerely thankful to Divya mol, Sandhya and Varghese chettan Research Associates, of our department for their relentless help and assistance.

I wish to express my gratitude to senior friends, Gayathri chechi, Ambika chechi, Vidhu chechi, Latha madam and Karuppaiyan sir for their ever willing help, great support and kind co-operation rendered through out my work. I gratefully thank the assistance by juniors Shareesh, Kishore and Divya.

I am deeply indepted to my class mates Aswathi, Vishnu and Marimuthu and all friends Remya V.M., Shibi, Dhanya, Grace, Margaret, Lincy, Divya, Shajna, Sree Rekha, Nisha Jose and Sameera, P. who have helped me very much during my course work.

The award of KAU Junior Research Fellow ship is duly acknowledged.

I thank Santhosh chettan of Students Computer Club, College of Horticulture, Vellanikkara for valuable help rendered in the typesetting of the thesis.

I am thankful to Valsala chechi and Mani chettan for helping me in planting the stock plant in the field and taking observations in trees.

I am in dearth of words to express my gratitude, appreciation and indebtedness to my father, V.V. George, mother, N.V Aleyamma, brother, Aji. V. George, sister-in-law, Bobby Varghese, niece Grace mol and sister, Ancy George for their constant encouragement, untiring support, love, constant prayer and warm blessings showered on me.

Sani

Sani George

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

6-Benzyl Adenine	- BA
Benzyl Amino Purine	- BAP
1-Naphthalene Acetic Acid	- NAA
2,4- Dichloro Phenoxy Acetic Acid	- 2,4-D
Indole 3-Acetic Acid	- IAA
Indole 3-Butyric Acid	- IBA
Murashige and Skoog Medium	- MS
Schenk and Hildebrandt Medium	- SH
Woody Plant Medium	- WPM
Ethylene Diamine Tetra Acetic Acid	-EDTA

INTRODUCTION

1. INTRODUCTION

India enjoys the privilege of having time tested traditional systems of medicines based on natural products. The traditional remedies of the ancient world were based on natural products, e.g. morphine from opium for use as narcotic and analgesic, cocaine from cocoa leaf as potential local anesthetic, ergot for obstetric use, castor oil and senna as laxatives, turmeric as antiseptic, garlic for blood and heart remedies. *Cassia fistula* use as hepatoprotective, antitussive, wound healing and anti-inflammatory (Bhakta, *et al.*, 1998). *Pongamia pinnata* (seeds), *Delonix regia* (bark and flowers), *Psidium guajava* (leaves) and *Aegle marmelos* (bark) use as therapeutic agents in inflammatory conditions.

The traditional Indian system of medicine namely Ayurveda involves dispensing of plant products in various forms such as powders, extracts and decotions and dates back to the Vedic period. Bulk of raw material (90%) for herbal medicines is produced in Asia, Africa and Latin America and some in Europe and USA (10%). About 90 per cent of the marketed material is collected from wild resources and there is some cultivated in China, India, USA, Germany, France, Italy and Eastern Europe (Sharma, 2004).

Gmelina arborea Roxb. commonly known as white teak was first introduced from Myanmar as a fast growing tree species. This is a multipurpose tree with edible fruits. The leaves are regarded as good fodder. Flowers produce good quality honey. Both wood ash and fruit yield a very persistent yellow dye. Bark leaves and roots contain traces of alkaloids, and are used medicinally in its native range, such as in Hindu medicine (Rao and Rao, 1970).

In order to understand the dynamics of natural population of any crop species and to initiate any crop improvement programme a thorough knowledge of reproductive biology is essential. Such studies are rather limited in *kumizhu.* The trees can be propagated by seeds, cuttings and grafting. However, the seeds of

Gmelina are not always easily available and their germination is also poor (Surendran, 1990).

In Dashamula, roots of tree species are used in preparation of ayurvedic formulations and hence the destruction of plants is severe and planting of seedlings for these species is almost negligible. Clonal propagation through macro and micropropagation is practised in a limited scale for *Gmelina arborea* and *Aegle marmelos.* Research reports are negligible in species like *Oroxylum indicum, Premna integrifolia* and *Stereospermum suaveolens.* Research efforts for genetically improving the species for higher production of the active components or increasing the production of the compounds under *in vitro* culture conditions are essential and it is practised in several medicinal plant species. The application of *in vitro* culture for the propagation of this Dashamula tree species will help in the sustainable availability of propagules and plant products (Yasodha, *et al.*, 2004).

The approximate annual requirement of dried roots of *kumizhu* alone in Kerala is about 300 tones. The techniques of clonal propagation are not sufficient to meet the demand of planting material and there is no appropriate conventional method for large-scale multiplication of *Gmelina.*

In this context, the present study was envisaged with the following objectives,

1. To elucidate the flowering pattern, reproductive biology, determination of anther dehiscence, stigma receptivity and palynology in *kumizhu*
2. To identify best explant, medium and growth regulator combination for *in vitro* culture establishment, proliferation and rooting
3. To standardise the protocol for direct and indirect regeneration of *kumizhu*

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Medicines are the second most essential requisite after food for mankind. Medicinal plants are the important sources of raw drugs. Plant based medicines are often based on long histories of traditional use and the vegetable drug or preparation there of is regarded as one active ingredient in its entity, whether or not the constituents with therapeutic activity are known.

In developed countries, demand for herbal medicines has increased dramatically due to a growing realisation of the limitations of isolated compounds for many problems, particularly those of a chronic nature, increased interest in all things "natural", advances in production capabilities and government recognition and support of this aspect of health care in countries such as Germany, Japan and France. In developing countries such as India, China and Indonesia 80 percentage of the people depend upon traditional system of medicines and commercially produced traditional herbal medicines have major markets integrated with western forms of health care. About 95 percentage of the industrial requirement of raw materials is met through indiscriminate collection from wild.

The genus *Gmelina* Linn. was named by Carl Linnaeus in honour of Johann Gottlich Gmelin (1709-1755) a German traveler and Professor of Botany at Tubingon (Tewari,1995). The genus belongs to the family Verbenaceae. It comprises of about 30 species and their sub specific taxa. This species is native to tropical and sub tropical Asia from Pakistan, Bhutan, Nepal, India to Thailand, Indochina and Malaya, north to southern China, eastward through the Philippines and Indonesia and south to northern Australia.

There are approximately 40 different species in the genus *Gmelina* (Mabberley,1997) some of which are medium to large trees, like *G.moluccana*, which can reach 33m in height and 1.5 m in diameter in Southern Islands (Lonnie, 1991). Other species in *Gmelina* genera are thorny bushes or vines.

In Indian region the genus is represented by four species, namely *G. philippensis, G. asiatica, G. elliptica.*ex Jack and *G. arborea. G. philippensis* Cham. is indigenous to Myanmar, Thailand and Malaya and has been introduced to India. *G. asiatica* Linn. is a shrubby species and often thorny. This species is distributed in the Deccan Peninsula of India and often planted in gardens for its bright yellow flowers and succulent leaves or as a hedge plant. *G.elliptica* Sm. is a shrub or small tree distributed in Nicobar Islands. *G.arborea* Roxb. is a widely spread tree throughout the greater part of India, Nepal, Bhutan, Bangladesh, Myanmar, Sri Lanka and Philippines (Tebbs, 1982).

G. arborea is known as white teak in English, Gumhar in Hindi, Gamhari in Sanskrit and Kumadi in Tamil (Tewari, 1995).

The systematic position of *kumizhu* is as follows,

Kingdom	- Plantae
Sub kingdom	- Tracheobionta
Division	- Magnoliophyta
Class	- Magnoliopsida
Subclass	- Asteridae
Order	- Lamiales
Family	- Verbenaceae

2.1 Botany

Gmelina arborea is an un armed, moderately sized to large deciduous tree with a straight trunk. It is wide spreading with numerous branches forming a large shady crown, attains a height of 30 m or more and a diameter of upto 4.5 m. Bark smooth, pale ashy-grey or grey to yellow with black patches and conspicuous corky circular lenticels. Leaves opposite-decussate, mostly rather soft and limp petioles cylindrical, 5-15 cm long, glabrous, leaf blades broadly ovate, 10-25 cm x 7-20 cm wide, caudate, usually cordate basally, with a short cuncate attenuation into the petiole, glanduliferous just above the petiole in the basal attenuation. Flowers are

abundant, reddish brown or yellow, in terminal and axillary 1-3 flowered cymes on the panicle branches, which are about 8-40 cm long. There are two peak periods for floral bud burst, which may vary from year to year and with local climatic conditions. In India, species flower around February-March and fruits ripen from the end of April to June (Tewari, 1995).

2.2 Therapeutic uses of *Kumizhu*

Gmelina arborea Roxb. locally called *kumizhu* is an accepted source of drug through out India. The plant parts are used for the treatment of various ailments like tuberculosis, gonorrhoea, cough, headache and for ulcer (Chopra et al., 1956). *Kumizhu* is an important ingredient of the group dashamula, which enters into the composition of many ayurvedic formulations (Kolammal, 1978). Its roots are an ingredient of dashamula; a herbal preparation and its fruits are used for fever and gastric problems (Ambasta, 1986). The young leaves are demulcent, chiefly used in gonorrhoea and catarrh of bladder (Singh and Khan, 1990). The roots are used for medicinal purposes to cure a number of ailments in India (Punjani and Kumar, 2002). The flowers are used as colouring and flavouring ingredients for cakes and other foods by the Dai minority in southern China and also have been used for pigment extraction (Wang, 2003).

2.3 Reproductive biology

The efficiency of sexual reproduction leading to tree fertility depends upon genetical and environmental factors affecting it. Studies on flowering, pollen production, pollination mechanisms, fertilization, seed set, seed shed, seed quality, fruit and seed ripening and seed dispersal are important to tree improvement. Knowledge of reproduction is crucial to our understanding of causes of rarity and for conservation of tree plant taxa (Drury, 1974, Ayensu, 1981, Kruckeberg and Rabinowitz, 1985). Egenti (1978) stated that although reproductive mechanism of temperate forest tree species is fairly well known, they are poorly understood in tropical tree species. Eventhough *kumizhu* is important as a medicinal plant and

timber source, the floral biology, breeding systems, pollinators and seed dispersal in its natural geographic regions have been studied by only a few scientists. The available literatures on *kumizhu* are limited. So literatures regarding other related plants are also included in this review.

2.3.1 Flowering seasons

Crop	Characteristics	References
Verbenaceae *Gmelina arborea*	Flowering begins between three and four years of age in most locations, but a full flush unlikely before 10 years. Flowering patterns are inconsistent at low latitudes	Wong, 1987
	Dry season bloomer, flowers during February – April	Raju and Rao, 2006
Tectona grandis	Flowering during rainy season and trees tend to flower synchronously. In Thailand flowering occurs between June and September	Sedgley and Griffin, 1989
Sapotaceae *Madhuca latifolia*	Leaf fall from February-April and flowering during March -April	Ram and Prasad, 1991
Fabaceae *Acacia mangium*	Flower buds appear on early November. Flowering between January and May	Hopkins and Graham, 1989
Tamarindus indicus	Blooms during mid May to mid July	Sasidharan *et al.*, 1999
Erythrina falcata	Flowering during dry season from late August to late October	Etcheverry and Aleman, 2005

2.3.2 Inflorescence

Crop	Characteristics	References
Verbenaceae *Gmelina arborea*	Flowers are arranged in terminal and axillary 1-3 flowered cymules on the panicle branches of about 8-40 cm long	Tewari, 1995
	Flowers showy yellow tinged with brown outside in dense terminal pedunculate panicles	Sivarajan and Indira, 2004
	Cymes are paniculate at ends of twigs, 15-30 cm long, branched and densely hairy	Stock *et al.*, 2004
	Flowers produced in paniculate cymes which arise at terminal and lateral shoots	Raju and Rao, 2006
Tectona grandis	Flowering period of one inflorescence is 2-4 weeks in which up to 300 flowers appear and 300 flowers open one day after sunrise	Keiding, 1972
Vitex negundo	Inflorescence is peduncled cymes, terminal often compound and pyramidal panicles	Banerjee, 1989
Petrea	Inflorescence is polytelic type and racemes with decussate bracts	Drewez and Martinez, 1999
Premna	Terminal, pedunculate cymes often many branched	Mactarlane *et al.*, 2002
Fabaceae *Bauhinia ungulata*	Terminal or pseudo lateral racemes	Bawa and Webb, 1984
Caesalpinia eriostachys	Racemes are terminal or sub terminal	Bawa and Webb, 1984
Dalbergia retusa and *Pterocarpus rohea*	Inflorescence is raceme, appearing as terminal or axillary panicle	Bawa and Webb, 1984
Pterocarpus macrocarpus kurl.	Inflorescence is raceme with average thirty flowers	Yupa and John, 2002
Bixaceae *Cochlospermum vitifolium*	Terminal panicles	Bawa and Webb, 1984
Bignoniaceae *Tabebula rosea*	Terminal panicles	Bawa and Webb, 1984

2.3.3. Floral Morphology

Crop	Features	References
Verbenaceae *Gmelina arborea*	Calyx bell shaped, five millimeter long, five toothed, corolla bright orange yellow, and four stamens in two pairs. Four celled ovary with one ovule in each cell	Stock *et al.*, 2004
	Flowers are large, brownish yellow, bisexual, zygomorphic and nectariferous	Raju and Rao, 2006
Tectona grandis	Flowers are actinomorphic hermaphroditic and has six petals in single whorl. Ovary with four ovules and a style with forked stigma	Bryndum and Hedegrat, 1969
Vitex trifolia	Calyx, three-five millimeter long, obscurely two lipped, with five small teeth, corolla blue to purple or violet, throat villous inside	Capareda, 1999
Rutaceae *Aegle marmelos*	Flowers have four recurved, fleshy petals (green outside, yellowish inside) and fifty or more, greenish yellow stamens	Thampman, 1993
Pilocarpous pennatifolius	Actinomorphic, dichlamydous, nectariferous and monoclinous flowers, dispersed in pendulous racemes	Souza *et al.*, 2003
Fabaceae *Pterocarpus rotundifolius*	Flowers are 15 cm long, petals yellow, crinkly	Hogberg, 1986
Sophora fernandeziana	Zygomorphic, bowl shaped, pubescent, fleshy calyx with fine small teeth	Bernardello *et al.*, 2004
Combretaceae *Terminalia superba*	Flowers sessile, small, greenish white, calyx tube saucer shaped, with short triangular lobes	Groulez and Wood, 1985

Euphorbiaceae *Sacaca cajucara* Benth.	Male flower has two perianths. Pentamerous simple and valvar. Androecium is polystemonous. Female flowers are monoperianthial, with a quincunical prefloration. The gynoecium consists of three carpels, three ovules, with a superior sericeous ovary	Conceica *et al.*, 2002

2.3.4 Anthesis and anther dehiscence

Crop	Features	References
Verbenaceae *G.arborea*	Anthesis during 08.00-11.00hr.Anther dehisces by longitudinal slitting of anther lobes, an hour after anthesis.	Raju and Rao, 2006
Rutaceae *Aegle marmelos*	Maximum anthesis occurs between 6.00 and 8.00hr	Srivastava and Singh, 2000
Fabaceae *Pterocarpus santalinus*	Anther dehisces around 00.30hr. Anther dehisces asynchronously after anthesis. Two anthers an hour later, three anthers again after one hour and other five again after one and half hour	Rao and Raju, 2002
Acacia nilotica	Anthesis occur between 18.00- 24.00 hr and anther dehiscence is in between 8.00-12.00 hr	Singh *et al.*, 2003
Plantaginaceae *Plantago ovata*	Anthesis is in between 5.30 and 7.00hr	Dutta *et al.*, 2004

2.3.5 Stigma receptivity

Crop	Features	References
Verbenaceae *G.arborea*	Stigma become receptive at the time of anther dehiscence and remains receptive until the evening of second day	Raju and Rao, 2006
Tectona grandis	Stigma of teak flowers are viable for one day after anthesis	Egenti, 1978
	The papillate stigma of teak is wet type with a hollow style and has a short receptive period (19.00-13.00hr)	Tangmitcharroen and Owens, 1997

Myristicaceae *Myristica fragrans*	Stigma is receptive for 2-3 days after which it turns dark brown	Armstrong, 1986
Myrtaceae *Syzygium aromaticum*	Shows maximum stigma receptivity 48 hr after anthesis	Pooll and Bermawiel, 1986
Fabaceae *Pterocarpus santalinus*	Stigma become receptive when first two anthers dehisce and remain until late evening of same day	Rao and Raju, 2002
Combretaceae *Prunus dulcis*	Pistil is receptive for 48 hrs after anthesis	Egea *et al.*, 2004

2.3.6 Self incompatibility

The site of incompatibility barrier in most forest trees is not known however it is known that in many species the barrier operates neither at stigma or style but somewhere in the ovary either before or after fertilization (Bawa, 1979).

Crop	Features	References
Verbenaceae *G. arborea*	Post zygotic self incompatibility	Bolstad and Bawa, 1982
	In India artificial selfing occasionally produces sound seeds	Bowen and Euesbio, 1983
Tectona grandis	Self incompatibility and protrandous nature	Hedegrat, 1973
	Late acting gametophytic self incompatibility	Tangmitcharroen *et al.*, 1996
Bixaceae *Cochlospermum religiosum*	Post zygotic self incompatibility	Rao and Reddi, 1994
Cochlospermum vitifolium	Post zygotic self incompatibility	Roubik, 1995
Sophora fernandeziana	Ovarian self incompatibility	Gibbs and Bianchi, 1999
Sterculiaceae *Sterculia chicha*	Incompatibility mechanism is based on non fusion of gametes in embryo sac	Neusa and Gibbs, 2005
Ranunculaceae *Delphinium nelsonni*	Partial self incompatibility either from inbreeding depression or maternal control	Waser *et al.*, 1987

2.3.7 Pollen morphology and viability

Crop	Features	References
Sapotaceae *Madhuca indica*	Pollens are roughly spheroidal, 4-5 colporate, exine thin, slightly reticulate, 30-60 micro meter in size	Reddi, 1976
Fabaceae *Uraria crinita*	Tricolporate and two celled at the time of shedding	Chin *et al.*,1999
Fabaceae *Bauhinia* sp.	Tricolporate pollens	Zou *et al.*, 2003
Rhamnaceae *Ziziphus*	Tricolporate, psilate or faintly reticulate exine or ornamentation	Gupta *et al.*, 2002
Solanaceae *Cestrun* sp.	Medium sized. Size ranged from 20 to 50 microm oblate, spheroidal to sub prolate three colporate pollens	Silva *et al.*, 2003
Acoraceae *Calamus* sp.	Pollen aperture is dicolporate and tectum of exine is reticulate	Lixiu *et al.*, 2004
Plantaginaceae *Plantago* sp.	Pollens are symmetrical, apolar, porate and spheroidal	Anjum and Qaiser, 2004
Pollen viability		
Viola cerasifolia	Ranged from 66.1 to 79.6 per cent	Freitas and Sasima, 2003
Withania sominifera	Pollen viability estimated by acetocarmine test and was 90.5 per cent during peak flowering season.	Kaul *et al.*, 2005

2.2.8 Fruit characteristics

Crop	Features	References
Verbenaceae *G. arborea*	Drupes are ovate or pyriform, 2-2.5 cm long smooth, orange yellow, yielding 1-4 seeds in egg shape	Little, 1983
	Gmelina seeds germinate in 7-21 days	Kijar, 2002
	Seeds from natural populations in India generally do not germinate as well as from other countries like Thailand and Myanmar.	Hodge and Dvorak, 2003

Fabaceae *Pterocarpus indicus*	Fruits are disc shaped, flat and have winged margins	Little and Wadsworth, 1964
Combretaceae *Terminalia arjuna*	Obvoid, oblong, dark brown to reddish, brown fibrous woody indehiscent drupe	Kumar and Kumar, 1994
Bignoniaceae *Stereospermum* sp.	Flat capsule, cylindrical, greenish, reddish brown to dark brown	Bein, 1996

2.4 Propagation in *kumizhu*

Gmelina is amenable to both sexual as well as asexual methods of propagation. This tree can be propagated by seedlings, rooted cuttings and grafting.

