In vitro regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg. cv. RRII 105) from immature anther explants via somatic embryogenesis

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By

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Declaration

I hereby declare that the thesis entitled "***In vitro* regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg. cv. RRII 105) from immature anther explants via somatic embryogenesis"** submitted to the M.G. University, Kottayam, for the award of the degree of **Doctor of Philosophy in Botany**, is the result of the research work carried out by me at the Biotechnology Division under the supervision of Dr. A. Thulaseedharan, Deputy Director (Biotechnology), Rubber Research Institute of India, Kottayam. I further declare that the thesis has not been previously formed the basis for the award of any degree.

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CERTIFICATE

This is to certify that the thesis entitled **"*In vitro* regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg. cv. RRII 105) from immature anther explants via somatic embryogenesis"** is an authentic record of original research work carried out **by Smt. P. Kumari Jayasree.**, at the Rubber Research Institute of India, Kottayam- 686 009 under my supervision for the award of the degree of **Doctor of Philosophy in Botany** under the Faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in this thesis has not been submitted earlier for any other degree or diploma.

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Abstract

With the ever increasing demand of natural rubber on one hand and the major limitations of conventional breeding on the other, efforts on research and developments are directed towards rubber breeding programmes to generate improved clones through biotechnology. Advances in cellular and molecular biology opened up an alternative and new avenue for crop improvement by the introduction of DNA transfer technology. However, the utilization of this technology to plant breeding is dependent on the availability of an efficient *in vitro* regeneration pathway compatible to the gene transfer method. Plant regeneration through somatic embryogenesis is currently receiving much attention, since this is a very useful technique for the exploitation of the natural gene transfer system mediated by *Agrobacterium*. In view of the above, an attempt has been made to develop a high frequency plant regeneration system through somatic embryogenesis. The present work has also made attempts on the histological, molecular and biochemical aspects of somatic embryogenesis.

The suitability of utilizing immature anther as explants were tried out and proved as an ideal explant for somatic embryogenesis. Of the four auxins, 2, 4-D, NAA, IAA and IBA tested, 2, 4-D was found as the potent auxin for callus induction. Maximum callus induction was obtained on modified MS medium supplemented with 2.0 mg/l 2, 4-D and 0.5 mg/l KIN. Among the four auxins tested on embryo induction, NAA showed a positive response and maximum number of embryos were produced on medium supplemented with 0.2 mg/l NAA and 0.7 mg/l KIN.

Explant pretreatment in liquid medium reduced the duration of callus induction. Immature anthers pretreated in liquid medium for 10 d followed by 25 d culturing on solid callus induction medium was found to be the most suitable ideal time. After primary callus induction, when the calli was maintained in 2, 4-D containing callus induction medium for another 15 d (35 d + **15 d**), influenced embryogenic capacity of callus and was found as the ideal time of callus transfer for embryogenesis. Polyamines had no significant stimulatory effect on embryogenesis. Inclusion of amino acids such as alanine and arginine showed no effect on enhancement of embryo induction. However, with asparagine, all tested concentrations allowed embryo differentiation similar to that of control. Addition of glutamine increased embryogenesis and maximum response was obtained at higher concentration

(200 mg/l). Casein hydrolysate also improved embryogenesis and maximum number of embryos was produced when medium was enriched with 400 mg/l casein hydrolysate.

Mature embryos were converted into plantlets and 27% of embryos were germinated into full plantlets on hormone free medium. Inclusion of cytokinins combined with GA_3, the somatic embryo germination and plantlet development was significantly enhanced. Of the four cytokinins (BA, ZEA, KIN, TDZ) tested, the response of TDZ at 0.25 mg /l concentration was found to be more effective for embryo germination as well as plant regeneration followed by BA and ZEA. Response with KIN was found to be low compared with other cytokinins.

A subculture interval of 50 d was found to be ideal for long term maintenance of embryogenic cultures. Using embryogenic callus aggregates, the embryogenic potential could be maintained up to 3 years. When proline was included in embryo induction medium, embryo induction frequency was increased with maximum being at 100 mg/l. Charcoal was found to be essential for long term maintenance of cultures and embryogenesis was reached maximum at 0.1% concentration. Embryogenic lines could also be maintained by inducing secondary embryos. Among different developmental stages of embryos, secondary embryos were higher in immature cotyledonary stage embryos.

The studies on incubation conditions indicated that *Hevea* needed continuous darkness till the acquisition of embryogenic competence or embryo induction. For embryo development and maturation as well as plant regeneration, light is a critical factor.

During hardening, non sterile potting mixture was found unsuitable. Of the three sterile potting mixtures, highest survival was obtained with sand: soil: cowdung mixture. IBA pretreated plantlets also survived well with a higher survival rate. SEM studies revealed that the amount of wax was low in leaves of weak plants. With healthy plants, epicuticular wax was started to appear during pre hardening and deposition was continued to increase during and after hardening.

Histological examinations indicated that embryogenic calli consisted of small cells with prominent nuclei and a thickened outer wall. In contrast, non embryogenic cells were characterized by the large size containing prominent nuclei and thin cell wall. Histochemical examinations revealed that at early stage, embryogenic calli was

filled with few starch grains, lipid and protein bodies. At late stage, almost all cells were densely accumulated with starch and number and quantity of lipids and protein bodies were more abundant. In contrast, non embryogenic calli were poorly filled with starch grains and both lipid content and protein bodies were less in number or quantity.

Cytological studies showed that all the tested plants having a chromosome number of 2n= 36. RAPD profiles of somatic plants detected no variation with all tested primers, while with the same primers, monoclonal seedlings showed detectable variation. Results of cytogenetic studies revealed no alteration in chromosome number from 3 year old callus cultures. RAPD analysis of long term embryogenic callus cultures displayed genetic similarity with short term callus as well as mother plant.

Electrophoretic analysis of protein profiles of different developmental stages of somatic embryogenesis displayed variation in their protein profiles. Among the stages, more protein bands were observed in embryos. Embryogenic calli and non embryogenic calli significantly varied in their protein profiles. No clear protein bands were observed in non embryogenic calli. The protein profiles of somatic embryos at different stages of development revealed that the overall pattern of proteins was similar in all 3 embryos, however, varied in their relative expression. Peroxidase zymogram at the five sequential stages revealed increased activity on embryogenic calli as well as in embryos. Three types of embryos exhibited same banding pattern with an increase in peroxidase activity. With esterase enzyme, embryos and plantlets had a very similar esterase pattern. Among the 3 types of embryos, globular embryos showed less activity and when reached to later stages such as torpedo and cotyledonary, embryos showed maximum activity.

Key Words

Acclimatization, Callus induction, Genetic transformation, *Hevea brasiliensis*, Immature anther, *In vitro* culture, Long term embryogenesis, Plant regeneration, Rubber, Secondary embryogenesis, Somatic embryogenesis, Somaclonal variation

Preface

Since 1958, with the beginning of differentiation of somatic embryos from carrot cell cultures, this phenomenon has recognized as an efficient method for regeneration of whole plant from somatic cell. In many crop plants, somatic embryogenesis has been regarded as the *in vitro* system of choice with the potential of mass propagation, protoplast work and synthesis of artificial seeds. Recently, there has been an increasing interest in the induction of somatic embryogenesis especially by the advent of genetic transformation.

In *Hevea*, during the past few decades, although great progress has been made for crop improvement, the narrow genetic base is a strong limitation for classical breeding. Further, the perennial nature of the crop and long breeding cycle makes it imperative to seek unconventional methods including genetic engineering. A reliable plant regeneration pathway via somatic embryogenesis is one of the prerequisite for genetic manipulation studies. The present study is an effort to develop a high frequency plant regeneration system through somatic embryogenesis. The protocol developed in the study should serve as a valuable breeding tool for genetic improvement of *Hevea* in the future.

The contents of the thesis are arranged in six chapters. The first chapter introduces the topic and describes the main objectives of the study. The second chapter provides an opportunity to give a brief overview of the crop and basic topics like plant tissue culture and somatic embryogenesis. This chapter also covers the review of the related work presented in the thesis. The experimental details and procedure followed in the study are given in the third chapter. The fourth chapter explains the results of the experiments and results obtained were discussed in the fifth chapter. In the sixth chapter the results are concluded and summarize the conclusion and prospects of the study.

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Abbreviations

ABA	:	Abscisic acid
ALF	:	Abnormal leaf fall
APS	:	Ammonium per sulfate
BA	:	6-Benzyladenine
CHAPS	:	3–[3–(cholamidopropyl) dimethylammonio]-l-propane sulfonate
d	:	Day (s)
°C	:	Degree centigrade
dNTPs	:	Deoxynucleotide triphosphates
2, 4-D	:	2, 4 - Dichlorophenoxyacetic acid
ECA	:	Embryogenic callus aggregates
EDTA	:	Ethylene diamine tetra acetic acid
g/l	:	Gram/Litre
GA_3	:	Gibberellic acid
h	:	Hour (s)
IAA	:	Indole - 3- acetic acid
IBA	:	Indole-3 – butyric acid
kDa	:	Kilodalton
KIN	:	Kinetin
l	:	Litre
µl	:	Microlitre
µg	:	Microgram
µM	:	Micromolar
ml	:	Milliliter
mM	:	Millimole
mg/l	:	Milligram per litre
min	:	Minute (s)
α-NAA	:	α- naphthaleneaectic acid
PAGE	:	Poly acrylamide gel electrophoresis
PCR	:	Polymerase chain reaction
PEG	:	Polyethylene glycol
RAPD	:	Random amplified polymorphic DNA

rpm	:	Rotation per minute
RRII	:	Rubber Research Institute of India
SDS	:	Sodium dodecyl sulfate
Taq	:	*Thermus aquaticus*
TEMED	:	Tetramethylethylene diamine
TDZ	:	Thidiazuron
TPD	:	Tapping panel dryness
UV	:	Ultra violet
v/v	:	Volume per volume
w/v	:	Weight per volume
ZEA	:	Zeatine

Chapter-1

INTRODUCTION

The life strategies of multicellular plants are strikingly different from animal species. During post embryogenic development, due to the absence of cell migration and the need for continuous organogenesis, plants maintain organ forming cell files called the meristems, which are highly dependent on environmental as well as developmental or hormonal factors. The ontogenic program of a plant is thus highly flexible and linked to the reversibility of the differentiation state of the somatic plant cells. Under extreme conditions, these cells have to change their fate, either to die or divide and dedifferentiate depending on the needs of the system. In general, the developmental program of a plant is much more open to alternative pathways compared to that of animals and this is manifested at the level of cellular differentiation. In addition to the natural *in vivo* forms of embryogenesis, there are three types of embryo development from *in vitro* cultured plant cells namely *in vitro* fertilization (Kranz 1999), microspore embryogenesis (Reynolds 1997) and *in vitro* somatic embryogenesis (Dudits *et al.* 1995).

In angiosperms, development of zygotic embryos from a unicellular fertilized egg occurs through a series of morphologically identical stages beginning with globular to heart, torpedo and cotyledon stages. Although the morphological description of the different stages of zygotic embryogenesis has been extensively studied using microscopy, studies at molecular and biochemical levels has been significantly blocked by the physical inaccessibility. The introduction of somatic embryogenesis thus provides a versatile model system for investigating different biological events occurring during plant embryogenesis (Zimmerman 1993). Since the initial description of somatic embryo induction from carrot cells for 50 years (Steward *et al.* 1958), this unique developmental potential has been recognized as an efficient method for regeneration of whole plants from a somatic cell by a phenomenon of totipotency of cells. In many crop plants, somatic embryogenesis is the main regeneration pathway and has been

protoplast work and production of synthetic seeds. The prospects of using somatic embryos as artificial seeds may revolutionize the area of plant propagation, germplasm storage and seed production. When integrated with conventional breeding programmes and molecular and cellular biology techniques, somatic embryogenesis provides a valuable tool for increasing the pace of genetic improvement of commercial crop species via genetic engineering, in particular for tree crop improvement, compared to annual crops. In addition to widening the pool of useful genes, recent years have seen a dramatic increase in the application of genetic engineering which allows the use of molecular farming for the production of a variety of high value recombinant proteins.

The rubber tree (*Hevea brasiliesis*) occupies a prime position worldwide for the production of natural rubber, regarded as nature's most versatile raw material. Chemically, natural rubber (cis 1, 4 polyisoprene), is a high molecular weight isoprenoid polymer and is valued for its high performance characteristics. Since several centuries, the usage of rubber is tied up with humankind and material mobility. Practically every movement of life requires this fascinating material ranging from the very basic personal articles to today's transportation including defense and civilian purposes. At the beginning of 20^{th} century, a series of technological developments in processing research revolutionized the uses of rubber and thereby vastly expanded its application. More than 50,000 rubber based products including tyres, engineering components and latex products are now being manufactured all over the world.

From the native environment of Brazil, rubber tree was introduced to South East Asian countries in 1876, by Sir Henry Wickham (Wycherley 1968). This material with a narrow genetic base has served as the base material for the subsequent spread of today's millions of rubber plantation across Asia and Africa. Presently, rubber is cultivated in over 40 countries with more than 9.5 millions ha. In India, the rubber cultivation marked the beginning of commercial planting in 1902. Now rubber is grown over an area of 5.97 lakh ha with 4.47 lakh ha under tapping and an annual production of 8.02 lakh tonnes (Indian Rubber Statistics 2007). During the last five decades, rubber plantation sector in India has achieved a spectacular growth in area, production and most notably in productivity. At present, India occupies the 4^{th} position in terms of total

production and more significantly attained the first position in productivity. Today, production of natural rubber in India is the highest among major natural rubber producing countries and the country occupies 4th position in production and consumption of natural rubber in the world.

In *Hevea,* hybridization coupled with vegetative propagation and clonal selection is considered as the most important conventional method of genetic improvement. In India, genetic improvements of *Hevea* were initiated during 1954. *Hevea* breeding programmes are aimed to synthesize ideal clones with high production potential combined with desirable secondary attributes like high initial vigor, thick bark having a good latex vessel system, good bark renewal, high growth rate after opening and tolerance to major diseases. In addition to maximum yield, attention has to be paid on producing clones with low incidence of tapping panel dryness. Additionally, we should also take into consideration to produce location specific clones. Clones with early attainability of tapping girth and high initial yields are also preferred in the later phase of exploitation.

Crop improvement of perennial crop in general and rubber in particular, by conventional breeding is, however, very complicated and time consuming as in many other tree crops. The major limitations are its very narrow genetic base, seasonal nature of flowering and low fruit set, lack of fully reliable early selection methods and pronounced interaction of genotype and environment. Furthermore, traditional *Hevea* breeding is extremely difficult due to the long gestation period up to 6-7 years to attain tappable girth of 50 cm and highly heterozygous nature of the tree. The genetic base for the millions of rubber plantations in the east is very narrow, limited to a few seedlings originally collected from a miniscule of the genetic range in Brazil (Schultes 1977). This lack of genetic diversity also leaves the crop highly susceptible to pathogenic attack. In addition to the original narrow genetic base, the vigorous unidirectional selection practiced over the years has further narrowed down the genetic base resulting a slow down in genetic advancement in recent years (Varghese *et al.* 2000). Moreover, the commercially accepted practice of propagation is budgrafting, which involves the grafting of homogenous scions from high yielding trees into unselected rootstocks. However, the rootstocks which are derived from open pollinated highly heterozygous seedlings cause stock scion

interaction leading to intra- clonal variation in field performance. When considering genetic improvement, vegetative propagation is regarded as an important method for achieving higher genetic gain in a shorter time. In *Hevea* breeding, wider adaptation of clonal propagation by bud grafting is also a serious constraint of conventional breeding. In this context, *Hevea* breeders across the world had realized the scope for broadening the narrow genetic base through biotechnological intervention.

Although classical *Hevea* breeding was limited due to certain constraints, over the past few decades, *Hevea* breeders have focused considerable attention in genetic improvements for evolving high yielding clones and have resulted in the release of outstanding clones for commercial planting. Among them, RRII 105 is a very promising clone with wide popularity (Nazeer *et al.* 1986) in the rubber planting sector having a high yield. Recently, a few clones, RRII 414 and RRII 430 having high yield than RRII 105 were released. However, until now, RRII 105 established the viability of natural rubber cultivation in the traditional rubber growing regions with both small and large growers. Most of the high yielding clones, including the clone RRII 105, were highly susceptible to tapping panel dryness, a major physiological disorder seen in the tapping panel of trees characterized by the partial or complete cessation of latex flow. Since 1904, several studies have been focused on the incidence of TPD. Perhaps, these studies have not result any conclusive information neither on the cause nor prescribe any remedial measure to this syndrome. Development of modified clones tolerant to TPD is therefore one of the pre-requisite in genetic improvement programmes. Abnormal leaf fall caused by P*hytophthora spp* is the most devastating fungal disease affecting rubber plantations in India. While traditional breeding has resulted in promising clones like RRII 105 with relative tolerance to ALF, fully tolerance to *Phytophthora* has not been developed so far. Further, susceptibility to other diseases like powdery mildew and leaf spot are also concern to the rubber plantations. Thus improving the clone with the specific objective of disease resistance without compromising on yield and productivity is a task of considerable dimension in crop improvement. Nowadays even in traditional areas, drought stress is very common in fields. Besides wide scale planting in the traditional belts of Kerala and Kanyakumari, rubber cultivation in India is being

extended to different environments like the non traditional zones of Karnataka, Maharastra, Orissa, West Bengal and North East states which are exposed to a wide range of abiotic stresses. Therefore, the genetic plasticity conferring the adaptation of rubber trees to diverse agroclimatic and soil conditions deserves more attention. There is no doubt that conventional breeding programmes will lead the rubber trees to increased latex yield by the release of high yielding clones. However, it is quite possible that the rate of rubber biosynthesis within the trees becomes a limiting factor (Arokiaraj *et al.* 2002). At this point, the prospect of developing rubber trees with enhanced latex yield is quite promising. Additionally, the success of producing transgenic *Hevea* plants will open up exciting possibilities to utilize the transgenic rubber trees as a versatile chemical factory to produce high value proteins where the recombinant proteins can be extracted continuously and nondestructively by tapping the rubber tree. The search for various biotechnological techniques including tissue culture and genetic engineering therefore hold a great potential in crop improvement programmes.

Genetic engineering provides the plant breeders with new tools to complement and supplement sexual hybridization for improvement of existing clones or the creation of totally new germplasm by the insertion of genes coding for useful agronomic traits. This new approach has provided enormous scope to make specific genomic changes in the elite clones in a relatively short period of time without the loss of any of the desired traits of the parental line. This process involves the insertion of well characterized gene(s) into regenerable cells and subsequent recovery of fully fertile plants with the genes integrated into their genome. Although several useful genes conferring resistance against bacterial and fungal are now available, genetic transformation using these genes of interest is still in infancy stage or rather difficult including *Hevea,* due to the lack of a regeneration system. Central to any transgenic technology is, therefore, the availability of a highly efficient reliable *in vitro* plant regeneration system. While an efficient regeneration protocol is essential, micropropagation is important for the transfer of large number of genetically modified plants to the field within a short span of time. Plant regeneration through somatic embryogenesis is considered as a powerful tool for propagation. Due to the high proliferation

potential and low risk of chimeric plant development, somatic embryogenesis is also a desirable component of plant transformation regeneration protocol (Ammirato 1989). The efficacy of such a system for plant production depends on the efficiency of multiplication and conversion rate of somatic embryos. However, efficient and high frequency somatic embryogenesis pathway for the regeneration of a large number of plantlets from *Hevea,* for rubber clone RRII 105 is still lacking. So far, no histological, biochemical and molecular studies were carried out to understand the somatic embryogenesis events in this clone. An attempt on these aspects will thus help us to understand not only the full mastry of somatic embryogenesis, but also enhances the regeneration frequency. In this scenario, realizing the urgent need for an *in vitro* regeneration system via somatic embryogenesis in *Hevea brasiliensis* (clone RRII 105, a popular high yielding Indian clone) and the potential use of this system in genetic improvement programmes, the present study was undertaken with the following objectives:

- To develop a high frequency plant regeneration system through somatic Embryogenesis utilizing immature anther as explants
- To optimize various parameters affecting the efficiency of callus induction, somatic embryogenesis and plant regeneration
- To initiate a long term embryogenesis system
- To study the biochemical changes associated with somatic embryogenesis

REVIEW OF LITERATURE

2. 1. The Para Rubber tree (*Hevea brasiliensis*)

In the plant kingdom, although, over 12,500 plant species belonging to 900 genera produce latex, natural rubber is found only in the latex of over 2000 species of 79 families. However, the wide variation of rubber content in these species may limit their consideration as sources of natural rubber (Raghavendra 1991). Of all the latex yielding genera, genus *Hevea* belonging to the botanical family *Euphorbiaceae* is considered as most important. The genus *Hevea* comprises ten species, all growing wild in the Amazon basin were strongly outcrossing and monoecious (Schultes 1990). *Hevea brasiliensis* (Muell. Arg. 2n= 36), the para rubber tree is a well known species of this genus and is the only commercially cultivated species as a source of natural rubber due to its high rubber content, yield of acceptable quality and convenience of harvesting (Wycherley 1992). Rubber trees (Fig. 1) grow best at temperature of 20-28° C with an annual rainfall of 2000 mm and growing on most soils with adequate drainage. This tree upon tapping, a process of controlled wounding of bark, produces copious amount of latex in the milky cytoplasm of highly specialized cells called laticifer or latex vessels. Rubber tree is widely accepted as a best natural machine for carbon sequestration. In the plantation industry of India, rubber tree occupies a place of pride among various crops. Moreover, a large population depends either directly or indirectly on the production of rubber for their livelihood and also provides significant employment opportunities. Furthermore, rubber plantation industry is contributing substantially to off set some of the socio-economic problems like deforestation, depletion of soil and environmental pollutions.

2. 2. *Hevea* breeding: Objectives, achievements and limitations

The ever increasing demand for natural rubber mainly in tyre industry and the rising price was the main impetus for the rapid expansion of rubber cultivation worldwide. Hence the breeders have been tailored in tune with the interest of growers as well as rubber industry to release high yielding cultivars. In

Figure 1: A mature rubber plantation

India, more than 50 years ago, using the original Wickham gene pool, *Hevea* breeding attempts were initiated. The breeding objectives are mainly focused on developing clones with high production potential associated with secondary characters like high initial vigor and growth, good latex vessel number, bark renewal and tolerance to major diseases. Moreover, clones with high latex and low incidence of TPD are another priority of breeding. Concerning climatic adaptation, there is a need to breed clones to adapt under different environmental conditions which is specific to particular locations (Varghese and Mydin 2000). In addition, clones with early attainment of tappable girth and high initial yield are also considered. During the past few decades, the breeders has paid rich attention for evolving high yielding clones and the systematic breeding, selection and evaluation have resulted in several outstanding clones with a substantial enhancement in productivity. One of the most significant achievements of breeding programmes in the Rubber Research Institute of India is the release of a high yielding clone, RRII 105. This clone is evolved by the hybridization and selection with Tjir (primary clone evolved by Tjir and ji Estate, Indonesia) x GL_1 (Glenshie I Estate, Malaysia) as parents. Recently, few clones (RRII 414 and 430) with better yield than RRII 105 was also released. However, the clone RRII 105 is still extremely popular in the traditional regions. In India, the growth of rubber plantation industry has been mainly through the expansion of rubber cultivation in Kerala and more than 98% of rubber was produced from the traditional belts of Kerala and Tamil Nadu.

Hevea breeding programmes have been based on a narrow genetic base. Development of new clones by conventional breeding mainly required three methods *viz.*, introduction, selection and hybridization. The most promising method is controlled hybridization between selected parental clones, evaluation of hybrids and selection of promising recombinants for further evaluation. In this tree crop, the juvenile period is relatively long ranging from 5 to 7 years depending on environmental conditions and management practices. In the traditional growing tract of India, flowering is restricted to few months from January to March. Non-synchronization of flowering in some of the selected parental clone is limiting the possibility of attending all possible cross combinations in an active breeding program. The low percentage of fruit set

following controlled pollination is another bottleneck limiting the hybridization progress. Moreover, perennial nature of the crop and high heterozygosity makes conventional breeding of *Hevea* rather difficult. Genetic advance gained in the early breeding phase seems to have slowed down in the more recent phases of breeding (Varghese *et al.* 2000) because of long breeding and selection cycle and absence of early selection parameter. Insufficient availability of land for field experimentation and pronounced interaction of genotype and environment are also makes the conventional breeding very difficult. Due to these limitations, the quick release of new clones is a long and tedious task and required more than 25 years. Vegetative method of propagation is also considered as another constraint of breeding due to stock scion interaction.

2. 3. Need for biotechnology in genetic improvements of *Hevea*

The worldwide practice of propagation of rubber for commercial planting is by budgrafting which is assumed to show less variability than seedlings. Budded plants exhibited uniformity due to the uniform genetic constitution of the scion. However, in all rubber plantations, tree to tree variation in yield is existing and this may be attributed to the heterozygous rootstock material leading to stock scion interaction which may greatly influence the latex yield of rubber trees. To achieve maximum productivity from high yielding clones, the possibility of uniform rootstocks is greatly needed. Moreover, considerable variation is also existed in the tolerance of rootstock to adverse soil conditions. In addition, the rootstock materials influenced the disease tolerance as well as the growth performance of the tree. From the early centuries itself, it was well documented the part played by rootstock on the production potential of rubber trees. In olden days in India, monoclonal seeds from selected high yielding clones were used for raising seedlings as rootstocks. Later, when the rubber cultivation was promoted into very extensive areas in the traditional and non traditional regions, the utilization of available assorted seeds became a general practice. However, assorted seeds showed more variability than monoclonal seeds, the assorted seedlings always displayed an appreciable spectrum of variation due to its preferential out crossing nature. In this back drop, production of uniform plants via somatic embryogenesis offers an effective means to utilize as rootstock. Since

uniform growth and early tapping due to reduced heterogeneity induced by the rootstock is expected from tissue culture plants. Somatic plants may also be utilized for crop improvement through transgenic approaches.

In spite of all major constraints of conventional breeding, a great progress has been made on the release of high yielding clones including RRII 105. However, the production potential is influenced by various plant physiological conditions and diseases. TPD is one of the most serious problems worldwide in rubber plantations, causing severe crop loss which accounts for 20-40% (Chen *et al.* 2003) of the annual rubber production. Occurrence of this syndrome was reported in Asian plantations since the beginning of 20th century. Concerning *Hevea* clones, RRII 105 was highly susceptible to this syndrome. Since 1904, studies at anatomical, nutritional, physio-biochemical, molecular and pathological levels have been focused on the incidence of TPD. Excessive tapping and intensive ethylene stimulation as well as rootstocks and genetic divergence between rootstock and scion are also suspected the reasons for TPD. However, stock scion interaction may not be a single factor contributing the occurrence of TPD (Thulaseedharan *et al.* 2006). Although, several studies have been focused on the incidence of TPD, no conclusive information is available to this syndrome. All this constantly necessitated the need for transgenic research to overcome TPD problem by producing genetically engineered *Hevea* plants with over expression of stress tolerant genes.

During various stages of growth, rubber trees are susceptible to a number of fungal diseases and thus make *Hevea* an excellent candidate for improvement through genetic engineering. In the traditional rubber growing tracts of India, the unfavorable climatic conditions like cloudy weather, low temperature and very high humidity caused the outbreak and rapid spread of many diseases. Abnormal leaf fall caused by *Phytophthora,* is a severe disease of rubber plantations in the traditional growing belt of India. Since the clone RRII 105 is most widely cultivated in the traditional growing areas, this clone is moderately affected by this disease. In nursery plants, leaf fall and shoot rot cause die back leading to retarded growth, loss of vigor and extended the period of immaturity. However, in mature plantations, extensive defoliation results in the reduction of growth and latex yield. The severity of the incidence of crop loss due to ALF has been found

more pronounced when left unsprayed for one disease season (Edathil *et al.* 2000). Although the clone RRII 105 exhibits relative tolerance to this pathogen, severe infection was also noticed in some locations (Jacob 2002). Prophylactic spraying of copper fungicides is a preventive measure against this disease however, these chemical measures are not sufficient for fully controlling the disease. *Oidium heveae* is becoming another most destructive fungus of *Hevea.* The mature rubber trees are attacked by this fungus during defoliation after wintering. In plantations at high elevations, the disease is prevalent throughout the year. In India, this tree crop causes significant crop loss in certain areas of south India and also in north east India (Edathil *et al.* 2000). Almost all clones including RRII 105 are susceptible to this disease. Application of sulphur dusting can enhance resistance, however, insufficient in seasons. Leaf disease of rubber caused by *corynespora cassiicola* is also frequently seen in rubber plantations. Clone RRII 105 is highly susceptible to this disease and this clone causes serious concern due to the extent of large area under this clone. Lack of effective control measures and the use of fungicides to maintain the health of susceptible rubber trees have proved to be uneconomic. Thus the development of plants fully tolerant to these diseases is also very significant.