2.4.1 Seed propagation

Yellow and yellow greenish fruits (the more mature ones) germinated best, but colour/maturity did not influence seedling growth (Mindawati and Rohayat, 1994). The relative growth rate of seedlings of *G. arborea* raised from small-sized seeds was more than that from large-sized seeds (Agboola, 2000). Large seeds had higher rate and percentage of seed germination than smaller seeds (Larrea *et al.*, 2000). Fresh seeds have high germination percentage, but stored seeds can loose viability rapidly (Galan and Vargas, 2000). It is suggested that it is better to collect greenish yellow fruits for seed extraction to produce quality seedlings (Pandey *et al.*, 2002).

2.4.2 Vegetative propagation

Stamp planting stock (bare root seedling production) method was described by Greaves (1981) in *Gmelina*. Root trainers are used when large quantities of planting stocks are required (Sanchez and Diaz, 1998). Zeaser (1998) stated that *G. arborea* can be successfully cleft grafted or bud grafted. Balza and Melendez

(2001) found that the best and most economical substrata for *Gmelina* rooted cuttings was a mixture of 50 per cent perlite and 50 per cent charred rice hulls.

2.4.3 *In vitro* propagation

Very few attempts have been made to propagate *G. arborea* through *in vitro* techniques. Sen *et al.* (1992) reported successful propagation of juvenile plants of *G. arborea* by *in vitro* methods. Multiple shoot formation was obtained by juvenile source on MS medium containing 1.0 mg /l BA and 0.1 mg/l NAA. Multiple shoots were produced from nodes, internodes, leaf pieces and cotyledons cultured on MS medium with NAA and BA at different concentrations (Yang *et al.*, 1992). Yang *et al.* (1993) reported *in vitro* clonal propagation of cell suspension culture of *G.arborea*. Multiple shoots were obtained from single node explants of mature *kumizhu* on MS medium supplemented with BA (Kannan and Jasrai, 1996). Multiple shoots were produced through axillary bud culture on Mc Cown's medium (Thirunavoukkarasu and Debata, 1998). Gamboa and Abdelmour (1999) cultured the node of *in vitro* germinated plantlets on MS medium with normal salt concentration and 0.5 mg/l BAP.

2.4.3.1 Factors influencing success of *in vitro* propagation

Success of *in vitro* propagation depends on several factors directly or indirectly. These factors include genotype of source plant, size, age and type of explant, surface sterilisation, presence or absence of other additives, nitrogen sources and concentrations, physical conditions of medium, pH, quality and intensity of light, temperature and relative humidity (Brown and Thorpe, 1986).

2.4.3.1.1 Genotype

Great differences exist in organogenesis, embryogenesis and regeneration of plantlet among plant species, varieties and even individuals of the same variety (Ahuja, 1983). Besides region dependent variation, shoot cultures of *G. arborea* also showed variation over successive subcultures (Dhiraj *et al.*, 2003). Mc Comb and Bennet (1982) observed that there was large difference in the capacity of explants from different selection of mature *Eucalyptus marginata* tree to survive in culture. Variation in *in vitro* response has also been shown in mature tree tissues from different regions of other species (Colman and Ernst, 1989 and Tang *et al.*, 2001) in oak seedlings from different regions (Juncker and Favre, 1989) as well as in different mulberry seedlings from same region (Sharma and Thorpe, 1990).

2.4.3.1.2 Season for collecting explants

Thakar and Bhargava (1999) reported that in *G.arborea* the axillary buds collected during summer responded better to *in vitro* culture than in winter. In teak, explants have to be collected in sprouting season when they are fresh and green (John *et al.*, 1997). Successful plantlets production in chestnut was obtained with shoots taken during mid May (Chavuin and Salesses, 1988). Yu (1991) observed that the test material, from *Litchi chinensis* taken after ten continuous rainy days had a low contamination rate (20%). In *Aegle* the nodal segments collected during March- April from current season shoots responded better to *in vitro* culture (Nazeem *et al.*, 2005). Tamarind shoot bud broke when explants where collected during April to June (Mahale *et al.*, 2005).

2.4.3.1.3 Age of explant

Thakar and Bhargava (1999) reported maximum twenty per cent response from nodal segments of mature trees of *G.arborea.* Various forest tree species have been cloned successfully by taking nodal and terminal bud explants from mature

tree species such as *Tectona grandis* (Gupta *et al.*, 1980), *Dalbergia sissoo* (Datta and Datta, 1983) and *Azadirachta indica* (Eeswara *et al.*, 1997). But several workers like Hackett (1985), Marri *et al.* (1986) in pear, Paily and D'Souza (1986) in *Lagestromea flosreginae*, Rajmohan and Kumaran (1988) in jack, Sumana and Kaveriappa (2000) in *Lagestroemia reginae* and Karale *et al.* (2005) in *Emblica officinalis* have reported slow growth, low propagation rate and weak *in vitro* performance of mature explants as compared to juvenile shoots.

2.4.3.1.4 Explant size and its position on the mother plant

Explants from the crown region of *G.arborea* are not ideal for multiple shoot formation (Thirunavoukkarasu and Debata, 1998). Sukartinigsih *et al.* (1999) reported that one to two centimeters long single nodal segments and nodal segments with axillary bud of *G.arborea* are suitable as explants.

In *Lagerstroemia speciosa*, Ho and Lee (1985) observed, nodal segments to be superior to shoot tips when cultured artificially. Rahman and Blake (1988) reported that nodal explants proliferate more vigorously than shoot tip in jack. Pseudo terminal buds of *Betula uber*, approximate 5 mm long opened after 4 to 5 days of culturing and produced three leaves in a week, whereas 3-4 mm axillary bud took 10-20 days to increase to the same size (Vijaya kumar *et al.*, 1990). High frequency organogenesis and multiple shoot regeneration were induced from rhizome explants of *Kaempferia galanga* Linn. on MS medium supplemented with 0.5 mg/l IAA and 2.5 mg/l BA (Swapna *et al.*, 2004). In peach shoot tips are superior in shoot induction compared to nodal explants (Sharma *et al.*, 2005). In *Citrus aurantifolia*, Kamble *et al.* (2005) reported that cotyledonary segment was the best explant when compared to root tip, hypocotyl stem, leaf, epicotyl stem and shoot tip of *in vitro* grown seedling.

2.4.3.1.5 Surface sterilisaton

Surface sterilisation is done to remove the microorganisms present on the explants with minimum damage to plant parts. Sodium hypochlorite (0.5 to 2.0 per cent w/v), calcium hypochlorite (filtered 5.0 to 10.0 per cent w/v) and mercuric chloride (0.05 to 0.1 per cent w/v) are the commonly used surface sterilants. As the surface sterilants are toxic to the plant cells, it is necessary to wash the tissues twice or thrice in sterile distilled water to ensure dilution of chemical (Hu and Wang, 1983). Addition of Tween 80 increase the efficiency of sterilant and rinsing the tissue with 70 per cent ethanol serve as a good surfactant (Dhawan, 1993).

Surface sterilisation of *G.arborea* was good in 0.1 per cent (w/v) mercuric chloride solution for 10 minutes followed by thorough washing with sterile distilled water (Thirunavoukkarasu and Debata, 1998). Sukartiningsih *et al.* (1999) sterilised explants of *G.arborea* under running tap water for one hour, followed by 70 per cent ethanol (v/v) for 30 seconds and by 10 per cent hydrogen peroxide (v/v) with three drops of Tween 80 solution per 200ml for 10 minutes, then rinsed five times in sterile distilled water.

Harda and Murai (1996) surface sterilised the buds of *Prunus mume* (Japanese apricot) with 70 per cent ethyl alcohol in one minute and then with 0.25 per cent Tween 20 for 15 minutes followed by three washes with sterile distilled water.

2.4.3.1.6 Systemic contaminants

During subculture of explants of *G.arborea* it was observed that the existence of bacterial contamination was like mucus, which sticked on leaves (Sukartiningsih *et al.*, 1999). Mathias and Anderson (1987) reported that bacteria, fungi or viruses on the surfaces of the bark, glandular hairs at the nodes and internal tissues could cause contamination in the explants. Mallika *et al.* (1992) suggested

that fungal infection of field explants of cocoa could be controlled substantially by prior fungicidal treatments of mother plants.

2.4.3.1.7 Culture medium

Selection of culture medium depends on the plant species and purpose of culturing. Many researchers used MS medium for culturing *G.arborea* (Yang *et al.*, 1992, Kannan and Jasrai, 1996 and Gamboa and Abdelnour, 1999). Thirunavoukkarasu and Debata (1998) used Mc Cown's medium for culturing *G.arborea*.

MS medium was used for in vitro culture of many medicinal plants like *sarpagandha* (Koshta and Bansal, 2002). *Calophyllum apetalum* (Nair and Seeni, 2003), *Vitex negundo* (Das *et al.*, 2005) and *Holarrhena antidysenterica* (Kumar *et al.*, 2005). *In vitro* culture of *sarpagandha* (*Rauvolfia serpentina*) produced multiple shoots in MS medium while roots were developed in half strength MS medium (Mahatre *et al.*, 2004).

Krishnan and Seeni (1994) observed that shoot tip explants from *Woodfordia fruticosa* in the SH medium established well in *in vitro* condition. *Woodfordia fruticosa* explants responded maximum to Schenk and Hildebrandt (SH) medium (Gayathri, 2005).

2.4.3.1.8 Exudations from the explants

Lethal browning of explants and exudations on the culture medium hampers the establishment of *in vitro* cultures of several plant species, especially woody plants. In *G.arborea* phenolic exudations from explants of crown region has been reported by Thirunavoukkarasu and Debata, (1998). Earlier reports also revealed that explants from the crown region of some trees like *Eucalyptus teretecornis*, *E.camaldulensis* and *Tectona grandis* exhibited exudation of phenolic compounds

more often than from the basal region (Das and Mitra, 1990, Devi *et al.*, 1994 and Gill and Gill, 1995).

Mathew *et al.* (1987) and Mathew (1995) have reported poly phenol interference in clove. Krishnan and Seeni (1994) observed browning of medium due to poly phenol exudation from different explants of *Woodfordia fruticosa* while Gayathri (2005) reported polyphenol exudation from the cut ends of *thathiri* explants into medium within 48 hours of culturing.

2.4.3.1.8.1 Methods to overcome poly phenol interference under *in vitro* conditions

Reduction of light intensity was found to reduce exudation of poly phenols effectively (Forrest, 1969, Hu and Wang, 1983 and Ziv and Halevy, 1983). Several workers like Bajaj (1978), Stevensons and Haris (1980) and Zaid and Tisserat (1983) have reported that addition of one-two per cent activated charcoal or 0.5 to 1.0 per cent poly vinyl pyrrolidone in the culture medium prevented the accumulation of poly phenols in the culture medium and the subsequent browning of explants. In *Tectona grandis* terminal and axillary buds were suspended in 2.0 per cent sucrose solution and 0.7 per cent soluble poly vinyl pyrrolidone (PVP) in 100ml conical flask on a semisolid medium (Gupta *et al.*, 1980). Pretreatments like soaking explants in water (Gupta *et al.*, 1980) or in anti oxidant solution like ascorbic acid or citric acid (Gupta *et al.*, 1980 and Zaid and Tisserat, 1983) or in adsorbant like 0.7 per cent poly vinyl pyrrolidone (Gupta *et al.*, 1980) or in a solution of antioxidant and sucrose (Gupta *et al.*, 1981) reduce polyphenol exudation and consequent oxidation. Cysteine at 250 mg/l prevents meristematic tip necrosis in *Pyrus* (Dhawan, 1993).

2.4.3.1.9 Plant Growth Regulators

Commonly used growth regulators in tissue culture include four groups such as auxins, cytokinins, gibberellins and retardants like abscissic acid. The requirement of specific concentration and combination of auxins and cytokinins for shoot multiplication of several tree species including *G.arborea* have been reported by Roy (1995). Yang *et al.* (1993) suggested that MS medium supplemented with 0.01mg/l and 5mg/l of BAP was effective for inducing of multiple shoots from node explants. Kannan and Jasrai (1996) found that MS medium supplemented with 1.1μM BAP was effective for inducing multiple shoots from node explants of mature *G.arborea*. This indicated that BAP is appropriate for multiple shoots induction in *G.arborea*. BA alone at 1.0mg/l and in combination with NAA (0.5mg/l) resulted in profuse callusing with very few or no shootlets formation (Thirunavoukkarasu and Debata, 1998) and Ailanthus malabarica (D' Silva and D' Costa, 1992). Higher concentration of BAP caused pronounced callus formation at the cut end and fewer axillary shoots elongation (Sukartiningsih *et al.*, 1999).

BA has been used in preference to other cytokinins to induce multiple shoots. Kinetin or kinetin with BA has given good results with *Azadirachta indica* (Muralidharan and Mascarenhas, 1989) and *Ailanthus malabarica* (D'Silva and D'Costa, 1992). Higher concentration of BAP caused pronounced callus formation at the cut end and fewer axillary shoots elongation in *Artocarpus heterophyllus* (Amin and Jaiswal, 1993) and *Morus serrata* (Pattnaik and Chand, 1997).

The levels of endogenous cytokinin and auxins in teak epicormic shoot explants are just adequate for multiple shoot formation *in vitro* (Devi *et al.*, 1994). Krishnan and Seeni (1994) reported that the induction and proliferation of multiple shoots in the *in vitro* culture of *Woodfordia fruticosa* depended solely on the presence of BAP in the medium. For *Oroxylum indicum* NAA 5mg/l and 2,4-D 1mg /l was found to be the best combination in terms of callus induction from cotyledon and leaf explants. However for explants taken from root NAA 2.5mg/l and 2, 4-D

0.5mg/l proved to be the best formulation (Parveen *et al.*, 2005). BAP at 0.2mg/l was found to be optimum for inducing multiple shoots in *thathiri* (Gayathri, 2005).

2.4.3.1.10 Media components

2.4.3.1.10.1 Carbon and Energy sources

Thirunavoukkarasu and Debata (1998) used sucrose 20g/l in Mc Cown's medium as carbon source for *in vitro* culture of *G. arborea.* Many workers used sucrose 30g/l as the carbon energy source (Sukartiningsih *et al.*, 1999 and Naik *et al.*, 2003) for micropropagation of *kumizhu.*

It has been reported that sucrose was the only sugar necessary for bud induction in *Pinus contorta* (Von and Erikkson, 1981). In *Catharanthus* cell culture catharanthine production was doubled by using fructose instead of sucrose (Jung *et al.*, 1992). Sucrose is the most commonly used carbohydrate in plant tissue and cell culture (Vu *et al.*, 1993). The majority of *in vitro* studies have concluded that sucrose supports near optimal rate of growth and it is relatively inexpensive (Swedlund and Locy, 1993). Among the carbon sources, sucrose was optimum for shoot formation on *in vitro* regeneration of *Centella asiatica* L. in the presence of BA or kinetin. Other carbon sources were less effective than sucrose both in BA and kinetin containing media (Hossain *et al.*, 2005).

2.4.3.1.10.2 Other media components

Einset (1978) found that the *in vitro* growth of explants from several *Citrus* species was greatly stimulated by the addition of orange juice to the medium. Polyphenolic compounds like phloroglucinol in the medium have been found to have a beneficial effect on organogenesis and growth (Hunter, 1979). Conger (1981) reported the role of complex organic compounds like casein hydrolysate, coconut water, yeast, malt extract and fruit and vegetable juice for successful

growth of tissues and organs. Activated charcoal added to liquid MS reduced IAA and IBA concentration by over 97 per cent (Scott *et al.*, 1990).

2.4.3.1.11 pH

During the course of growth of plant cells in media, pH may drop as low as four and rise to seven (Martin and Rose, 1978). Such changes can be expected to have an impact on the metabolism of cells. Sanenz *et al.* (1993) stated that changes in medium pH could change the permeability of cell membranes, resulting in the release of products into the culture medium.

2.4.3.1.12 Culture environment

During *in vitro* growth, plants face unnatural environmental conditions such as high humidity, low photon flux density and low CO_2 concentration. This leads to developmental and physiological alterations in plants, which impairs their ability to survive when transferred to *ex vitro* conditions (Ziv, 1995).

According to Murashige (1977) the optimum day light required is 16 hrs for wide range of plants. In teak, the optimum temperature was found to be 28±2°C. At lower temperature suppression of growth and at higher temperature browning of shoots was observed (Mascarenhas *et al.*, 1993).

2.4.3.1.13 Rooting of *in vitro* formed shoots

IBA was effective for root induction and root system development of shoots of *G.arborea* (Yang *et al.*, 1993 and Kannan and Jasrai, 1996). Combination of IBA and NAA was better for root system development in *G.arborea* than IBA alone (Sukartiningsih *et al.*, 1999). The best rooting response was noticed in about 80 per cent of the shootlets cultured on medium containing 1.5 mg/l of IBA (Thirunavoukkarasu and Debata, 1998).

Ancora *et al.* (1981) reported that among the different auxins, NAA was the most effective for root induction. A combination of auxins gave better response for *in vitro* rooting in teak and clove (Gupta *et al.*, 1980 and Mathew and Hariharan, 1990).

Activated charcoal has profound influence on the rooting of shoots *in vitro*. Activated charcoal absorbs toxic substances in the medium there by improving root regeneration and development of more roots (Jaisal and Amin, 1987).

2.4.3.1.14 Hardening and planting out

Transferring plants from the culture vessel to the potting mixture require careful and step by step procedure. Success in acclimatisation depends not only on post transfer conditions but pre transfer conditions as well (Ziv, 1986). In *G.arborea* plantlets were maintained in high relative humidity (70-90%) at 30°C under fluorescent light with 16 hr photoperiod in a growth chamber with humidity control for four to eight weeks and 90 per cent survival rate was obtained (Sukartiningsih *et al.*, 1999). The plantlets of *G.arborea* were planted in 7×10 cm poly bag containing a mixture of brick and coarse river sand (1:2) and irrigated with water and after three weeks shifted to green house. Twenty to sixty per cent survival has been reported (Dhiraj *et al.*, 2003).

Lee *et al.* (1987) used a mixture of perlite, vermiculite and peat in a ratio 1:1:1 by volume to harden the *in vitro* produced plantlets of *Lagerstroemia indica*. In *Agave sisalana*, Das (1992) reported that rooted plantlets transferred to a mixture of soil and sand in a ratio of 1:1 survived better when placed in green house for 90-100 days. In teak *in vitro* rooted shoots were planted in a 3:3:1 soil: sand: compost mixture in small poly bags, covered with glass sheets (John *et al.*, 1997). The rooted plants of *thathiri* were planted in earthen pots filled with fine river sand which had been autoclaved previously and kept covered with polythene sheets (Gayathri, 2005).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present investigation was carried out in the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during the period 2004-2006.

3.1 Reproductive biology

Two trees maintained in the Dr. T. V. Viswanathan Memorial Herbal Garden attached to the Department of Plant Breeding and Genetics were utilized for this study.

3.1.1 Time taken for inflorescence development

Time taken for full development of inflorescence from visual observation stage to bud formation was studied by tagging five flowering shoots in two trees.

3.1.2 Duration of blooming

Time taken for the entire inflorescence to bloom that is, from the opening of first flower in the inflorescence to the last flower in the same inflorescence.

3.1.3 Floral morphology

Five fresh flower buds and flowers were collected. Hand sections, both L.S and T.S were taken and examined under microscope and description of morphological features like size, colour and number of floral parts, androecium and gynoecium were undertaken.

3.1.4 Anthesis

Five inflorescence were selected in trees and flower buds were observed at one hour interval between 7pm and 9am of the next day. The peak time of anthesis was recorded.

3.1.4 Time of anther dehiscence and stigma receptivity

The colour and appearance of anthers were examined with hand lens at one hour interval in fully mature flower buds of each inflorescence to find out the time of anther dehiscence in a flower. The stigmatic surface was also observed for any change in colour or appearance in the same buds, at same interval of time to find out the stigma receptivity.

3.1.5 Pollen morphology and fertility

For determining morphology, pollen samples were taken from fully opened new flowers and acetolysed. Then the sculpturing on the exine was examined under the microscope.

Fertility of pollen was assessed on the basis of stainability of pollen grains in acetocarmine glycerine mixture. Pollen grains were collected from newly opened flowers and stained in a drop of acetocarmine mixture on a clean slide and kept aside for one hour. All the pollen grains that were well filled and stained were counted as fertile and others are sterile. Two fields of five slides were prepared and observed under microscope and the values expressed as percentage.

Pollen diameter was measured using an ocular micrometer, after calibration.

3.2 Fruit and seed characteristics

3.2.1 Fruit characteristics

Time for fruit set was taken by tagging fully opened flowers in five inflorescence. Fruit shape, colour and size were noticed.

3.2.2 Seed characteristics

Seed colour, shape and seed coat characteristics were observed.

3.3 *In vitro* propagation technique

The experiments to standardise *in vitro* propagation technique in *kumizhu* was done at the Tissue Culture Laboratory attached to the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during the period 2004-2006. The study was undertaken through the conduct of the following two experiments.

1: Direct regeneration

2: Regeneration through callus mediated organogenesis

3.3.1 Source of explants

One year old rooted cuttings were brought from the College of Forestry at Vellanikkara and were planted in earthen pots of diameter 45 cm and depth 35 cm which were filled with a mixture of sand, soil and dry cow dung in the ratio 1:1:1. The plants were kept in a net house covered with polythene sheets and watered on alternate days. Bavistin was sprayed once a week to control fungal diseases.

3.3.2 Culture medium

3.3.2.1 Composition of the medium

The response of the explants in three different basal media was studied in the present experiment. Culture media used were Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962), Schenk and Hildebrandt (SH) medium

(Schenk and Hildebrandt, 1972) and Woody Plant Medium (WPM) (Lloyd and Mc Cown, 1980). Compositions of these three different media are given in Table 1. The basal media were supplemented with different levels of auxins like IAA, NAA and 2, 4,-D and cytokinins that included BAP, Adenine and Kinetin singly or in combination, in different experiments to study the effect of different growth regulators on culture establishment.

3.3.2.2 Preparation of the stock solutions

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the medium. Stock solution of the major and minor nutrients were prepared first by dissolving the required quantity of chemicals in double distilled water and stored under refrigerated conditions in amber coloured bottles (Appendix I, II and III). Stock solutions for the major and minor nutrients were prepared afresh every three months. The vitamin stock solution was prepared every six to eight weeks and those of growth regulators were prepared fresh every four weeks.

Table1. Composition of various media tried for *in vitro* culture of *kumizhu*.

Ingredients (mg/l)	MS	WPM	SH
Inorganic constituents			
$(NH_4)NO_3$	1650	400	
$(NH_4)\ H_2PO_4$			300
KNO_3	1900		2500
K_2SO_4		990	
KH_2PO_4	170	170	
$Ca\ (NO_3)_2.4H_2O$		556	
$CaCl_2.2H_2O$	440	96	200
$MgSO_4.7H_2O$	370	370	400
$FeSO_4.7H_2O$	27.8	27.8	15
Na_2EDTA	37.3	37.3	20
$MnSO_4.4H_2O$	22.3	22.3	
$MnSO_4.H_2O$			10
$ZnSO_4.7H_2O$	8.6	8.6	1
H_3BO_3	6.2	6.2	5
KI	0.83		1
$Na_2MoO_4.2H_2O$	0.25	0.25	0.1
$CuSO_4.5H_2O$	0.025	0.25	0.2
$CoCl_2.6H_2O$	0.025		0.1
Organic constituents			
Myoinositol	100	100	1000
Nicotinic Acid	0.5	0.5	5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.1	1	5
Glycine	2	2	
Sucrose	30000	30000	30000

3.3.2.3 Preparation of the culture medium

Specific quantities of the stock solution were pipetted out into a beaker. The required quantity of plant hormones was mixed. Sucrose and inositol were added fresh and well dissolved and volume made up to the required level using double distilled water. The pH of the solution was adjusted at 5.8 using 1.0N NaOH or 0.1N HCl. For obtaining a semisolid medium, agar was added at 0.75 per cent level and the medium was boiled till a clear solution was obtained. About 20 ml of this molten medium was dispensed into the culture tubes (15x2.5cm or 20x2.5cm s).

3.3.2.4 Sterilisation of the culture medium

The tubes were plugged with non absorbent cotton and auto claved at 121° C and 15 psi (1.06 kg/cm^2) for 20 minutes. The medium was allowed to cool to room temperature and stored in a cool dry place.

3.3.3 Preparation of the explants

Shoot tips, nodal segments and leaf bits were taken as explants for study. Shoot tips of approximately 0.5 to 1.0 cm in length were excised carefully from the plants and tender leaves were clipped off and were used as explants. Both mature and tender stem segments upto 2 cm were taken and cut into nodal segments with axillary buds and used as explants. Tender leaves were cut into small bits and used as explants.

The explants were immersed in 1.0 per cent Teepol solution for five minutes and were thoroughly washed in running tap water to remove all traces of chemical. Explants were kept in Bavistin 0.1 per cent solution for 30 minutes and thoroughly washed with distilled water.

3.3.4 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilisation of the explants, preparation and inoculation of the explants and subsequent sub culturing were carried out in a clean laminar air flow chamber. The working table of the laminar air flow chamber was initially surface sterilised with absolute alcohol and then by switching on the ultra violet light for 30 minutes. The petridishes, forceps, knives and other inoculation aids were initially autoclaved and then flame sterilized before each inoculation. The hands were washed thoroughly with soap under running tap water. After drying, they were wiped with absolute alcohol before inoculation.

3.3.5 Culture establishment

3.3.5.1 Surface sterilisation

Surface sterilisation was carried out under perfect aseptic conditions in the laminar airflow chamber. The washed explants were put into the sterilant and kept immersed for the required period. They were continuously agitated manually to ensure thorough contact of the explants with the chemical. The different sterilisation treatments tried for the explants are listed in Table 2. The explants after surface sterilisation were rinsed five times thoroughly with sterilised distilled water to remove traces of the sterilant from the surface of the explant.

3.3.5.2 Inoculation

The explants that were surface sterilised were inoculated under perfect aseptic conditions into the different basal media supplemented with varying quantities of growth regulators and cultured (Plate 1).

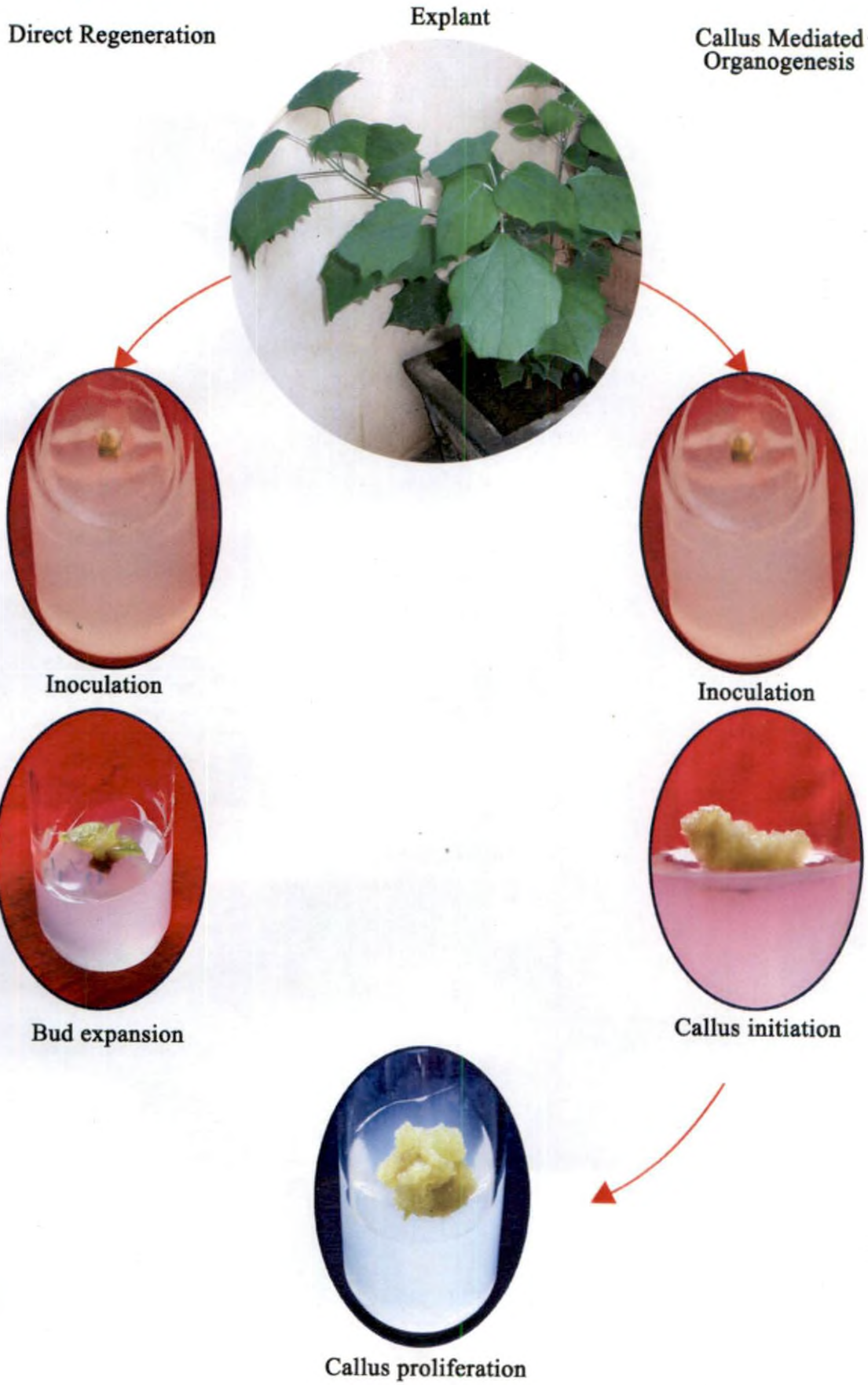


Plate 1: Protocol for micropropagation

3.3.5.3 Culture conditions

The cultures were incubated at 25 ± 2° C in an air conditioned culture room with 12 hours period (1000 lux) supplied by cool white fluorescent light. Relative humidity in the culture room varied between 60 and 80 per cent according to the climate prevailing.

3.3.5.4 Standardisation of basal medium and explant

The best basal medium for *in vitro* culture of *kumizhu* was identified by inoculating the explants into the three basal media viz. MS medium, SH medium and WPM which were supplemented with growth hormones to initiate culture in *kumizhu* (Table 3). Shoot tips, nodal segments and leaf bits were employed for establishing cultures. The best media and explant were identified, based on their capacity for direct regeneration and indirect regeneration through callus mediated organogenesis.

3.3.6 Direct regeneration

On the basis of the studies conducted for standardisation of basal medium and explants, the best basal medium and explant will be utilized for direct regeneration study.

3.3.6.1 Shoot induction

3.3.6.1.2 Effect of medium supplements

Studies were conducted to determine the effect of various growth regulators on the induction of multiple shoots from explants. Details of the treatments conducted are presented in Table 4. Surviving cultures were sub cultured at an interval of 3-4 weeks to the same medium in which they were inoculated, for a period of three months. The response of the explants in each sub culture was observed and recorded.

Table 2. Surface sterilisation treatments in *kumizhu*

Sterilant	Concentration (%)	Duration
Ethyl alcohol	70	10seconds
	70	15seconds
	70	30seconds
	70	45seconds
	70	60seconds
$HgCl_2$	0.1	1minute
	0.1	2 minutes
	0.1	4 minutes
	0.1	6 minutes
	0.1	8 minutes
Ethyl alcohol+$HgCl_2$	70 + 0.1	30 seconds+4minutes
	70 + 0.1	30 seconds +6 minutes
	70 + 0.1	30 seconds +8 minutes
	70 + 0.1	60 seconds+4 minutes
	70 + 0.1	60 seconds +6 minutes
	70 + 0.1	60 seconds +8 minutes

Table 3. Basal media employed for *in vitro* culture in *kumizhu*

Treatment	Media
T1	MS + BA 1 mg/ l
T2	MS + BA 2 mg/ l
T3	MS + Kinetin 1 mg/ l
T4	MS + Kinetin 2 mg/ l
T5	MS + 2,4-D 2 mg/ l
T6	SH + BA 1 mg/ l
T7	SH + BA 2 mg/ l
T8	SH + Kinetin 1 mg/ l
T9	SH + Kinetin 2 mg/ l
T10	SH + 2,4-D 2 mg/ l
T11	WPM + BA 1 mg/ l
T12	WPM + BA 2 mg/ l
T13	WPM + Kinetin 1 mg/ l
T14	WPM + Kinetin 2 mg/ l
T15	WPM + 2,4-D 2 mg/ l

3.3.7 Regeneration through callus mediated organogenesis

Based on the studies conducted for standardization of basal medium and explants, thebest basal medium and explant will be utilized for callus mediated organogenesis studies.

3.3.7.1 Effect of growth regulators in callus induction

MS medium was supplemented with different levels of auxins to induce callusing in the cultures. NAA, 2, 4-D alone and in combination with BA were used to induce calli and their effect studied. Details are given in Table 5.

Cultures were incubated in 16 hr photoperiod light at 25 ± 2 °C at a relative humidity of about 60 to 80 per cent depending on the external climatic conditions. The relative performance of different explants for callus induction and proliferation was observed. Callus Index (CI) was worked out as below.

$$\mathbf{CI = P \times G}$$

Where,

P = percentage of cultures initiating callus and

G = growth score

Scoring was done based on the spread of callus and a maximum score of four was given to those calli that have occupied the whole surface of the media, three months after incubation.

Score 1 = callus occupies ¼ of the media surface

Score 2 = callus occupies ½ of the media surface

Score 3 = callus occupies ¾ of the media surface

Score 4 = callus occupies complete media surface

3.3.7.2 Effect of media supplements in organogenesis /embryogenesis from the callus

Calli obtained from the nodal segments were sub cultured in media containing different combination of cytokinins and auxins for shoot induction. The various treatments tried to induce multiple shoots from the calli are presented in Table 5. Response of the calli was observed and recorded at fortnightly intervals.

Table 4. Growth regulators for multiple shoot induction in *kumizhu*

Treatment	Medium
T16	MS + BA 1 mg/l
T17	MS + BA 1.5 mg/l
T18	MS + BA 2 mg/l
T19	MS + BA 3 mg/l
T20	MS + BA 4.5 mg/l
T21	MS + BA 5 mg/l
T 22	MS + BA 5.5 mg/l
T23	MS + Kinetin 1 mg/l
T24	MS + Kinetin 2 mg/l
T25	MS + Kinetin 4 mg/l
T26	MS + Kinetin 4.5 mg/l
T27	MS + Kinetin 5 mg/l
T28	MS+ BA 1 mg/l +NAA 0.5 mg/l
T29	MS+BA 2 mg/l +NAA 0.5 mg/l
T30	MS+BA 3 mg/l +NAA 0.5 mg/l
T31	MS+BA 4 mg/l + NAA 0.5 mg/l
T32	MS+BA 5 mg/l +NAA 0.5 mg/l
T33	MS+BA 5 mg/l +NAA 1 mg/l
T34	MS+BA 1 mg/l + Kinetin 2 mg/l + NAA 0.5 mg/l
T35	MS+ Kinetin 0.5 mg/l + NAA 0.5 mg/l
T36	MS + Kinetin 4 mg/l + NAA 0.5 mg/l
T37	MS + BA 0.5 mg/l + Kinetin 0.5 mg/l
T38	MS + BA 0.5 mg/l + IAA 0.5 mg/l
T39	MS + BA1 mg/l + IAA 0.5 mg/l
T40	MS + BA 1 mg/l + IAA 1 mg/l
T41	MS + BA 1.5 mg/l + IAA 2 mg/l
T42	MS + Kinetin 0.5 mg/l + IAA 0.5 mg/l
T43	MS + Kinetin 1 mg/l + IAA 0.5 mg/l
T44	MS + Kinetin 1 mg/l + IAA 1 mg/l
T45	MS + Kinetin 1.5 mg/l + IAA 2 mg/l

Table 5. Growth regulators for callus mediated organogenesis in *kumizhu*

Treatment	Medium
Callus Induction	
T46	MS + 2,4-D 0.5 mg/l
T47	MS + 2,4- D 1mg/l
T48	MS + 2,4- D 1.5 mg/l
T49	MS + 2,4- D 2 mg/l
T50	MS + NAA 0.5 mg/l
T51	MS + NAA 1 mg/l
T52	MS + NAA 1.5 mg/l
T53	MS + NAA 2 mg/l
T54	MS + 2,4-D 1 mg/l +BA 0.3 mg/l
T55	MS + 2,4- D 1 mg/l + BA 0.5 mg/l
T56	MS+ 2,4-D 1 mg/l + BA 1 mg/l
T57	MS + 2,4- D 1 mg/l + NAA 1 mg/l
Callus Regeneration	
T58	MS + BA 4.5 mg/l
T59	MS + BA 5 mg/l
T60	MS + BA 1 mg/l + NAA 0.5 mg/l
T61	MS + BA 2 mg/l + NAA 0.5 mg/l
T62	MS + BA 3 mg/l + NAA 0.5 mg/l
T63	MS + BA 4 mg/l +NAA 0.5 mg/l
T64	MS + BA 4.5 mg/l +NAA 0.5 mg/l
T65	MS + BA 5 mg/l +NAA 0.5 mg/l
T66	MS + BA 5 mg/l +NAA 1 mg/l
T67	MS + BA 0.5 mg/l +Kinetin 0.5 mg/l
T68	MS + Kinetin 1 mg/l +IAA 1 mg/l
T69	MS + Kinetin 0.5 mg/l +IAA 0.5 mg/l
T70	MS + Kinetin 1 mg/l +NAA 0.5 mg/l
T71	WPM + Kinetin 0.5 mg/l +IAA 1 mg/l
T72	WPM+ Adenine 1 mg/l
T73	WPM+ BA 1 mg/l +IAA 1 mg/l

RESULTS

4. RESULTS

The results obtained in the study are grouped into two major heads.

4.1 Reproductive biology

4.2 Micro propagation

4.1 Reproductive biology

4.1.1 Season of flowering

Blooming started in January when new flushes sprouted out and continued upto the end of March (Plate 2).

4.1.2 Inflorescence characters

Inflorescence is a terminal cyme and on an average 10-15 flowers were presented in each inflorescence (Table 6). Blooming started about 7 days after the appearance of visible buds and lasted one week (Table 7 and 8). Blooming is from the base of inflorescence to tip in an irregular manner (Plate 3).

4.1.3 Floral Morphology

The flowers are short, stalked, pubescent, large, bisexual, scented and 4.45-5cm long (Table 9). The calyx is tubate and five lobed at tip. The corolla is brownish yellow and has a short tube of girth 1.53 cm (Table 9), with the upper lip formed by two petals and the lower lip by one broad central and two lateral petals (Plate 4).