When plants grown under stressful conditions, in addition to other stresses, availability of nutrient uptake and utilization of nutrient elements are disturbed and as a result growth, development and yield of the crop are affected due to changes in the whole plant physiology. In the case of *Hevea*, the susceptibility to different climatic variations is reflected in fluctuations in yield. In traditional regions, suitable land for expansion has been almost exhausted and thus attempts were made to cultivate in the non traditional areas. However, nowadays, rubber cultivation in India is faced with various climatic constraints such as drought, extreme temperature and high light intensity limiting growth and yield performance of rubber trees in the traditional as well as non traditional areas (Jacob *et al.* 1999). Drought combined with high temperature in central India and the chilling winter temperature in north-eastern regions also adversely affects the plant survival in field (Alam and Jacob 2002). Recognizing the severe climatic limitation faced by rubber growing regions in particular non traditional areas and

total crop loss, scope for the introduction and expression of genes conferring resistance to these abiotic stresses may offer a path of resistance to rubber trees.

Added to this, the classical breeding has potentially benefited from genetic engineering by producing genetically engineered rubber plants by incorporating latex synthesizing genes to enhance rubber biosynthesis. Over the last decade, increased improvement has been made in the productivity of rubber trees and released elite clones with increased latex yield. However, it is quite possible that the rate of rubber biosynthesis within the trees becomes the limiting factor (Arokiaraj *et al.* 2002). Rapid progress has already been made all over the world to identify and isolate the rubber biosynthesis genes (Priya *et al.* 2006). In this situation, the scope for altering rubber biosynthesis pathway of high yielding clones by integrating latex synthesizing genes is also highly desirable.

Another attractive possibility will be to use the transgenic technology for the production of biopharmaceutical molecules and other valuable recombinant proteins in a host plant via molecular farming or bio-farming. However, the extraction and purification from complex plant systems were difficult and the system involves the destruction of whole plant or a portion of it (Arokiaraj *et al.* 2002). Also after every harvest, the plant takes time for the new growth and as a result, protein recovery may be batch wise rather than a continuous process. To overcome this problem, one such crop that could be usefully exploited is rubber tree, in which the proteins synthesized in the lacticiferous system are easily extracted by tapping. From a commercial view, the transgenic rubber trees are thus an excellent biological factory for the production of proteins in large quantities. A pre-requisite for the production of all said transgenics by integrating desired genes into rubber trees is the availability of a suitable *in vitro* regeneration system via somatic embryogenesis.

To explore the potential use of somatic embryogenesis in crop improvement programmes via genetic transformation, attempts have already been made worldwide rubber research laboratories and produced genetically modified rubber plants for clones GL 1 (Arokiaraj *et al.* 1994; 1996) and PB 260 (Blanc *et al.* 2006) with *Gus* gene. Later, transgenic rubber plants that synthesizing human serum albumin in the serum fraction of rubber latex is produced with clone GL 1 (Arokiaraj *et al.* 2002). Recently, few numbers of genetically

modified rubber trees with MnSOD gene was achieved for clone RRII 105 from anther derived calli (Jayashree *et al.* 2003; Sobha *et al.* 2003). However, protocols for efficient and reproducible somatic embryogenesis and plant regeneration are essential to exploit the full potential of genetic modification. Since *Hevea* proved to be recalcitrant to tissue culture, regeneration by somatic embryogenesis required in depth studies, particularly for clone RRII 105, which could be further utilized in transgenic research which is still in its infancy stage.

2. 4. Plant tissue culture and *in vitro* morphogenetic pathways

Haberlandt, although, started his first attempt in plant tissue culture in 1902 (Bhojwani and Razdan 1983), he did not succeed due to technical problems, however, his ideas attracted many scientists to pursue this line of investigation. Later, the experiments of many scientists leads to the progress in the culturning of plant cell, tissue and organ on artificial medium under controlled environmental conditions and gave birth to plant tissue culture by exploiting the ability of plant cells to form complete plantlets, a phenomenon known as totipotency of cells. During *in vitro* culture of an explant, new signals from the medium created the formation of new gradients which induces a series of molecular and physiological events leading to dedifferentiation of explants resulted in unorganized cell division and growth followed by the formation of the callus. When new gradients are formed, different signals were functioned leading to reorganization of cell division followed by the formation of meristematic growth centers which in turn induced organized morphogenetic expression and eventually redifferentiation occured. This resulted in the formation of unipolar structures called organs through organogenesis or bipolar structures named embryos through somatic embryogenesis. During the morphogenetic events, two main phases are involved, primarily an induction phase in which the cells are induced to acquire organogenic or embryogenic competence and secondly expression phase, in which the expression of this potential occurred in the development of either an organ or embryos (Ammirato 1985). Regeneration through somatic embryogenesis is preferred over organogenesis because of a single cell origin of the somatic embryos (Merkle *et al.* 1995). Although somatic embryos have always been assumed to have a single cell origin (Merkle *et al.*

1995), somatic embryos could also araise either from single cells or from group of cells (Williams and Maheswaran 1986).

2. 5. Somatic embryogenesis: Concept and Definition

One of the finest endeavors in the history of plant tissue culture has been the cultivation of free cells and cell groups derived from higher plants, especially in angiosperms, in a chemically defined medium. Subsequently, by the change in the hormonal balance of the medium induced their regenerative potential. A type of regeneration frequently observed is the development of embryo-like structures that recapitulate with a high degree of precision of the typical stages in the embryogenesis of a fertilized egg cell. Since the embryos are formed from the somatic cells of the plants, as opposed to gametophyte or germ cells, the phenomenon is referred to as somatic embryogenesis. According to Williams and Maheswaran (1986), somatic embryogenesis is a process by which diploid cells develop into differentiated plants through characteristic embryological stages identical to zygotic embryogenesis without fusion of gametes. This ability to produce normal embryos and indeed whole plants from somatic cells resides uniquely within the plant kingdom.

With the beginning of differentiation of somatic embryos in cell culture of carrot (Steward *et al.* 1958), this phenomenon attracted the attention of developmental botanists. During the last 3-4 decades, somatic embryogenesis has dominated the research activities in the field of plant morphogenesis because of its potential role in basic and applied plant sciences. Generally two types of somatic embryogenesis have been distinguished by Sharp *et al.* (1980) *viz.*, direct and indirect. Direct somatic embryogenesis is considered as the development of an embryo directly from the explant source without an intervening callus. In indirect somatic embryogenesis, the explant passes through a callus phase and embryos were derived from callus or cell suspension or from cells or a group of cells. In other words, in an appropriate *in vitro* environment, the explants undergoes a permissive pattern of development from cells that already possess an embryogenic competence or cells are induced to acquire embryogenic competence. Sharp *et al.* (1980) have described the presence of pre-embryogenic determined cells (PEDC) which followed the direct somatic embryogenesis and

generally, the event does not require the presence of plant growth regulators in the medium. On the other hand, indirect somatic embryogenesis was occurred from the induced embryogenically determined cells (IEPC) and this type of embryogenesis strongly depends upon the presence of growth regulators in the medium, in particular, the presence of auxin.

2. 6. Somatic embryogenesis in *Hevea*

Soon after the discovery of somatic embryogenesis in carrot (Steward *et al.* 1958), an ever increasing number of species and explants have induced to form somatic embryos. Over the past few decades, somatic embryogenesis was well documented for almost all plant species including woody tree crops. However, the progress in the field of somatic embryogenesis, in particular, for woody perennials has been quite noteworthy in the past decade (Jain *et al.* 1999). Research on *Hevea* somatic embryogenesis was initiated almost simultaneously by two groups of researchers, a Chinese team at Rubber Cultivation Research Institute, Baodao, and a Malaysian team at Rubber Research Institute of Malaysia, using anther wall as the explant source. Paranjothy (1974) was the first to report somatic embryogenesis in *Hevea* from anther wall derived calli although complete plants could not be regenerated. Paranjothy and Rohani (1978) subsequently succeeded in shoot development of the embryos. Since then, plant development was achieved from anther wall (Wang *et al.* 1980; 1984; Wan *et al.* 1982) and integumental tissue (Asokan *et al.* 1992). Later, plantlets were developed from stamen cultures (Wang and Chen 1995). Simultaneously research on somatic embryogenesis was initiated at CIRAD, France and Carron and Enjalric (1985) regenerated plantlets from inner integumental tissue of immature fruits and successfully established in the soil. According to the authors, during somatic embryogenesis, four stages were involved *viz.*, callogenesis, differentiation of embryos, multiplication of embryos and germination of embryos and plantlets. Although several clones were tested, successful embryogenesis and plant regeneration has been achieved for a few genotypes. Among them, only one clone, PB 260, was chosen (Carron *et al.* 1989) for its embryogenic potential and this clone thus has been the choice for many studies on somatic embryogenesis. Since then, several investigators have worked

extensively on somatic embryogenesis and it was observed that the embryogenic potential was affected by polyamines and exogenous hormones (El Hadrami *et al.* 1989; 1991), timing of subculturing (Michaux- Ferriere and Carron 1989) and water status of medium and explant (Etienne *et al.* 1991). Detailed investigation on embryo maturation was carried out by Etienne *et al.* (1993) and reported that slow desiccation with 351 mM sucrose along with 1 mM ABA enhanced germination ability and conversion into plantlets. Effect of ABA and ABA with PEG on embryo development was also described by Veisseire *et al.* (1994) and (Linossier *et al.* 1997). Montoro *et al.* (1993; 1995) studied the influence of sucrose and calcium and reported that a higher levels of sucrose (351 mM) and calcium (12 mM) improved somatic embryogenesis. Etienne *et al.* (1997a) promoted somatic embryogenesis using a temporary immersion technique in a high calcium enriched medium. Later, Etienne *et al.* (1997b) used a pulsed air immersion technique for embryo enhancement. Engelmann *et al.* (1997) successfully used a freezing protocol for cryopreservation of calli and observed a rapid regrowth of calli and embryo induction from cryopreserved calli. In recent years, Martre *et al.* (2001) also attempted a temporary immersion technique and improved somatic embryo production. Blanc *et al.* (1999; 2002) investigated the possible role of the relation of zygotic and somatic embryos in conversion ability and Lardet *et al.* (2007) displayed better regeneration with calli precultured on 1 mM $CaCl_2$. Although, the regeneration efficiency via somatic embryogenesis has been greatly improved and promising results have been obtained, the system still remains difficult or a fleeting phenomenon due to the frequent browning of calli leading to tissue degeneration and a loss of embryogenic competence (Veisseire *et al.* 1994). Further, low rates of germination and plant conversion (Cailloux *et al.* 1996; Linossier *et al.* 1997) and insufficient embryogenic yield as well as the origin of the plant material considerably affected the somatic embryogenesis phenomenon (Blanc *et al.* 1999) for clone PB 260.

In *Hevea*, successful embryogenesis and plant regeneration has been restricted to a few genotypes including 3 Chinese clones (Wang *et al.* 1980), clones like PB 260, PR 107, PB 235 and RRII 600 (Carron *et al.* 1989) and GL 1 (Asokan *et al.* 1992). According to Carron *et al.* (1989) success of regeneration especially via somatic embryogenesis is highly dependent on genotypes.

However, Montoro *et al.* (1993) reported that callus structure and morphogenetic capacities are not strictly specific to genotype but rather to genotype x medium interaction, revealing the necessity for optimizing the cultural conditions in order to achieve somatic embryogenesis for each genotype in *Hevea*. The first preliminary report on somatic embryogenesis for the clone RRII 105, using immature anther as explants was made by Kumari Jayasree *et al.* (1999). Later, Sushamakumari *et al.* (2000) reported a regeneration system from immature inflorescence, however, conversion of the somatic embryos into full plantlets has got several constraints like the low percentage of embryo maturation and the occurrence of abnormal embryos incapable of regenerating into plants (Sushamakumari *et al.* 1999). Since then, there is not much information available except reports by Kala *et al.* (2006) from *in vitro* leaf and Sushamakumari *et al.* (2006) from *in vitro* roots. The present study exploited the capacity of immature anthers, for the first time, to regenerate plantlets via somatic embryogenesis.

2. 7. Plant regeneration via somatic embryogenesis: A general view

2. 7. 1. Factors affecting callus induction, somatic embryogenesis and plant regeneration

Generally, the process of somatic embryogenesis employs a culture medium which contains mineral salts, vitamins, carbohydrate source and plant hormones. Among these, the most important one is plant hormones. Auxin was the crucial growth regulator used in the induction of embryonic calli. Once the embryogenic cells were induced, the requirements for auxin were decreased, even in the absence of auxin, the embryogenic cells develop into somatic embryos. However, if the embryos are exposed to high concentration of auxin during development, they fail to accumulate storage protein and germination frequency will be lower (Stuart *et al.* 1985). Removal of auxin from the culture therefore considered to inactivate several genes or synthesise new gene products for the completion of plant development (Zimmerman 1993). Thus, auxin available to the culture is generally believed to be crucial for somatic embryogenesis (Zimmerman 1993). 2, 4-D is considered as a potent auxin for initiating callus in many plant species including citrus (Tao *et al.* 2002), cotton (Mishra *et al.* 2003), cereals (Kaur and Kothari 2004) and garlic (Luciani *et al.* 2006). In *Hevea* 2, 4-D or a combination of 2, 4-D and NAA were used for callus induction (Carron and

Enjalric 1985; Chen *et al.* 1979; Sushamakumari *et al.* 2000). Concentrations of 2, 4-D played a significant role in quality of the calli with regard to their subculture ability and regeneration potential. Calli amount, texture and colour were varied depending on the type and concentration of auxins especially 2, 4-D (Martin 2003). 2, 4-D concentrations in the medium influenced the callus nature and consequently different types of calli were obtained during callus induction. During somatic embryogenesis of wheat, Filippov *et al.* (2006) induced two types of callus, type I, a non embryogenic and type II, an embryogenic calli. According to Mishra *et al.* (2003), in cotton, hard tightly organized compact calli is non embryogenic while grainy textured friable callus is embryogenic. In *Hevea,* a compact calli with highly embryogenic nature was obtained from integumental tissue of clone PB 260 (Montoro *et al.* 1993).

Auxin type and concentration can influence development and morphology of somatic embryos in several species like peanut (Chengalrayan *et al.* 1997) and sesame (Mary and Jayabalan 1997). Moreover, embryogenic frequency as well as number of somatic embryos was dependent on auxin type and concentration. NAA (2.0 mg/l) was optimum for high frequency of somatic embryos where as IAA provided maximum number of somatic embryos (Mandal and Gupta 2003). Junaid *et al.* (2006) reported that, in *Catharanthus,* once callus was induced in 2, 4-D containing medium, NAA was required for embryo induction. Earlier, influence of NAA on embryogenesis from mature anther of *Hevea* was shown by Chen *et al.* (1979). On medium containing 0.1 mg/l NAA, maximum somatic embryos were obtained, in contrast, with 2, 4- D containing medium, where only globular embryos were obtained during somatic embryogenesis in *Sesbania sesban* (Shahana and Gupta 2002). Parthasarathy *et al.* (2001) showed that, in woody plants, induction of somatic embryos was influenced or enhanced by 2, 4-D. By contrast, embryo induction frequency was decreased sharply when the concentration of 2, 4-D was higher than 9 μM (Wang and Wei 2004).

Embryogenesis is a complex multi-stage process involving a lengthy time period from explant inoculation to plant regeneration. Embryogenic callus formation is the first and crucial step during somatic embryogenesis and moreover the frequency of somatic embryogenesis depends on the initial callus.

In majority of plant species, the synthetic auxin 2, 4-D is the most commonly used auxin for inducing callus. Culturing the explants under the influence of 2, 4-D results in the increase in endogenous auxin levels in explants (Michalczuk *et al.* 1992) and therefore a long time culturing on auxin may detrimental to further morphogenesis. In order to reduce the length of time or to expedite the whole process of embryogenesis, an ideal strategy is to use a medium with minimum addition of hormones or to minimize the exposure period to hormones. In this context, a liquid based system is preferred. According to Sharma *et al.* (2007), a 20 µM 2, 4- D pulse treatment for 1 h yielded a significantly higher number of somatic embryos. In cucumber, before culturing on standard medium, an initial culture on high concentration of 2, 4-D increases the production of embryogenic calli formation. However, a decrease in embryogenic calli formation was observed by culturing for longer periods on high 2, 4- D medium (Kuijpers *et al.* 1996). When contact of embryo explants with callus induction was increased upto 21 d, the rate of somatic embryogenesis and number of regenerated wheat plants was increased, however, prolonged contact upto 28 d delayed shoot development of embryos (Filippov *et al.* 2006). All these results clearly indicated that initial culture of explants as well as time exposure to auxin is greatly influenced embryogenic calli formation and further embryo induction.

In *Hevea*, little is known about the effects of various factors affecting somatic embryogenesis and plant development. The diammine putrescine and the polyamines spermine and spermidine are the main polyamines in plants acting in various processes like cell division, somatic embryogenesis, root formation, floral initiation and fruit development, nitric oxide metabolism, secondary metabolism, senescence and abiotic and biotic stress responses (Bais and Ravishankar 2002; Catarina *et al.* 2007). Several reports have shown the involvement of polyamines particularly in their free forms in somatic embryogenesis. Exogenous supply of polyamines seems have a positive effect on somatic embryogenesis (Kevers *et al.* 2002; Rajesh *et al.* 2003). Among the polyamines, spermidine is more effective and the beneficial role of spermidine in somatic embryogenesis was reported in *P. ginseng* (Monteiro *et al.* 2002). In *Hevea* El Hadrami *et al.* (1989) reported that the exogenous supply of polyamines increased both embryogenic calli formation and embryogenesis. In oil palm, although both putrescine and

spermidine significantly increased the rate of somatic embryos, efficiency of the induction of somatic embryos, secondary somatic embryos and plant regeneration was higher in putrescine containing medium (Rajesh *et al.* 2003). External application of polyamines induces browning of callus with a dimished capacity of embryo initiation (Kevers *et al.* 2002). In *Gnetumula*, spermine and spermidine helped proliferation of embryogenic callus but had no effect on embryogenesis (Augustine and Souza 1999). By contrast, Adkins *et al.* (1998) reported that when polyamine was applied during callus phase in coconut has enhanced somatic embryo induction. More recently, few studies have demonstrated the possible role of polyamines and nitric oxide metabolism in somatic embryogenesis (Catarina *et al.* 2007). According to Catarina *et al.* (2007), the polyamines, spermine and spermidine seem to interfere with the growth and development and morphogenetic evolution of somatic embryos, through alterations in the endogenous nitric oxide and polyamine metabolism in the species.

Nitrogen is a major element for *in vitro* morphogenesis (Halperin 1995) and different nitrogen balances and sources in the culture medium can promote somatic embryo induction. By contrast, Merkle *et al.* (1995) reported that the addition of amino acids to the culture medium might inhibit or promote the somatic embryo development and conversion to plant. The supply of amino acid in the culture medium can cause increased protein reserves in somatic embryogenesis and facilitate maturation (Morcillo *et al.* 1999). According to Merkle *et al.* (1995) storage proteins are the markers for embryo maturation and consequently the quality marker of the regenerated plant. In carrot somatic embryos, the total protein content increased three times when glutamine was added (Dodeman 1995) and in oil palm somatic embryos, glutamine enhanced the accumulation of 7S globulins (Morcillo *et al.* 1999). In *Feijoa,* the induction of somatic embryos was increased significantly when medium was supplemented with 4 mM glutamine, asparagine or arginine (Das Vesco and Guerr 2001). Addition of alanine and glutamine does not stimulate embryogenesis, but it reduces or even inhibits the embryogenesis (Hita *et al.* 2003). During androgenesis of *Cucumis sativus,* amino acids glutamine, arginine and asparagine individually at 2.0 mM enhanced embryo induction (Ashok Kumar and Murthy 2004). Glutamine alone or in combination with the organic or inorganic forms of

nitrogen has been used in different phases of somatic embryogenesis (Garin *et al.* 2000). In *Feijoa,* glutamine (4 mM) dramatically increased the number of somatic embryos (Das Vesco and Guerr 2001) however; glutamine did not show any enhancement on embryo induction at higher concentration (5 mM) but favoured compact callusing (Vikrant and Rashid 2002). Similarly, casein hydrolysate improves embryogenesis (Wang *et al.* 1999; Tang 2000; Hita *et al.* 2003). Combination of glutamine and casein hydrolysate increased the number of somatic embryos in *Eucalyptus* (Muralidharan and Mascarenhas 1995) and in Japanese larch (Kim and Moon 2007). In contrast, glutamine and casein hydrolysate has no role or response in direct somatic embryogenesis in *Oncidium gower* (Cheng and Chang 2002).

Germination is a process by which both root and shoot development on an embryo with an intact hypocotyl occurred and conversion describes the development of expanded trifoliolates and branched roots on an embryo. Despite the interchangeable uses of the terms germination and conversion, conversion is more appropriate way of assessing the quality of embryos. The term germination usually refers to only root elongation which may not be necessarily followed by shoot growth. Thus the success of plant regeneration cannot be based on germination alone. Conversion, on the other hand, refers to growth of a functional shoot and root system and it is through this process that viable plantlets are produced. Usually mature embryos germinated and converted into a full plant on hormone free medium with a low sucrose concentration. During germination, the percentage of root emergence is generally higher than shoot growth. It is therefore, apparent that encouraging organized cell division, the shoot pole germinating embryos would benefit the conversion processes. It was reported that, though the somatic embryogenesis frequency was high, conversion rate was very low due to the presence of incomplete somatic embryos that lacked shoot poles (Liliane *et al.* 2001; Fernando *et al.* 2003). In *Hevea*, the low germination and further regeneration is one of the major constraints during somatic embryogenesis limiting the application of this system in clonal propagation (Cailloux *et al.* 1996; Linossier *et al.* 1997; Sushamakumari *et al.* 2000).

It is well documented that exogenous cytokinins mainly aminopurines play a major role in germination of somatic embryos in plant species like rose

wood (Muralidhar Rao and Lakshmisita 1996), *Acacia catechu* (Rout *et al.* 1995), *Swietentia macrophylla* King (Maruyama and Ishii 1999). Thidiazuron, one of the several substituted ureas such as N-N,-diphenyl urea has been investigated for cytokinin activity (Lu 1993) and was found as a best cytokinin for tissue culture of legumes and woody plants especially recalcitrant species (Lakshmanan and Taji 2000; Lu 1993). However, reports on its potential role on woody plant somatic embryogenesis and germination are limited (Lu 1993; Sreenivasu *et al.* 1998; Xie and Hong 2001). TDZ is known to induce cytokinin like effects in a number of plant species particularly in woody species depending on the concentrations used (Barrueto Cid *et al.* 1999). Replacing 6 BA and KIN with TDZ, increased peanut plant recovery through somatic embryogenesis (Chengalrayan *et al.* 1997).

2. 8. Long term embryogenesis

Somatic embryogenesis is a linear developmental process from the very young stage to a mature somatic embryo. However, in many cases, somatic embryogenesis is a circular process and a somatic embryo develops up to a certain stage and then reverts to the earlier stages of the embryogenic pathway. That is, once a cell or a group of cells is determined to follow the embryogenic pathway, they will start to divide in a co-ordinated manner. When this co-ordinated behavior is lost, then individual cells or group of cells re-enter the embryogenic pathway and form a new generation of embryogenic tissues. If this is repeated, it results in a continuously proliferating embryogenic culture (Raemakers *et al.* 1999). Long term embryogenesis could be maintained either through cyclic secondary somatic embryogenesis or by using embryogenic callus cultures. The process is also known in different terminology like repetitive embryogenesis or recurrent embryogenesis. This system has certain advantages over short term embryogenesis, such as high rate of multiplication, independence of an explant source and repeatability. Additionally, embryogenic potential could be maintained for over long periods. Long term embryogenesis has been described for many woody species including *Camellia sinensis* (Kato 1996) *and Quercus suber* (Guijarro *et al.* 1995) by secondary embryogenesis and *Thevetia peruviana* (Sharma and Kumar 1994) and *Juglans cinerea* (Pijut 1995) by

utilizing both ECA and secondary embryos. In grape, Motoike *et al.* (2001) maintained the embryogenic cultures of several *V. vinifera* cultivars for 2 years on medium containing 2, 4-D. Martinelli *et al.* (2001) demonstrated plant regeneration from 10 year old embryogenic calli of *V. rupestris*. Recently, Gray *et al.* (2006) maintained a cell line of *V. lomgi* for 18 years however, these cultures have lost their embryogenic competence. In *Hevea*, repetitive embryogenesis has been induced from primary somatic embryos cultured on B_5 medium supplemented with 0.5 mg/l NAA (Asokan *et al.* 2002).

Several factors including genotype (Carron *et al.* 1989), auxin like 2, 4-D (Motoike *et al.* 2001; Asokan *et al.* 2002), sucrose (Asokan *et al.* 2002), calcium content (Etienne *et al.* 1997) and activated charcoal (Motoike *et al.* 2001) affected embryogenesis from long term cultures. More recently, it was reported that both the level of sucrose and developmental stage of explanted embryos strongly influenced the embryogenic capacity from long term cultures (Nair and Gupta 2006). Carron *et al.* (1989) tested different genotypes of *Hevea* on somatic embryogenesis and among them only one clone exhibited embryogenic competence for longer periods. Etienne *et al.* (1997) studied the influence of calcium for long term embryogenesis and observed that high $CaCl_2$ concentration (9 mM) in the embryo induction greatly favoured somatic embryo development. Asokan *et al.* (2002) reported the presence of 2, 4-D at 4.0 mg/l and 50 g/l sucrose in embryo induction medium enhanced repetitive embryogenesis for clone GT1. The effect of charcoal on culture establishment and plant regeneration has been reported in various species. However, according to Motoike *et al.* (2001), during long term maintenance of cultures, charcoal may remove ingredients from culture medium that are essential for maintenance of proembryogenic mass. In *Hevea,* for clone RRII 105, although embryos were effectively induced in the presence of charcoal (2.0 g/l), the effect of charcoal from long term cultures is not known.

2. 9. Effect of light

The effect of physical factors such as light and dark has an important role in every phase of plant growth and development. However, dark and low light conditions are commonly used for somatic embryogenesis in wheat (Wang and

Wei 2004), barley (Ganeshan *et al.* 2003) and oat (Nuutila *et al.* 2002). Absolute darkness was needed for embryogenesis in poplar (Michler and Bauer 1991). In eucalyptus, the highest frequency of embryogenesis was observed under low light condition (16/8 h : light/dark) while under dark condition, the percentage of embryogenesis was found to be very low (Prakash and Gurumurthi 2005). However, dark treatment was necessary for the induction of somatic embryos from protoplasts (Saji and Sujatha 1998). By contrast, somatic embryogenesis in *Manioc gladioli* was completely suppressed by light (Joseph *et al.* 2000).

2. 10. Hardening and scanning electron microscopy study

Acclimatization may be defined as a process by which the *in vitro* derived tissue culture plants adapted to the new external uncontrolled environment. During hardening, the regenerated plantlets are susceptible to transplantation shock leading to high mortality rate. Under *in vitro* culture, the plantlets not only live in a high relative humidity (95-100%), but also depend upon the medium containing sugars and all other essential nutrients. Transferring the plantlets from such an environment to *ex vitro* is a critical step for the survival of plantlets. Hardening and acclimatization are the major hindrance for *in vitro* grown plantlets after transplantation (Bandyopadhyay *et al.* 2004). For woody species, a very few reports are available for a detailed hardening procedure (Chabukswar and Deodhar 2005). In *Hevea* also, no detailed studies have been so far reported for a successful hardening process and consequently this process still continues to be a bottleneck for successful field establishment.