Plate 2: Tree in Bloom

Plate 3: inflorescence

Table 6. No. of flowers in an inflorescence

Sl. No.	No. of inflorescence	No. of flowers
1	1	10
2	1	12
3	1	11
4	1	13
5	1	15
	Mean±SE	12.16 ±0.848

Table 7. Time taken for inflorescence development

No. of inflorescence	Tagged date	Date of blooming	No. of days
1	19/1/06	24/1/06	6
2	19/1/06	25/1/06	7
2	20/1/06	26/1/06	6
1	21/1/06	26/1/06	6
2	21/1/06	27/1/06	7
2	23/1/06	30/1/06	8
2	23/1/06	30/1/06	8
1	30/1/06	09/2/06	11
4	01/2/06	10/2/06	10
1	15/2/06	21/2/06	7
1	16/2/06	22/2/06	7
		Mean±SE	8±0.055days

Table 8. Blooming duration

No. of inflorescence	Starting of blooming	Completion of blooming	No. of days taken
1	24/1/06	30/1/06	6
1	25/1/06	30/1/06	5
1	25/1/06	31/1/06	6
1	26/1/06	31/1/06	5
2	26/1/06	01/2/06	6
1	27/1/06	04/2/06	8
1	30/1/06	07/2/06	8
2	30/1/06	08/2/06	9
1	09/2/06	18/2/06	9
4	10/2/06	18/2/06	8
		Mean±SE	7±0.953 days

The stamens are four and epipetalous. The anterior pair is longer while the posterior pair is short. The anthers are dithecous and dehisce by longitudinal slits (Plate 5). The ovary is bicarpellary with four ovules. The style is long and terminated with short stigma with two unequal lobes (Plate 7). The stigma lies between anthers of the long and short pair of stamens. The nectar is produced and protected in short corolla tube (Table 10).

Calyx is persistent with fruit. After anthesis corolla tube falls off, but style remains with ovary for one day.

4.1.4 Anthesis

A preliminary study revealed that there was no flower opening between 9 am and 9 pm. So hourly observations on flower opening were taken from 9 pm to 9 am the next day. The percentage of flower opening at periodic intervals is presented in Table 11. Anthesis started from late mid night and lasted up to 3.00 am.

4.1.5 Anther dehiscence and stigma receptivity

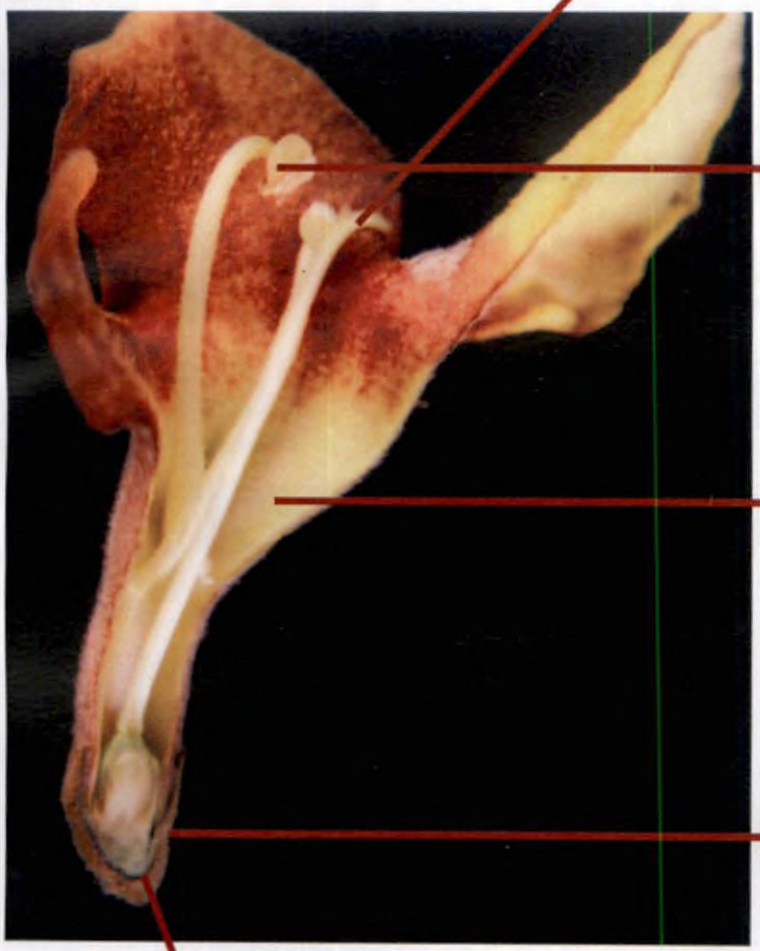
Fully mature flower buds were observed for anther dehiscence and stigma receptivity at periodic intervals (one hour) (Table 12).

Anther dehisced early in the morning, between 8 and 9 am. Anthers dehisce by longitudinal slits. Stigma was receptive during anthesis and continued to be receptive for 4-5 hours (Figure 1).

4.1.6 Pollen morphology and fertility

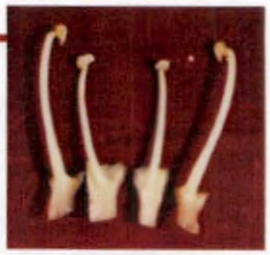
4.1.6.1 Pollen morphology

The morphology of pollen collected from fully mature buds were studied by acetolysis method. To the naked eye, pollen is creamy white, tricolpate, and oval

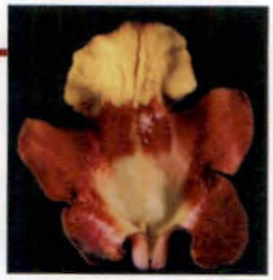


L. S. of flower

Gynoecium



Androecium



Corolla

Calyx

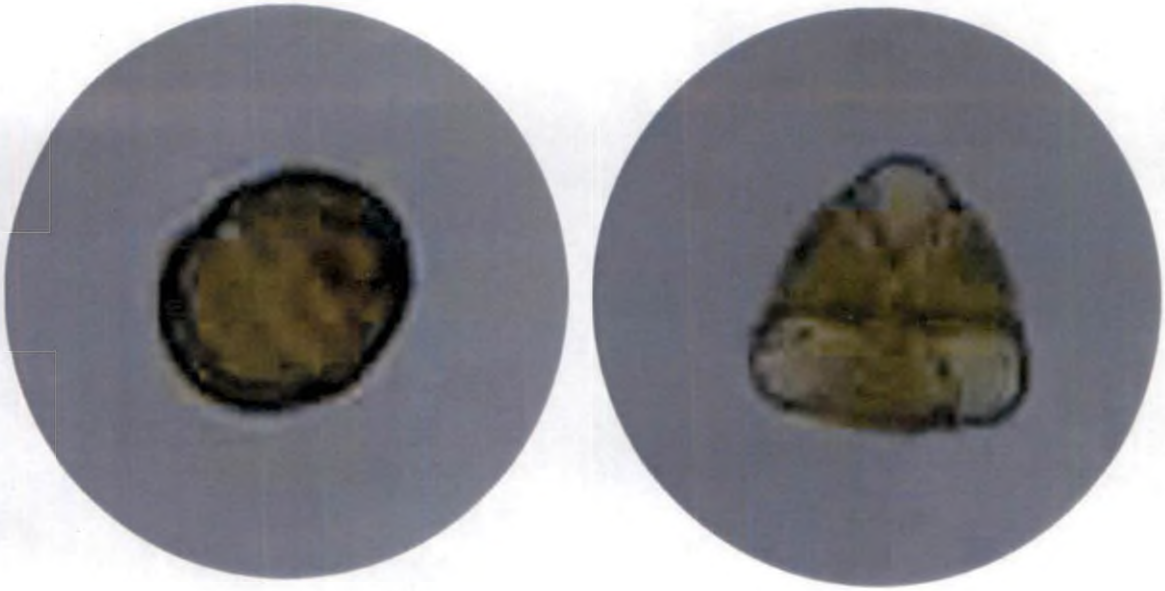
Nectary

Plate 4: Floral morphology

Ventral view

Dorsal view

Plate 5: Anther lobe of *G.arborea*



Before acetolysis

After acetolysis

Plate 6: Pollen grains of *G. arborea*

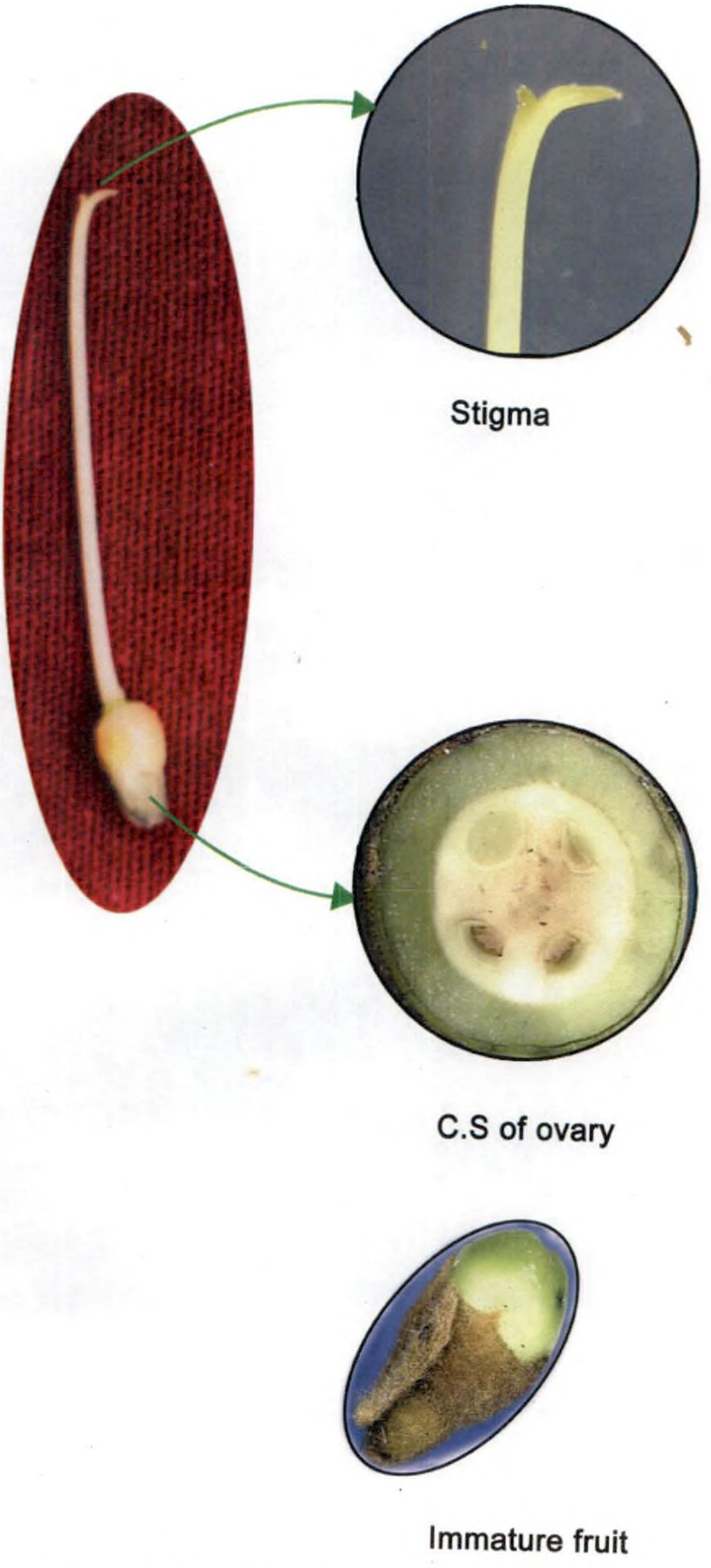


Plate 7: Gynoecium of *G. arborea*

with smooth exine. Average size of pollen is 26.88 micrometer length and 23.96 micro meter breadth (Table 13) (Plate 6).

4.1.6.2 Pollen fertility

The fertility of pollen collected from fully mature buds was assessed by acetocarmine staining, which revealed cent per cent fertility (Table 14).

4.1.7 Pollination system

Five inflorescence were covered with butter paper a day before starting of anthesis and observed for self pollination. No fruit set occurred.

In natural open pollination up to 16.5 per cent fruit set recorded (Table15). Immature fruit fall was common. Birds, honeybees, *Xylocopa*, ants, beetles and small insects were common pollinators.

4.1.8 Fruit and seed characteristics

4.1.8.1 Fruit features

Immature fruits are initially brick red, later green and turn yellow when ripe. The ripe fruits are smooth with leathery shining pericarp, pulp and one stony seed. Average fruit size is 3.10 × 9.26 cm (Table 16) (Plate 8).

4.1.8.2 Seed Features

Seed is with rough and hard seed coat, oval shaped, tapering to one end. Size is 2.04×4.32 cm (Table16) (Plate 9).

Table 9. Flower size

Sl.no.	Length (cm)	Girth (cm)
1	4.5	1.5
2	4.0	1.4
3	4.4	1.5
4	4.5	1.5
5	4.5	1.5
6	4.8	1.8
Mean ±SE	4.45±0.25 cm	1.53±0.056 cm

Table 10. Floral parts

1	Bracts	present
2	Calyx	green, tubular with five lobes, persistent, nectariferous glands present
3	Corolla	bright orange yellow, tubular with five petals
4	Androecium	stamens - four
		epipetalous
		didynamous
		anthers are dithecous
5	Gynoecium	long style
		stigma bifid
		ovary bicarpellary
		ovules - four

Table11.Anthesis time

Time (hrs)	No. of flowers opened	Percentage
9-11pm	0	-
11-1 am	1	3.33
1-3am	26	86.6
3-5am	3	10.00
5-7am	0	-
7-9am	0	-

(No. of flowers studied =30)

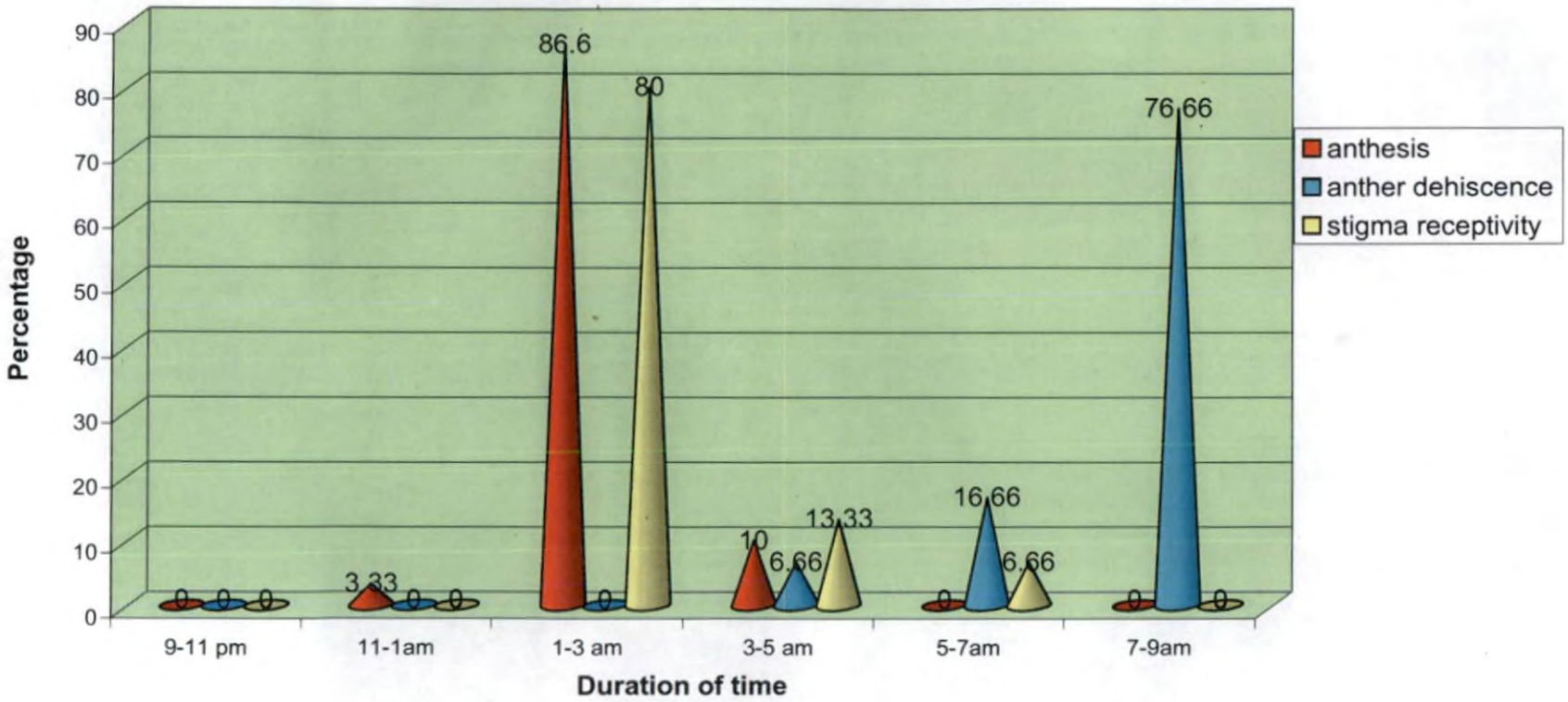


Figure1: Time of anthesis, anther dehiscence and stigma receptivity

Table 12. Anther dehiscence and stigma receptivity

Time in hours	No.of flowers dehisced	Percentage of anther dehiscence	No. of receptive stigma	Percentage of stigma receptivity
9-11 pm	0	-	0	-
11-1 am	0	-	0	-
1-3 am	0	-	24	80.00
3-5 am	2	6.66	4	13.33
5-7 am	5	16.66	2	6.66
7-9 am	23	76.66	0	-

(Number of flowers observed-30)

Table 13. Pollen size

Sl.no	Length (micro meter)	Breadth (micro meter)
1	32.69	25.42
2	36.32	25.42
3	32.69	25.42
4	29.06	29.06
5	25.42	25.42
6	21.79	21.79
7	29.06	25.42
8	21.79	21.79
9	18.16	18.16
10	21.79	21.79
Mean±SE	26.88±1.892 µm	23.96±0.968µm

Table 14. Pollen fertility

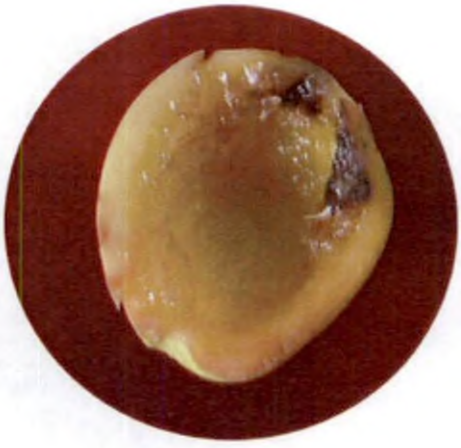
Sl.no	No.of pollen	No.of stained pollen	Percentage
1	104	104	100
2	144	144	100
3	49	49	100
4	52	52	100
5	72	72	100

Table 15. Fruit set in open pollination

Sl.no.	No.of flowers	No.of mature fruits	Percentage fruit set	Percentage fruit fall
1	15	5	33.33	66.67
2	13	3	23.07	76.93
3	11	2	18.18	81.82
4	12	1	8.33	91.67
5	13	0	-	100
Mean±SE			16.50±5.82	83.50±5.49

Table16. Fruit and seed size

Sl.no.	Length cm	Girth cm
Fruit size		
1	3.0	9.5
2	3.0	9.5
3	3.5	9.0
4	3.0	9.0
5	3.0	9.3
Mean±SE	3.10±1.5cm	9.26±0.111 cm
Seed size		
1	2.0	4.5
2	2.0	4.0
3	2.0	4.6
4	1.9	4.0
5	2.3	4.5
Mean±SE	2.04±0.07cm	4.32±0.131cm



C. S of fruit

Plate 8 : Mature fruit of *G. arborea*

Plate 9 : Seed of *G. arborea*

C. S of seed of *G. arborea*

L.S of seed of *G. arborea*

4.2 Micro propagation

The results of various experiments carried out for the standardisation of *in vitro* propagation technique in *kumizhu* (*G. arborea* Roxb.) by direct regeneration and regeneration through callus mediated organogenesis are presented in detail.