During *in vitro* cutlture, the formation of abnormal characteristics like leaf morphology, altered mesophyll structure, poor photosynthesis, non functional stomata and marked decrease in epicuticular wax were very common (Ziv 1986). Several reports have indicated that epicuticular wax present in tissue culture plants are inadequate and are considered to be a major factor responsible for excessive water loss resulting in low survival rate of *in vitro* plants (Sutter 1988). Epicuticular wax is a natural raw material having many functions including modification of water loss and wettability of leaf surfaces. Presence of epicuticular wax helps in reducing cuticular transpiration and stomatal transpiration (Mercy *et al.* 2005). In *Hevea* to elucidate the role of epicuticular wax and survival of somatic plants, SEM study was carried out during pre and post hardening with both normal and abnormal plants.

2. 11. Histochemical characterization

During *in vitro* culture, the somatic cells or tissues acquire embryogenic potential after a dedifferentiation process (Fambrini *et al.* 1997). During this transition, cells have to activate their cell division cycle, reorganize their physiology, metabolism and gene expression patterns (Feher *et al.* 2003) and thereby drastic alterations may be occurred in the cell. Studies using light and electron microscopy have provided detailed descriptions of the morphological and cellular changes that characterize embryogenic competence (Namasivayam 2007). Based on histological observations on various plant system including pearl millet and cork oak, the embryogenic cells that form somatic embryos are characterized generally as small, isodiametric in shape, have large and densely staining nuclei and nucleoli and are densely cytoplasmic (Namasivayam 2007). However, in *Hevea*, relatively few studies have been reported on the histology of somatic embryogenesis (Carron *et al.* 1992; Michaux- Ferriere *et al.* 1992). In these reports, the authors were focused on the origin and ontogeny of somatic embryos. According to Carron *et al.* (1992) more emphasis were made on the histological and biochemical analyses of plant material and culture media in relation to water, atmospheric gases in culture vessels, minerals, polyamines, phenols and growth regulators. Histological analysis of friable calli obtained from integumental tissue allowed Michaux-Ferriere *et al.* (1992) to attribute both unicellular and multicellular mode of origin to somatic embryos and observed that culture conditions favoured either uni or multicellular mode of embryogenesis in *Hevea*. But in these studies, the histological aspects of embryogenic nature of callus were briefly mentioned without description of the relationship with storage reserves. Additionally, the histological features of non embryogenic callus were not examined. For these reasons, our objectives were to characterize the histological features of embryogenic calli.

2. 12. Genetic stability of somatic plants

An important aspect of any *in vitro* propagation protocols is the genomic stability of regenerants. It is expected that all cloned individuals will have the same genotype as the mother plant. Despite the advantages of the system, genetic instability has been observed among *in vitro* derived plants. Genetic variability is sometimes

beneficial and highly desirable for crop improvement, however, when utilizes the system for mass multiplication and genetic transformation, random changes are not desirable. Therefore, any system which eliminates or significantly reduces tissue culture generated variations can be of much practical utility. Regeneration via somatic embryogenesis in general, direct embryogenesis is expected to produce less number of variation. However, in many cases, plantlets regenerated from *in vitro* culture might exhibit variations called somaclonal variation (Larkin and Scowcroft 1981) which is often heritable. Somaclonal variation often arises during *in vitro* culture as a consequence of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture conditions (Larkin and Scowcroft 1981). The source of explant and mode of regeneration either via somatic embryogenesis or organogenesis or axillary / bud multiplication are known to play a major role in determining the presence or absence of variation (Damasco *et al.* 1996). According to Vasil and Vasil (1980), the species, explant type, the donor genotype, the composition of culture medium, conditions of the physical culture and the duration between successive subcultures all affect the frequency of somaclonal variants. In addition, various molecular events like modifications in the chromosome number or methylation pattern, chromosome breaks, transposons, activation, deletions, genome rearrangements, endopolyploidy or nucleotide substitutions also lead to genetic instability (Larkin and Scowcroft 1981).

Several strategies can be used to assess the genetic purity of *in vitro* derived plants such as morphological descriptions, physiological and cytological studies, molecular and biochemical studies. They provide a convenient method for detecting genetic changes. However, molecular markers offer several advantages since they are stable and detectable in all tissues and not affected by the environment. Of the available techniques, random amplified polymorphic DNA (RAPD) analysis which uses a single arbitrary sequence of 10 base oligonucleotide (Williams *et al.* 1990) to generate DNA fragments, is the most useful and rapid technique. To confirm the genetic stability of the material propagated *in vitro,* RAPD is referred as an appropriate tool (Rout *et al.* 1998; Goto *et al.* 1998; Lattoo *et al.* 2006; Ray *et al.* 2006). By means of RAPD analysis, polymorphism was detected in callus derived plants of tumeric (Salvi *et al.* 2001) and tomato (Soniya *et al.* 2001). By contrast, *Pinus* plantlets derived from callus via organogenesis were also shown to be homogenous (Goto *et al.* 1998). Genetic stability /

homogeneity have also been reported among meristem culture derived plants in ginger (Rout *et al.* 1998) and micropropagated plants of *Chlorophytum arundinaceum* Baker (Lattoo *et al.* 2006). However, the reports of Damasco *et al.* (1996) and Ray *et al.* (2006) showed that plants derived from meristem culture may not always be genetically true- to-type. Thakur *et al.* (1999) observed no somaclonal variation in *Quercus serrata* somatic seedlings. Similar observations were reported with *Panax* somatic embryos where amplification products of 21 primers were monomorphic (Shoyama *et al.* 1997). However, in spruce, Heinze and Schmidt (1995) concluded that a low frequency of genetic instability was present in the population of somatic derived plantlets. In several *Citrus* species, somatic embryo derived plants have been reported to genetically stable with respect to morphology, cytology and physiology. Also genetic stability is reported for willow (*Salix* sp.) and date palm (*Phoenix dactylifera*) plants regenerated through somatic embryogenesis (Akhar *et al.* 2000).

Long term maintenance have proven a viable tool for propagation, however, frequent subculturing on solid medium though enhanced somatic embryo and plant regeneration, the risk of occurrence of somaclonal variation increases with increasing culture duration (Wang *et al.* 2004). Therefore, it is necessary to maintain the genetic constitution through its life span. The application of higher concentration of growth regulators and recurrent subculture for indefinite period hinders the maintenance of genetic fidelity (Sahijram *et al.* 2003). It is unclear whether prolonged culture of embryogenic calli will result in mutation or other genetic abnormalities. Epigenetic changes or mutations almost certainly accumulate which negatively affects the competence of embryogenic cultures. Medium components, culture age, explant tissue and even plant genotype affected chromosomal instability of tissue culture regenerated plants (Peschke and Phillips 1992). The cytological characteristics commonly noticed in somaclonal variation are changes in chromosome number and structure. Considering chromosome number, polyploidy is the most frequently observed chromosome abnormality in the regenerants. Many factors are involved in chromosomal abnormalities includes plant growth regulator. However, it has been observed that growth regulator added to the medium can influence frequency of abnormality. Chromosomal differences reflect the fundamental changes in morphological, physiological and biochemical characters that result

from different gene action and expression (Sharma and Sen 2002). In *rhubarb*, the chromosomal changes have resulted in morphological variation (Zhao *et al.* 2005). According to Lee and Phillips (1988), as the age of the culture increased the frequency of mutation in regenerated plants are also increased. Radic *et al.* (2005) reported that culture age and added plant growth regulators showed no effect on cytogenetic stability from long term cultures of C. *ragusina.* However, Al Zahim *et al.* (1999) observed genetic variation in plants regenerated from 14 month old calli of garlic. Taking into consideration, the present study has made an attempt to maintain and retain the embryogenic competence for long periods. The study also tested the influence of proline and activated charcoal on long term embryogenesis. This report also examined the genetic stability of long term cultures by RAPD approaches and chromosome number by cytological analysis.

2. 13. Biochemical characterization during somatic embryogenesis

The ability of somatic plant cells in culture to regenerate entire plants by somatic embryogenesis is a remarkable biological phenomenon. The transition of somatic cells into cells that are capable of forming an embryo is the most important process of somatic embryogenesis and also the area least understood. A better understanding of the biochemical events leading to the morphogenetic differentiation of somatic cell to a whole plant not only enhances our understanding of the mechanism but also enhances the embryo induction and plant regeneration frequency. Studies on total protein and isozyme analysis (Samantaray *et al.* 1999) provide a relatively convenient tool for examining biochemical changes associated with morphogenesis. Biochemical difference between embryogenic and non embryogenic callus with respect to proteins and isozymes has been reported by many authors Blanco *et al.* (1997) and (Alves *et al.* 1994; Asokan *et al.* 2001).

Proteins are valuable indicators of differentiation that could be used as biochemical markers to understand the events of morphogenesis. Proteins differ not only with respect to their function but also in terms of their timing and extent of expression during plant development (Reinbothe *et al.* 1992). Interestingly, functionally related proteins seem to be encoded by groups of co-ordinately expressed genes. The molecular aspects of embryogenesis in carrot have been studied and several proteins like lipid transfer protein (EP2), acidic endochitinase

(EP3) and cationic peroxidase have been shown to play a key role in carrot somatic embryogenesis (De Jong *et al.* 1995). Storage compounds are important biochemical markers of physiological quality of somatic embryos and may affect their final developmental stages and conversion to plants (Cailloux *et al.* 1996). There are several studies on changes in protein pattern during somatic embryogenesis including birch (Hvoslef-Eide and Corke 1997). Tchorbadjieva *et al.* (1992) identified several stage specific extracellular glycoproteis during *D. glomerata* L. somatic embryogenesis. Nielsen and Hansen (1992) and Stirn *et al.* (1995) have reported the role of secreted proteins and their use as biochemical markers on embryogenic potential in monocots. Accordingly, 30 extracellular polypeptides and two extracellular glycoproteins accompanying somatic embryogenesis were identified and these proteins were correlated with embryogenic capacity and the loss of regeneration capacity in barley (Nielsen and Hansen 1992; Stirn *et al.* 1995). In coffee, differences between embryogenic and non embryogenic calli with respect to proteins are reported. Also during the ontogeny of coffee somatic embryogenesis, at globular, heart and torpedo stages, somatic embryos showed changes in some proteins that are progressively accumulated throughout the development (Yuff *et al.* 1994). However, embryoids at globular stage had significantly accumulated higher levels of total proteins which decreased gradually with embryo maturation. Variations in the protein levels in embryogenic cultures were reported for *Laris – leptoeuropeae* which showed high protein levels in the 2nd week of culture and decreasing significantly in the 5th week (Catarina *et al.* 2003). Similarly, the O. *catharinensis* embryogenic cultures showed high frequencies of embryos in 1-2 weeks old cultures and high protein levels (Gutmann *et al.* 1996). Also during maturation, a decrease in protein content by 25% has shown by soybean somatic embryos (Chanprame *et al.* 1998). Recently, analysis of total protein pattern at all developmental stages of sandal wood somatic embryos revealed the presence of an array of proteins. Accordingly, a relative abandunce of low molecular weight proteins were predominant in friable embryogenic calli where the induction of embryo development was predominant (Suma and Balasundaran 2004).

The application of isozymes as markers in embryogenic culture has been reported in many studies (Alves *et al.* 1994; Asokan *et al.* 2001). Devi and Radha (1997) used

isozymes to distingusish between embryogenic and non embryogenic calli. Peroxidase and esterase have been proved as useful markers for the detection of somatic embryogenesis (Alves *et al.* 1994) and embryogenic potential (Martinelli *et al.* 1993). Peroxidase attributed vital roles in regulation of cell growth, differentiation and embryogenesis. It is well known that 2, 4-D influences the activity and the pattern of peroxidase isozyme (Suma and Balasundaran 2004). Although the precise function of esterase has yet to be elucidated, this enzyme enhance pectin gelling and plays a promotive role in embryogenesis. Esterase isoforms have been shown to be involved in the regeneration process in cereals (Bapat *et al.* 1992). Changes in esterase isozyme pattern in monocots have been used to analyse different stages of somatic embryogenesis (Bapat *et al.* 1992). On the other hand, in *D. glomerata* L, the esterase isozyme did not change significantly during somatic embryogenesis (Tchorbadjieva and Odjakova 2001). In a recent study, the embryogenic calli and embryos showed maximum peroxidase activity during somatic embryogenesis with *Hevea* integumental tissues. Similar pattern was obtained with esterase isozyme also. However, there was a marked difference in isozyme pattern between embryogenic and non embryogenic calli (Asokan *et al.* 2001). More recently, it was reported that peroxidase and esterase isozymes displayed uniform activity in all developmental stages of sandal somatic embryogenesis. However, in this case, increased peroxidase activity was seen in friable embryogenic calli and the activity was low in developing embryos. By contrast, esterase activity was stimulated in later stages of development. Among embryos, immature and mature embryos are characterized by more isoforms in sandal somatic embryos (Suma and Balasundaran 2004).

MATERIALS AND METHODS

In the traditional rubber growing belts of South India, followed by wintering, flowering will be started from December to February. Flowers of *Hevea* are short stalked and both male and female flowers are seen in the same inflorescence. Male flowers are differentiated from female by their small size and more in number. There are 10 stamens / anthers arranged on the staminal coloum in two whorls of five each. Young flowers were collected from 10-12 year old healthy trees of *Hevea brasiliensis*, clone RRII 105, growing in experimental fields of RRII.

3. 1. Somatic embryogenesis and plant regeneration

3. 1. 1. Explant preparation

After collection, flowers were stored in plastic bags kept in ice buckets and immediately brought to the laboratory. Initially the developmental stage of flower buds was examined and chromosome counting was made. Based on these observations, in the present study, floral buds at the diploid developmental stage were excised and used for the study. To remove the dust and other surface contaminants, the floral buds were transferred to a beaker containing one drop of Tween 20 and then washed thoroughly in running tap water for 5 min. Prior to surface sterilization, to minimize explant contamination infected explants were eliminated by cutting the stalk from surrounding explants as soon as they were observed. All operations from surface sterilization were carried out under sterile condition in a laminar air flow hood. After 5 min wash in running tap water, immature floral buds were surface sterilized for 5 min with 0.5% sodium hypochlorite solution or with 0.1% (w/v) mercuric chloride solution containing a few drops of Tween 20 (1 drop for 50 ml). Sterilized explants were then rinsed 5-6 times in sterile distilled water to remove the sterilant completely.

3. 1. 2. Induction of callus and embryos

Immature anthers, at diploid stage, were dissected out under a dissection microscope (Nikon, Japan). To prevent explant browning, few drops of sterile solution of ascorbic acid (100 mg/l) were spread over the floral buds. Dissected

Table 1: Composition of Modified MS medium

Nutrients	Concentrations (mg/l)
Macro	
NH_4NO_3	1000
KNO_3	1900
$CaCl_2$ anhydrous	333
$MgSO_4$ anhydrous	181
KH_2PO_4	170
Micro	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO.\ 2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
KI	0.83
$FeSO_4.\ 7H_2O$	27.85
$Na_2EDTA\ 2H_2O$	37.25
Vitamins	
B5	
Myo inositol	100
Sucrose	50 g/l

anthers were kept in ascorbic acid solution till inoculation. Dissected anthers were also kept in sterile distilled water and observed the influence of ascorbic acid on explant browning and callus induction. For inducing callus, MS basal medium (Murashige and Skoog 1962) as well as a modified MS based medium (Table 1) was tried out. MS medium was modified by lowering NH_4NO_3 to 1.0 g/l, replacing MS vitamins with B_5 (Gamborg *et al.* 1968) and used 5% sucrose (w/v). For identifying the suitable auxin for callus induction, initially four auxins namely 2, 4-dichlorophenoxyaectic acid (2, 4-D), α-naphthaleneaectic acid (NAA), indole-3-aectic acid (IAA) and indole-3-butyric acid (IBA) were tested at two concentrations (1.0 and 2.0 mg/l) in combination with kinetin (KIN) (0.5 and 1.0 mg/l). Eight anthers per tube were inoculated onto all the trial combinations containing approx. 10 ml. of callus induction medium. After two months of

inoculation, cultures were evaluated for callus induction. Among the four auxins tested, 2, 4-D was selected as the potent auxin for inducing callus and the optimum concentration of 2, 4-D was evaluated. To optimize the level of 2, 4-D, a separate experiment was further carried out with different concentrations of 2, 4-D ranging from 0.0-3.0 mg/l with an increment of 0.5 mg combined with 0.5 mg/l KIN using modified MS as the basal medium. 8 anthers were placed onto each combination. Observations were made at every 7 d of culture. Data on callus growth (callus scoring) were recorded after 60 days of culture. Efficiency of callus induction was scored on the basis of amount of callus induced by each treatment.

Once after callus induction, primary calli were subcultured for embryogenesis on medium containing modified MS basal salts and 7% sucrose (w/v). Four auxins such as IAA, IBA, 2, 4-D and NAA in combination with KIN were tested for its ability on the induction of somatic embryogenesis. Thirty factorial combinations of KIN with auxins were separately tested. Equal amount of callus (approx. 150 mg) was subcultured on these combinations. Cultures were evaluated at 2 month as well as after 4 months of culture and observations were taken. All experiments were repeated at least two times with three replications. Data on percentage of explants producing callus and embryos was based on visual observation. To analyze the factorial effect of KIN / NAA, analysis of variance (ANOVA) was carried out. Treatment means were separated using least significant difference (LSD) test at the 5% probability level.

3. 1. 3. Factors influencing callus and embryo induction efficiency

3. 1. 3. 1. Explant pretreatment in liquid medium

With the aim of reducing the time requirement of callus induction, before inoculation to solid callus induction medium, the dissected anthers were pretreated in liquid callus induction medium. After dissection, 6 anthers for each treatment were transferred to 15 ml of liquid medium in Erlenmeyer flask (100 ml). The cultures were shaken at 80 rpm using a rotary shaker. Anthers were pretreated for a period of 0, 5, 10, 15 and 20 d. After treatment, anthers from each flask were filtered and then placed on semi-solid callus induction medium. Immediately after placing the anthers to both liquid and solid medium, the cultures were incubated under darkness. Modified MS basal salt supplemented with 2.0 mg/l 2, 4-D and 0.5 mg/l KIN being more responsive for callus

induction, the same was used in these experiment also. There were five treatments and each treatment was replicated five times. Each flask containing six anthers was considered as one replication and the experiment was repeated twice. The percentage efficiency of calli formation was calculated based on the amount of friable embryogenic callus induced by each explants to the total number of explants cultured.

3. 1. 3. 2. Callus age / subculturing time for embryogenesis

Primary calli induced in callus induction medium were proliferated by maintaining in the same medium. To investigate callus age or the ideal time of callus transfer for embryogenesis, primary calli induced in callus induction medium were maintained in the same medium for different time intervals, 0, 15, 30, 45 and 60 d. (Primary callus induced from 35 d (10 +25 d) culture was used as control in this study). At every time interval, callus from callus induction medium was subcultured onto embryo induction medium for embryogenesis. Cultures were maintained under darkness and evaluated after 50 d of culture. For each treatment, five replicate cultures were raised and the experiment was performed twice. Percentage of callus induction was calculated based on visual observation in respect of amount of friable highly embryogenic callus induced by each callus clumps to the total callus clumps cultured. Variables such as calli colour and texture are also considered.

3. 1. 3 . 3. Effects of polyamines and amino acids on embryo induction

Using modified MS basal medium containing 0.7 mg/l KIN with 0.2 mg/l NAA (control), the following experiments were conducted separately to assess the effects of diverse media constituents on somatic embryogenesis. To study the influence of polyamines, spermine and spermidine was tested at four concentrations (0. 0, 0.5, 1.0 and 2.0 mg/l). Spermine and spermidine was filter sterilized through 0.22 µM membranes (Millipore) and added to autoclaved medium. For investigating the influence of amino acids, different experiments were carried out with various amino acids such as alanine, asparagine, arginine and glutamine. These amino acids were introduced in the basal medium at 5 different concentrations 0, 50, 100, 150 and 200 mg/l. Effect of casein hydrolysate on embryo induction was also evaluated by adding different concentrations of casein (0, 100, 200, 400 and 600 mg/l) to the medium.

Equal amount of calli (approx. 150 mg) were transferred to all combinations and cultures were kept under darkness. Experiment was repeated three times with three replications for each treatment. Data was taken after two months of culture and efficiency of embryogenesis was calculated by analysis of variance (ANOVA).

3. 1. 4. Development of plantlets

For embryo maturation, cotyledon stage embryos were maintained in the same embryo induction medium for 2 more months. After maturation, individual cotyledonary embryos were transferred onto 20 ml. of plant regeneration medium for embryo germination and conversion to plantlets. The plant regeneration medium consisted of the same basal medium as in embryo induction medium, however, the medium was devoid of growth regulators and supplemented with 3% sucrose (w/v).

3. 1. 4. 1. Effect of cytokinins on plant regeneration

In an attempt to increase the plant regeneration frequency, the response of four cytokinins (BA, ZEA, KIN and TDZ) was evaluated. Cytokinins BA, ZEA and KIN were tested at 5 concentrations (0.0, 0.5, 1.0, 1.5, 2.0 mg/l) and TDZ was tested at 6 concentrations (0.0, 0.25, 0.5, 0.75, 1.0, 2.0 mg/l). All cytokinins were added to the medium before autoclaving. White opaque cotyledonary stage embryos were placed on 20 ml of plant regeneration medium comprising 2.0 mg/l GA_3 coupled with the above mentioned concentrations of cytokinins. Cultures were evaluated at 10 and 60 d of culture. Data was made based on thirty replications from three experiments and the percentage of germination and full plant recovery were recorded. The percentage effect was analyzed using Z test. The plantlets derived were evaluated for their initial growth in terms of plant height, shoot length and number of leaves before and after four months of acclimatization and the data were statistically analyzed using ANOVA.

3. 2. Long term embryogenesis

3. 2. 1. Long term maintenance of embryogenic callus aggregates

The embryogenic callus cultures (derived from primary somatic embryogenesis) consisted of embryogenic calli bearing small embryos was termed as embryogenic callus aggregates. This embryogenic callus aggregates was used for

initiating long term embryogenesis. To maintain the embryogenic cultures for a long time, a preliminary experiment was carried out to identify the subculture interval. Embryogenic callus cultures were cultured to the fresh medium in every 25, 50 and 75 d and identified the optimum subculture interval. The embryogenic callus aggregates were carefully detached from the medium. At each passage, approx. 100 mg of tissue was cultured on medium containing glutamine (200 mg/l), casein hydrolysate (400 mg/l), NAA (0.2 mg/l), KIN (0.7 mg/l) and GA_3 (2.0 mg/l).

3. 2. 2. Effect of proline and charcoal on long term embryogenesis

For embryo induction from long term cultures, two different factors like proline and activated charcoal in the medium were tested. Proline effect was investigated by enriching the medium with different concentrations (0.0, 50, 100, 200 and 300 mg/l). To investigate the influence of charcoal, embryo induction medium was supplemented with the following concentrations of charcoal (0, 0.05%, 0.1%. 0.2% and 0.3%). Approx. 100 mg embryogenic callus aggregates (8 month old) were cultured into these combinations and cultures were incubated under darkness at 25 ± 2° C. Cultures were maintained in the same medium combinations up to 3 years with a regular subculture interval of 50 d. Mature embryos were converted into plantlets. For all experiments, thirty replicate cultures were raised and experiment was repeated thrice. Embryo induction efficiency was calculated and compared. The data were subjected to analysis of variance.

3. 2. 3. Long term maintenance by secondary embryos

Embryogenic lines were also be maintained by secondary embryogenesis. To induce secondary somatic embryos, different developmental stages of embryos *viz*., globular, heart/torpedo, immature and mature cotyledon embryos and germinated somatic embryos were carefully detached and cultured on embryo induction medium. Cultures were maintained in darkness. Embryos were germinated and regenerated into plants.

3. 3. 1. Stock solutions and media preparations

All stock solutions of major and minor were prepared according to standard procedure. Stock solutions of all hormones (M/S sigma chemicals, USA) were prepared by dissolving the hormones in a small amount of either 70%

ethanol or 1N KOH / 1N HCl and then adding distilled water to final volumes. The growth regulators were added prior to autoclaving unless otherwise mentioned. Coconut water (5% v/v) was included in all medium and was obtained from tender green coconuts. Coconut water was boiled to 80° C, cooled and then filtered using vacuum pump and added before adjusting the pH. Before adding agar, pH of the medium was adjusted to 5.6 with 1N KOH or 1N HCl. All media were solidified with gelrite (0.21% w/v). Activated charcoal (0.2% w/v) was added in all medium except in callus induction medium and added after melting the medium using electric hot plates. All media were dispensed as 10 or 20 ml aliquots in glass test tubes (25 x 15 cm) and plugged with nonabsorbent cotton plugs wrapped in muslin cloth. Media were sterilized by autoclaving for 15 min at 121° C.

3. 3. 2. Culture conditions

For callus and embryo induction, cultures were placed in darkness at 25±2°C. Cultures for plant regeneration were maintained in light under 16 h photoperiod with light intensity (40 $\mu E/m^{-2}/S^{-1}$) provided by cool white fluorescent tubes of 40 W (Philips, India) and at temperature of 25±2°C with 80-90% relative humidity, unless otherwise specified.

3. 4. Effect of light

A separate experiment was undertaken to determine the influence of incubation conditions on the induction of callus and embryos as well as plant regeneration. Immature anthers were inoculated on callus induction medium and cultures were incubated in complete darkness and continuous light (20 $\mu E/m^{-2}/s^{-1}$) provided by Philips white fluorescent tubes at a temperature of 25±2°C. Induced calli were proliferated on the same callus induction medium and cultured for embryo induction. Embryos were matured and converted into plantlets. Influence of light on callus induction and proliferation, embryo induction and maturation and conversion to plantlets were evaluated.

3. 5. Hardening and SEM study

3. 5. 1. Hardening

When the plantlets having two fully matured leaves with 2-3 cm shoot length and a well developed tap root, the plants were taken out from the culture

tube and the medium sticking to them is gently washed off with tap water. The plantlets were then transplanted to small polybags containing different planting substrates *viz.*, 1) non sterile soilrite, 2) sterile soilrite, 3) sterile sand and soil (1:1) and 4) sterile sand, soil and dried cow dung (1:1:1). During hardening, to reduce mortality rate, an *in vitro* hardening technique was tested. In this procedure, the plantlets were transplanted to sterilized big culture tubes filled with sterile sand and soil and irrigated with ½ strength modified MS basal. Mouth of the tubes was covered with sterile aluminum foil and the plantlets were maintained under *in vitro* conditions in light. In another experiment, before transplantation, the plantlets were transferred to liquid medium which was devoid of sucrose and supplemented with 1.0 mg/l IBA and kept the cultures for 10 d in light. The plantlets were then transplanted to small polybags containing sterile sand, soil and cow dung. After transplantation to small polybags, to maintain high relative humidity (>80%), the planted bags were covered with another polythene bag in which water was sprinkled. Finally the polythene bags were removed and once the plants were acclimatized, the plants were replanted to large polybags filled with mixture of sand, soil and dried cow dung and kept in shade house. During hardening period, the plants were irrigated with ½ strength modified MS salts every alternate days and after acclimatization tap water was used.

3. 5. 2. Scanning electron microscopy

To examine the correlation of epicuticular wax with survival rate of *in vitro* derived plants, scanning electron microscopy study was attempted. SEM study was performed with somatic plants during pre and post hardening process. Both healthy (normal) and weak (abnormal) plants were subjected for the study and were compared with budgrafted control plant. For this study, 3 month old acclimatized somatic plant and 3 year old field grown control plant was used. Leaves from all samples were dried using critical point dryer. The samples were finally coated with gold and coated samples were viewed through a Jeol JSM 35C scanning electron microscopy.

3. 6. Histological studies

To ascertain the embryogenic nature of callus cultures, histological studies were performed on small sections of friable calli producing embryos and calli which could not produce embryos. The embryogenic calli and non embryogenic calli were selected by visual observations. Two weeks before the

study, samples were subcultured onto fresh medium and actively dividing cell cultures were selected. Samples were stained overnight in Iodine Potassium Iodide (Johansen 1940), Sudan Black B (Ruzin 1999) and Oil Red dye (Philip and Reghu 2003) and Mercuric Bromophenol (Mazia *et al.* 1953) for the detection of starch, lipids and total proteins. Samples were also stained with 0.1% Toludine blue for cell characterization. After staining, the samples were washed with distilled water. Stained materials were pressed for uniform smearing and slides were prepared by mounting on glycerol. Slides were examined and quantitative image analysis was done using Leica Q Win V.2.1 image analysis software.

3. 7. Cytological and molecular analysis of somatic plants

3. 7. 1. Ploidy level

Diploid nature of regenerants was confirmed by counting the number of chromosomes using acetocarmine (2%) squash method. Somatic plants derived from primary somatic embryogenesis were taken out from culture tube and washed in sterile water to remove the agar. Tips (4-5 mm) from healthy roots of five randomly selected somatic plants were excised and fixed in a Carnoy solution (glacial acetic acid: ethanol - 1:3) for 4 hrs. The samples were then stained and squashed in 2% acetocarmine for 1hr at 60-80°C and used for chromosome counting. Slight heating and pressing was required for obtaining good results. All observations were recorded from fresh preparations.

3. 7. 2. Analysis of genomic stability

For the analysis of genetic stability of somatic plants by RAPD technique, ten plants derived from primary somatic embryogenesis were randomly selected. Somatic plants after one year of acclimatization was used for the study. RAPD profile of somatic plants was compared with budgrafted control plant. Control plants were raised by budgrafting of buds taken from mother plants. To confirm the genomic stability of somatic plants, 10 monoclonal seedling plants were also used as controls.

3. 7. 2. 1. Raising monoclonal seedlings

Healthy seeds were collected from monoclonal garden, *Hevea* breeding station, Kanyakumari Dist, Tamil Nadu. Immediately after collection, seeds were

packed in powdered charcoal for retaining viability. Seeds were then grown in well drained beds with periodic watering. Germinated seeds were collected from beds when the radical comes just out of the seed and planted in polybags and maintained in the green house and used for the study.

3. 7. 2. 2. Genomic DNA isolation

Total genomic DNA was isolated following the modified CTAB procedure (Doyle and Doyle 1990). Young, light green, uninfected leaves were collected from somatic plants, control plant and monoclonal seedlings. After collection, leaf samples were packed in polybags kept in icebox. The leaves were washed thoroughly in tap water and then rinsed with sterile distilled water. 2 g of leaf tissues were ground to a very fine powder with liquid nitrogen using sterile mortar and pestle and then transferred into 50 ml centrifuge tube. 20 ml 2X CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 0.1% polyvinylpolypyrrolidone (PVPP) and 0.1% ß-mercaptoethanol) was added and incubated in a water bath for 30 min at 55°C with occasional shaking. The homogenate was mixed gently and centrifuged at 8000 rpm for 10 min to pellet the cell debris. The supernatant was transferred to a new tube and added equal volume of Tris-saturated phenol: chloroform: isoamyl alcohol (25:24:1) and spun at 10,000 rpm for 10 min. The aqueous phase was carefully removed to a new tube and to remove RNA, samples were incubated for 2 h at 37°C with 5.0 µl of DNAase free RNAase (10 mg/ ml, M/S Sigma Aldrich, USA). The proteins were extracted by adding equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged for 10 min at 10,000 rpm at room temperature. Aqueous phase was then re-extracted with equal volume of chloroform and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a fresh tube and DNA was precipitated with 0.6 volume of ice cold isopropyl alcohol. Tubes were then kept in ice for 20 min and the precipitated DNA was pelleted by centrifugation at 8, 000 rpm for 10 min at 4°C. The pellet was washed in 70% ice cold ethanol and then air dried by keeping in an inverted position over a blotting paper. The pelleted DNA was re-suspended in TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA pH 8.0). DNA samples were stored at 4°C at - 20 °C.

3. 7. 2. 3. Agarose gel electrophoresis

Agarose (0.8 g) was mixed with 100 ml running buffer (0.5X TBE) and boiled in a microwave oven until the agarose was completely melted. The melted agarose was allowed to cool to 50°C and ethidium bromide solution (1 mg/ml - stock) was added to

give a final concentration of 5 µg/ml. The mixture was then poured into a gel casting tray. When the gel was solidified the comb was removed carefully. The gel was then placed in the electrophoresis tank filled with running buffer (0.5X TBE). One µl of loading buffer (0.25% bromophenol blue, 30% glycerol in TE buffer pH 8.0) was mixed with 2 µl of DNA. Samples were then loaded in 0.8% agarose gel wells. Gels were run at 50 V for 4h or until bromophenol dye front has migrated to the bottom of the gel. DNA bands were visualized in a UV transilluminator and were photographed under UV light utilizing EDAS 290 gel photodocumention system (M/S Kodak, USA). DNA concentration from each samples were estimated by comparing the band intensities with DNA standards and appropriate dilutions were made so as to get 20 ng DNA/µl of the sample.

3. 7. 2. 4. PCR amplification

In a preliminary screening with 20 primers based on amplification, 13 arbitrary decamer primers (Operon Technologies, Alameda, California, USA) from kit A, B, C and D were selected. These primers producing clear, reproducible banding patterns were used for RAPD analysis following standard protocol of Williams *et al.* (1990). Amplification was performed in a DNA Thermal Cycler (Perkin Elmer, USA) in volumes of 20 µl consisting of 1 x reaction buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM dNTPS-dATP, dGTP, dCTP and dTTP (Amersham-Pharmacia, UK), 0.5 units of Taq DNA polymerase enzyme (Bangalore Genei, Bangalore), 20 ng of template DNA and 250 nM of RAPD primers. All reaction mixtures were prepared as master mixes for each primer to minimize the measurement errors. In order to avoid evaporation, the reaction mixture was over layered with one drop of sterile mineral oil (Sigma Aldrich, USA). Following initial denaturation at 94°C for 3 min, the amplification programme was set to 36 cycles of denaturation at 94°C for 1min, annealing at 38°C for 1 min and extension at 72°C for 2 min with a final extension at 72°C for 10 min. On completion of the programme, DNA samples were stored at -20°C. Chloroform (70 µl) was added to the PCR tubes to ease the recovery of samples. The amplified samples were fractionated in 1.5% agarose gel. Loading dye (7 µl) was added to each PCR tube and mixed well and 10 µl was loaded and electrophoresis was performed at 50 V. The molecular marker used was the DNA double digested with *EcoR*I and *Hind* III restriction enzymes. Gels were stained in an ethidium bromide solution for 20 min and the gels were viewed and photographed under UV light. PCR amplification was repeated thrice to check the reproducibility.

3. 8. Analysis of genetic stability of long term cultures

3. 8. 1. Chromosome counting

To evaluate the cytogenetic stability of long term cultures, the chromosome counting from embryogenic calli were made following hematoxylin staining method (Grosser and Gmitter 1990). The material for cytological analysis comprised of samples derived from 3-year-old cultures (long term calli). Three year old calli were subcultured in fresh medium two week before chromosome counting. Actively dividing cells from these callus cultures were harvested and pretreated with 1, 4 - dichlorobenzene for 3 h. The samples were then washed with distilled water and fixed in Camoy solution for 16 to 20 h. After overnight fixing, the tissues were softened in 5 M HCl for 12 min. The tissue was then washed with distilled water and treated with 4% ammonium sulfate for 12 min followed by treatment with 4% ammonium sulfate for 1h. The rinsed materials were stained with 0.5% hematoxylin for 3 h and then smeared for chromosome examination. The experiment was repeated with samples from five different tubes.

3. 8. 2. RAPD analysis

RAPD technique was used to determine the genetic stability from long term embryogenic calli. Two groups of embryogenic calli were tested, one group was obtained from 8-month-old embryogenic calli (short term calli) where as the other group was derived from 3 year old embryogenic calli (long term calli). Genomic DNA was extracted from the calli of two populations. DNA was also extracted from leaves of mother plant. DNA was isolated following the modified procedure of Doyle and Doyle (1990) as described earlier (3.7.2.2). 10 arbitrary decamer primers producing easily scorable bands were selected for polymerase chain reaction. DNA amplification was performed as mentioned earlier with the same PCR conditions (3. 7. 2. 4.). The PCR products were separated on 1.5% agarose gel and gels were analyzed under UV light and photographed. Amplification was repeated thrice with samples from five different tubes.

3. 9. Biochemical studies during somatic embryogenesis

3. 9. 1. Total soluble protein extraction and electrophoresis

Tissues at five developmental stages of somatic embryogenesis were selected for the study. Callus (C), appearing on the explants at 6 weeks of culture on callus induction medium, embryogenic callus (EC) selected from a population of embryogenically

competent cells differentiating into a mass of embryos, non-embryogenic callus (NC), the friable calli which never produce embryos, embryos (E), a number of embryos at different developmental stages and plantlets (P), the fully developed plant were selected for qualitative separation of total soluble proteins. Somatic embryos at 3 different stages *viz*, globular embryos (GE), heart/torpedo embryos (TE) and cotyledon embryos (CE) were also used for the study. All samples were frozen in liquid nitrogen and stored at -20° C until use. Frozen tissue samples of 500 mg each were crushed into a fine powder in liquid nitrogen with 1.5 ml of ice cold dithiothrietol (2%). After centrifugation in the cold at 10,000 rpm for 20 min, the supernatants were mixed with 8 volumes of ice cold acetone and the homogenate was incubated in ice for 2 h. The protein was precipitated by centrifugation and then gently washed the precipitate twice with ice cold 100% (v/v) acetone. The final pellet was air dried and solubilized in a small volume of buffer containing 0.5 ml of the following (8 M Urea, 4% CHAPS, 50 mM EDTA). Proteins from each sample were analyzed on 12% 1.5 mm thick SDS-PAGE as described by Laemmli (1970) in a vertical electrophoresis apparatus. After washing, the glass plates were rinsed with distilled water and then with acetone. The plates were sandwiched with two 1.0 mm spacers in between. Both sides of the assembly were sealed using cello tape. The sandwich was then locked and transferred on to the boat, which is filled with 2% agar solution. The assembly was laid vertically in the agar solution such that the lower end of sandwich is sealed by the agar. The entire assembly was left undisturbed for 15 min to allow the agar to solidify. Separating gel solution was poured in between the glass plates carefully without air bubbles. The top of the gel was covered with water saturated isobutyl alcohol to prevent contact with air and kept the gel for polymerization for 30 min at room temperature.

After polymerization, the isobutanol was poured off and the plates were rinsed with 1X Tris HCl buffer pH 8.8 and the traces of buffer were removed using filter paper. Stacking gel was then prepared and carefully poured over the separating gel. A comb was 1 mm thick was carefully inserted between the plates in to the stacking gel and left the solution to get polymerise. After polymerization, the comb was removed carefully and the wells were rinsed with distilled water. Traces of water were removed with filter paper. Electrophoresis buffer (1X) was added to the lower buffer tank of the vertical apparatus. Then polymerized gel was mounted and the upper buffer tank was also filled with the buffer. Protein samples to be analyzed were mixed with the loading buffer (3:1).

For SDS-PAGE, all reagents containing SDS were used and the samples were boiled for 4 min in a boiling water bath for the breaking down of proteins into polypeptides. Samples were loaded to the bottom of the well with apex gel loading tips. Each well was loaded with 40 µg of sample extract except for non embryogenic calli (80 µg). Electrophoresis was carried out initially at 20 mA for 1h. After the entry of the proteins into the gel, the electrophoresis was continued at a constant voltage 50V. Electrophoresis was performed until the marker dye bromophenol blue reached the bottom of gel. After electrophoresis, gels were fixed and put in staining solution containing (0.5%) (w/v) Coomassie Brilliant Blue R-250, isopropanol and acetic acid. Staining was done for 6-7 h and then put for destaining in methanol with acetic acid solution. Destaining was continued for 2-3 d with occasional shaking till clear bands were obtained. After complete destaining, the gels were photographed. Experiments were repeated with samples taken from three different tubes. Protein molecular weight markers phosphorylase b (97.44 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α--lactalbumin (14 kDa) (Genei, Bangalore, India) was used as protein standards.

Separating gel solution (10%)-20 ml	**Stacking gel solution (3.5%) - 5 ml**
Water - 8. 33 ml Tris-HCl/SDS (4X, pH 8.8) - 5.0 ml (1.5 M Tris-HCl, 0.4% SDS) APS (10%) - 200 µl 30% acrylamide solution - 6.66 ml TEMED - 20 µl (APS was freshly prepared just before the gel solution transferred to the gel plate)	Water - 3. 05 ml Tris-HCl /SDS (4X, pH 6.8) - 1.25 ml APS (10%) - 100 µl 30% acrylamide solution - 0. 65 ml TEMED - 20 µl
Loading / sample buffer (6X)	**Electrophoresis buffer (5X)**
Tris-Cl (4X) pH 6.8 - 7 ml Glycerol - 3 ml SDS - 1 g Bromophenol blue - 1.2 mg Diluted to 2x before use	Tris base - 15.1 g Glycine - 72 g SDS - 5.0 g Made up to 1L with distilled water diluted to 1X before use

3. 9. 2. Isozyme studies

Samples at five developmental stages *viz,* callus, non embryogenic callus, embryogenic callus, embryos and plantlets as well as embryos at three different developmental stages viz, globular embryos, heart/ torpedo embryos and cotyledon embryos were used for isozyme study. Isozymes, peroxidase (Prx) and esterase (Est) were selected. Tissues were ground to a fine powder in liquid nitrogen with 1.5 ml of ice cold dithiothrietol (2%). The homogenate was centrifuged at 10,000 rpm for 20 min and the supernatants were used for isozyme analysis. Isozymes were analyzed using native PAG electrophoresis (Laemmli 1970). The electrophoresis buffer consisted of 0.025 M Tris and 0.192 M glycine. The acrylamide concentrations in the stacking gel was 4% incorporating Tris-HCl pH 6.8 (0.5 M) and for running gel, the acrylamide concentration was 7.5%. Each well was loaded with 50 µg of sample extract except for non embryogenic calli (100 µg). Electrophoresis was carried out at 50 V for 30 min followed by 100V. All operations were carried out at 4°C and kept overnight at room temperature. All experiments were repeated with thrice. After electrophoresis, gels were removed and stained with specific stains. For peroxidase, gels were stained with Benzidine-H_2O_2 (Vallejos 1983). Staining solutions were prepared by mixing 0.1% (w/v) benzidine hydrochloride with 0.4 ml glacial acetic acid and left the filtrate in dark until use. 0.2 ml of hydrogen peroxide was added at the time of gel incubation and stained for 10 min. After the bands became clear, drained off the staining solution and washed the gels. For esterase, the staining procedure of Lebrun and Chevallier (1988) was followed. Sodium phosphate buffer (0.1 M pH 7.0) was pre incubated at 4°C in dark. The gels were first washed with 0.1 M phosphate buffer (pH 7.0) and then pre incubated phosphate buffer for 20 min at 4°C in dark. After draining the buffer, napthyl acetate solution was added and left at 4°C for 10 min. Buffer was again drained and the gels were then covered with Fast Blue RR salt for 5 min. After staining, the gels were incubated at room temperature with gentle shaking until the bands were distinguished. The gels were then fixed in water: ethanol: acetic acid: glycerol (2:1:1:1). Finally the gels were washed in distilled water, viewed and photographed.

RESULTS

4. 1. Utilization of immature anthers as explants

In the present study, with the view of developing a reliable *in vitro* protocol for rubber, the suitability of immature anther as a source of explant for *Hevea* somatic embryogenesis was explored. Immature flower buds (Fig. 2 A) before microsporogenesis were selected for the study. During dissection of immature anthers with ascorbic acid 60 %, anthers remain fresh, white in colour and inducing callus. Although slight browning was also occurred in low frequency (Table 2). It was observed that among 40 % brownish anthers, callus was induced from 30 % of brownish anthers (Fig. 2 B, C) suggesting that browning was not having a complete effect on callus induction, however, required a long time for callusing. Without ascorbic acid or when sterile water was used, tissue browning was very severe. All explants turned to dark and only 10% explants induced callus and no callus was induced from remaining darkened anthers (Fig. 2 D).

Table 2: Effect of ascorbic acid on tissue browning and callusing

Treatments	No of anthers	% of browning	% of callus induction
Ascorbic acid	100	40 (slight)	30
Water	100	100 (dark)	10

4. 2. Induction of callus and embryos

The response of two basal media and different auxins are illustrated in Table 3. In the present study, among the two basal media studied, modified MS medium was found to be more effective. On MS medium, callus was produced when 2, 4-D is provided as the auxin along with 0.5 mg/l KIN. With other auxins (NAA, IAA and IBA), no callus was induced even after two months of culturing. In these treatments, initially swelling of anthers was observed however, later they get shrivelled and dried.

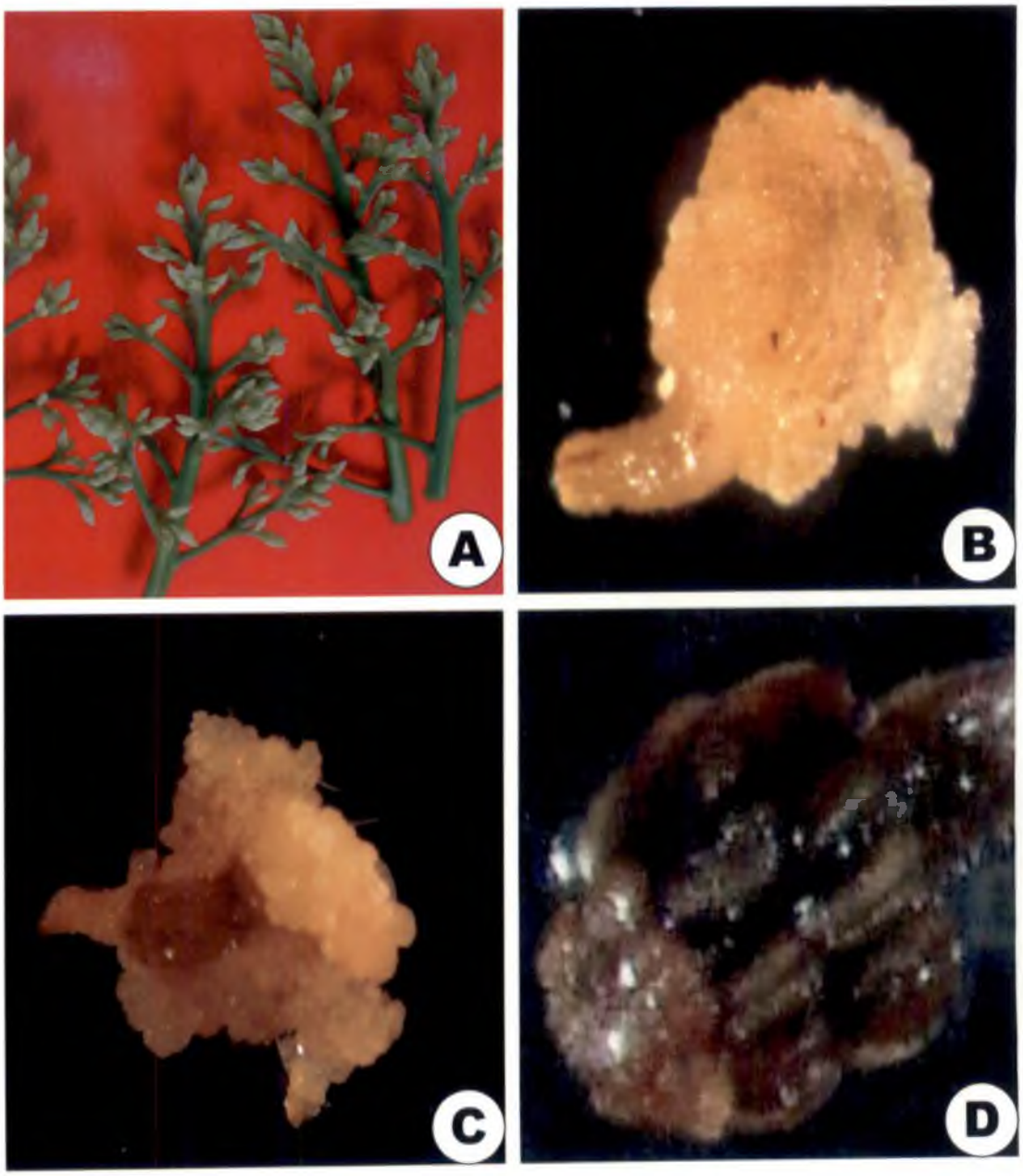

Figure 2A-D: Explant nature and callusing response. A. Immature flower buds; B & C. Callus initiation from brown anthers; D. A view of darkened anther

When KIN concentration was increased to 1.0 mg/l, for all auxins, no callus was induced. However, with modified MS medium, callus was induced on medium containing 1.0 mg/l and 2.0 mg/l 2, 4-D along with 0.5 mg/l KIN. When KIN level was increased to 1.0 mg/l, callus induction was decreased. Other auxins IAA and IBA, at 2 tested concentrations along with KIN, were totally ineffective for callus induction but resulted in explant swelling. However, when NAA was used as auxin, little callus was induced at higher concentration (2.0 mg/l NAA+ 0.5 mg/l KIN).

Table 3: Response of basal media and auxin on callus induction

Basal medium	Auxins/KIN (mg/l)	2,4-D	NAA	IAA	IBA
MS medium	1.0 + 0.5	+	Swelling	Swelling	Swelling
	2.0 + 0.5	+	Swelling	Swelling	Swelling
	1.0 + 1.0	Swelling	Swelling	Swelling	Swelling
	2.0 + 1.0	Swelling	Swelling	Swelling	Swelling
Modified MS medium	1.0 + 0.5	++	Swelling	Swelling	Swelling
	2.0 + 0.5	++++	Swelling	Swelling	Swelling
	1.0 + 1.0	+	Swelling	Swelling	Swelling
	2.0 + 1.0	+	+	Swelling	Swelling

Callus scoring + = Little callus ++ = Medium callus +++ = Profuse callus

To find out the optimum level of 2, 4-D required for callus induction, a one way factorial experiment was carried out and the results are presented in Table 4. Results indicated that in 2, 4-D containing medium, about 80% of the explants responded positively and produced callus. However, depending on 2, 4-D concentrations, the rate of callus was differed. At all concentrations of 2, 4-D, swelling of anthers was seen after 2 weeks of culture (Fig. 3 A, B) followed by callus initiation at 50 days of culture (Fig. 3 C, D). No callus was induced in the absence of 2, 4-D. At lower concentrations ranging from 0.5-1.0 mg/l, amount

of callus induced was less. When 2, 4-D concentration was further increased (1.5 and 2.0 mg/l), callus rating was also increased. On the other hand, at still higher concentrations such as 2.5 and 3.0 mg/l, maximum callus was induced. The morphology of callus was differed depending on 2, 4-D concentration. Based on morphology, the callus produced by different treatments was classified into three types (Table 4).

Table 4: Effect of 2, 4-D and KIN on callus induction

2,4-D+ Kn	Callus morphology	Callus type	Callus scoring
0. 0 + 0.5			-
0.5 + 0.5	Friable, watery, soft, white creamy	Type I	+
1.0 + 0.5	Friable, watery, soft, white creamy	Type I	+
1.5 + 0.5	Semi friable or compact pale yellow	Type II	++
2.0 + 0.5	Compact, shiny surface, pale yellow	**Type II**	+++
2.5 + 0.5	Compact, hard or dry, pale yellow	Type III	+++
3.0 + 0.5	Compact, hard or dry, pale yellow	Type III	++++

Callus rating - = No callus, + = 1 - 20%, ++ = 21 - 50%, +++ = 51 - 80%, ++++ = 81 - 100%

Callus belonging to Type I was soft, watery and friable (Fig. 3 E) which are found to be non embryogenic based on microscopic observation. This type of callus was originated from medium containing lower levels of 2, 4-D (0.5-1.0 mg/l). Type II callus (Fig. 3 D) was initiated at concentrations of 1.5 and 2.0 mg/l. This type of callus was semi friable or compact and embryogenic in nature with shiny texture under microscope. Beyond this, as the concentration of 2, 4-D was increased to 2.5-3.0 mg/l, friability was reduced. This type of callus, termed type III (Fig. 3 F), was produced mainly in higher concentrations of 2, 4-D. Although the callus rating was higher at this concentration, the callus was compact and hard. Among the three types, Type II alone was effective for embryogenic callus induction. The optimal concentration of 2, 4-D for embryogenic callusing was 2.0 mg/l combined with 0.5 mg/l KIN. In this combination, pale yellow compact aggregates of cells with a shiny surface was observed and callus rating was also higher compared with 1.5 mg/l 2, 4-D.

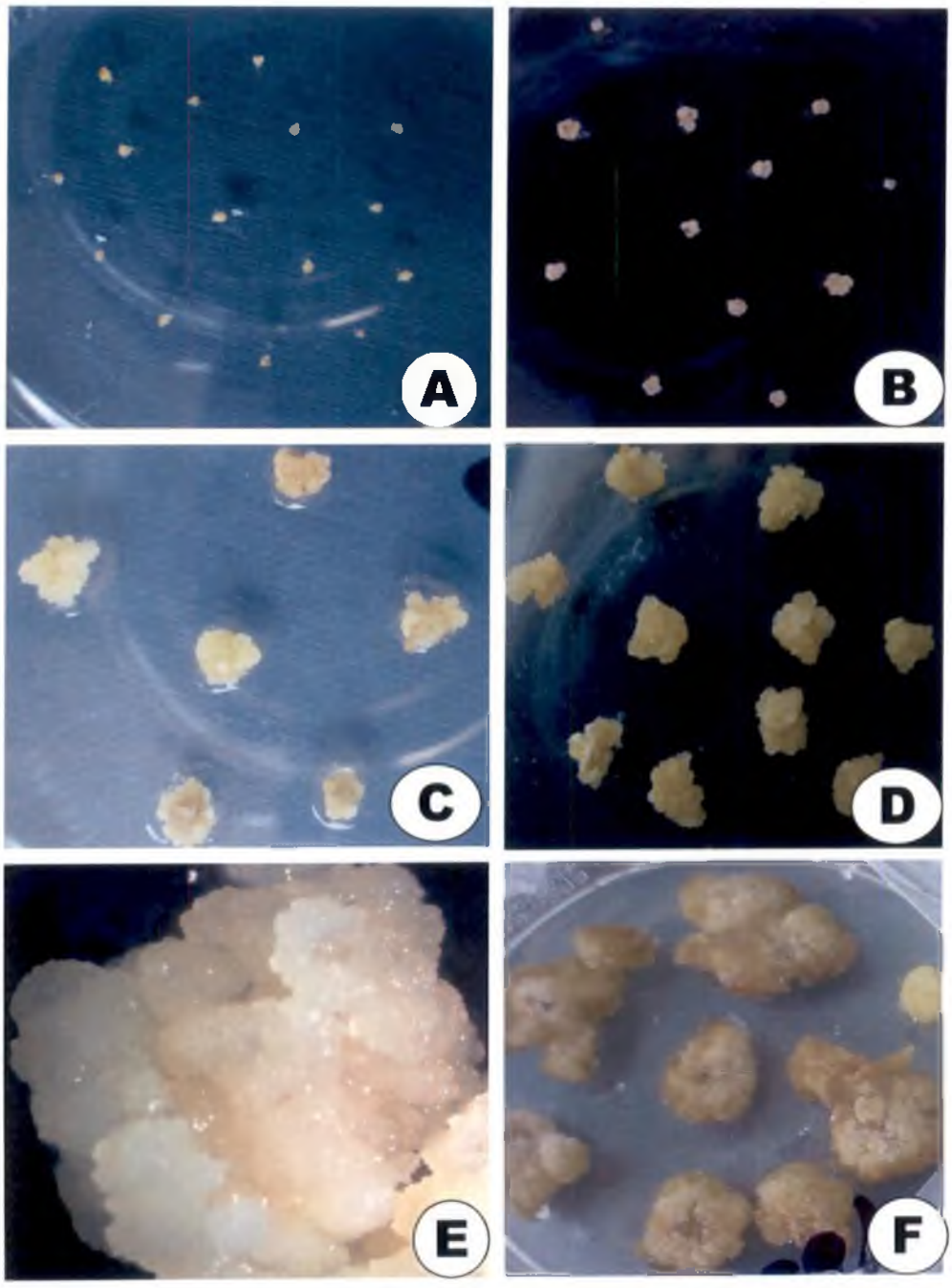

Figure 3 A-F. Callus induction and types of callus. A. Immature anthers at 7 d of culture; B. 15 d of culture; C. 40 d of culture; D. 60 d of culture (Type II callus); E. Type I callus; F. Type III callus

Before the transfer of callus to embryo induction medium, the colour of callus ranges from creamy to yellow and callus texture ranged from friable to hard (Table 4). After callus transfer to embryo induction medium, colour of primary callus was changed to brownish (Fig. 4 A). The morphogenic responses are summarized in Table 5. After 2 months of culture, a yellowish, highly friable and mucilaginous embryogenic callus (Fig. 4 B) started forming from the older brownish callus of Type II. Embryoids (Fig. 4 C) were induced from these embryogenic mass after 4-5 months of culture. This friable embryogenic mass displayed active morphogenic growth and then successively developed into globular embryos. Different stages of embryos *viz*, globular (Fig. 4 D), heart / torpedo (Fig. 4 E) and cotyledonary stages (Fig. 4 F) were observed in the same medium. In addition to the normal dicotyledonous embryos, abnormal embryos with swollen / fused cotyledons were also noticed. Induction of somatic embryogenesis was favoured only by Type II callus. After transfer of Type I callus to embryo induction medium, yellow friable but translucent callus induced from brownish callus and no embryos was induced from this callus. However, with type III callus, friable callus was not produced, instead the calli remains as brownish.