4.2.1 Culture establishment

4.2.2 Surface sterilisation

Effect of surface sterilisation treatments on culture establishment in *kumizhu* is presented in Table 17.The most effective sterilisation was achieved by the combination treatment of soaking the explants in 70 per cent alcohol for 30 seconds followed by soaking them in $HgCl_2$ 0.1 per cent for four minutes or eight minutes, where the percentage of surviving cultures was 80 and 20 per cent were dead. But combination of 70 per cent alcohol for 30 seconds with 0.1 per cent $HgCl_2$ for four minutes was selected as best treatment to avoid longer exposure of explants to 0.1 per cent $HgCl_2$. Explants were contamination free when treated with 0.1 per cent $HgCl_2$ alone for six and eight minutes. But survival of explants was less (60%) (Figure 2).

Treatment with ethyl alcohol alone or $HgCl_2$ alone was not sufficient for surface sterilisation. Increasing the time of soaking adversely affected the explants survival.

4.2.3 Effect of different basal media in the culture establishment

The effect of three different basal media in the culture establishment of explants of *kumizhu* is presented in Table 18. The results show that culture responded best to Murashige and Skoog (MS) medium (63%). The explants responded to WPM medium (28%) also but to a lesser extent compared to MS medium. The response was lowest in SH medium (18%). MS medium was

identified as best medium for *in vitro* culture of *kumizhu* and hence was used in further studies (Figure 3).

4.2.4 Effect of different explants cultured on the culture establishment

Among the three explants tried namely shoot tip, nodal segments with axillary buds and leaf bits, maximum response was shown by nodal segments with axillary buds (47%). Shoot tips responded to 41 per cent. Bud expansion and callus formation were also good for nodal segments with axillary buds compared to shoot tips. Leaf bits did not respond in any media. Table 18. So nodal segments with axillary buds were used for further experiment (Figure 4).

Table 17. Effect of surface sterilants on explants

Sterilant	Concent-ration (%)	Duration	Contamination (%)	Un contaminated cultures	
				Dead %	Live %
Ethyl alcohol	70	10 seconds	70	0	30
	70	15 seconds	100	0	0
	70	30 seconds	30	30	40
	70	45 seconds	70	0	30
	70	60 seconds	60	10	30
$HgCl_2$	0.1	1 minute	40	40	20
	0.1	2 minutes	40	60	0
	0.1	4 minutes	30	40	30
	0.1	6 minutes	0	40	60
	0.1	8 minutes	0	30	70
Ethyl alcohol + $HgCl_2$	70 + 0.1	30 seconds + 4 minutes	0	20	80
	70 + 0.1	30 seconds + 6 minutes	0	40	60
	70 + 0.1	30 seconds + 8 minutes	0	20	80
	70 + 0.1	60 seconds + 4 minutes	50	40	10
	70 + 0.1	60 seconds + 6 minutes	10	80	10
	70 + 0.1	60 seconds + 8 minutes	0	90	10

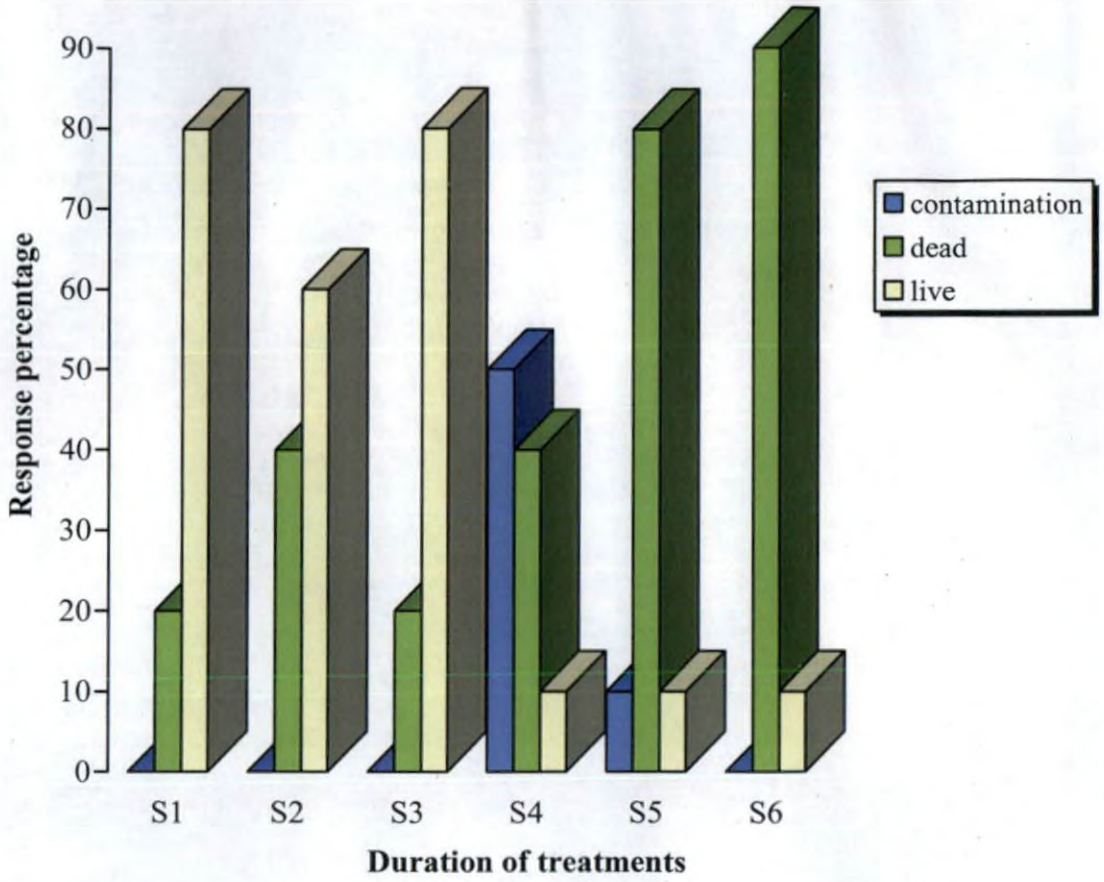


Figure 2. Effect of surface sterilant combination of Ethyl alcohol (70%)+$HgCl_2$ (0.1%) on *kumizhu* explants

Table 18. Effect of basal media on culture establishment

Treatment	Explants	Cultures responding %	Response
T1	S	30	bud expansion
	N	70	bud expansion
	L	nil	
T2	S	40	bud expansion
	N	75	callus
	L	nil	
T3	S	52	bud expansion
	N	65	bud expansion
	L	nil	
T4	S	80	bud expansion
	N	70	bud expansion
	L	nil	
T5	S	65	callus
	N	82	callus
	L	nil	
T6	S	nil	
	N	nil	
	L	nil	
T7	S	nil	
	N	10	bud expansion
	L	nil	
T8	S	nil	
	N	nil	
	L	nil	
T9	S	nil	
	N	nil	
	L	nil	
T10	S	nil	
	N	25	callus
	L	nil	
T11	S	10	bud expansion
	N	20	callus
	L	nil	
T12	S	40	bud expansion
	N	30	callus
	L	nil	
T13	S	nil	
	N	nil	
	L	nil	
T14	S	30	bud expansion
	N	25	callus
	L	nil	
T15	S	20	callus
	N	50	callus
	L	nil	

S-shoot tip, N-nodal segments with axillary buds, L-leaf

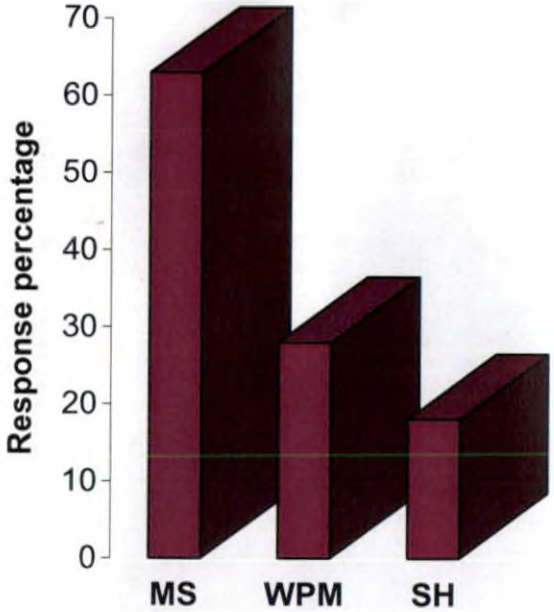


Figure 3.Response of medium in *in vitro* culture of *kumizhu*

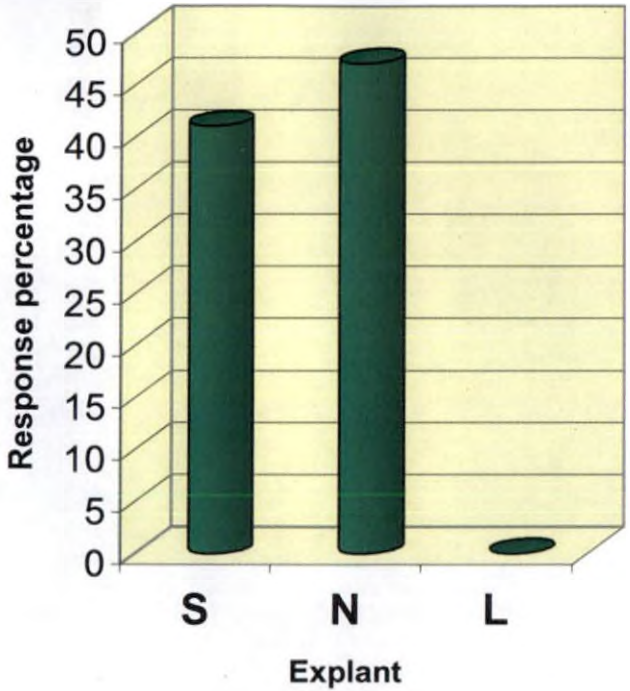


Figure 4.Explant response of *kumizhu* for *in vitro* culture

4.3 Direct Regeneration

4.3.1 Enhanced release of multiple shoots

Detailed experiments were conducted to identify an ideal growth regulator combination for multiple shoot induction and proliferation in *kumizhu*. Different levels of cytokinins like BA and kinetin alone and in combination with auxins like NAA and IAA were used.

4.3.1.1 Effect of cytokinins

BA and kinetin were used in different concentrations for multiple shoot induction using MS as basal medium. The results are presented in Table 19

The treatments T20, T21, T22, T24, T25, T26 and T27 showed bud expansion. Ninety two per cent of the inoculated culture of T27 showed bud expansion within 7 days of inoculation. T22 and T26 produced 90 per cent bud expansion 13 and 9 days after inoculation respectively. T24 showed least response to bud expansion (60%) taking the maximum duration of 16 days for response.

Among the twelve treatments, treatments T16, T17, T18, T19 and T23 showed a tendency for callusing. Hence these treatments were repeated under callus mediated organogenesis.

Table 19. Effect of cytokinins on multiple shoot induction

Treatments	Bud expansion %	Days taken
T16		
T17		
T18		
T19		
T20	83	12
T21	85	14
T22	90	13
T23		
T24	60	16
T25	65	15
T26	90	9
T27	92	7

4.3.1.2 Effect of combinations of cytokinins and auxins

The response of nodal segments for multiple shoot induction in different combinations of auxins and cytokinins are given in Table 20.

Among the eighteen treatments, T32, T33, T34 and T43 showed bud expansion. Maximum percentage of cultures showing bud expansion was in T33 (80%) while least was in T43 (10%). Days taken for bud expansion by these treatments ranged from 11 (T33) to 21 days (T43).

T28, T29, T30, T31, T35, T36, T38, T40, T42, T44, and T45 showed a tendency for callusing and hence these treatments were repeated under callus mediated organogenesis.

4.4 Regeneration through callus mediated organogenesis

4.4.1 Callus induction and proliferation

The effect of different media combinations on callus induction and proliferation from the nodal segments with axillary buds is given in Table 21. Among the twelve treatments T50 and T51 did not show any response.

Among treatments using BA alone T19 took the least number of days for callus induction (7 days) while T16 took maximum number of days for callus induction (19 days). T18 had highest callus index (70) but took 12 days for callus induction. Although combination of growth regulators between BA+NAA, BA+IAA and kinetin+IAA showed a tendency for callusing, there was less percentage of callusing.

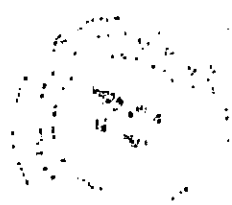


Table 20. Combination of growth regulators for multiple shoot induction

Treatment	Bud expansion %	Days taken
T28		
T29		
T30		
T31		
T32	71	12
T33	80	11
T34	55	18
T35		
T36		
T37		
T38		
T39		
T40		
T41		
T42		
T43	10	21
T44		
T45		

Callus induction ranged from 90 per cent (T47) to 56 per cent (T46). T57 recorded a callus induction percentage of 80. Days taken for callus induction ranged from 6 days (T57) to 30 days (T46). Based on callus index scores T47 (180), followed by T57 (160) and T49 (150) were adjudged as good treatments for induction of calli in *kumizhu*.

Callus produced by treatments from T38 to T49 was friable and cream. Callus formed in treatments from T16, T17, T18, T19, T23, T28, T29, T30, T31, T35, T36, T52, T53, T54, T55, T56 and T57 was light greenish in colour.

4.4.2 Callus Regeneration

Calli were sub cultured in media with various concentrations of BA and kinetin along with NAA and IAA. The details are furnished in Table 22.

Callus proliferation was noticed in all treatments except T60, T67 and T70.The number of cultures responding to various treatments varied from 88 per cent (T59) to 20 per cent (T68). The duration taken for response ranged from 15 (T73) to 36 days (T61). The calli formed were friable and green in colour. When callus was sub cultured to WPM medium, proliferation started around 19 days after subculturing. The callus formed was white and friable.

Table 21. Effect of growth regulators on callus induction

Treatments	Days for callusing	Percentage	Growth score	Callus index	Nature
T16	19	65	1	65	Friable, light green
T17	16	62	1	62	Friable, light green
T18	12	70	1	70	Friable light green
T19	7	60	1	60	Friable, light green
T23	11	60	1	60	Friable, light green
T28	18	40	1	40	Friable, light green
T29	20	40	1	40	Friable, light green
T30	16	60	1	60	Friable, light green
T31	14	55	1	55	Friable, light green
T35	15	10	2	20	Friable, light green
T36	16	25	1	25	Friable, light green
T38	16	10	1	10	Friable, cream
T40	8	20	1	20	Friable, cream
T42	8	20	1	20	Friable, cream
T44	20	20	1	20	Friable, cream
T45	18	10	1	10	Friable, cream
T46	30	56	1	56	Friable, cream
T47	19	90	2	180	Friable, cream
T48	17	60	1	60	Friable, cream
T49	16	75	2	150	Friable, cream
T50	Nil				
T51	Nil				
T52	10	60	1	60	Friable, light green
T53	27	60	1	60	Friable, light green
T54	25	70	1	70	Friable, light green
T55	21	75	1	75	Friable, light green
T56	8	60	1	60	Friable, light green
T57	6	80	2	160	Friable, light green

Table 22. Effect of growth regulators on callus regeneration

Treatments	Callus proliferation %	Days taken	Colour
T58	20	30	Friable, green
T59	88	30	Friable, green
T60	Nil		
T61	55	36	Friable, green
T62	50	35	Friable, green
T63	58	29	Friable, green
T64	48	34	Friable, green
T65	45	30	Friable, green
T66	50	23	Friable, green
T67	Nil		
T68	20	18	Friable, cream
T69	55	19	Friable, cream
T70	Nil		Friable, cream
T71	70	19	Friable, white
T72	75	18	Friable, white
T73	50	15	Friable, white

DISCUSSION

5. DISCUSSION

Gmelina arborea is a valuable medicinal tree with good timber value. Despite its importance *Gmelina arborea* has not been studied for its floral biology, breeding systems, pollination and seed dispersion in its native geographical regions (Raju and Rao, 2006). Reliable information on pollinating vectors and flower development, is important for the design of seed orchards, for making decisions on mating designs (open pollination or controlled pollination) determining isolation distances between orchards and sub lines and maintenance of adequate insect population in desired areas (Dvorak, 2004). The tree can be propagated by seeds, cuttings and grafting, but these conventional propagation techniques are not sufficient to meet the demand of planting material (Kannan and Jasrai, 1996).

The above references indicate the importance of studies on reproductive biology and micro propagation in *kumizhu*. Hence the results obtained from this study are discussed here under as follows:

5.1 Reproductive Biology

5.2 Micro propagation

5.1 Reproductive Biology

5.1.1 Season of flowering

In the natural distribution area with a seasonal climate, flowering starts in the dry season when trees are leafless (Rachmawati *et al.*, 2002). The trees in Dr.T.V.Viswanathan Memorial Herbal Garden started blooming in January when new flushes sprouted out and continued upto the end of March. Similar reports have been made by Stock *et al.* (2004) in *G. arborea* trees raised in Venezula region, while Raju and Rao (2006) reported that the trees of *G.arborea* occurring in the Eastern Ghats forests of Vishakapatnam flowered during February-April.

5.1.2 *Inflorescence characters*

Inflorescence is a terminal cyme blooming in an irregular manner. Around 10-15 flowers are present in an inflorescence and blooming lasts upto a week Tewari (1995), Stock *et al.* (2004) and Raju and Rao (2006) have opined the inflorescence of *G.arborea* as cymes.