Table 5: Callus morphology and morphogenic response

Callus type	Morphology	Morphogenic response
Type I	Creamy white, friable calli, loose	No embryos
Type II	Yellow, highly friable, embryogenic, mucilaginous	**Embryos**
Type III	Brown calli, no new callus formation	No embryos

For the induction of somatic embryos, combinations of KIN with different auxins were used. Among the 4 auxins (IAA, IBA, 2, 4-D and NAA) tested, IAA and IBA induced a higher rate of browning with lower proliferation of calli (Table 6). These auxins resulted in sporadic formation of embryos either in single form or as clusters. When 2, 4-D was used as auxin, proliferation rate was increased but resulted in low calli browning and embryo development. On 2, 4-D containing medium, most of embryos remained in the globular stage only. However with NAA, the proliferated calli were differentiated into somatic embryos.

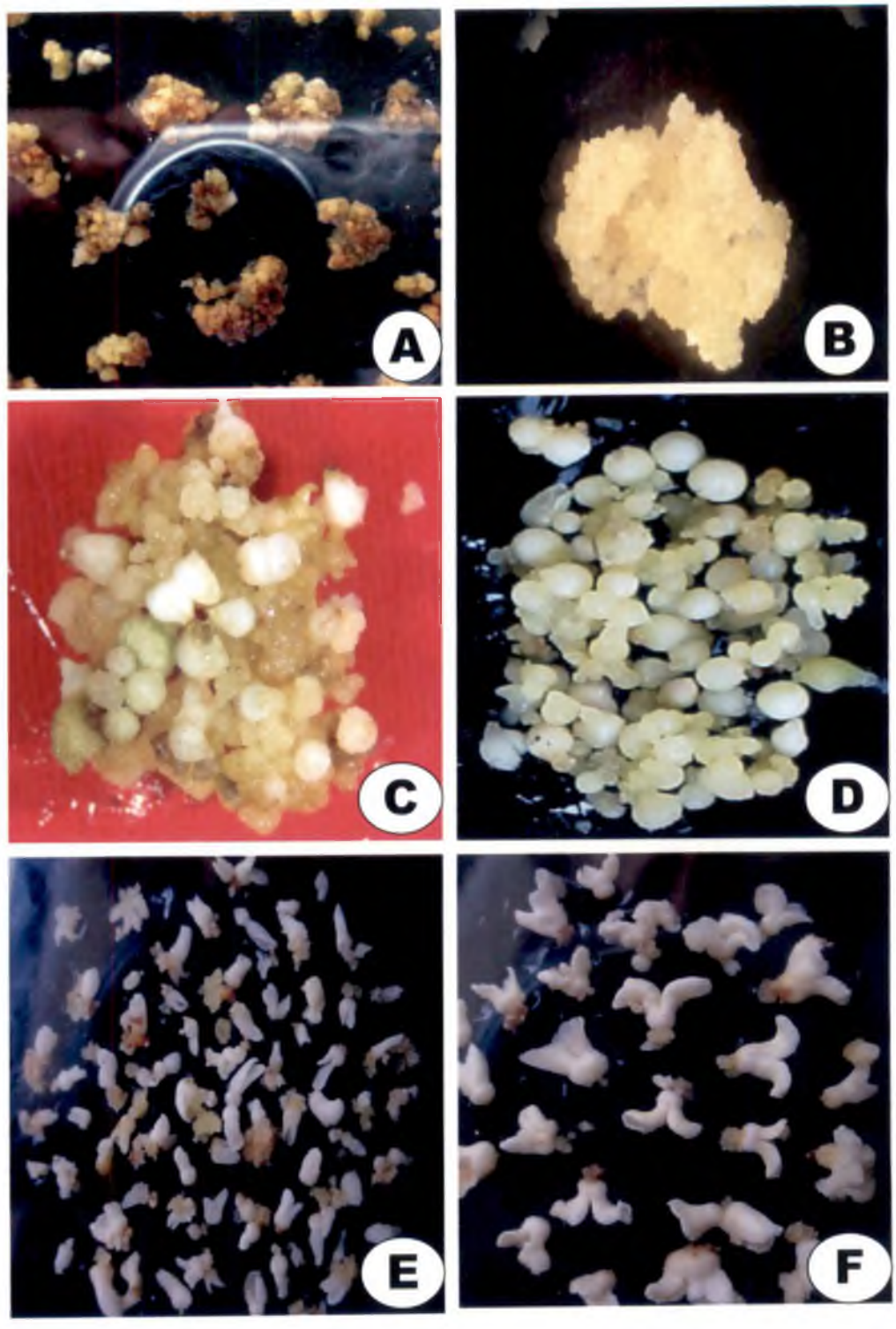

Figure 4 A-F: Stages of embryo induction. A. Formation of brown callus On transfer of yellow callus to embryo induction medium; B. Yellow calli emerged from brown calli; C. Emerging of embryoids; D. Globular embryos; E. Heart/Torpedo embryos; F. Cotyledon embryos

Table 6: Effect of different auxins and KIN on embryo induction

Auxins / Cytokinin 0.0-0.4 / 0.5-1.0 (mg/l)	Nature of response		
	Callus browning	New calli proliferation	Embryo formation
IAA / KIN	High	20% FEC + slow growth	Sporadic
IBA / KIN	High	20 % FEC + slow growth	Sporadic
2,4-D / KIN	Low	70 % FEC + Fast growth	Infrequent
NAA / KIN	**Medium**	**40 % FEC+ slow growth**	**Frequent**

Table 7 shows the effect of KIN and NAA on embryo induction. Among the 30 combinations tested, embryogenesis was observed in most of the combinations containing both NAA and KIN. However, no somatic embryos were induced on medium without NAA and KIN. Lower concentrations of NAA were found to be more effective for embryogenesis. By increasing the concentrations of NAA, a gradual decrease in embryo induction was noticed and above 0.3 mg/l strongly inhibited embryogenesis. Similarly, the cytokinin KIN was influenced embryo induction. Maximum embryos were produced on medium containing 0.7 mg/l KIN combined with 0.2 mg/l NAA. Irrespective of NAA level, higher KIN concentrations did not enhanced embryo induction, but embryo production was drastically decreased.

Table 7: Influence of KIN and NAA on somatic embryo induction

NAA (mg/l)	KIN (mg/l)					
	0.5	0.6	**0.7**	0.8	0.9	1.0
0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.1	4.67±1.75	10.83±1.33	15.33±1.63	11.83±1.72	5.67±0.82	0.70±0.82
0.2	10.0±1.67	18.0± 2.37	**23.83±1.72**	20.67±2.58	20.67±2.58	10.30±1.75
0.3	2.83±1.72	9.00±1.79	14.17± 2.9	11.83±1.72	4.67±1.51	1.00±0.89
0.4	0.50±0.84	3.17±1.47	6.00±1.26	3.83±1.47	1.33±1.75	0.00±0.0

VR= 10.92; CD (5%) = 1.95

4. 3. Parameters influencing callus and embryo induction frequency

4. 3. 1. Explant pretreatment in liquid medium

The results indicated that by direct culture on solid medium, the initial sign of explant swelling followed by callus formation from immature anthers was observed after 50 days of culture. At this time, the induced calli were embryogenic in nature. When the dissected anthers were pretreated in liquid medium, the time requirement for callusing was reduced. During an initial period of 5 d culture in liquid followed by subculture on solid medium, only a total of 45 d was required for callus induction. However, 10 day pretreated explants underwent callus formation after 25 days of culture on solid medium. When explants were cultured on liquid medium for 15 days, duration for callus induction was reduced to 30 and 25 d respectively. However, the percentage of calli with embryogenic nature was affected during long explant pretreatment (Table 8). Each of the treatment was scored for callus initiation based on number of explants producing good quality callus with smooth shiny texture under microscope / number of explants cultured. With 5 day pretreated explants, calli formation was increased significantly. When explants were pretreated for 10 days, calli formation was decreased slightly. With this treatment, only 35 days was required for callus initiation as compared to control where 50 days was required. Prolonged culture in liquid medium (15 and 20 days), callus induced were compact and hard in nature and mainly resulted in decreased embryogenic calli formation.

Table 8: Effect of liquid pretreatment on callus induction

Liquid medium (d)	Solid medium (d)	Total (d)	Calli formation (%)
0	50	50	80
5	40	45	85
10	**25**	**35**	**80**
15	15	30	60
20	5	25	50

4. 3. 2. Callus age / time of subculturing for embryogenesis

To study the optimal time for first subculture of primary callus for embryogenesis, the calli (Fig. 5 A) induced on 35 d was used (10 + 25 d) as control. After callus induction, when the primary calli were subcultured to embryo induction medium, the calli turned brownish and thereafter friable highly embryogenic, yellow calli were produced at a frequency of 30% (Table 9). When the calli (Fig. 5 A) maintenance in callus induction medium was increased from 35 to

50 days, calli proliferation (Fig. 5 B) as well as friable embryogenic calli production (after transfer to embryo induction medium) was increased from 30 to 50%. However, exposure of calli to callus medium was further increased, the calli gradually looses its friability (Fig. 5 C) and turns to hard. Such calli during transfer to embryo induction medium, the formation of friable highly embryogenic calli was decreased. Prolonged maintenance on 2, 4-D containing callus induction medium was resulted in decreased embryogenic calli production. But in these treatments, the primary callus was proliferated effectively.

Table 9: Effect of callus age (subculturing time) on embryo induction

Maintenance on CIM (d)	Calli proliferation rate*(%)	Friable calli formation (%)
0 + 35	2	30
15 + 35	**5**	**50**
30 + 35	7	40
45 + 35	9	25
60 + 35	10	10

* = rating scale (1-10): 1= low, 5= medium, 10= profuse

4. 3. 3. Effect of polyamines

The effects of polyamines, spermine and spermidine on embryo induction are shown in Table 10. Results clearly show that the addition of spermine in all tested combinations does not improve embryogenesis. Inclusion of spermine on medium allowed embryo induction, but embryo production remains more or less same as compared to that of control. On the other hand, treatment with spermidine was effective when used in a concentration of 0.5 mg/l, although the difference is very marginal. When the concentration was increased, the somatic embryo production was slightly reduced. However, when both spermine and spermidine was incorporated, the calli proliferation (visual observation) was found to be enhanced.

Table 10: Effect of polyamines on embryo induction

Polyamines	Concentrations (mg/l)	Number of Embryos (Mean)
Spermine	0.0	21.44 (a)
	0.5	20.33
	1.0	21.00
	2.0	20.33
Spermidine	0.0	21.44 (a)
	0.5	**22.67**
	1.0	20.44
	2.0	20.22

(a)=VR=1.18, CD (5%) = NS (not significant), (b)=VR= 4.60, CD (5%)=1.49

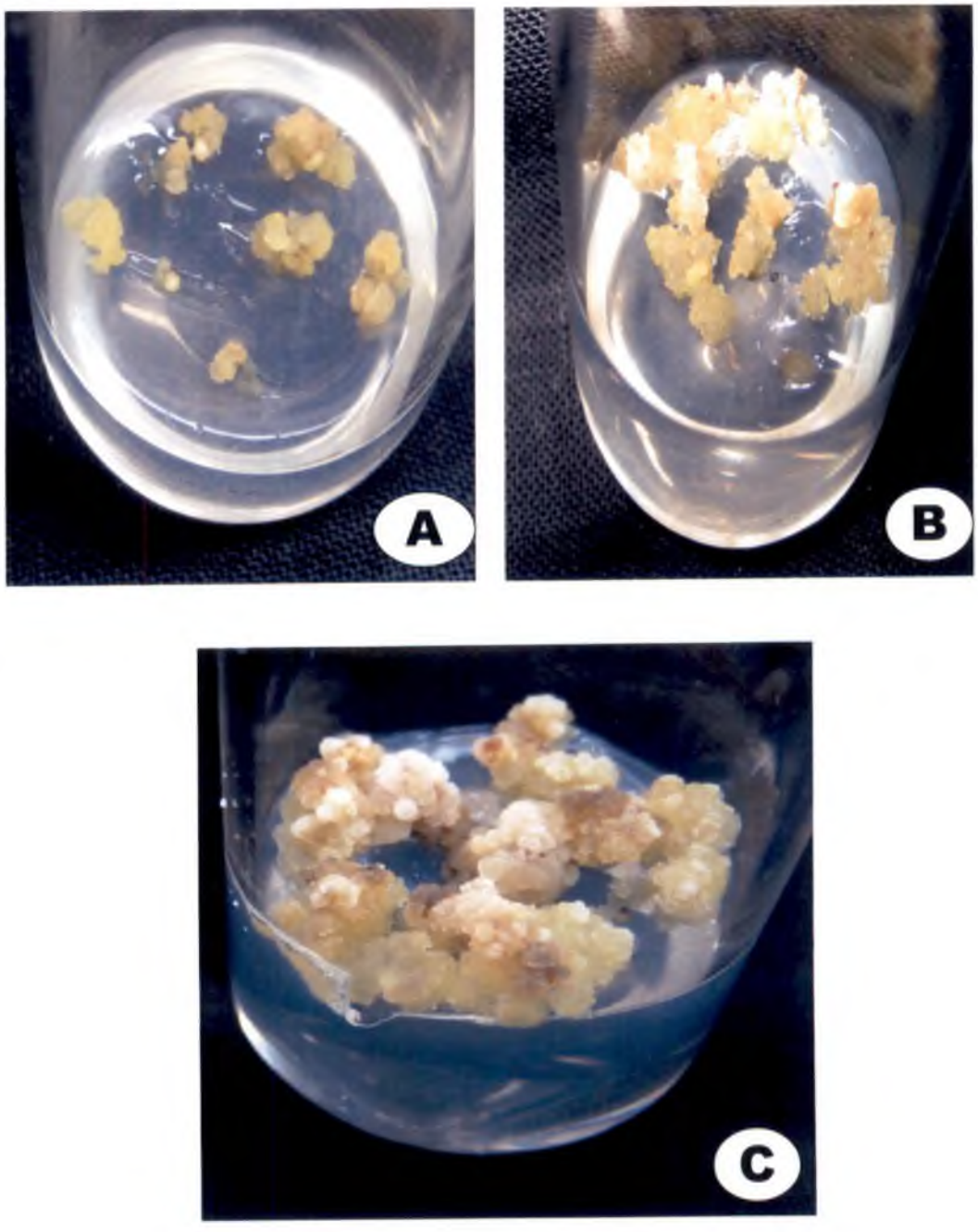

Figure 5A-C: Callus response and effect of subculturing time. A. Callus induced from explants of 10 d pretreatment in liquid +25 d culture on solid medium ; B. Callus from 50 d maintenance on 2,4-D medium; C. Callus from 65 d maintenance

4. 3. 4. Effect of different amino acids on embryo induction

There are many reports on the positive role of amino acids on embryo induction in many plants, however, in *Hevea* little information is available on its influence on embryo induction. From the results obtained in our study, not all amino acids applied to the culture medium improved somatic embryogenesis. Among the four amino acids, alanine, asparagine, arginine and glutamine tested, the amino acid alanine does not appear to have any beneficial effect on embryo induction (Table 11).

Table 11: Effect of different amino acids on embryo induction

Amino acids	Concentrations (mg/l)	Embryos (Mean)
Alanine	0.0	22.00 (a)
	50	18.22
	100	15.22
	150	12.11
	200	9.22
Asparagine	0.0	22.00 (b)
	50	21.22
	100	21.11
	150	21.22
	200	20.33
Arginine	0.0	22.00 (c)
	50	20.22
	100	20.22
	150	16.33
	200	14.00
Glutamine	0.0	21.12(d)
	50	24.25
	100	30.08
	150	42.25
	200	44.75

(a), VR = 78.29, CD(5%) = 1.62, (b), VR = 2.41, CD(5%) = NS
(c), VR = 37.62, CD (5%) = 1.53, (d), VR = 136.51, CD (5%) = 2.51

Instead, its supplementation on medium resulted in decreased embryo induction. Even at 50 mg/l, the embryo induction frequency has reduced compared with the control. By increasing the alanine concentration, the embryo induction frequency has gradually decreased and at higher concentration (200 mg/l) the embryo induction was slightly inhibited. On the other hand, asparagine had no stimulatory effect on somatic embryogenesis. At all concentrations tested, more or less same effect on embryo induction was obtained compared to that of control. With arginine, a decreased effect was observed. When arginine was added in the medium in concentrations ranging from 50-100 mg/l, the response was

more or less same as compared to that of control. By increasing the levels from 150- 200 mg/l, embryo induction was decreased. Supplementation of glutamine resulted in significant improvement in embryo induction frequency. In all treatments from 50-200 mg/l, both friable embryogenic calli (visual observation) as well as embryos were produced. It was found that the frequency was increased with increasing concentrations of glutamine. Highest number of somatic embryos was produced on medium with 200 mg/l glutamine.

Beneficial effects of casein hydrolysate on embryo induction are indicated in Table 12. When medium was enriched with casein hydrolysate, there was an increase in frequency of embryogenesis induction. At lower concentrations, casein did not give much influence on embryo induction. However, when concentration was increased to 400 mg/l, a gradual increase in embryogenesis was obtained and maximum number of embryos was produced at 400 mg/l. Beyond this concentration, a decrease in embryogenesis was noticed. In contrast, the formation of highly friable embryogenic calli was increased with increasing concentrations (visual observation).

Table 12: Effect of casein hydrolysate on embryo induction

Organic additive	Concentration (mg/l)	Embryos (Mean)
Casein hydrolysate	0	20.00 (a)
	100	23.25
	200	25.62
	400	**33.12**
	600	27.12

(a) = VR = 48.45, CD (5%) = 2.03

4. 4. Plantlet regeneration

After 2-3 weeks of growth, mature embryos showed bipolarity. It was observed that during embryo germination, simultaneous development of root and shoot was occurred occasionally. In most cases, root induction occurred first followed by the formation of shoot apex. However, shoot apex growth was not observed in certain cases. 27% of normal embryos converted into plantlets upon transferring to hormone free medium.

4. 4. 1. Effects of cytokinins on plant regeneration

By transferring the mature embryos (Fig. 6 A) into plant regeneration medium containing 2.0 mg/l GA_3, irrespective of the type and concentration of cytokinins used, the germination started within 7-10 days of culture. Usually the germination was initiated by the expansion of cotyledons and changing the colour to green. The middle and upper portion of

hypocotyl turned green while lower portion remained as white (Fig. 6 B). The elongated somatic embryos developed to normal root and shoot (Fig. 6 C) followed by primary leaf emergence (Fig. 6 D). However, the germination frequency and plantlet development was significantly enhanced by the inclusion of cytokinins.

After 10 days of culture, embryo germination frequency was significantly higher (70%) for medium containing 0.5 mg/l BA. By maintaining the cultures for another 50 days, the germinated embryos developed into full plantlets (Fig. 6 E, F). At this concentration, 23% embryos produced only root without shoot (Fig. 6 G). However, with 1.0 mg/l although the germination was decreased, the full plant development was slightly increased than with 0.5 m/l. When the BA concentration was increased to 1.5 mg/l, the germination as well as full plant development was decreased. At higher concentrations of BA (2.0 mg/l), a further decrease on both germination and plant development was observed (Table 13). In all combinations of BA tried, maximum response was obtained with 0.5 mg/l.

Table 13: Effect of BA + 2.0 mg/l GA_3 on plantlet development

BA (mg /l)	Embryos (%)			Plant recovery (%)
	nongerminated	root only	germinated	
0.0	13 a	47 NS	40 a	50 a
0.5	6.6 ab	23	**70 b**	80 b
1.0	0.0 b	40	60 ab	83 c
1.5	6.6 ab	33	60 ab	67 abcd
2.0	10 ab	43	47 ab	57 d

Table 14: Effect of ZEA + 2.0 mg/l GA_3 on plantlet development

ZEA (mg /l)	Embryos (%)			Plant recovery (%)
	nongerminated	root only	germinated	
0.0	6.6 NS	57 NS	37 a	45 a
0.5	6.6	40	40 abcd	50 d
1.0	3.0	43	53 c	56 abcd
1.5	0.0	40	60 d	72 b
2.0	0.0	33	**67 b**	80 c

Table 15: Effect of KIN + 2.0 mg/l GA_3 on plant development

Kn (mg /l)	Embryos (%)			Plant recovery (%)
	Nongerminated	root only	germinated	
0.0	13 NS	47 NS	40 NS	42
0.5	13	47	40	42
1.0	10	43	47	50
1.5	6.6	43	50	47
2.0	3.0	43	53	56

Data within a column followed by a common letter are not significantly different at the 5% level of significance
Data within a column indicated NS, all are not significant

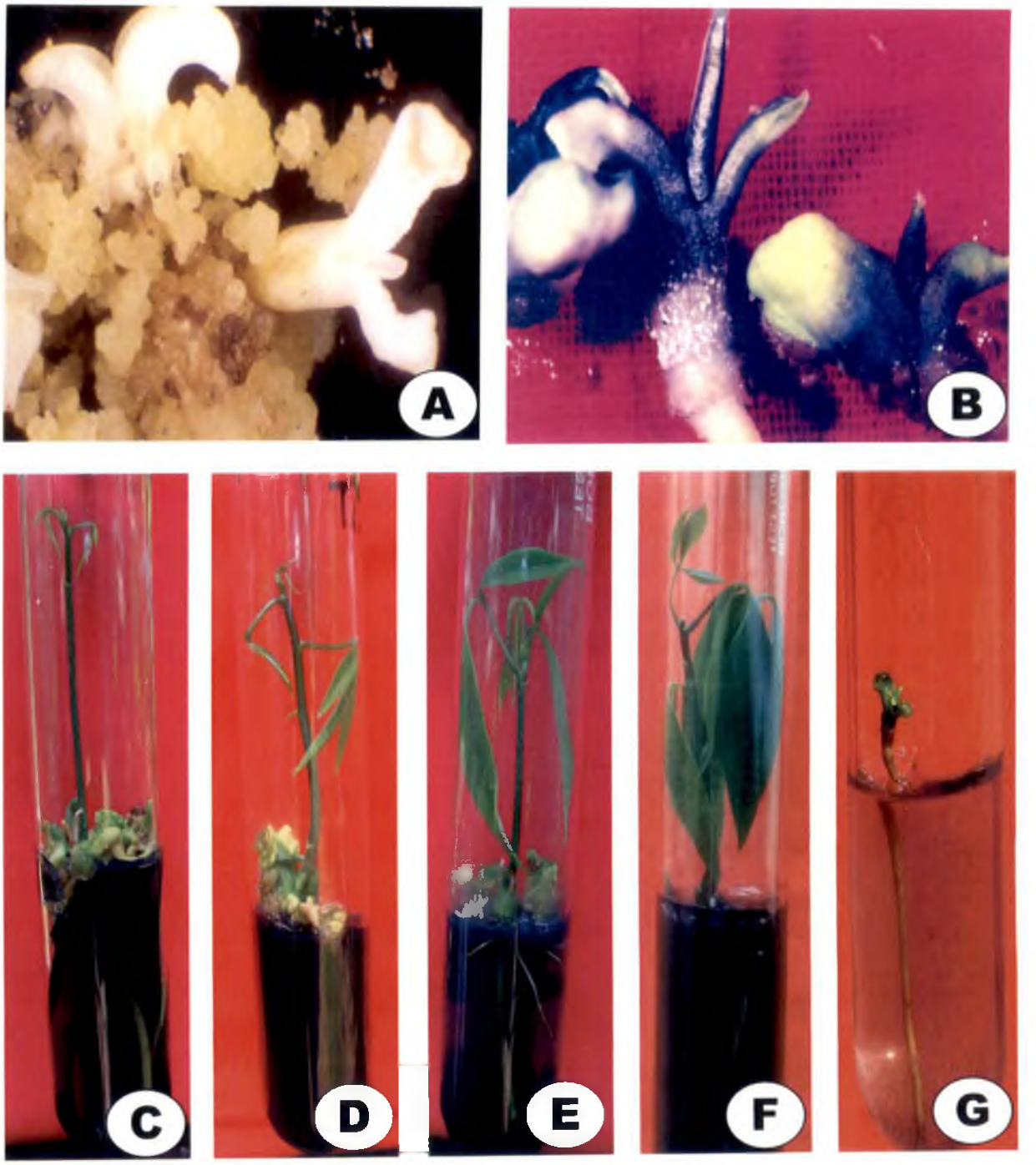

Figure 6 A-G: Stages during somatic plant regeneration.
A. Mature embryos; B. Bipolar embryos; C. Plant with elongated shoot and root; D. Leaf emergence; E-F. Fully developed plantlets; G. embryo with root only

By replacing BA with ZEA, at lower concentrations (0.5 mg/l), the response was lower. If left these cultures for another 50 days in the same medium, no difference in response in terms of germination and full plantlet development was noticed (Table 14). By increasing the concentrations of ZEA to 1.0 mg/l, the response was also increased. However, beyond 1.0 mg/l, a increased effect was observed and reached maximum at 2.0 mg/l concentration. When KIN was added in the medium, comparatively lower response was obtained. No difference in response was noticed between control and medium supplemented with a lower concentration of KIN (0.5 mg/l). At this concentration, the percentage of germinated embryos without shoot was very higher (Table 15). However, even after 50 days of culture on the same medium, shoot apex was never emerged from these embryos. Even at higher concentration, embryo germination and recovery of full plantlet was only 53% and 56% with KIN.

Table 16: Effect of TDZ+ 2.0 mg/l GA_3 on plant development

TDZ (mg /l)	Embryos (%)			Plant recovery (%) (B)
	nongerminated	root only	germinated (A)	
0.00	6.6 NS	50 a	43	38
0.25	3.0	17 b	80	83
0.50	3.0	37 ab	60	67
0.75	3.0	37 ab	60	67
1.00	6.6	43 ab	50	53
2.00	10	56 c	30	40

Data within a column followed by a common letter are not significantly at different at the 5% level of significance

Data within a column indicated NS, all are not significant

A where treatments 1-2, 2-5, 2-6, 3-6 and 4-6 are significantly different

B where treatments 1-2, 1-3, 1-4, 2-5, 2-6, 3-6 and 4-6 are significantly different

By the addition of TDZ, embryo germination was started 7 days after culture and maximum germination was occurred when medium was supplemented at a concentration of 0.25 mg/l (Table 16). After 50 days, the germination frequency was increased. When the concentration was further raised, the frequency of germination and plantlet development was decreased. All concentrations above this level showed a decreased effect and a minimum response was occurred at 2.0 mg/l. Among the cytokinins, plantlets produced by TDZ showed a maximum height of 11.0 cm and were vigorous in growth compared to BA and ZEA derived plants (Table 17). Initial growth of plants at four months of acclimatization was also higher for plants produced from TDZ containing medium than from BA and ZEA (Table 18).