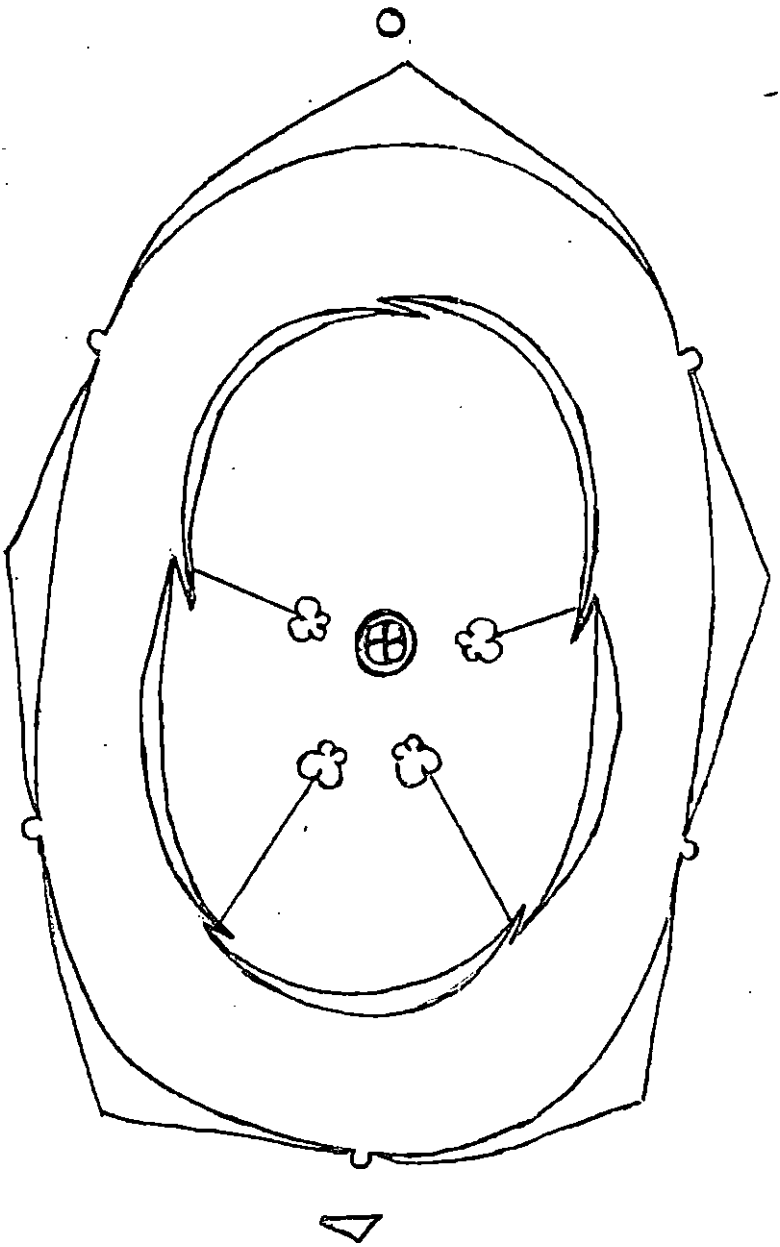
5.1.3 Floral morphology

The flowers are short stalked, pubescent, large, bisexual, scented and 5 cm long. Calyx persistent, tubate and five lobed at tip. Corolla brownish yellow and has a short tube with the upper tip formed by two lateral petals. Corolla tube falls off after anthesis. Stamens four, epipetalous, anterior pair longer while posterior pair is short. Anthers dithecous and dehisce by longitudinal slits. Ovary bicarpellary with four ovules (Plate 10). Style long and remains with ovary for one day. Stigma short with two unequal lobes. Stigma lies between anthers of the long and short pair of stamens. The nectar produced is protected in short corolla tube. These findings are in concurrence with the results obtained by Tewari (1995), Stock *et al.* (2004) and Raju and Rao (2006).

5.1.4 Anthesis, anther dehiscence and stigma receptivity

Anthesis started from late midnight and lasted upto 3.00 am. Anther dehisces by longitudinal slits. Raju and Rao (2006) reported longitudinal sliting of anthers. Stigma receptive during anthesis and continued to be receptive for 4-5 hours.

Gmelina arborea Roxb.



$$\hat{\varphi}\ \oplus\ K_{(5)}\ \overline{C_{(5)}\ A_{4}}\ G_{(4)}$$

FLORAL FORMULA

Plate 10: FLORAL DIAGRAM

5.1.5 Pollen morphology and fertility

Pollen is creamy white, tricolpate, oval with smooth exine and 100 percent fertile. Average size of pollen is 26.88 micrometer length and 23.96 micrometer breadth. The pollen of *Phryma* is tricolpate, tectate-perforate to microreticulate, and has simple columellae (Chadwell, 1992).

5.1.6 Pollination system

Conflicting information exists on whether *G.arborea* is self incompatible. Bowen and Euesbio (1983) and Raju and Rao (2006) have indicated that artificial selfing occasionally produced seeds in Indian conditions while, Bolstad and Bawa (1982) in a study on trees in Costa Rica have reported complete incompatibility. In the present study it was noticed that on selfing there was no fruit set, while under natural cross pollination upto 16.5 per cent fruit set was recorded. Raju and Rao (2006) have also concluded that the natural fruit set rate is low when compared to very high flower production.

Honeybees, ants, beetles, *Xylocopa*, small insects and birds were common pollinators. Bolstad and Bawa (1982) observed native bees. The species is predominately outcrossed and flowers are pollinated by large bees (Rachmawati *et al.*, 2002). Dvorak (2004) reported the presence of hymenopteran insects. Raju and Rao (2006) recorded *Amegilla, Apis, Xylocopa* and birds as pollinators.

5.1.7 Fruit and seed characteristics

Immature fruits are initially brick red, later green and turn yellow when ripe. The fruit is a drupe (Bolstad and Bawa, 1982) with smooth leathery shining pericarp, pulp and one strong seed. The average size of fruit is 3.10 x 9.26 cm. Oval shaped seeds taper to one end. Seed coat is rough and hard. Size 2.04 x 4.32 cm. Similar results have been observed by Little (1983) and Tewari (1995).

5. 2 Micropropagation

5.2.1Culture establishment

5.2.1.1 Surface sterilisation

The most effective sterilisation was achieved by the combination treatment of soaking explants in 70 per cent alcohol for 30 seconds followed by soaking them in $HgCl_2$ 0.1 per cent for four minutes. Combination of both chemicals was recommended by Kannan and Jasrai (1996) and Naik *et al.* (2003) in *G.arborea.*

Increasing the time of exposure to chemicals ($HgCl_2$ 0.1 %) more than eight minutes led to the death of explants. In *thathiri* the exposure of explants to chemicals more than five minutes was found to be deleterious (Gayathri, 2005).

Rinsing of explants four or five times with sterile distilled water, removed all traces of the chemical. Sukartiningsih, (1999) in *G.arborea* and Krishnan and Seeni (1994) in *thathiri* have recommended the need for rinsing the explants five to six times in sterile distilled water before inoculation.

5.2.1.2 Basal medium

For *in vitro* propagation of *kumizhu* the best medium identified was Murashige and Skoog medium (1962). MS medium was used as basal medium by Yang *et al.* (1992), Kannan and Jasrai (1996), Sukartiningsih *et al.* (1999) and Naik *et al.* (2003) in *G.arborea.*

In contrast, Thirunavoukkarasu and Debata (1998) reported better response with Mc Cown's medium. However in the present study response of explants to WPM (28%) was to a lesser extent and negligible in SH medium (18%).

5.2.1.3 Explant

Nodal segments have been widely used as explants for clonal propagation of trees owing to their high multiplication rates and genetic stability. In our study using different explants it was noticed that nodal segments gave higher response for both callusing and bud expansion. Hence nodal segments with axillary buds were selected for plant regeneration studies. Studies on micropropagation in *G. arborea* by workers like Yang *et al.* (1993), Kannan and Jasrai (1996), Thirunavoukkarasu and Debata (1998), Sukartiningsih *et al.* (1999) and Thakar and Bhargava (1999) have supported the use of nodal segments as explants. The use of nodes in preference to shoot tips for proliferation of stem is also reported in other medicinal species like *Adathoda beddomei* (Sudha and Seeni, 1994), *Aegle marmelos* (Ajithkumar and Senni, 1998), and *Ulteria salicifolia* (Ganaprasad *et al.*, 2003). However, inferences in all these studies were based on relatively better morphogenetic responses with nodal explant cultures compared to shoot tip cultures and not on exclusive regenerative capacity of the former.

Shoot tips were identified as best explants for micropropagation of *thathiri* (Gayathri, 2005). In the present study shoot tip explants showed leaf bud expansion alone. Leaf bits did not show any response.

5.2.2 Direct regeneration

Generally, cytokinins have been utilized to overcome the apical dominance of shoots to enhance the branching of the lateral buds from leaf axils (Murashige, 1977). The direct effect of cytokinins in tissue culture may vary according to the particular compound used, the type of cultures and the plant species from which it was derived (George and Sherrington, 1984). Different levels of cytokinins like BA and kinetin and in combination with auxins like NAA and IAA were used in the present study for direct regeneration. Higher concentration of BA and kinetin resulted in bud expansion. Yang *et al.* (1993) and Kannan and Jasrai (1996) have

reported that higher concentration of BA alone resulted in pronounced axillary shoot elongation in *G.arborea.*

When combination of growth regulators were used leaf bud expansion were observed. Eighty per cent of cultures containing BA 5 mg/l and NAA 0.5 mg/l produced leaf bud expansion. Least response (10%) was given by media containing kinetin 1mg/l and IAA 0.5 mg/l. Highest bud expansion was shown by media containing high dose of BA (5mg/l). This has also been reported in *Gmelina arborea* by Thirunavoukkarasu and Debata (1998) where higher concentrations of BA in combination with NAA (0.5mg/l) resulted in very few or no shootlets. Combination of NAA and BA was found best for shoot induction in *Rawolfia serpentina* by Gajbhiye *et al.*, 2006).

From the present study it is deduced that *in vitro* response in *G.arborea* is better when auxins are added at lower concentration and that NAA has a better response over IAA.

5.2.3 Regeneration through callus mediated organogenesis

BA upto 3 mg/l and kinetin upto 1 mg/l induced callusing. Yang *et al.* (1993) and Kannan and Jasrai (1996) have reported that higher concentration of BA alone resulted in pronounced callus formation in *G.arborea.*

- Pronounced callus formation was observed when the explants were inoculated on medium containing 2, 4-D at various levels (0.5 mg/l to 2 mg/l). The callus produced was friable in nature. Hasgawa (1980) reported that a high, concentration of auxin induces callus formation. Similar observations have been made by Datta *et al.* (2002) in rose. Although callus formation was high, the duration for callusing was more than a fortnight.

When auxins were used in combinations, 2,4-D + NAA combination at 1 mg/l each was found to be very effective with a callus index of 160 in just 6 days.

The growth score for this treatment is 2. Hence it can be safely concluded in this case that combination of auxins (2,4-D and NAA) was better over media containing 2,4-D alone.

The callus obtained was subcultured in regeneration medium with different levels of various growth regulators. Both MS and WPM media were tried for callus regeneration.

Among the different treatments tried with MS media it was noticed that BA at 5 mg/l alone resulted in good callus proliferation. Around 30 days duration was taken to obtain 88 per cent callus proliferation. Similar reports were given by Thirunavoukkarasu and Debata (1998) in *G.arborea* and in *thathiri* by Gayathri (2005). Around 55 per cent callus proliferation was noticed in MS media containing kinetin 0.5 mg/l and IAA 0.5 mg/l in about 19 days.

In general the period taken for callus proliferation treatments containing WPM was almost half when compared with the duration taken for response in treatments containing MS media. WPM supplemented with adenine recorded maximum callus proliferation (75 %).

5.3 Future thrusts

In order to understand the dynamics of natural population of any crop species and to initiate any crop improvement programme a thorough knowledge of reproductive biology is essential. Studies on floral biology, breeding systems, pollinators and seed dispersers are rather limited. Seeds from natural populations in India generally do not germinate, which may be due to local anomalies in the reproductive biology process, genetics (in breeding) climatic effects or other (Dvorak, 2004). The future depends on hybrid crosses between *G.arborea* and other species of *Gmelina*, which if successful, may produce new combination with better drug value, drought resistance, wood properties and greater disease resistance.

In the present study, an attempt has been done to describe the reproductive biology of *G.arborea*. The floral biology has been explained but many factors like, factors responsible for failure of seed set under artificial selfing, site of incompatibility have not been investigated in detail. In addition breeding system, pollinators and seed dispersal have not been observed experimentally. The result presented in the study is the result of observations recorded for one year only. Further observations are not possible within the scope of this study. Hence relevant studies in this direction may be continued in the future.

Propagation methods are not sufficient to meet the demands on planting materials. Hence in this study an attempt was made to develop a protocol for micropropagation in *G.arborea*. However, during the course of the study, only callusing was obtained on culture. Hence different treatments may be attempted to obtain callus regeneration and subsequently field planting of *in vitro* produced plantlets in *kumizhu*. In addition callus obtained can be utilized for cell suspension culture for secondary metabolite extraction. Large scale extraction of alkaloids will be highly beneficial.

SUMMARY

6. SUMMARY

The present investigation was carried out during the period 2004-2006 in the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara. An attempt has been made to study the reproductive biology and to standardize *in vitro* propagation technique in *G.arborea*. The trees in the Dr.T. V. Viswanathan Memorial Herbal Garden attached to the Department of Plant Breeding and Genetics were used for observing the reproductive biology. The micro propagation studies were conducted in the tissue culture laboratory of the Department of Plant Breeding and Genetics. Seedlings of *Gmelina* planted in pots were used as source of explants. The salient findings of the investigation are presented below.

1. The trees in Dr.T.V.Viswanathan Memorial Herbal Garden started blooming in January when new flushes sprouted out and continued upto the end of March.
2. Inflorescence is a terminal cyme blooming in an irregular manner. Around 10-15 flowers are present in each inflorescence and blooming lasts upto a week
3. The flowers are short stalked, pubescent, large, bisexual, scented and 5 cm long.
4. Calyx persistent, tubate and five lobed at tip. Corolla brownish yellow and has a short tube with the upper tip formed by two lateral petals, corolla tube fall off after anthesis.
5. Stamens four, epipetalous, anterior pair longer while posterior pair is short. Anthers dithecous and dehisce by longitudinal slits
6. Ovary is bicarpellary with four ovules. Style long and remains with ovary for one day. Stigma short with two unequal lobes. Stigma lies between anthers of the long and short pair of stamens. The nectar produced is protected in short corolla tube.
7. Anthesis started from late midnight and lasted upto 3.00 am. Anther dehisces by longitudinal slits. Stigma was receptive during anthesis and continued to be receptive for 4-5 hours.

8. Pollen creamy white, tricolpate, oval with smooth exine and 100 per cent fertility. Average size of pollen is 26.88 micrometer length and 23.96 micrometer breadth.
9. On selfing there was no fruit set, while under natural cross pollination upto 16.5 per cent fruit set was recorded
10. Honeybees, ants, beetles, *Xylocopa*, small insects and birds were common pollinators.
11. Immature fruits are initially brick red, later green and turn yellow when ripe
12. The fruit is a drupe with smooth leathery shining pericarp, pulp and one strong seed. The average size of fruit is 3.10 x 9.26 cm
13. Oval shaped seeds tapering to one end. Seed coat is rough and hard.
14. Among the different explants tried, it was noticed that nodal segments gave higher response for both callusing and bud expansion
15. Among the different basal media tried MS medium was identified as the best media
16. Surface sterilization of the explants was carried out effectively by soaking the explants in 70 per cent alcohol for 30 seconds followed by soaking them in $HgCl_2$ 0.1 per cent for 4 minutes.
17. Increasing the time of exposure to chemicals more than eight minutes led to the death of explants.
18. On assessing the effect of cytokinins on direct regeneration, it was observed that higher concentration of BA and kinetin resulted in bud expansion.
19. When combination of growth regulators were used BA 5 mg/l and NAA 0.5 mg/l produced 80 per cent leaf bud expansion
20. Pronounced callus formation occurred when explants were inoculated on medium containing 2, 4-D at various levels (0.5 mg/l to 2 mg/l). The callus produced was friable in nature. BA upto 3 mg/l and kinetin upto 1 mg/l also led to callusing
21. When auxins were used in combinations, 2,4-D and NAA combination at 1 mg/l each was found to be very effective with a callus index of 160 in just 6 days

22. The callus obtained was sub cultured in different regeneration media like MS and WPM and among the different treatments tried with MS media it was noticed that BA at 5 mg/l alone resulted in good callus proliferation. The duration taken for callus proliferation was 30 days.

23. WPM was found to be more suitable for callus proliferation as the period taken for callus proliferation was almost half when compared with the duration taken for response in treatments containing MS media. WPM supplemented with adenine recorded the maximum callus proliferation.

REFERENCES

REFERENCES

Agboola, D.A.2000. Studies on the germination inhibitors in the fruits of four tropical tree species. *Global J.Pure Appl.Sci.* 6 (1): 27-30.

Ahuja, M.R.1983. Somatic cell genetics and rapid clonal propagation of aspergillus. *Silva Genetica.* 32: 131-135.

Ajithkumar, D. and Seeni, S. 1998. Rapid clonal multiplication through *in vitro* axillary shoot proliferation of *Aegle marmelos* (L.) Corr., a medicinal tree. *Pl. Cell Rep.* 17: 422.

Ambasta, S.P.1986.*The Useful Plants of India.* Council of Scientific and Industrial Research, New Delhi, 280 p.

Amin, M.N. and Jaiswal,V.S.1993. *In vitro* response of apical bud explants from mature tree of jack fruit (*Artocarpus heterophyllus). Pl.Cell Tissue Organ Cult.* 33: 59-65.

Ancora, G., Belli-Donini, M.L. and Cuozzo, l. 1981. Globe artichoke plants obtained from shoot apices through rapid *in vitro* micro propagation. *Sci.Hort.* 14: 207-213.

Anjum, P. and Qaiser, M. 2004. Pollen flora of Pakistan. Plantaginaceae. *Pakist. J. B.* 36 (1): 199-204.

Armstrong, E. J. 1986. Floral biology of *Myristica fragrans* Houtt. (Myristicaceae) the nutmeg of commerce. *Biotropica.*18(1): 32-38.

Ayensu, E.S.1981.Assessment of threatened plant species in the United States. In: Synge, H. (ed.), *The Biological Aspects of Rare Plants Conservation,* Wiley, New York, pp 9-58.

Bajaj, Y.P.S. 1978. Regeneration of haploid tobacoo plants from isolated pollen grown in drop culture. *Indian J.Exp.Biol.*16: 407-409.

*Balza, G. and Melendez, D. 2001. Evaluacion de diferentes mezelas de substrato para la propagacion vegetative del hibrido *E.grandis* X *E.urophylla* (urograndis) *Gmelina arborea.* Informe de investgacion No.36. Smurfit Carton de Venezuela. 4 p.

*Banerjee, A.K. 1989. Shrubs in tropical forest ecosystems: examples from India. *World Bank, Technical Paper* No: 103, The World Bank, Washington D.C.80p.

Bawa, K.S.1979. Breeding system of tree in tropical wet forest.*N.Z.J.Bot.*17: 521-524.

Bawa, K.S. and Webb, C.J. 1984. Flower, fruit and seed abortion in tropical forest trees. Implications for the evolution of paternal and reproductive patterns. *Amer. J. Bot.* 71 (5): 736-751.

Bein, E. 1996. *Useful trees and shrubs in Eritrea Regional Soil Conservation Unit (RSCU).* Eritrea Regional Soil Conservation Unit. Nairobi, Kenya, 250 p.

Bernardello, G., Aguilar, R. and Anderson, J. 2004. Reproductive biology of *Sophora fernandeziana* (Leguminosae), an vulnerable endemic species from Isla Robinson Crusoe. *Am. J. Bot.* 91(2): 198-206.

Bhakta, T., Mukerjee, P.K., Saha, K., Pal, M. and Saha, B.P.1998.Studies on antitussive activity of *Cassia fistula* (Leguminosae) leaf extract. *Pharma.Biol.* 36:140-143.

Bolstad, P.V. and Bawa, K.S. 1982. Self incompatibility in *G.arborea* (Verbenaceae). *Silvae Genetica.* 31:19-21.

*Bowen, M. R. and Euesbio, T.V.1983. *G.arborea* flowering and seed studies. *Forest Genet. Res. Inf.* 12: 27-28.

Brown, D.W. and Thorpe, T.A.1986. Plant regeneration by organogenesis. In: Vasil, I.K. (ed.), *Cell Culture and Somatic Cell Genetics of Plants-Plant Regeneration and Genetic Variability* Vol.3, Academy Press, New York, pp 49-66.

Bryndum, K. and Hedegrat, T. 1969. Pollination of teak (*Tectona grandis* L.). *Silvae Genetica.* 18(3): 57-96.

Capareda, E.P.1999. Plant Resources of South East Asia. No 12(1). *Medicinal and Poisonous Plants.* Prosea Foundation, Bogor and Indonesia, 502p.

Chadwell, T.B., Steven, J.W.and Philip, D.C.1992.Pollen morphology of *Phryma* and some putative relatives. *Systematic Bot.* 17 (2): 210-219.

Chavuin, J. E. and Salesses, G. 1988. Advances in chestnut micropropagation. *Acta Hort.* 277: 339-342.