Table 17: Morphological characters of plantlets (Values are means ± SD)

Cytokinin (mg/l)	Plant height (cm)	Shoot length (cm)	Leaves (nos)
TDZ - 0.25	11.45 ± 0.30	6.29 ± 0.19	3.21 ± 0.17
BA - 0.50	10.38 ± 0.32	5.56 ± 0.21	2.81 ± 0.18
ZEA – 2.0	08.47± 0.32	4.91± 0.21	2.56 ± 0.18

Table 18: Morphological characters at 4 months after acclimatization

Cytokinin (mg/l)	Shoot length (cm)	Leaves (nos)
TDZ - 0.25	9.14 ± 0.58	7.71 ± 0.48
BA - 0.50	16.40 ± 0.69	7.20 ± 0.56
ZEA – 02.0	14.60 ± 0.69	7.00 ± 0.56

Table 19: Effect of subculture interval on embryo induction (8 month old calli)

Subculture interval (days)	Embryo induction (Mean)
25	39.10
50	52.43
75	33.50
	CD (5%) = 1.30

4. 5. Long term embryogenesis

An attempt was made to initiate long term embryogenesis using embryogenic callus aggregates and embryos, derived from primary somatic embryogenesis, as the target tissue. The preliminary experiment with different subculture intervals revealed that when subculturing embryogenic callus aggregates with an interval of 25 d, new callus growth was visible within 15 days and subsequently, proliferation occurred along with the differentiation of embryos (Table 19). When culture period was prolonged to 50 d, maximum embryo induction was observed. By prolonging the subculture interval further to 75 d, the new embryogenic callus continued to grow vigorously and above 50 d, the yellow colour of callus was gradually disappeared and callus became creamy / whitish in colour. Moreover, after this period, callus at the upper surface was not in contact with the medium and thereby reduced embryo differentiation. During culture, the brownish embryogenic callus aggregates (Fig. 7 A) induces yellowish callus (Fig. 7 B) from which several embryos emerged (Fig. 7 C). Embryos at different developmental stages were observed (Fig. 7 D) and embryos reached up to colyledonary stages could be readily converted into plantlets during that period. The remaining cultures continued in the same manner and showed embryogenic competence up to three years.

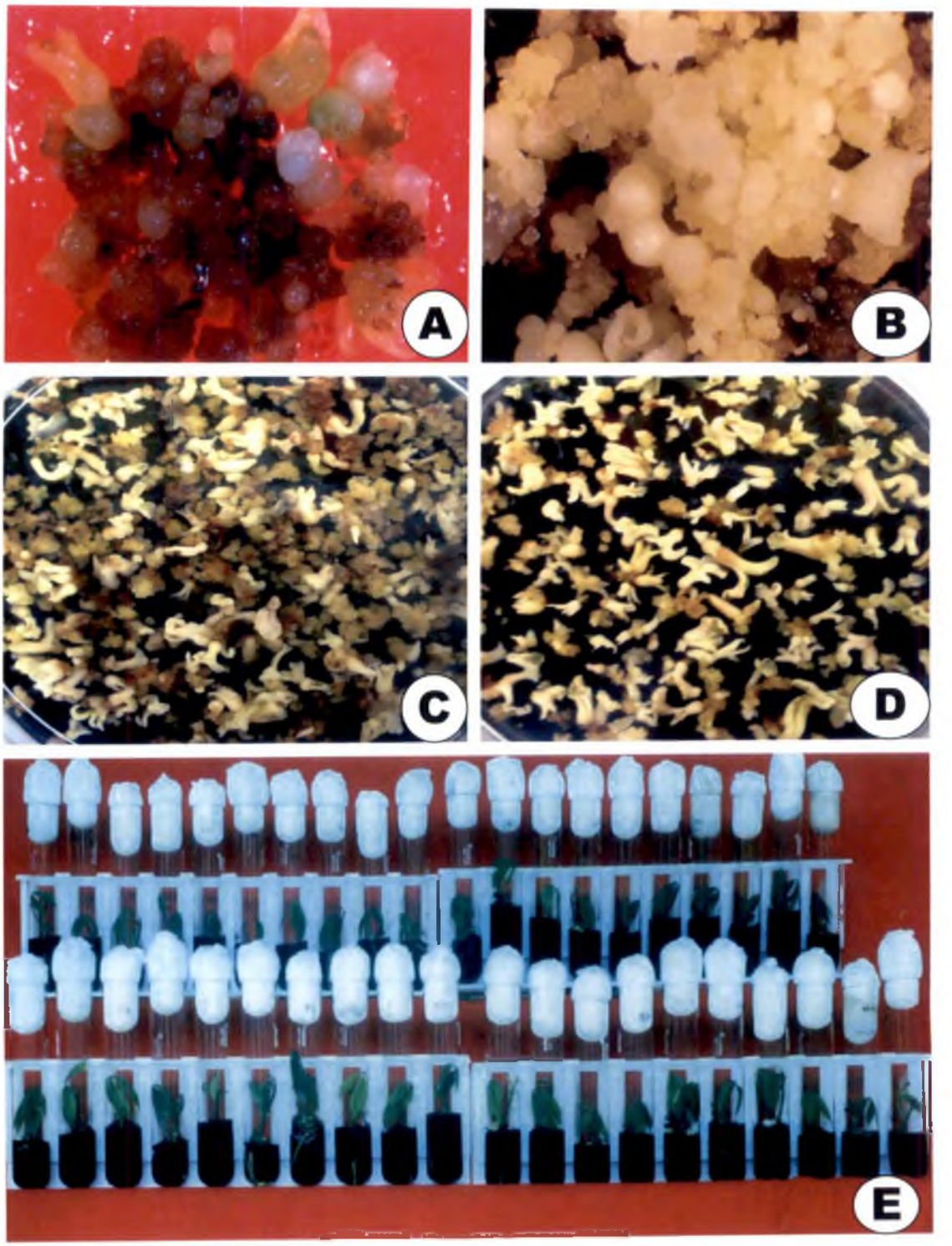

Figure 7 A-E: Initiation of long term embryogenesis.
A. Embryogenic callus bearing embryos; B. Emerging of yellow callus; C. Induction of embryos; D. Embryos at different stages; E. Plantlets

4. 5. 1. Effect of proline and charcoal on long term embryogenesis

To study the effect of proline and charcoal on embryo induction from long term cultures, separate experiments were attempted. 8 month old ECA was subcultured on different combinations of proline containing medium. In the reported study, proline showed a positive effect on embryogenesis (Table 20). When proline was included in the medium, embryo induction was increased. But at higher concentrations, a decrease in frequency was observed. Among the tested concentrations, highest number of embryos was produced at 100 mg/l proline. Beyond this level, a decreased effect was resulted. However, induction and proliferation of new callus was increased (visual observation) up to 200 mg/l. At very high concentration (300 mg/l), both callus induction and embryogenesis was reduced. By maintaining the cultures up to 3 years, proline containing medium showed more or less comparable response on embryogenesis.

Table 20: Effect of proline on embryogenesis from 8 month (a) and 3 year old (b) callus

Proline (mg / l)	Mean number of Embryos (a)	Mean number of Embryos (Mean) (b)
0	50.43	48.70
50	55.83	56.60
100	**60.50**	**62.43**
200	55.50	52.96
300	51.67	50.50
	CD (5%) = 1. 30	CD (5%) = 1. 81

Table 21: Effect of charcoal on embryogenesis from 8 month (a) and 3 year old (b) callus

Charcoal (%)	Embryos (Mean) (a)	Embryos (Mean) (b)
0.00	30.77	21.36
0.05	45.80	56.50
0.10	**58.37**	**66.86**
0.20	46.33	54.30
0.30	38.90	48.50
	CD (5%) = 2.28	CD (5%) = 1.71

AC had a significant effect on long term embryogenesis. In the absence of AC, the embryo induction frequency was very low (Table 21). When added at 0.05%, a gradual increase in embryo induction was observed and reached maximum at 0.1%. With 0.2% AC, even though embryogenesis was occurred, the frequency was found to be reduced and at 0.3% embryo induction declined significantly. Compared to AC enriched medium, callus induction and proliferation was high in the absence of AC. When maintained the cultures up to 3 three years, it was observed that in medium without AC, embryo induction frequency was further reduced. However, compared to 8 month old cultures, embryogenesis was slightly higher from long term cultures for all combinations. Mature cotyledonary embryos were converted into plantlets upon transferring of embryos to germination medium containing either BA (0.5 mg/l) or TDZ (0.25 mg/l) along with GA_3. 70-80% plant recovery was observed during this period (Fig. 7 E).

On the other hand, secondary embryos were also induced from all stages of embryos. However, the frequency was differed depending on developmental stages. Both globular and heart / torpedo stages responded for the production of secondary somatic embryos (Fig. 8 A, B). Secondary embryos were higher in immature cotyledon stage embryos (Fig. 8 C). Mature cotyledon embryos (Fig. 8 D) and germinated embryos (Fig. 8 E) also produced secondary embryos.

Usually secondary somatic embryos were emerged from the root pole of the embryos or the hypocotyl region of the germinated embryos. The secondary somatic embryos were grown into full cotyledon embryos when maintained in the same medium without subculture for 1 month. After maturation, embryos were converted into plantlets. Remaining embryos underwent for secondary embryogenesis in the same manner and embryogenic lines have been maintained for 1 year by secondary embryogenesis.

4. 6. Effect of light

Environmental variables are considered as one of the important factors affecting the morphogenic response during *in vitro* culture. In the present experiment, the influence of light and darkness on callus and embryo induction as

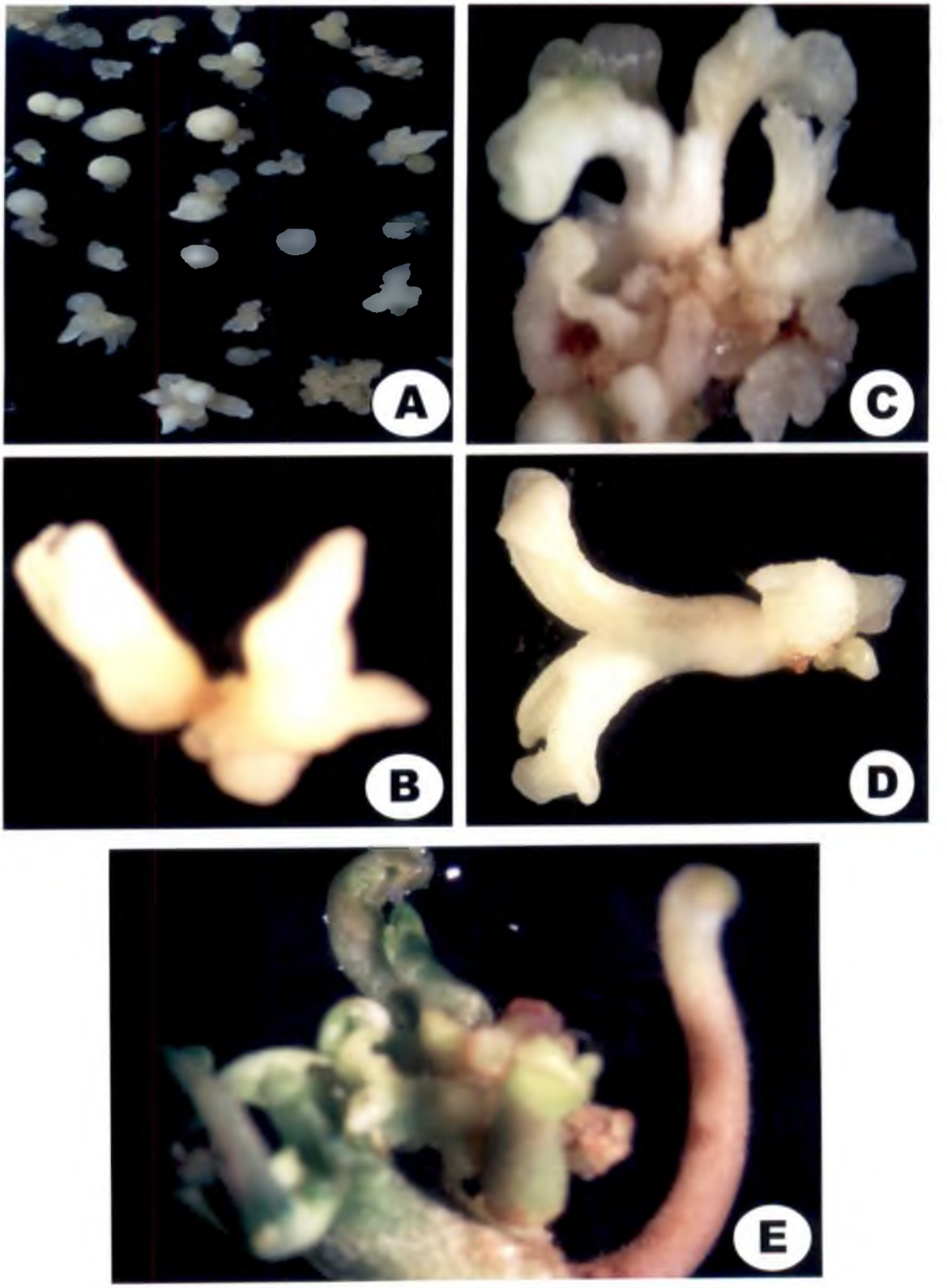

Figure 8 A- E: Induction of secondary embryos. A. From globular; B. Heart/Torpedo; C-D. Immature and mature cotyledon; E. Germinated embryos

well as plantlet regeneration were tested and the results are discussed. When inoculated cultures were incubated under light and dark conditions, although callus was induced from explants kept under both conditions, dark incubation was found to be most suitable. Moreover, further proliferation of calli was also affected. Callus cultures incubated under darkness progressed callus growth and proliferation and a callus rating of 80% proliferation was observed. While with light incubated cultures, only 10% proliferation was obtained. During light incubation, cultures exhibited a green pigmentation. Upon subculturing of this callus to embryo induction medium, either friable embryogenic calli or embryos were not produced. On the other hand, callus induced under darkness produced highly friable embryogenic calli upon subculturing to embryo induction medium. Once the callus acquired embryogenic competence under darkness, further differentiation to embryos occurred either in darkness or light. That is somatic embryo development was not affected by incubation conditions. Moreover, though, embryo maturation was also occurred under both conditions, light conditions favoured maturation. Light is very essential for plant regeneration. Under dark incubation also embryos were germinated at a low frequency. Embryos induced healthy vigorous thick tap root and an elongated shoot. But the leaf system was not fully differentiated under darkness.

4. 7. Hardening and SEM studies

4. 7. 1. Hardening

The regenerated plantlets planted in different sterile potting mixtures and the IBA pretreated plants survived with frequencies ranging from 10-72%. Transplanting of somatic plants into non-sterile soilrite was found to be unsuccessful and in this condition only 10% of plants was survived after 30 d. Among the different sterile potting mixtures, sand: soil: cowdung mixture was found to be the most effective and in this mixture 62% of regenerated plants survived after 30 d. Survival in soilrite was also effective followed by sand and soil (Table 22). However, plantlets when pretreated in liquid medium (-sucrose) containing IBA, resulted in higher rate of acclimatization (72%).

Moreover, the observations indicated that IBA pretreated plantlets produced longer root and having lateral roots. However, weak plantlets with abnormal morphology showed poor acclimatization with a maximum survival on sand: soil: cowdung mixture (20%). Irrespective of planting substrate, weak plants showed poor survival. The present study also indicated that hardening under *in vitro* condition was totally ineffective. In *in vitro* hardening technique, the plantlets get wilted after one week of

Table 22: Effect of potting mixture on hardening of somatic plants

Potting mixture / nature of plants	No. of plants	Survival rate (%) 30 days	60 days
Non-sterile soilrite - healthy	50	10	4
Sterile soilrite - healthy	50	50	42
Sterile sand:soil - healthy	50	46	40
Sterile sand:soil:cowdung-healthy	50	62	54
Sterile sand:soil:cowdung +IBA- healthy	50	72	60
Sterile soilrite - weak plant	25	10	8
Sterile sand:soil - weak plant	25	12	8
Sterile sand:soil:cowdung - weak plant	25	20	12

transfer due to severe fungal contamination. During the hardening process, initially the plants were covered with polythene bag (Fig. 9A) for about two weeks so as to maintain high relative humidity (80-90%). After two weeks, the humidity was gradually lowered down by making holes in polythene bags and then the holes may be enlarged thereby setting the plants to adapt lower humidity. Finally after one month, the polythene bags were removed and the plants (Fig. 9 B) were kept in glass house condition for one more month. During 2 months of transplantation, irrespective of potting mixture, survival rate was significantly decreased (Table 22). During hardening, the plants were irrigated with ½ strength modified MS salts in every alternative day and after acclimatization plain tap water was used. Once the plants were acclimatized, the plants were replanted to large polybags (Fig. 9 C) filled with a mixture of sand, soil and dried cow dung and kept in shade house for field planting (Fig. 9 D).

4. 7. 2. SEM study

Healthy plants (Fig. 10 A) had dark green leaves arranged on the stem with more internodal gaps whereas abnormal / weak plants (Fig. 10 B) possessing thin slender leaves or with a stunted shoot. SEM studies revealed that all the samples possessed epicuticular wax and invariably there was more wax on the abaxial side. However, the amount of wax varied with the source of leaf. Leaves from weak plants at the time of hardening showed low epicuticular wax (Fig. 10 D, H) and deposition was not increased during hardening also. SEM study with acclimatized weak plant was not included in this study, because the leaves were wrinkled and wilted during processing. In the case of healthy plants, epicuticular wax was started to appear during pre hardening (Fig. 10 E, I) and deposition was continued to increase during and after hardening (Fig.10 F, J). in the acclimatized plants (Fig.10 C), the pattern of wax deposition was almost identical to that of field grown plants (Fig.10 G, K).

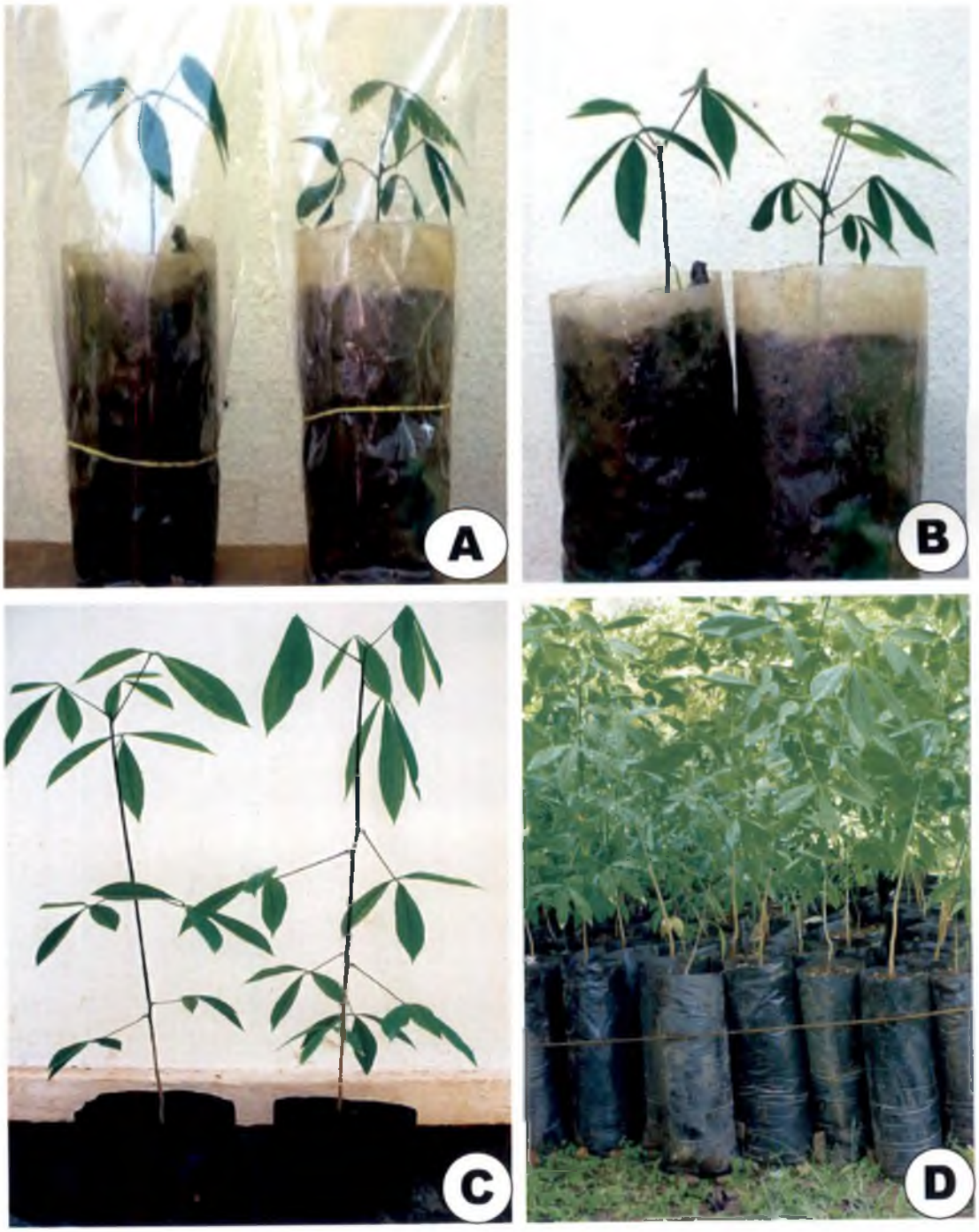

Figure 9 A-D: Hardening of somatic plants. A. Plants covered with polythene bags; B. Plants after removal of polythene bags; C. Acclimatized plants growing in big poly bags; D. Plants ready for field planting

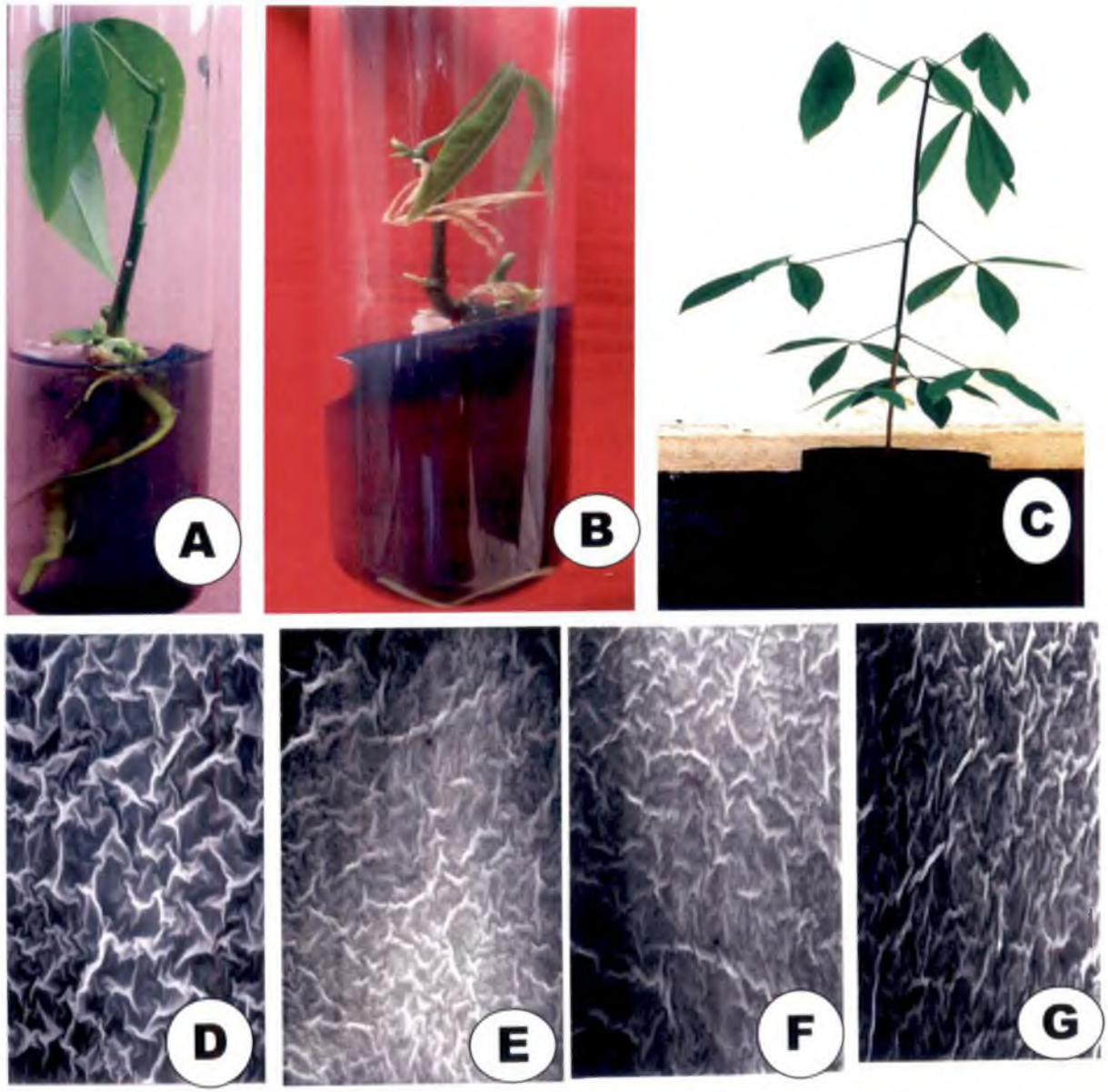

Figure 10 A-C. Somatic plants used for SEM study. A. Healthy B. Weak plant before hardening; C. Acclimatized plant. **D-G.** SEM photographs showing distribution of epicuticular wax on the upper surface of leaves. D. Weak; E. Healthy plant before hardening; F. Acclimatized healthy plant ; G. Bud grafted control plant.

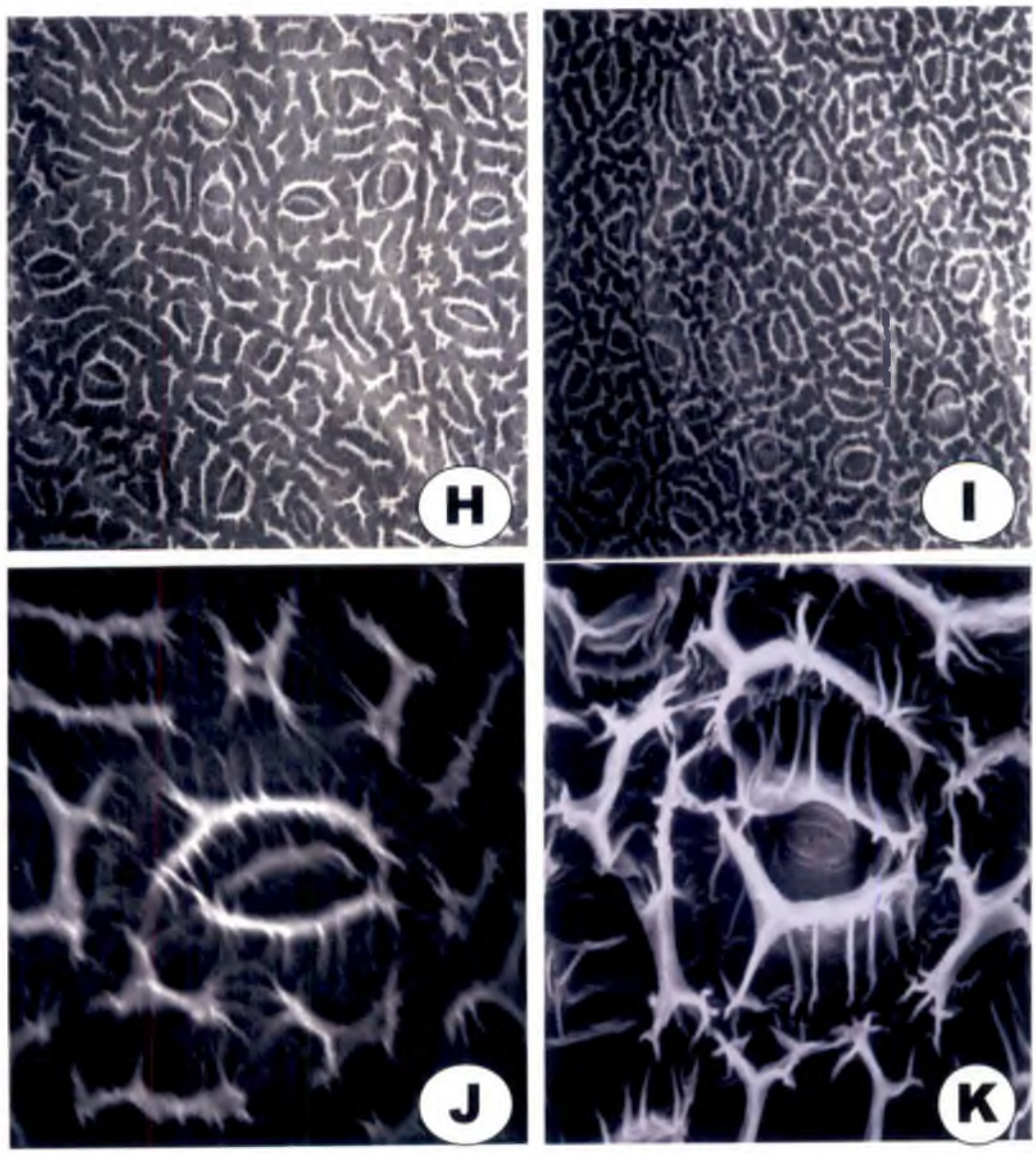

Figure 10 H-K: SEM photographs showing distribution of epicuticular wax on the lower surface of leaves. H. Weak; I. Healthy plant before hardening; J. Acclimatized healthy plant ; K. Bud grafted control plant

4. 8. Histological studies

Histological examinations indicated that there was histomorphological difference between embryogenic and non embryogenic calli. Observations revealed that embryogenic calli (Fig. 11 A) consisted of small cells with actively dividing stages. Cells could be easily separable and delineated by a thickened outer wall. Embryogenic callus consisted of cells with prominent nuclei (Fig. 11 B). In contrast, non embryogenic cells (Fig. 11 C) were divided very fast, characterized with large cells containing prominent nuclei (Fig. 11 D) and thin cell wall. Histochemical characterization for the detection of storage reserves revealed that embryogenic calli accumulated considerable amount of starch, lipids and proteins compared with non embryogenic calli. At early stage, embryogenic calli usually contained few starch grains with small size and could be seen randomly distributed (Fig. 11E). However at late stage, in embryogenic cells, starch granules increased both in size and number (Fig. 11 F). At this stage, almost all cells were densely accumulated with starch. By contrast, in non embryogenic calli, cells were poorly filled with starch grains (Fig. 11 G). Figure (Fig. 11 H) shows the lipid content in cell aggregates at early stage. Lipids were seen in small droplets and the number of droplets was more at late stage (Fig.11 I, J), however, the lipid content was less in number or quantity in non embryogenic calli (Fig. 11 K). Regarding total proteins, notable difference in number / quantity were seen among the two stages. In later stages, protein bodies appear to be significantly more abundant (Fig. 11 M) than in early stage (Fig. 11 L). Also protein bodies were less in number or quantity in these cells (Fig. 11 N).