*Chin, C.I., Chieng, H.T., Liu, C.C. and Huang, T.C. 1999. Microsporogenesis and exine sub structure in *Uraria crinita* (Fabaceae). *Grana.* 38(5): 277-283.

Chopra, R.N., Nayar, S.L. and Chopra, I.C. 1956. *Glossary of Indian Medicinal Plants*. CSIR, New Delhi, 300p.

Colman, G.D., and Ernst, D. 1989. *In vitro* shoot regeneration of *Populus deltoides,* effect of cytokinin and genotype. *Pl. Cell Rep*. 8: 459-462.

Conceicao, C.C.C., Mota, M.G.C., Nasmento, M.E. and Vieira, I.M.S.2002. Flowers, fruits and seedlings morphology of *Sacaca cajucara* Benth. (Euphorbiaceae). *Acta Hort.* 569: 167-171.

Conger, B.W.1981. *Cloning Agricultural Plants via In vitro Techniques*. CRC Press, Florida, 74p.

Das, T.1992. Micropropagation of *Agave sisalana. Pl. Cell Tissue Organ Cult.* 31 (3): 253-255.

Das, T. and Mitra, G.C. 1990. Micropropagation of *Eucalyptus teriticornis* . *Pl. Cell Tissue Organ Cult.* 22:95-103.

Das, S., Sharma, K and Agarwal, V.2005. *In vitro* clonal propagation of *Holarrhena antidysentrica* (L.) Wall through nodal explants from mature trees. *In Vitro Cell Developmental Biol. Pl.* 41(2):137-144.

Datta, K. and Datta, S.K.1983. Acezin induced regression of forest trees *Dalbergia sissoo* Roxb., through tissue culture. *Curr. Sci.* 52:432-436.

Datta, S.K., Chakrabarthy, D., Deepthi, Mandal, A.K., Misra, P. and Saxena, M. 2002. *In vitro* petal culture and callus formation in *Rosa* species. *Indian J. agric. Sci. 72*(5): 271-276.

Devi, Y.S., Mukherjee, B.B. and Gupta, S.1994. Rapid cloning of elite teak (*Tectona grandis*) by *in vitro* multiple shoot production. *Ind.J.Expt.Biol.* 32: 668-671.

Dhawan, V.1993. Tissue culture of hard wood species. In: Prakash, J. and Pierik, R.L.M. (eds.), *Biotechnology, Commercial Prospects and Problems.* Oxford and IBH Publishing Co. Pvt. Ltd. New Delhi, pp 43-65.

Dhiraj, N., Vartak, V. and Bhargava, S. 2003. Provenance and sub culture dependent variation during micropropagation of *Gmelina arborea*. *Pl.Cell Tissue Organ Cult.*73: 189-195.

Drewez, S.I. and Martinez, S.1999. Morphology of inflorescence in Verbenaceae-Verbenoidae11, Tribe Petreae. *Darwiniana.* 37(3-4): 209-218.

*Drury, W.H. 1974. Rare Species of Plants. *Rhodora.* 82 (3): 48.

D'Silva and D'Costa, L.1992. *In vitro* propagation of *Anacardium occidentale* L. *Pl.Cell Organ Cult.*29: 1-6.

*Dutta, G., Dutta, A., Das, N.D. and Ghosal, K.K.2004. Studies on floral biology of Isabgul (*Plantago ovata* F.) in new alluvial zone of West Bengal. *J. Inter Academician.* 8(4): 630-633.

Dvorak, W.S. 2004. World view of *Gmelina arborea*: opportunities and challenges. *New Forester.* 28 (2-3): 1-16.

Eeswara, J.P., Stuchbury, T., Allan, E.J. and Mordue, L. 1997. A standard procedure for the micro propagation of the neem (*Azadirachta indica*). *Pl. Cell Rep.* 17(3): 215-219.

Egea, J., Ortega, E., Canovas, J.A. and Dicenta, F. 2004. Pistil receptivity in self compatible almond cultivars. *Israel J. Pl. Sci.* 52(2): 149-153.

Egenti, L.C. 1978. Pollen and stigma viability in Teak (*Tectona grandis* L.f.). *Silvae Genetica.* 27(1): 29-32.

Einset, J.W. 1978. Citrus tissue culture stimulation of fruit explant cultures with orange juice. *Pl. Physiol.* 62: 885-888.

Etcheverry, A.V. and Aleman, C.E.T. 2005. Reproductive biology of *Erythrina falcata* (Fabaceae): Papillionoidae, *Biotropica.* (37): 54.

Forrest, G.J. 1969. Studies on the polyphenol metabolism of tissue cultures, derived from the tea plant (*Camellia sinensis* L.). *Biochem. J.* 113: 765-772.

Freitas, L. and Sasima, M. 2003. Floral biology and Pollination mechanism in two *Viola* species - from nectar to pollen flowers. *Ann. Bot. 45*: 311-317.

Gajbhiye, V.R., Dhoneekshe, B.L. and Janbhale, N.D.2006. *In vitro* root regeneration from leaf derived callus of the Indian snake root (*Rawolfia serpentina* (L.) Benth ex. Kurz.). *Indian J. Genet.* 66(2): 165-166.

*Galan, R. and Vargas, J. 2000. Tratmientos para estimulary homogenizer la germinacion en semillas de *Gmelina arboea.* Informe de Invesstigacion No.5 smurtit Carton Y Papl de Mexico, 15p.

*Gamboa, J.P. and Abdelnour, A.1999. Micropropagation of *Gmelina* (*Gmelina arborea* Roxb.). *Agronomia Costarricense.* 23 (1): 69-76.

Gamborg, O.L. and Shyluk, J.P. 1981.Nutritional media and characteristics of plant cell and tissue cultures. In: Thorpe, T.A. (ed.), *Plant Tissue Culture, Methods and Applications in Agriculture.* Academic Press. New York, pp 21-44.

Gangaprasad, A., Nair, L.G., Radhakrishnan, K., Seeni, S., Nair, G.M. and Pushpangadan, P. 2003. Micropropagation of *Utleria salicifolia* endemic

and endangered ethnomedicinal plant of the Western Ghats. *J. Medicinal Aromatic Pl. Sci.* 25: 19-24.

Gayathri, G.2005. Standardisation of *in vitro* Propagation Techniques in *thathiri (Woodfordia fruticosa* (L.) kurz.). MSc.(Ag.) thesis, Kerala Agricultural University,Thrissur,76p.

George, E.F. and Sherrington, P.O. 1984. *Plant Propagation by Tissue Culture.* Exegetics Limited, Basing Stoke, 690p.

Gibbs, P.E. and Bianchi, M.B. 1999. Does late acting self incompatibility show family clustering? Two more species of Bignoniaceae with LSI: *Dolichandra cyananchoides* and *Tabebuia nodosa. Ann. Bot.* 84:449-457.

Gill, R.T.S. and Gill, S.S. 1995. *In vitro* exudation of phenolics in *Eucalyptus* . *Indian For.* 120: 504-509.

*Greaves, A.1981. *Gmelina arborea. Forestry Abstracts.* Common wealth Forest Bureau, (42): 227-258.

Groulez, J. and Wood, P.J. 1985. *Terminalia superba, a monograph.* Center Technique Forestrier Tropical, France and Commonwealth Forestry Institute, England, 250p.

Gupta, P.K., Nagdgir, A.F., Mascarenhas, A.F. and Jagannathan, V. 1980. Tissue culture of forest trees-clonal propagation of *Tectona grandis* L. (teak) by tissue culture. *Pl.Sci.Lett.* 17:259-268.

Gupta, P.K., Mascarenhas, A.F. and Jagannathan, V. 1981. Tissue culture of forest trees: Clonal propagation of mature tree of *Eucalyptus citridiora* Hook. by tissue culture. *Pl. Sci. Lett.* 21:195-201.

Gupta, R.B., Sharma, S. and Goyal, S.C. 2002. Study on pollen morphology of genus *Ziziphus*. *J.Phytological Res.* 15(2): 217-220.

Hackett, W.P.1985.Control of phase change in woody plants. In: Little, C.H.A. (ed.), *Control of Shoot Growth in Trees,* Proceedings of the joint workshop of international union of forest research organization working parties on xylem and shoot growth physiology, Federictton, Canada, pp 257-272.

Harda, H. and Murai, Y.1996. Micro propagation of *Prunus mume*. *Pl.Cell Tissue Organ Cult.* 46:265-267.

Hasgawa, P.M. 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. *J. Am. Soc. Hort. Sci.* 105: 216-220.

Hedegrat, T. 1973. Pollination of Teak (*Tectona grandis* L.f). *Silvae Genetica.* 22:124-128.

Ho, L.C.L. and Lee, S.K. 1985. Micropropagation of *Lagerstroemia speciosa* (L.) Pers. (Lythraceae). *Gardens Bulletin.* 38(2): 175-184.

Hodge, G.R. and Dvorak, W.S. 2003. The CAMCORE international provenance progeny trials of *Gmelina arborea* genetic parameters and potential gain. In: Dvorak, W.S., Hodge, G.R., Woodbridge, W.C. and Romero, J.L. (eds.), *Recent advances with Gmelina arborea,* CD-ROM, CAMCORE, North Carolina University, Raleigh, North Carolina, USA.

Hogberg, P.1986. Nitrogen fixation and nutrient relations in Savanna wood land trees (Tanzania). *J. Appl. Ecol.* 233 (2): 675-688.

Hopkins, M.S. and Graham, A.W. 1989. Community phenological patterns of a low land tropical rain forest in north eastern Australia. *Aust. J. Ecol.* 14:399-413.

Hossain, M.A., Hossain, M.T., Ali, M.R. and Rahman, S.M.M.2005. Effect of different carbon sources on *in vitro* regeneration of Indian penny worts (*Centella asiatica*). *Pakist.J.Biological Sci.* 8(7): 963-965.

Hu, C.Y. and Wang, P.J.1983. Meristem, shoot tip and bud cultures. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Amada, Y. (eds.), *Hand book of Plant Cell Culture Vol.I-Techniques for Propagation and Breeding.* Mac Millan Publishing Co. NewYork, pp 177-227.

Hunter, C.S. 1979. *In vitro* culture of *Cinchona ledgerina. J.Hort.Sci.* 54: 11-114.

Jaisal, V.S. and Amin, M.N.1987.*In vitro* propagation of guava from shoot cultures of mature trees.*J.Pl.Physiol.* 130(1): 7-12.

John, C.K., Nadgadad, R.S. and Mascarenhas, A.F. 1997.*Teak-Tissue culture of Economic Plants*. Center for Science and Technology of Non Aligned and other Developing Countries, New Delhi and Common Wealth Science Council, London, pp 110-142.

Juncker, B. and Favre, J.M. 1989. Clonal effects in propagating oak trees via *in vitro* culture. *Pl.Cell Tissue organ Cult.* 19: 267-276.

Jung, K.H., Kwak, S.S., Kin, S.W., Lee, H., Choi, C.Y., and Lis, J.K.1992. Improvement of the *Catharanthine* productivity in hairy root cultures of *Catharanthus roseus* by using monosaccharide as a carbon source. *Biotech.Mol.Lett.* 14: 695-700.

Kamble, A.B., More, T.A., Karale, A.R. and Pattil, S.C. 2005. Callus culture and somatic embryogenesis studies in *Citrus aurantifolia* Swingle. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.), *Biotechnological Interventions for Improvement of Horticultural Crops :Issues and strategies,* Proceedings of ICAR National Symposium, 10-12 January, 2005, College of Horticulture, Kerala Agricultural University, Thrissur, pp 109-110.

Kannan, R.V. and Jasrai, Y.T.1996. Micropropagation of *Gmelina arborea. Pl. Cell Tissue Organ Cult.* 96(3): 269-271.

Karale, A.R., Kadam, J.H., Kamble, A.B., Kaledhonkar, D.P., Shinde, K.G. and Pattil, M.T. 2005. Micropropagation of *Emblica officinalis* Gaertn. through callus culture. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.), *Biotechnological Interventions for Improvement of Horticultural crops: Issues and strategies,* Proceedings of ICAR National Symposium, 10-12 January, 2005, College of Horticulture, Kerala Agricultural University, Thrissur, pp 111-113.

Kaul, M.K., Kumar, A. and Sharma, A. 2005. Reproductive biology of *Withania sominifera* (L.) Dunal. *Curr.Sci.* 83(90): 1375-1377.

*Keiding, H.1972. Seed orchards *Hevea* and teak. In: Proceedings on *Symposium on Seed Orchards*. Scandinavian Association of Geneticists, Denmark Forest Tree Improvement, 4:109-123.

*Kijar, S.B.2002. *G.arborea* Roxb. In: Vozzo J.A (ed.) *Tropical Tree Seed Manual,* Uspa Forest Vice, pp 476-477.

Kolammal, M. 1978. *Pharmacognosy of Ayuervedic drugs*. Thiruvananthapuram, No: 10, 450p.

Koshta, N. and Bansal, Y.K.2002. Shoot tip culture for micropropagation of medicinal plant *surpagandha* (*Rauvolfia serpentina* Benth.Ex kurz.). *J.Phytological Res.* 15(1): 95-99.

Krishnan, P.N. and Seeni, S.1994. Rapid micropropagation of *Woodfordia fruticosa* (L.) Kurz. (Lythraceae), a rare medicinal plant. *Pl. Cell Rep.* 14(1): 55-58.

Kruckeberg, A.R. and Rabinowitz, D.1985.Biological aspects of endemism in higher plants.*Annu.Rev.Ecol.Syst.*16: 447-479.

Kumar, S. and Kumar, S.1994. Seed damage of tree *Terminalia arjuna* Bedd. by rose ringed parakeet (*Psitacula krameri* (Scopali). *Indian J. For.* 17(2): 151-153.

Kumar, R., Sharma, K. and Agarwal, V. 2005. *In vitro* clonal propagation of *Holarrhena antidysentrica* (L.) Wall through nodal explants from mature trees. *In vitro Cell. Developmental Biol.* 41 (2): 137-144.

*Larrea, G.R., Hernandez, V.J. and Laguna, R. 2000.Treatments to stimulate and homogenize germination in seeds of *Gmelina arborea* Roxb. *Revista Chapingo Serie Ciencias Forestales y del Ambiente.* 6(1): 21-28.

Lee, B. C., Lee, S.K. and Kim, J.H.1987.*In vitro* mass propagation of *Lagerstroemia indica* f. alba Rehder. *Research Report.* Institute of Forest Genetics, Korea Republic, 23: 128-137.

Little, J.E.L. 1983. *Common Fuel Wood Crops: A handbook for their Identification.* Mc Clain Printing Co. Parsons, WV, 180p.

Little, E.E. and Wadsworth, F.H.1964. Common trees of Puerto Rico and the Virgin Islands. *Agricultural Hand Book* No: 249.US Department of Agriculture, Washington DC. 315p.

Lixiu, G., Zhao, F., W. and Jie, Y.H. 2004. Pollen morphology of the genus *Calamus* L. from China. *J.trop. Subtropical Bot.* 12(6): 515-520.

*Lloyd, B. and Mc Cown, B. 1980. Commercially feasible micro propagation of mountain laurel *Kolima latifolia* by use of shoot tip culture. *Comb.Proc.int.Pl.Prop.Soc.* 30:421-427.

Lonnie, N.D.1991. The status of *Gmelina moluccana* in the Solomon Islands. *Forest Research Note* No: 72, Solomon Islands Forestry Division, 11p.

Mabberley, D.J.1997. *The Plant Book.* Cambridge University Press, Cambridge, UK, 858p.

Mactarlane, T.D., Watson, L. and Marchant, N.G. 2002. Western Australian Genera and Families of Flowering plants. *Western Australian Herbarium.* Available: http://florabase. calm. wa. gov. au. 11.7.2006

Mahale, S.M., Karale, A.R., Pattil, S.C., Kadam, J.H., Kamble, A.B., Khirsagar, D.B. and Desai, U.T.2005. Influence of season of explant collection on induction of multiple shoots from axillary buds of the tamarind. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Kesavachandran, R., Joseph, L. and John, P.S. (eds.), *Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies,* Proceedings of ICAR National

Symposium, 10-12 January, 2005. College of Horticulture, Kerala Agricultural University, Thrissur, pp 168-169.

Mallika, V.K., Sankaran, A.M., Sindhu, K., Rekha, C., Vijayakumar, N.K. and Nair, R.V. 1992. Plantlet Regeneration *in vitro* from nodal segments of cocoa (*Theobroma cocoa* L.) and field planting. *J. Plantn. Crops*. 20:114-122.

Martin, S.M. and Rose, D. 1978. Growth of plant cell (*Ipomea spp.*) suspension cultures at controlled pH levels. *Can.J.Bot.* 54:1264.

*Marri, K.A.L., Duron, M., Arnaud, Y. and Miginaic, E. 1986. Comparative study on the response to micropropagation of adult Passe Crassane pears and seedlings in meristem culture. Competes Rendus Academy, *Agric.France.* 72(5): 413-421.

Mascarenhas, A.F., Kendurkar, S.Y. and Khuspe, S.S.1993. Micropropagation of Teak. In: Ahuja, M. R. (ed.), *Micropropagation of woody plants*. Kluwer Academic Publishers, London. PP 247-267.

Mathew, S.1995. Standardisation of *in vitro* propagation technique in clove (*Syzigum aromaticum* (L.) Merr. and Perry. MSc.(Ag.) Thesis. Kerala Agricultural University, Thrissur, 131p.

Mathew, M.K.and Hariharan, M. 1990. Micropropagation in cloves (*Syzigium aromaticum* (L.) Merr. and Perry). *J.Plantn. Crops*.20: 308-309.

Mathew, M.K., Francis, M.S. and Hariharan, M. 1987. Development of callus in cloves (*Syzygium aromaticum* (L) Merr. and Perry). *J.Plantn.Crops.*15(2): 123-125.

Mathias, P.J. and Anderson, P.G. 1987. Bacterial contamination in tropical hard wood cultures. *Acta Hort.*12: 43-48.

Mc Comb, J.M. and Bennet, I.S. 1982.Vegetative propagation of *Eucalyptus* using tissue culture and its application to forest improvement in western Australia. In: Fujiwara, A. (ed.), *Plant Tissue Culture*, Japanese Association of Plant Tissue Culture, Tokyo, pp 721-722.

Mahatre, S.P., Dhumale, D.B. and Tule, D.S.2004. Response of axillary buds in various culture conditions for *in vitro* growth in sarpagandha. *Ann.Pl.physiol.*18(2):136-138.

*Mindawati, N. and Rohayat, N. 1994. Effect of fruit colour of Gmelina arborea L. on germination and seedling growth. *Bulletin Penelitian Hutan,* No. 560, pp 43-54.

Muralidharan, E.M. and Mascarenhas, A.F. 1989. Tissue culture of forest trees in India.*Curr.Sci.*58: 606-612.

Murashige, T.1977. Clonal propagation of horticulture crops through tissue culture. In: Bars, W., Reinhard, E. and Zenik, M.H. (eds.), *Plant Tissue Culture and its Biotechnological Application,* Springer Verlag, New York, pp 392-403.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol.Pl.* 15:487-497.

Naik, D., Vartak, V. and Bhargava, S. 2003. Provenance and subculture dependent variation during micropropagation of *Gmelina arborea. Pl. Cell Tissue Organ Cult.* 73:189-195.

Nair, L.G. and Seeni, S.2003. *In vitro* multiplication of *Calophyllum apetalum* (Clusiaceae) an endemic medicinal tree of the Western Ghats. *Pl. Cell Tissue Organ Cult.* 75(2): 169-174.

Nazeem, P.A., Smitha, P.S., Thomas, J. and Rathy, K. 2005.*In vitro* shoot regeneration in eagle wood-*Aquilaria agallocha* Roxb. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L. and John, P.S. (eds.), *Biotechnological Interventions for Improvement of Horticultural Crops: Issues and strategies,* Proceedings of ICAR National Symposium, 10-12 January, 2005. College of Horticulture, Kerala Agricultural University, Thrissur, pp 118-119.

Neusa, T. and Gibbs, P.E.2005. Floral biology and breeding system of *Sterculis chica* St.hil. (Sterculaceae). *Botanical J. Linnean Soc.*1: 49-69.

Paily, J.and D'Souza, C.1986. *In vitro* clonal propagation of *Lagerstroemia flosreginae* Retz. *Pl. Cell Tissue Organ Cult.* 6(1): 41-45.

Pandey, S., Bisht, S. and Jamaluddin,P .2002. Pericarp colour an indicator for seed quality in *Gmelina arborea* Roxb. *Indian Forester.* 128(4): 473-476.

Parveen, Prabha, B., Sharma, V.K. and Mandal, A.K.2005. *In vitro* propagation through epicotyl explants of *Oroxylum indicum* Vent. *J. Non Timber Forest products.* 12(3): 123-126.

Pattnaik, S.K. and Chand, P.K.1997. Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl., *Morus lhoukoiz* and *Morus serrata* Roxb., through *in vitro* culture of apical shoot buds and nodal explants from mature trees. *Pl.Cell Rep.* 16: 503-508.

Pooll, P.A. and Bermawiel, U.1986. Floral biology in the Zanzibar type clove in Indonesia. *Euphytica.* 35(1): 217-223.

Punjani, B.I. and Kumar, V. 2002. Traditional medicinal plant remedies to treat cough and asthmatic disorders in the Aravelli ranges in Northern Gujarat, India. *J.Nat. Remed.* 2 (2): 173-178.

Rachmawati, H., Irantoni, D. and Hansoen, C.P.2002. *Gmelina arborea* Roxb. *Seed leaflet,* No. 62, Danida Forest Seed Centre, Humleback, Denmark.

Rajmohan, K. and Kumaran, M.N. 1988. Influence of explant source on the *in vitro* propagation of jack (*Artocarpus heterophyllus* Lam.).*agric.Res.J* .,Kerala. 26(2): 169-174.

Rahman, M.A. and Blake, J. 1988. Factors affecting *in vitro* proliferation and rooting of shoots of jack fruit (*Artocarpus heterophyllus* Lam.). *Pl.Cell Tissue Organ Cult.*13: 179-187.

Raju, J.S. and Rao, S.P. 2006. Pollination by bees and Passerine birds and seed dispersal by monkeys in the white teak, *G. arborea*, a commercially important timber tree species in the eastern Ghats. *Curr. Sci.* 90(2): 232-236.

Ram, P. and Prasad, R.1991.Mahua: The tree of the poor. *J. trop. For.*7(3): 171-179.

Rao, S.P. and Raju, A. J.S.2002.Pollination ecology of Red Sanders *Pterocarpus santalinus* (Fabaceae), an endemic and endangered tree species. *Curr. Sci.* 8(9): 1144-1148.

Rao, D.V. and Rao, E.V.1970. Chemical components of leaves of *Gmelina arborea*. *Indian.J.Pharmacy.* 32:140-141.

Rao, B. C. and Reddi, S. C. 1994. Reproductive biology of *Cochlospermum religiosum. J.trop. Ecol.* 35:209-218.

Reddi, C.S. 1976. Floral mechanism, pollen productivity and pollen incidence in *Madhuca indica* Gmelin. with remarks on the mode of pollination. *New Botanist.* 3(1-2): 11-16.

Roubik, D.W. 1995. Pollination of cultivated plants in the tropics. *FAO Agricultural Services Bulletin,* 118p.

Roy, K.S.1995. *In vitro* micro propagation techniques of high quality planting stock of some timber trees. *Pl. Tissue Cult.* 5: 88.

*Sanchez, L.A. and Diaz, J. 1998.Crecimiento inicial en campo de *Gmelina arborea* producida en diferents tipos de envase.informe de Investigacion No.9.Smurfit Carton de Venezula, 11p.

Sanenz, C. L.A., Mardonado, I.E. Mendoza, O., Moraro V., Ciavitz, R., Meyer, L.M. and Logola, V.M. 1993. Effect of medium pH on release of secondary metabolites from roots of *Datura, Stramonium, Catharanthus roseus* and *Tagetes patula* cultured *in vitro. Appl.Biochem.Biotechnol.* 38:257-267.

Sasidharan, K.R., Nagarajan, B., Varghese, M., Nicodemus, A., Mahadevan, N.P., Durai, A. and Gireesan, K. 1999. Insect pollination versus enhanced fruit production in *Tamarindus indicus* and *Tectona grandis. J. Palynology.* 35:93-97.

Schenk, R.V. and Hildebrandt, A.C.1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can.J.Bot.* 50:199-204.

*Scott, E.S., Rao, A.N. and Loh, C.S. 1990. Tissue culture of *Dipterocarpous. Bulletin of National University.* Singapore, 20:175-179.

Sedgley, M. and Griffin, A.R. 1989. *Sexual Reproduction of Tree Crops.* Academic Press, London, 250p.

Sen, J., Islam, M.S., Roy, S.K. and Hadiuzzman, S.1992. Micropropagation of juvenile and adult *Gmelina arborea. Pl.Tissue Cult.* 2: 89-95.

Sharma, R. 2004. *Agro techniques for Medicinal Plants.* Daya Publishing House, New Delhi, 265p.

Sharma, K.K. and Thorpe, T.A. 1990. *In vitro* propagation of mulberry (*Morus alba* L.) through nodal segments. *Sci.Hort.* 42:307-320.

Sharma, A., Bhatia, S.K., Yadev, N.R. and Sharma, S. 2005. Effect of various modifications of MS medium on shoot induction and proliferation of peach. In: Rao, G.S.L.H.V.P., Nazeem, P.A., Girija, D., Keshavachandran, R., Joseph, L., and John, P.S. (eds.), *Biotechnological Interventions for Improvement of Horticultural Crops: Issues and strategies*, Proceedings of ICAR National Symposium, 10-12 January, 2005. College of Horticulture, Kerala Agricultural University, Thrissur, pp 114-115.

*Silva, S.N., Carvaiho, A.M.V. and Santos, F.A.R. 2003. Pollen morphology of F_2 (*Cesturm* L. (Solanaceae) species from hygrophilous forest in Bahia, Brazil. *Acta Scientianum Biological Sciences,* 25(2): 439-443.

Singh, V.K. and Khan, A.M.1990. *Medicinal Plants and Folklores.* Today and Tomorrow's Printers and Publishers, New Delhi, 250p.

Singh, V.K., Kaur, A. and Gupta, R.C. 2003. Floral biology, pollination mechanism and breeding systems in *Acacia nilotica. J.Tree Sci.* 22 (1&2): 1-8.

Sivarajan, V.V. and Indira, B. 2004. *Ayuervedic drugs and their Plant Sources*, Oxford and IBH Publishing Co. Pvt. Ltd. New Delhi, 570p.

*Souza, L.A., Mourao, K.S.M., Moscheta, I.S.and Rosa, S.M. 2003. Floral morphology and anatomy of *Pilocarpous pennatifolius* Lm. (Rutaceae). *Revista Brasileria de Botanica*. 26(2): 175-184.

Srivastava, K.K. and Singh, H.K. 2000. Floral biology of bael (*Aegle marmelos*) cultivars. *Indian J. Agric. Sci.* 70(11): 797-798.

Stevensons, J.H. and Haris, R.E. 1980. *In vitro* plantlet formation from shoot tip explants of *Fuchsia hybrida* var. Swing time. *Can.J.Bot.* 58:2190-2199.

Stock, J., Vargas, M., Angarita, K., Gonzalez, R. 2004. Seed production of *Gmelina arborea* by controlled pollination. *New Forest*.28:167-177.

Sudha, C.G. and Seeni, S. 1994. *In vitro* field multiplication and field establishment of *Adathoda beddomei* C.B.Clarke, a rare medicinal plant. *Pl. Cell Rep.* 13(3-4): 203-207.

Sukartiningsih, S., Nakamura, K. and Yuji, I. 1999. Clonal propagation of *Gmelina arborea* Roxb. by *in vitro* culture. *J.For.Res.* 4:47-51.

Sumana, K.R. and Kaveriappa, K.M. 2000. Micro propagation of *Lagerstroemia reginae* Roxb. through shoot bud culture. *Indian J.Pl.Physiol.* 5 (3): 232-235.

Surendran, C. 1990. Vegetative propagation of *Gmelina arborea* Roxb. through single nodded cuttings. *Indian J.For.* 13:162-164.

Swapna, T.S., Binitha, M. and Manju, T.S. 2004. *In vitro* multiplication in *Kaempferia galanga* Linn. *Applied Biochemistry and Biotech.*118 (1/3): 233-242.

Swedlund, B. and Locy, R.D. 1993. Sorbitol as the primary carbon source for the growth of embryogenic callus of maize.*Pl.Physiol.* 103:1339-1346.

Tang, W., Whetten, R. and Sedoroff, R. 2001. Genotypic control of high frequency adventitious shoot regeneration via somatic organogenesis in lobbly pine. *Pl.Sci.*161: 267-272.

Tangmitcharroen, S. and Owens, J.N. 1997. Floral biology, pollination, pistil receptivity and pollen tube growth of teak (*Tectona grandis* Linn. f.). *Ann. Bot.* 79 (3): 2278-241.

Tangmitcharroen, S., Owens, T. N., Dieters, M.J., Matheson, A.C., Nickels, D.G., Harwood, C.E., Walker, S.M. 1996. Floral biology, pollination and pollen tube growth in relation to low fruit production of teak (*Tectona grandis* linn.f) in Thailand. In: *Tree improvement for sustainable tropical forestry.* QFRI-IUFRO conference, 27 October - 1 November, Caloundra, Queensland, Australia, pp 265-270.

*Tebbs, M.C. 1982. *Verbenaceae*: *An enumeration of flowering plants of Nepal.* Trustee of British Museum (Nat. History), London, 298p.

Tewari, D.D. 1995. *A monograph on GAMARI (G.arborea Roxb.).* International Book Distributors, Dehradun, 126 p.

Thakar, J. and Bhargava, S. 1999. Seasonal variation in antioxidant enzymes and the sprouting response of *Gmelina arborea* Roxb. nodal sectors cultured *in vitro. Pl.Cell Tissue Organ Cult.* 59(3): 181-187.

Thampman, P.K.1993. *Trees and Tree Forming Peekay Tree crops*. Development Foundation, Kerala, 250p.

Thirunavoukkarasu, M. and Debata, B. K. 1998. Micro propagation of *Gmelina arborea* Roxb. through axillary bud culture. *Indian J.Pl.Physiol.* 3 (2): 82-85.

Vijayakumar, N.K., Ferret, P.P. and Sharik, T.L. 1990. *In vitro* propagation of the endangered Virginia round leaf birch (*Betula uber*) using dormant buds. *For. Sci.* 36(3): 842-846.

Von, A.S. and Eriksson, T.1981. *In vitro* studies of adventitious shoot formation in *Pinus contorta*. *Can.J.Bot.* 59:870-874.

Vu, J.C.V., Niedz, K.P. and Yelensky, G. 1993. Glycerol stimulation of chlorophyll synthesis, embryogenesis and carboxylation and sucrose metabolism enzymes in nucellar callus of Hamlin sweet orange. *Pl.Cell Tissue Organ Cult.* 50:39-44.

Wang, Z.2003. Cultivation and Utilization of *G.arborea* in South Yunnan, China. In: Dvorak, W.S., Hodge, G.R., Woodbridge, W.C. and Romero, J.L. (eds.), *Recent advances with Gmelina arborea,* CD-ROM, CAMCORE, North Carolina University, Raleigh, North Carolina, USA.

*Waser, N.M., Montalvo, A.M. and Gray, R.N. 1987. Female, male choice in a perennial herbaceous wild flower, *Delphinium nelsonii. Evolutionary Trends in Plants*. 1:29-33.

Wong, C.Y. 1987. Flower stimulation techniques for mature *G.arborea* trees. In:The International Workshop on *Reproductive Ecology of Tropical Forest Plants,* 8-12 June, 1987, Bangi, Malaysia, 14p.

Yang, J.C., Tsay, J.Y., Chung, J.D. and Chen, Z.Z. 1992. *Micropropagation of Gmelina arborea R.*, Special Publication, Taichung District Agricultural Improvement Station, 29: 213-218.

Yang, J.C., Tsay, J.Y., Chung, J.D. and Chen, Z.Z. 1993. *In vitro* clonal propagation and cell suspension culture of *Gmelina arborea. Research Bulletin.* Taiwan, Forestry Research Institute, 1:1-9.

Yasodha, R., Ghosh, M., Barthwal, S. and Gurumurthi, K. 2004. Importance of biotechnological research in tree species of Dashamula. *Indian Forester.*130(1): 79-88.

*Yu, T.Y.B.1991. Study on some factors in tissue culture of Lychee (*Litchi chinensis*). *Fujian agric.Sci.Tech.* 10(5): 17-18.

Yupa, D.A. and John, N.O.B. 2002. The Reproductive Biology and Reproductive Success of *Pterocarpus macrocarpus* kurl. *Biotropica.* 34(1): 58-67.

Zaid, A. and Tisserat, B.H. 1983. *In vitro* shoot tip differentiation in *Phoenix dactylifera* L. *Date palm J.* 2(2): 163-182.

Zeaser, D. 1998. *Vegetative propagation of melina (Gmelina arborea Roxb.).*CAMCORE International Tree Breeding Short Course Book. North Carolina State University, Raleigh, North Carolina, USA.140p.

Ziv, M. 1986. *In vitro* hardening and acclimatization of tissue culture plants. In: Winther, L.A. and Alderson, P.G (eds.), *Plant Tissue Culture and its Agricultural Application,* Butterworths, London, pp 187-196.

Ziv, M. 1995. *In vitro* acclimatization. In. Aitken, C.J., Kozai, T. and Smith (eds.), *Automation and Environmental control in Plant Tissue Culture,* Butterworths, London, pp 493-516.

Ziv, M. and Halevy, A.H.1983. Control of oxidative browning and *in vitro* propagation of *Strelitzia reginae*. *Hort. Sci.* 18(4): 434-436.

Zou, P., Xiang, Z.D. and Ping, Z.T. 2003. Pollen morphology of *Bauhinia species* endemic to China (Caesalpiniaceae). *J. trop. Subtropical Bot.* 11(3):249-254.

* originals not seen

APPENDICES

Appendix I. Preparation of MS medium stock solution

Chemicals	Stock concentration	mg/l stock solution	ml/l media
Stock 1	50 x		20
$(NH_4)NO_3$		82500	
KNO_3		95000	
KH_2PO_4		8500	
$MgSO_4.7H_2O$		18500	
Stock 2	50x		20
$CaCl_2.2H_2O$		11000	
Stock 3	100x		10
Na_2EDTA		3700	
$FeSO_4.7H_2O$		2800	
Stock 4	100x		10
$MnSO_4.4H_2O$		2230	
$ZnSO_4.7H_2O$		860	
H_3BO_3		620	
KI		83	
$Na_2MoO_4.2H_2O$		25	
$CuSO_4.5H_2O$		2.5	
$CoCl_2.6H_2O$		2.5	
Stock 5	100x		10
Glycine		200	
Nicotinic Acid		50	
Pyridoxine HCl		50	
Thiamine HCl		10	

Iron Stock Preparation

A quantity of 3.725 g of Na_2 EDTA and 2.785 g of $FeSO_4.7H_2O$ was dissolved separately in 100 ml of double distilled water. Both solutions were warmed up gently. The hot solution of Na_2 EDTA was added to the hot solution of $FeSO_4.7H_2O$ and the final volume is made upto 1000 ml.

Appendix II. Preparation of SH medium stock solution

Chemicals	Stock concentration	mg/l stock solution	ml/l media
Stock 1	100x		10
$(NH_4)\ H_2PO_4$		30000	
KNO_3		250000	
Stock 2	100x		10
$CaCl_2.2H_2O$		20	
Stock 3	100x		10
$MgSO_4.7H_2O$		40000	
Stock 4	100x		10
$FeSO_4.7H_2O$		1500	
Na_2EDTA		2000	
Stock 5	100x		10
$MnSO_4.H_2O$		1000	
$ZnSO_4.7H_2O$		500	
H_3BO_3		10	
$Na_2MoO_4.2H_2O$		20	
$CuSO_4.5H_2O$		10	
$CoCl_2.6H_2O$			
Stock 6	100x		10
Nicotinic Acid		500	
Pyridoxine HCl		50	
Thiamine HCl		500	

Appendix III.Preparation of WPM stock solution

Chemicals	Stock concentration	mg/l stock solution	ml/l media
Stock 1	50x		20
K_2SO_4		49500	
$(NH_4)NO_3$		20000	
KH_2PO_4		8500	
$MgSO_4.7H_2O$		18500	
Stock 2	50x		20
$Ca\ (NO_3)_2.4H_2O$		27.8	
Stock 3	50x		20
$CaCl_2.2H_2O$		4800	
Stock 4	100x		10
Na_2EDTA		3700	
$FeSO_4.7H_2O$		2800	
Stock 5	100x		10
$MgSO_4.7H_2O$		37000	
$ZnSO_4.7H_2O$		860	
H_3BO_3		620	
$CuSO_4.5H_2O$		25	
$Na_2MoO_4.2H_2O$		25	
Stock 6	100x		10
Glycine		200	
Pyridoxine HCl		50	
Nicotinic Acid		50	
Thiamine HCl		100	

STUDY OF REPRODUCTIVE BIOLOGY AND *IN VITRO* PROPAGATION TECHNIQUES IN *KUMIZHU* (*Gmelina arborea* Roxb.)

By

SANI GEORGE

ABSTRACT OF THE THESIS

submitted in partial fulfilment of the requirement
for the degree of

Master of Science in Agriculture

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Department of Plant Breeding and Genetics

COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2007

ABSTRACT

The present study 'Study of reproductive biology and *in vitro* propagation techniques in *kumizhu*' was undertaken in the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara during 2004-2006. The study comprised of two major experiments namely (i) Studies on reproductive biology and (ii) Studies on micro propagation

The trees started blooming in January when new flushes sprouted out and continued upto the end of March. Inflorescence is a terminal cyme blooming in an irregular manner. The flowers are short stalked, pubescent, large, bisexual and scented. Calyx persistent, tubate and five lobed at tip. Corolla brownish yellow and has a short tube with the upper tip formed by two lateral petals. Stamens four, epipetalous. Anthers dithecous and dehisce longitudinally. Ovary bicarpellary, style long. Stigma short with two unequal lobes. Anthesis from late mid night to 3.00 am. Stigma receptive during anthesis and the receptivity lasts for 4-5 hours. Fruit set was absent under artificial selfing while under natural cross pollination upto 16.5 per cent fruit set was recorded. Fruit drupe with one strong seed. Seeds oval shaped, tapering to one end.

MS medium was best medium suited for callus induction and bud expansion. Nodal segments were the explants for direct organogenesis and for indirect organogenesis. Surface sterilization was done by soaking explants in 70 per cent alcohol for 30 seconds, followed by soaking in $HgCl_2$ 0.1 for 4 minutes. MS+BA 5 mg/l+NAA 0.5 mg/l produced leaf bud expansion. Pronounced callus formation was observed when explants were inoculated on medium containing 2, 4-D at various levels (0.5 mg/l to 2 mg/l). BA upto 3 mg/l and kinetin upto 1 mg/l induced callusing. WPM was found to be more suited for callus proliferation as the time for response was almost half of that of MS medium. WPM supplemented with adenine recorded maximum callus proliferation.