4. 9. Cytological and molecular analysis of somatic plants

4. 9. 1. Ploidy level

In the present study, root tips of 5 regenerants randomly chosen were squashed for chromosome counts. Cytological analysis revealed that all tested plantlets showed the chromosome number ($2n = 36$) (Fig. 12A). Results of our study confirmed that the plantlets regenerated were diploid. Moreover, under microscopic observation, it was clearly indicated that callus was induced only from the anther wall (Fig. 12B).

In order to confirm whether the *Hevea* plantlets regenerated through somatic embryogenesis are genetically homogenous or not, their RAPD profiles was compared with that of control mother plant. An initial screening of 10 mer, 20 RAPD primers resulted in the selection of 13 primers which produced clear and reproducible banding patterns. For each sample, amplified products were scored for the presence or absence of bands. DNA bands with low intensity which could not be distinguished clearly were not scored. Each primer produced a unique set of amplification products and was differed in their size.

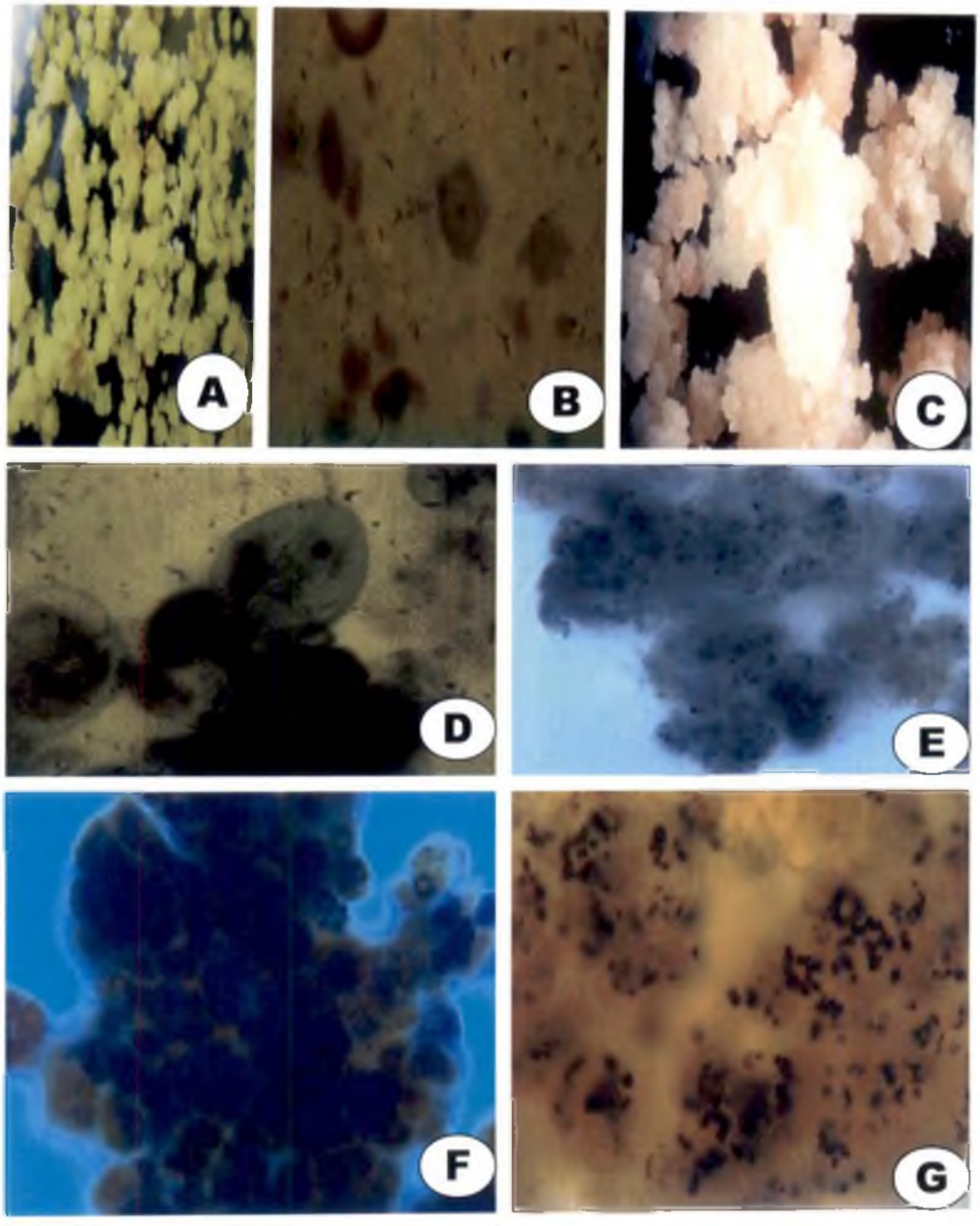

Figure 11 A-G: Histological observations. A. Embryogenic calli; B. Small cells with nuclei; C. Non embryogenic calli; D. Large cells prominent nuclei; E-G. Distribution of starch. E. Embryogenic calli at early stage; F. Late stage; G. Non embryogenic calli.

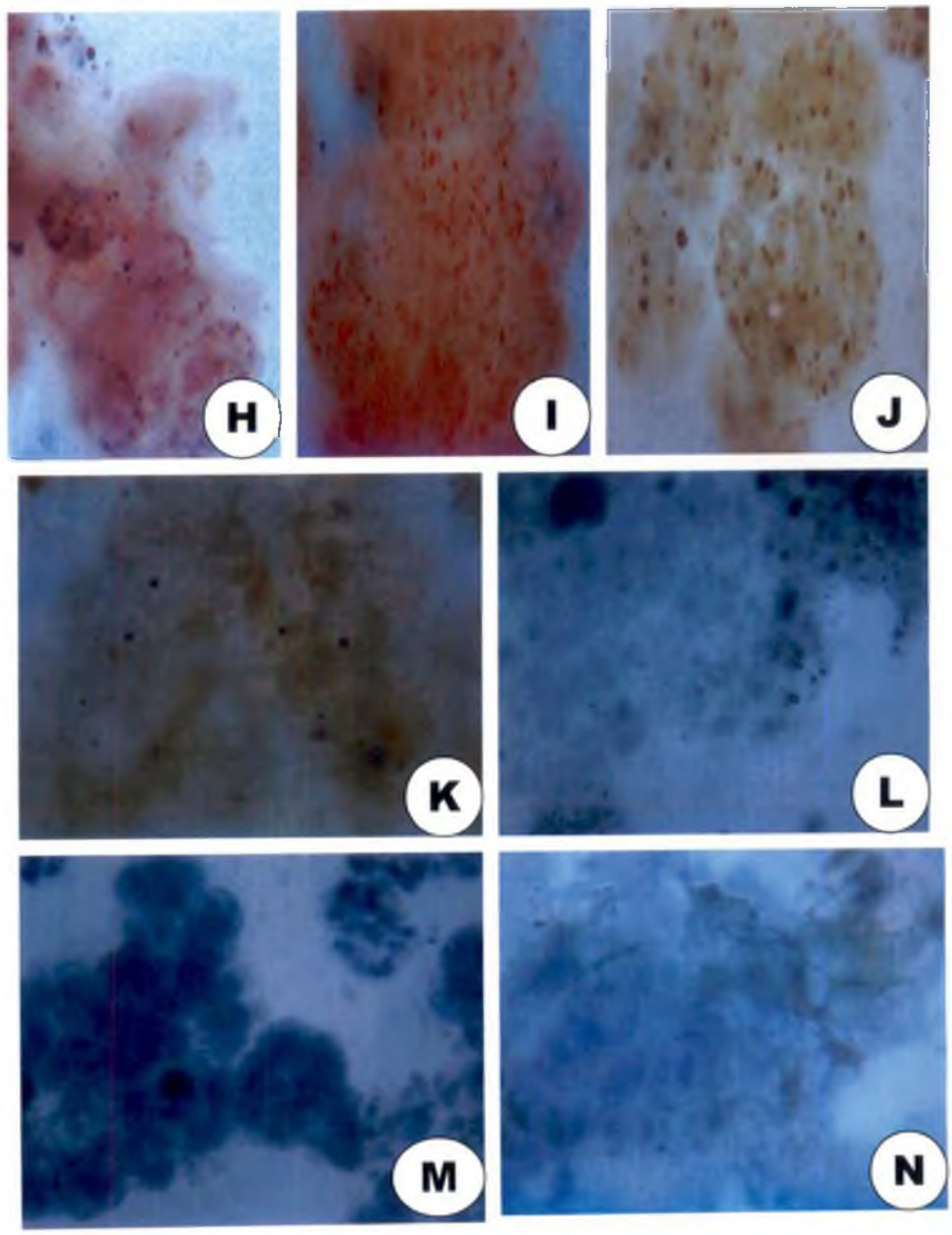

Figure 11: Histological observations. H-K. Lipid content. H. Embryogenic calli at early stage; I. Late stage with Oil red; J. with Sudan black; K. Non embryogenic calli. L-N. Distribution of protein. L. Embryogenic calli at early stage; M. Late stage; N. Non embryogenic calli

4. 9. 2. Analysis of genetic stability by RAPD

Table 23: Details of RAPD profiles in 10 somatic plants

Primers	Sequences (5' – 3 ')	No of bands	Total no of bands
OPA-04	AATCGGGCTG	8	96
OPA-07	GAAACGGGTG	10	120
OPA-08	GTGACGTAGG	5	60
OPA-12	TCGGCGATAG	10	120
OPA-20	GTTGCGATCC	8	96
OPB-06	TGCTCTGCCC	6	72
OPB-07	GGTGACGCAG	12	144
OPB-18	CCACAGCAGT	10	120
OPB-20	GGACCCTTAC	10	120
OPC-04	CCGCATCTAC	10	120
OPC-09	CTCACCGTCC	11	132
OPD-08	GTGTGCCCCA	11	132
OPD-18	GAGAGCCAAC	11	132

Table 24: Summary of RAPD analysis of monoclonal seedlings

Primers	No of bands	Polymorphic band	Non parental band
OPA-12	13	9	3
OPA-20	9	5	1
OPB-07	14	5	2
OPB-20	11	1	1
OPD-18	12	2	1

Figure (Fig. 12 B, C) shows a representative amplified banding pattern produced by 2 primers (OPA 20, OPB 20) in plantlets regenerated from somatic embryos. With all tested primers, no detectable variation was observed in somatic plants. All the amplification products generated were monomorphic in all 10 somatic derived plantlets as well as budgrafted control mother plant. However, the number of bands varied with respect to primers (Table 23). Banding pattern gives a minimum of 5 bands in OPA-08. With the primer OPB-06 also, the number of bands produced was found to low. However, with OPA-04 and OPA-20, 8 bands were produced. With all other primers, a maximum of 10-12 bands were produced. Also, we examined the genetic stability of monoclonal seedlings and compared this with RAPD profile of somatic plants. Among the 10 seedlings, a detectable variation was observed with all tested primers. 5 primers yielded 70 scorable bands including 22 polymorphic bands which were absent in the control and somatic plants, but were present in some of the seedlings. Also some bands observed in the control and somatic plants were absent in some of the seedlings. The number of bands varied from each primer (Table 24). With primer A12, seedlings showed wide variation among the plants itself. Primer A12 yielded 13 scorable bands including 9 polymorphic bands across seedlings. However, the polymorphic band numbers 3, 5 was present only in some of the seedlings, mother plant and somatic plants. Band numbers 6, 8 and 13 are also polymorphic bands and are absent in control and somatic plants and some of the seedlings (Table 25).

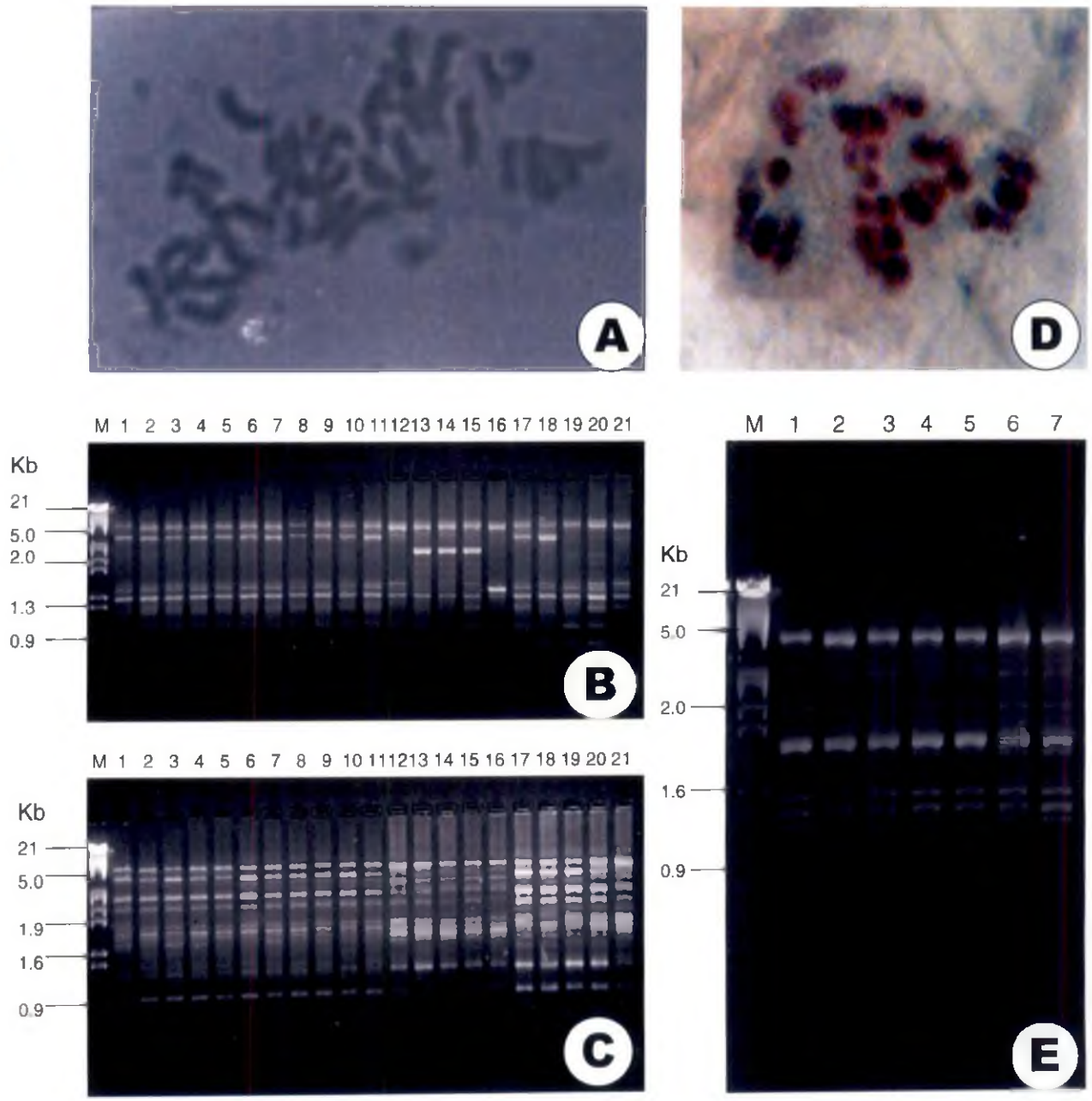

Figure 12 A-E: Chromosome number and RAPD profiles.

A. Somatic plant showing chromosome number 2n=36; B. DNA amplification obtained with primer OPA-20; C. primer OPB-20. M. Marker, λ DNA double digested ECoRI & Hind III; control plant (1); somatic plants (2-11); monoclonal seedlings (12-22). D. 3 year old calli showing chromosome number 2n=36. E. RAPD profile using the primer OPD-18; Marker (M); Control plant (lane 1); 8 month old calli (lane 2); 3 year old calli (lane 3-7)

Table 25: RAPD profiles with primer OPA12

B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
6	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+	-	+
7	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	+	-
8	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-
13	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-

Table 26: Presence of polymorphic bands with primer OPA 20

B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+
3	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 27: Presence of polymorphic bands with primer OPB-07

B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
6	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-	+
9	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

L1=Control plant, L2-12= Somatic plants, L12-21=Seedlings

Table 28: Presence of polymorphic bands with primer OPB-20

B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
3	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 29: Presence of polymorphic bands with primer D 18

B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

L1=Control plant, L2-12= Somatic plants, L12-21=Seedlings

With A20, among seedlings, polymorphism was detected at band no 2, 3, 5 and 7. However, this polymorphic band at 2, 5 and 7 was present in both control and somatic plants. Polymorphic band 3 was absent in control and somatic plant (Table 26). With primer B7 (Table 27), the band (at positions 6 & 9) was present in almost all seedlings except L 16 and L17 –L18 respectively. With B20, the parental band (number 2) was absent from L 12-L16 and the non parental band (No: 3) is present in all seedlings. These results revealed that with B20 primer (Table 28), uniformity is very high in seedlings. In the case of D18 (Table 29), non parental band (band no: 5) is present in all seedlings however, missing in control and in all somatic plants. Polymorphism is also very low in this case.

4. 9. 3. Cytogenetic analysis of long term callus cultures

Cytological observations were made to examine any changes in the basic chromosome number (2n = 36) from long term callus cultures. Callus cultures from 5 different tubes were subjected for ploidy analysis and the results revealed no alteration in chromosome numbers from 3 year old callus cultures (Fig. 12 D). From this observation we can conclude that callus cultures could maintain chromosomal balance at least up to 3 years without consequent loss of chromosomal number.

4. 9. 4. Genetic analysis of long term callus cultures by RAPD

In RAPD analysis of long term embryogenic callus cultures, samples produces distinct amplification profiles, displayed same banding pattern. Out of 20 random primers tested, only 12 primers produced scorable bands. Genetic similarity between the short term and long term callus cultures as well as mother plants was scored by comparing their RAPD profile for each of the primer. Samples from both short and long term cultures revealed a similar banding pattern with that of control (Table 30). Long term cultures produced a total of 550 bands. The number of bands ranged from 5 in OPA-08 and 6 in OPB-06 to 12 in OPB-07. All the bands were monomorphic in long term callus as well as in short term cultures and showed no detectable variation. Figure (Fig. 12 E) shows the amplified banding pattern with primer, OPD 18, from long term callus.

Table 30: RAPD profile details from long term callus cultures

Primers	Long term callus	Total bands
OPA-04	8	40
OPA-07	10	50
OPA-08	5	25
OPA-12	10	50
OPA-20	8	40
OPB-06	6	30
OPB-07	12	60
OPB-18	10	50
OPB-20	10	50
OPC-04	10	50
OPC-09	11	55
OPD-18	11	50

4. 10. Biochemical studies during somatic embryogenesis

4. 10.1. Protein changes associated with somatic embryogenesis

The sequential developmental stages of somatic embryogenesis revealed significant differences in protein profiles (Fig.13 A, lanes 1-6). During the initial callus phase (C) one prominent band, with molecular weight higher than 66 kDa was appeared. At the next stage, i.e, in embryogenic callus (EC), more proteins with higher quantity were produced with molecular

weight ranging from 14 to 97 kDa. However, the expression was higher for low molecular weight proteins, particularly, below 29 kDa. Compared to low molecular weight proteins, expression was lower for high molecular weight proteins. At embryo stage (E), there was a relative abundance of low and high molecular weight proteins. The expression level of proteins is also higher in this stage compared to other stages of development. More than 10-15 bands were clearly detected at this stage. Further, 2 polypeptides, in the mid zone with molecular weight ranging between 45 and 66 kDa were more intense. In addition, a few bands with high molecular weight proteins above 97 kDa were also seen. When the embryos proceeded to plantlet stage (P), the intensity of many bands was reduced. Only 4 bands were seen at this stage. However, clear difference was observed in electrophoretic analysis of proteins between embryogenic (EC) and non embryogenic calli (NC) and no bands in the detectable range were observed.

Figure (Fig. 13 A, lanes 7-9) shows the protein electrophoresis analysis of somatic embryos at different stages of development; globular (GE), heart / torpedo (TE) and cotyledon (CE). Nearly 40 polypeptides including both high and low molecular weight could be resolved. The overall pattern of proteins was more or less similar in all 3 samples. However, the expression level was different among the stages. Two bands with molecular weight approximately, 29 kDa and above are more prominently accumulated in globular embryos. In globular stage, the level of protein of 29 kDa and above was found to be maximum and then remained more or less similar up to cotyledon stage. These proteins expressed in a much higher intensity than other proteins. Another group corresponding to molecular weight within a range of 45 kDa and above also expressed in a much higher intensity, however, on subsequent stages, a diminished expression was observed. In general, we can categorize the proteins in three groups namely low molecular weight proteins below 29 kDa, another group ranging from 45 to 97 kDa and high molecular weight proteins above 97 kDa. In all three stages, almost all 3 groups of proteins are seen however, varied in their relative abundance. Among the three groups of proteins, the expression was higher for low molecular weight proteins. However, the high molecular weight proteins revealed a much lower expression level. When the three stages of development were compared, the same pattern of expression was observed. In conclusion, when embryos proceeded from globular to cotyledon stages of development, these proteins were expressed in a much lower intensity.

4. 10. 2. Isozyme studies

The isozyme analysis at the five sequential stages of embryogenesis revealed differences in activities as well as a remarkably distinct zymogram profile of peroxidase and esterase

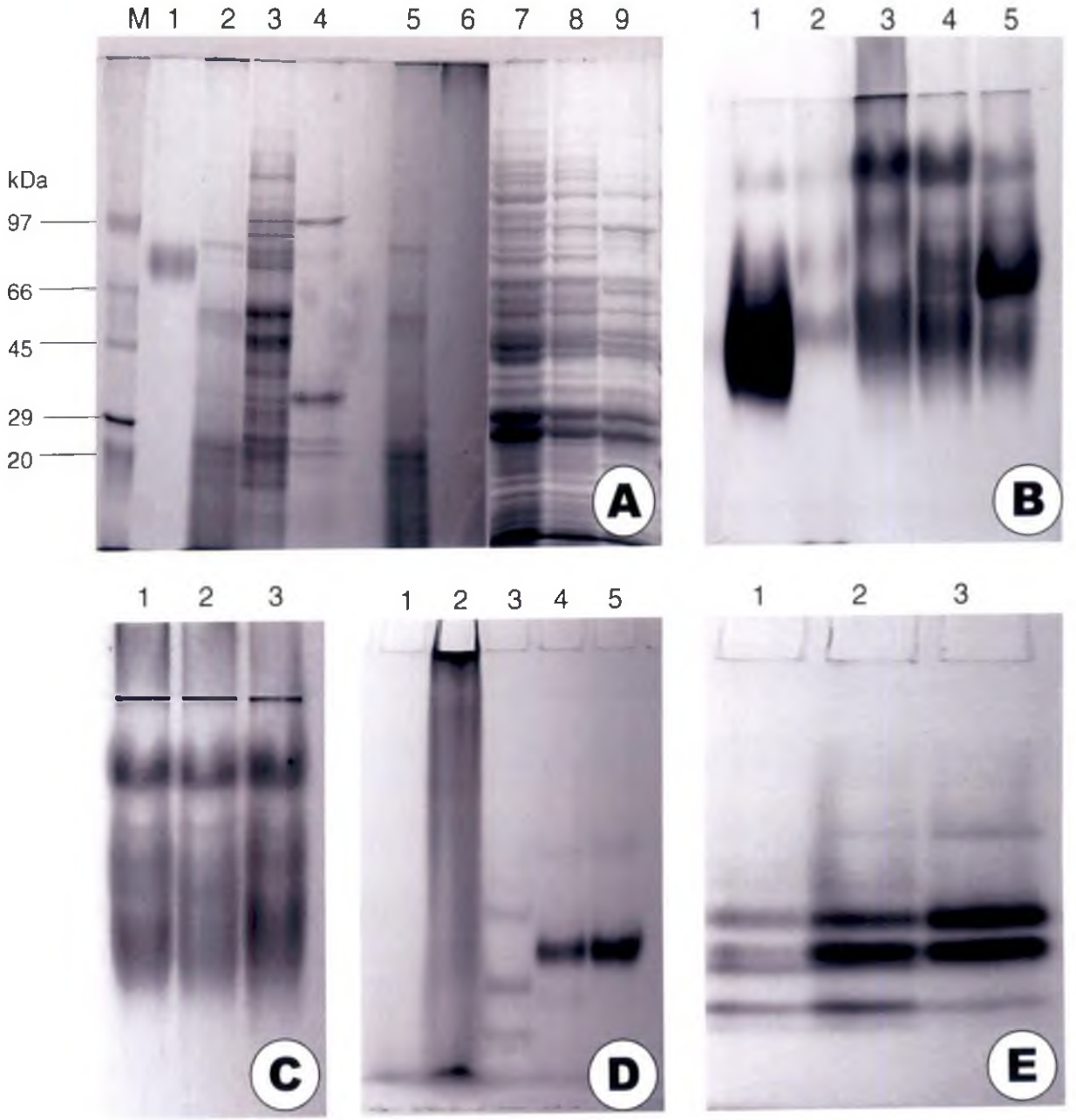

Figure 13A: Lane 1-9 represents protein profiles of callus; embryogenic callus; embryos; plantlets; embryogenic callus; non embryogenic callus; gloular ; heart/torpedo; cotyledon embryos; M. Marker. B. Peroxidase isozyme profiles of callus (1); non embryogenic callus (2); embryogenic callus (3) ; embryos; plantlets. C. Peroxidase isozyme profiles of embryos viz., globular (1); heart/torpedo (2); cotyledon (3). D Esterase isozyme profiles of callus (1); non embryogenic callus (2); embryogenic callus (3); embryos (4); plantlets (5). E. Esterase isozyme profiles of embryos viz., globular (1); heart/torpedo (2); cotyledon (3).

respectively. For isozyme peroxidase, a total of 5 bands were detected, however, varied at different developmental stages (Fig.13 B). For example, band 1 was prominent in embryogenic calli, but the intensity was decreased during differentiation of embryogenic calli to embryos and on subsequent differentiation to plantlet. Very weak activity or no activity was observed in primary callus and non embryogenic calli at the position of band 1. For callus, Prx zymogram showed high intensity in the fast migration zone where as no activity in slow migration zone. Embryogenic and non embryogenic calli showed clear differences in isozyme banding pattern. Embryogenic calli exhibited an increased enzyme activity, intensity and specificity of bands. But non embryogenic calli showed very faint activity. Latter stages of development *viz*, embryogenic calli, embryos, and plantlets revealed similar pattern for Prx, although differed in activities. The intensity of bands at 1 and 2 were gradually decreased as the development proceeded from embryogenic calli to embryo followed by plantlet formation. However, one major band was prominently present in plantlets, but in embryos, instead of one major band two minor bands were present bands where as in embryogenic calli the upper minor band was absent. Another major band in the fast migration zone was present in all stages of development. In the Prx zymogram, callus stage was characterized by high activity of this band, while non embryogenic calli exhibited less activity, but for the other 3 stages, a high activity was observed. Three types of embryos exhibited same banding pattern with an increase in activity of peroxidase (Fig. 13 C). As found in sequential stages, all 5 bands were present in developing embryos. Among the 5 bands, one band at the slow migration zone was prominent in all three types of embryos. However, among the different stages, peroxidase activity was slightly higher in cotyledon stage embryos.

The different developmental stages varied in isozyme pattern of Esterase (Est) and were characterized by a few band as shown in figure (Fig. 13 D). In callus, no activity was observed whereas in non embryogenic callus, a smear of Est activity was observed. Non embryogenic calli was distinguished from embryogenic callus by the absence of bands. In the Est profile, a total of 5 bands were observed. EC was characterized by 3 bands, one in mid and 2 in the fast migration zone. These bands were present in embryogenic calli but with minimum activity. These bands were absent in embryos and plantlets. Embryos and plantlets had a very similar Est pattern and was characterized by few bands one faint band in the mid migration zone and another prominent one in the fast migration zone. The Est zymogram of 3 types of embryos revealed a similar pattern, but differed with respect to activity as development proceeds to subsequent stages (Fig. 13 E). The globular embryos showed less activity and when reached to later stages, torpedo and cotyledon stages had maximum activity.

DISCUSSION

Global consumption of natural rubber is continuously increasing in line with the improvements of living standards. Due to this rapid growth, the rubber based products manufacturing industry all over world has emphasized the need for substantial increase in the total production of natural rubber. In *Hevea* breeding, though conventional methods have made steady improvement in agronomic traits such as yield, not much genetic diversity exists for further crop improvement, due to narrow gene pool. Biotechnology is contributing novel methods for genomic manipulation thus complementing conventional methods of *Hevea* breeding. The development of an efficient regeneration pathway is a key for the feasibility of such manipulation studies. In this study we have been searching for an efficient somatic embryogenesis pathway from immature anthers. Moreover, the influence of several parameters affecting the system that leads to a significantly higher embryo induction and plant regeneration frequency were also studied. Further, a detailed histological and biochemical investigation at different developmental stages of somatic embryogenesis was attempted to gain a better understanding of the cellular and biochemical changes associated with somatic embryogenesis.

5. 1. Utilization of immature anther as explants

It is well documented that somatic embryogenesis and plant regeneration is highly dependent on explant type, developmental stage and culture conditions and the success of plant regeneration is dependent on the combination of all these factors (Minocha *et al.* 1995). Therefore, optimization of regeneration conditions by choosing the correct type of explant tissue and its developmental stage is essential. In *Hevea*, considering the constraints experienced with different explants that have already been tested, it was essential to look for other suitable explants for *in vitro* culture with the view of developing a reliable *in vitro* protocol for rubber. Thus in this study, the suitability of exploring immature anther as a source of explants for *Hevea* somatic embryogenesis was tested. The source of explants for initiating *in vitro* culture is found to be very critical. Floral tissues particularly anthers are widely used as the explant for inducing

embryogenic cultures including *Hevea* (Chen *et al.* 1979) and grapes (Mauro *et al.* 1986). During dissection of immature anthers, ascorbic acid solution was found to be very essential to prevent browning as well as to get healthy explants. Without ascorbic acid, tissue browning was very severe and no callus or only few calli was induced from these explants. During culture initiation with integumental tissue of *Hevea*, it was reported that explant browning or darkening was very severe which subsequently lead to death of explants (Varghese *et al.* 2000). For most of the woody plants, culture initiation is a major problem due to explant browning and subsequent death, which is generally attributed to the production of phenolic compounds (Thomas and Ravindran 1997). Several antioxidants such as ascorbic acid combined with citric acid and cystein was reported to prevent tissue browning.

5. 2. Induction of callus and embryos

In plants, cell cycle regulation is linked to the developmental programs and responds rapidly to stimuli from both internal and external environment. Though the nature of stimuli that co-ordinate the interplay between environment, plant development and cell cycle programs are still unknown, phytohormones are known to be the major regulators of plant cell division (Evans 1984). During *in vitro* culture, the success of culture initiation and development depends mainly on the culture medium. Results of our experiments revealed that modified MS medium was found to be more effective than MS medium. This is in agreement with the observations of Chen *et al.* (1979) and Carron and Enjalric (1985) where they used modified MS medium for somatic embryogenesis of *Hevea.* Generally, most somatic embryogenesis protocols use a strong auxin such as 2, 4-D or a combination of auxin and cytokinin in the primary culture medium to support both callus induction and cell proliferation and also for induction of embryogenesis. Our results are in accordance with the hypothesis that for inducing callus from immature anthers, the strong auxin, 2, 4-D combined with KIN, is essential as growth regulators. Earlier reports are also reveals the requirement of 2, 4-D for callus induction from different explants like integumental tissue and inflorescence of *Hevea* genotypes, PB 260 and RRII 105, (Carron and Enjalric 1985; Sushamakumari *et al.* 2000). Similarly, 2, 4-D is used as a potent auxin for callus initiation in many crops including citrus (Tao *et al.* 2002), cotton (Mishra *et al.* 2003), cereals (Kaur and Kothari 2004) and garlic (Luciani *et al.* 2006). A combination of 2, 4-D and NAA was also found to be effective for callus induction from different explants

like inflorescence of rubber clone RRII 105 (Sushamakumari *et al.* 2000) and mature anthers of Chinese clones (Chen *et al.* 1979). However, the results of our studies show that no callus or very little callus was induced in the presence of auxins like IAA, IBA and NAA. On the other hand, Sushamakumari *et al.* (2000) initiated callus at higher concentrations of NAA, IBA and IAA from inflorescence explants of *Hevea* clone RRII 105, however, the callus proliferation was comparatively low. This suggests that eventhough same genotype was used, the hormonal requirement for callus induction in *Hevea* was found to be different. The differences may usually associated with the variation between genotypes in susceptibility to genetic programming and reprogramming of embryogenically competent cells by external factors or either developmental stage of explant or explant specificity effect.

According to Martin (2003), amount, texture and colour of calli varied depending on the type and concentration of auxin especially 2, 4 - D. Results of our experiments are in consistence with this hypothesis that callus rate, colour and morphology was differed depending on 2,4-D concentration. The present experiments have demonstrated that at lower concentrations, callus rate was low however, by increasing the concentrations of 2, 4-D, callus rating was increased. Our observations indicated that 2, 4-D concentration in the medium influenced friability of calli or calli nature and resulted in 3 types of callus. At low 2, 4-D concentrations, Type I callus was produced. However, medium with 1.5-2.0 mg/l 2, 4-D stimulated the growth of semi friable / compact embryogenic Type II callus. A similar kind of compact calli with highly embryogenic competence was obtained from integumental tissue of *Hevea* for clone PB 260 (Montoro *et al.* 1993). While concentrations at 2.5 mg/l and above, Type III callus were formed. On the other hand, in the present study, although Type III callus was also compact, the callus was hard and non embryogneic in nature. These findings may explain that high concentrations of 2, 4-D reduces friability or the embryogenic nature. The present study agrees the observations of Mishra *et al.* (2003), who observed hard tightly organized compact calli in cotton. According to him, this type of callus is non friable and never differentiated into embryos, however, grainy textured callus capable of undergoing somatic embryogenesis is made up of large cells loosely organized in a friable callus. Similarly, Filippov *et al.* (2006) also reported two types of callus, type I and type II, during somatic embryogenesis of wheat. Type I is watery, translucent, friable and non embryogenic where as type II is friable, yellowish and embryogenic producing embryos. Recently, Anuradha *et al.* (2006) induced

different types of calli namely fast growing friable calli, slow growing compact nodular calli and brownish nodular calli from *Nothapodytes foetida*, a medicinal tree. Accordingly, the callus tissue was classified into 3 types: type I callus is soft, friable and icy with wrinkled surface which proliferated as non embryogenic callus, type II callus is pale yellow coloured, smooth, nodular and compact callus and is embryogenic, type III was of mucilaginous, compact and non embryogenic. Among these types, only Type II callus was differentiated into embryos. Results of our study have also revealed that only Type II callus was induced embryos and this in agreement with the results of Mishra *et al.* (2003) and Anuradha *et al.* (2006). Our results do not coincide with the observations made by Kim *et al.* (2006) who observed 3 types of calli in *Alstroemeria*. One is soft and watery callus (non embryogenic) and among the other two, one is compact and other is friable however, both types are highly embryogenic and induced embryos.

Since somatic embryogenesis consists of several phases and each stage needed specific growth requirements, the level of hormones is very crucial (Christianson 1987). Auxin was the crucial growth regulator in the induction of embryogenic calli and embryos. Both auxin type and concentration was influenced the development as well as morphology of somatic embryos in several species (Chengalrayan *et al.* 1997; Mary and Jayabalan 1997; Mandal and Gupta 2003; Junaid *et al.* 2006; Shahana and Gupta 2002). In the reported study, all auxins combined with KIN induced embryos however, the optimum being depended on auxin type and concentration. Of the 4 auxins used, NAA showed the most positive effect on embryo induction. Earlier, influence of NAA on embryogenesis from mature anther of *Hevea* was shown by Chen *et al.* (1979). Our results indicated that 2, 4-D was found to be the less effective auxin for embryo induction than IAA and IBA. When 2, 4-D was used in embryo induction medium few embryoids were obtained and calli proliferation was found to be more. Moreover, the embryoids failed to grow further. A possible explanation for these results may be assumed that 2, 4-D is a highly stable auxin showing strong resistance to enzymatic degradation and conjugation in plant cell (Moore 1989) and therefore 2, 4-D persisted in medium. Exogenous auxins are needed for inducing embryogenic cells and once the embryogenic cells were induced, the requirements of auxin was decreased and even in the absence of auxin, the embryogenic cells develop into somatic embryos. From this point, our results indicated that once calli with embryogenically potential cells was formed in callus induction medium in the presence of a strong auxin like 2, 4-D, other auxins such as NAA were required to induce

embryos. Similar results, i.e., once callus was induced on 2, 4-D containing medium, NAA was required for embryo induction in *Catharanthus* (Junaid *et al.* 2006). Mandal and Gupta (2003) also used NAA (2.0 mg/l) for high frequency of somatic embryos. However, IAA was used for obtaining maximum number of somatic embryos. In *Sesbania sesban,* Shahana and Gupta (2002) obtained highest somatic embryos on medium containing 0.1 mg/l NAA, in contrast, with 2, 4- D containing medium, only globular embryos were obtained. Parthasarathy *et al.* (2001) showed that in woody plants, induction of somatic embryos was influenced or enhanced by 2, 4-D. By contrast, embryo induction frequency decreased sharply when the concentration of 2, 4-D was higher than 9 μM (Wang and Wei 2004).

5. 3. Parameters influencing callus and embryo induction efficiency

5. 3. 1. Explants pretreatment in liquid medium

The ability to form somatic embryos is not merely an intrinsic property of the species; instead, it is a property under genetic control such that individual genotypes within a species can differ in their ability to undergo somatic embryogenesis. Besides genetic factor, both epigenetic and medium constituents also influence the process very much. Since somatic embryogenesis being a complex multistage process, involving a lengthy time period from explant to plant regeneration, liquid based pulse treatment experiments were commonly used. In the present study, with the aim of reducing the duration of callus induction, we tested the influence of explant pretreatment in liquid medium. As compared to control, pretreated explants required comparatively low time period for callus induction. A 10 d pretreatment in liquid followed by 25 d culture on solid medium was found to be more effective. However, by prolonging the period of pretreatment, a reduction in total time requirement was observed. This may probably due to the fact that liquid culture changes the physiological nature of explants. Due to this, the explants are completely submerged in the liquid medium where explants uptake more nutrients and hormones that favours callus induction. Although the exact mechanism is not well understood, the absence of a gelling agent may have increased the availability of water and dissolved substances to the explant (Debergh 1983). Moreover, the embryogenic calli formation was significantly affected by preculture. The results of our experiments revealed that short duration pretreatments in liquid medium increased the production of embryogenic calli. In contrast, embryogenic calli formation was decreased when the explants are pretreated for longer periods. This may attributed to the assumption that during prolonged culture, liquid may cause increased availability of nutrients including

hormones, 2, 4-D. It can be concluded that even though embryogenic callus was formed from all pretreatments, the formation of embryogenic calli increased for 10 day pretreated explants where as only 35 days was required for callus induction. A similar finding was reported in cucumber by Kuijpersel *et al.* (1996) where increased embryogenic callus was obtained when explants were cultured for 10 days on 14 µM (3.0 mg/l) medium, but decreased during prolonged culture. These results are in coincidence with the observation of Sharma *et al.* (2007) who obtained a 20 µM 2, 4-D pulse for 1 h yielded a significantly higher number of somatic embryos compared to longer exposure periods.

5. 3. 2. Time of subculturing for embryogenesis

Embryogenic callus formation is the first and crucial step during somatic embryogenesis and moreover, the frequency of somatic embryogenesis depends on the initial callus. In majority of plant species, the synthetic auxin 2, 4-D is the most commonly used auxin for inducing callus. Culturing the explants under the influence of 2, 4-D results in the increase in endogenous auxin levels in explants (Michalczuk *et al.* 1992) and therefore a long time culturing on auxin may detrimental to further morphogenesis. The observations of our study clearly indicated that the auxin 2, 4-D present in callus induction has a prominent role in the formation of embryogenic calli formation. According to the present experiment, once after callus initiation, by increasing the maintenance of primary callus on 2, 4-D containing callus induction medium, the friability of callus was looses gradually leading to hard compact calli. Such calli during subculture to embryo induction medium resulted in decreased production of friable highly embryogenic calli. This is in agreement with Michaux-Ferriere and Carron (1989), who reported a reduction in embryogenic calli formation by frequent callus subculturing in callus induction medium. Filippov *et al.* (2006) concluded that when contact of explants with callus induction medium was increased to 21 days, rate of embryogenesis and plant regeneration was increased, however, maintenance up to 28 days delayed shoot development of embryos. From our study, it can be concluded that after callus initiation, calli proliferation for another 15 days were the ideal time for subculturing to embryo induction medium and if delayed, embryogenic calli formation was decreased suggesting that callus maintenance on medium with high 2, 4-D might have caused the loss of embryogenic cells.

5. 3. 3. Effect of polyamines

Polyamines are an important and interesting group of naturally occurring low molecular weight, polyatomic, aliphatic nitrogenous compound present in all

cells. Among the polyamines, spermine and spermidine are the main polyamines in plants. Modulation of polyamine metabolism has been studied in different system in relation to somatic embryogenesis demonstrating that PAS are crucial endogenous factors during *in vitro* embryo formation (Catarina *et al.* 2007). Several reports have shown the involvement of polyamines particularly in their free forms in somatic embryogenesis.

In our study, we examined the response of polyamines and results suggested that polyamines enhanced embryogenic callus formation, but does not enhance (except a slight enhancement in 0.5 mg/l spermidine) embryogenesis. A similar kind of observation is seen in *Gnetumula*, where spermine and spermidine helped proliferation of embryogenic callus but had no effect on embryogenesis (Augustine and Souza 1999). In our experiments, spermine at all tested concentrations did not improve somatic embryogenesis. On the other hand, when spermidine was supplemented, slightly higher number of embryos was produced on medium with 0.5 mg/l. This effect of polyamine is contradictory to the results obtained with *Hevea* (El Hadrami *et al.* 1989) where a supply of polyamines to the culture medium enhanced the production of embryogenic calli and frequently increases the number of embryos per callus. According to him, the insufficient availability of endogenous polyamines is one of the limiting factors for somatic embryogenesis in *Hevea*. This may attributed to the clonal variability in endogenous polyamine content (El Hadrami *et al.* 1989), explant source variability or may be controlled by the exogenous growth regulators. Kevers *et al.* (2002) and Rajesh *et al.* (2003) reported the positive effect of exogenous supply of polyamines on somatic embryogenesis. By contrast, browning of callus with a reduced capacity of embryo initiation was obtained by external application (Kevers *et al.* 2002). It was found that spermidine was the most effective polyamines and its beneficial role in somatic embryogenesis was reported in *P. ginseng* (Monteiro *et al.* 2002) and *Hevea* (El Hadrami *et al.* 1989). Rajesh *et al.* (2003) reported that both putrescine and spermidine significantly increased the rate of oil palm somatic embryos, however, efficiency of somatic embryos, secondary somatic embryos and plant regeneration was higher in putrescine containing medium.

5. 3. 4. Effect of amino acids

Compared to inorganic nitrogen, organic nitrogen sources applied to the culture medium influenced somatic embryo production. According to Merkle *et al.* (1995), the addition of amino acids to the culture medium might inhibit or promote the somatic embryo development and conversion. Our findings indicated that among various amino acids, alanine and arginine revealed no effect on embryo enhancement, however, inhibitory or reduces embryogenesis at higher concentrations. Asparagine had no stimulatory effect, but never showed any negative effect and all tested concentrations allowed embryo differentiation similar to that of control. Glutamine was the only amino acid improved embryogenesis. Maximum response on embryo induction was obtained at higher concentration of glutamine. From the current study, it can be concluded that amino acids, alanine, asparagine and arginine revealed any stimulatory effect on embryogenesis; however, glutamine significantly enhanced embryo induction. In contradiction, Das Vesco and Guerr (2001) and Ashok-kumar and Murthy (2004) reported the positive effect of both asparagine or arginine and glutamine which significantly increased embryo induction. This is not in agreement with the report of Hita *et al.* (2003) where alanine does not have any effect on embryogenesis similar to our studies. According to Hita *et al.* (2003) glutamine also showed no stimulatory effect, but reduces or even inhibits the embryogenesis. But according to our result, glutamine was the only most effective amino acid for embryo enhancement, similar in *Feijoa*, where 4 mM glutamine dramatically increased somatic embryos (Das Vesco and Guerr 2001). However, Vikrant and Rashid (2002) did not show any enhancement on embryo induction at higher concentration (5 mM) of glutamine, instead it favoured only compact callusing. It is suggested that glutamine enhances protein accumulation. According to Merkle *et al.* (1995) storage proteins are the markers for embryo maturation and consequently the quality marker of the regenerated plant. In carrot somatic embryos, the total protein content increased three times when glutamine was added (Dodeman 1995) and in oil palm somatic embryos, glutamine enhanced the accumulation of 7S globulins (Morcillo *et al.* 1999). Casein hydrolysate, a complex mixture of 18 amino acids has been widely used as an additive for embryogenesis. In the present investigation, inclusion of casein hydrolysate improved embryo induction and resulted in maximum embryo production at 400 mg/l. This is in accordance with the observation of Tang (2000) who obtained maximum

response of casein at 500 mg/l for somatic embryogenesis in *Panax ginseng*. Similarly, the positive effect of casein hydrolysate on somatic embryos production was reported by Wang *et al.* (1999) and Hita *et al.* (2003). A combined effect of casein hydrolysate with glutamine on somatic embryo enhancement was reported by Kim and Moon (2007). This is in contrast to Cheng and Chang (2002) who obtained no response with casein and glutamine in direct somatic embryogenesis in *Oncidium gower*.

5. 4. Plantlet regeneration

After maturation, germination and full plant recovery was achieved on hormone free medium. However, the conversion frequqency was found to be low (27%). Similarly, according to Kim and Moon (2007) after maturation, plantlets with well developed roots could be obtained and 35.5% conversion frequency was obtained.

Cytokinins are plant hormones promoting cell division and differentiation and have been shown to regulate a variety of biological activities in whole plants and in tissue cultures. It is well documented that amino purine cytokinins are identified as an important media component for germination of somatic embryos in many plant species including rose wood (Muralidhar Rao and Lakshmisita 1996); *Acacia catechu* (Rout *et al.* 1995); *Swietentia macrophylla* King (Maruyama and Ishii 1999); *Hevea brasiliensis* (Sushamakumari *et al.* 2000). Substituted ureas such as N- N- diphenyl urea with higher cytokinin activity, TDZ have been used as a best cytokinin for the *in vitro* regeneration of legumes and woody plants, especially, the recalcitrant species (Lakshmanan and Taj 2000) and Lu (1993).

In the present study, for all tested combinations of BA, embryo germination and full plant development was occurred. However, maximum response was obtained at lower concentration (0.5 mg/l). These results are in agreement with the observations of Muralidhar - Rao and Lakshmisita in rose wood (1996), in coconut palm (Verdeil *et al.* 1994) and in cassava (Sofiari *et al.* 1997) where 0.5 mg/l BA was required during germination and plantlet conversion.m In *Hevea,* it was reported that BA enhanced germination of integumental derived somatic embryos (Veisseire *et al.* 1994) and germination of inflorescence derived somatic embryos (Sushamakumari *et al.* 2000). However, in both of these studies, the germination frequency was only 27 and 36%. The reason for low germination percentage in these studies may be due to the very low (Veisseire *et al.* 1994) or high concentration of BA (Sushamakumari *et al.* 2000) used in the medium. This may be also due to explant specificity. In the present study, when BA was replaced with ZEA, maximum response was noticed with

the highest concentration (2.0 mg/l) tried. However, in S. *macrophylla* King, lower concentrations of ZEA were required for somatic embryo germination (Maruyama and Ishii 1999). The results of our study revealed that though BA and ZEA showed more or less similar response, the requirement for germination and post germination was different. Overall, BA induced higher germination than ZEA although the difference was very marginal. With KIN, higher concentrations were required for obtaining maximum response. Similarly in *Acacia catechu*, development of plantlets via somatic embryogenesis was achieved from immature cotyledon derived calli when medium was supplemented with 4.0 mg/l KIN along with NAA (Rout *et al.* 1995). However, in the present work, when TDZ was added in the medium, the maximum as well as a high frequency embryo germination and plant development was recorded with low concentration (0.25 mg/l). When TDZ concentration was gradually increased, the response was decreased. Similar reports were also made for somatic embryogenesis in pigeonpea (Sreenivasu *et al.* 1998). According to Sushamakumari *et al.* (1999) in *Hevea*, TDZ induced multiple shoots from somatic embryos at lower concentrations. In the present study also, maximum response of TDZ was obtained with lower concentrations. This is attributed to the hypothesis that TDZ is active at lower concentrations than the amino purine cytokinins (Mok *et al.* 1987). Initial growth of plants was also higher for plants produced from TDZ containing medium than BA and ZEA suggesting that TDZ might have exhibited a dual role as both auxin and cytokinin like activity as reported in ornamental species (Le and Duong 2000).

During *in vitro* culture, many factors were found to affect somatic embryo germination and full plant recovery. Our results also revealed that for all treatments, irrespective of cytokinin type and concentration, some embryos remained un-germinated. According to Hay and Charest (1999), many normal looking mature embryos failed to germinate because of the internal abnormalities such as intercellular air spaces. As the number and size of air spaces increased, there was a corresponding decrease in the formation of shoot apical meristem. The present studies have shown that although 90% of embryos produced long vigorously growing tap root, the induction of shoot apex was not observed for all embryos resulting shootless embryos. This may probably due to the most sensitivity of shoot apical meristem to cultural conditions than root pole. Moreover, according to Yeung and Stasolla (2000), it is observed that shoot apical meristem of somatic embryos is not stably determined as in their zygotic counter parts. However, root apical meristem of somatic embryos closely resembles to that of their zygotic embryos and therefore the percentage of root emergence was higher than shoot

differentiation. Also in most cases, presence of incompletely developed somatic embryos that lacked shoot poles is the reason for low germination / regeneration of plants (Liliane *et al.* 2001; Fernando *et al.* 2003). The results of the present study showed that all germinated embryos were not converted into viable plantlets. Since, germination and full plant recovery was defined as two different stages during morphogenesis, the reason of our results reflected the fact that although germination occurred, sometimes the germinated embryos did not have a viable apical meristem or well developed epicotyl formation or cotyledonary structures with or without a radicle on the opposite end (Maruyama and Ishii 1999).

5. 5. Long term embryogenesis

Embryogenic callus aggregates, used for initiating long term embryogenesis was known in different terminology *viz*, proembryogenic mass or embryogenic clumps by different authors. In this study, these embryogenic cultures have been maintained by subculturing on medium containing growth regulators KIN (0.7 mg/l), NAA (0.2 mg/l) and GA_3 (2.0 mg/l). However, for long term maintenance, subculture interval was found to be a critical factor. Experiment with different subculture intervals revealed that subculturing with an interval of 50 d induced maximum embryos. When subculture interval was proloned to 75 d, callus at the upper surface was not in contact with the medium and thereby reduced embryo differentiation. This clearly indicated that transfer to fresh medium in every 50 d was needed to ensure the embryogenic competence of callus for long periods. According to our results, a continous exposure to growth regulators was needed for long term maintenance and induction of embryos. In grapes (*V. vinifera*), embryogenic calli of several cultivars were maintained for 2 years or even 4 or more years on medium containing the auxin 2,4-D (Motoike *et al.* 2001). Similarly, embryogenic calli were maintained for long term by continuous transfer on medium containing auxin with or without cytokinin or by subculture on semi solid medium without growth regulators (Gray *et al.* 2006). On the contrary, long term maintenance on medium lacking growth regulators have been reported by many authors (Cailloux *et al.* 1996) and (Guijarro *et al.* 1995).

5. 5. 1. Effects of proline on embryogenesis from long term cultures

Earlier reports shows that osmotic stress was associated with an increased accumulation of storage reserves in embryos similar to zygotic embryos (Brisibe *et al.* 1994). This amino acid, proline seems to play an important role on osmotic stress as well as in the control of cell pH. Moreover, it was observed that total protein content of embryogenic callus

was higher in the presence of proline (Santos *et al.* 1996). Addition of proline to the culture medium can be an important factor in achieving a high efficiency of somatic embryo production as demonstrated in several studies (Bela and Shetty 1999; Vikrant and Rashid 2002). Contradictory results are also obtained with peanut (Murch *et al.* 1999) by the addition of proline.

Although, embryogenesis was effectively observed in the embryo induction medium with short term cultures, in the present study, the influence of proline on embryo induction from long term cultures was assessed. Our studies indicated that as the concentration was increased from 50-200 mg/l, the formation of embryogenic mass from brownish ECA was also increased. Enhanced effect of proline on embryo induction was obtained at all concentrations compared to control. However, maximum effect was obtained at lower concentration (100 mg/l). Vikrant and Rashid (2002) reported that addition of proline at 5 mM concentration favoured differentiation of embryos significantly; however, Hita *et al.* (2003) greatly increased the embryogenic efficiency by the addition of 2.5 mM proline. In our study, with higher concentrations (200-300 mg/l), the response was however, decreased. This was in agreement with the assumption that proline is acting as an additional nitrogen supply and higher concentrations (300 mg/l) combined with high salted medium, the total nitrogen supply was too high which might have a decreased effect. Cultures maintained for 3 years on proline containing medium allowed to induce embryogenic calli and embryos. All cultures exhibited comparatively same respone or slightly higher response at lower concentration (100 mg/l) though the difference is very marginal. This reflects the fact that nitrogen originating from amino acid is stimulated quickly into carbonic skeletons during the metabolism and synthesis of the protein when compared to other inorganic nitrogen sources. Therefore, from the study we could conclude that if proline is present in the medium in lower concentrations for longer periods will not be inhibitory to embryogenesis.

5. 5. 2. Effects of charcoal on embryogenesis from long term cultures

In the preliminary studies, AC was used (0.2%) in embryo induction medium and embryos were effectively induced on this medium. However, it is necessary to understand whether to maintain the embryogenic cultures for a long period, AC is needed throughout the culture period and if required at what level AC influenced embryo production. In *Hevea*, no information is available on its effect from long term cultures and in the present experiment our attention is focused on this matter. According to the investigation, absence of AC looses the embryogenic nature of calli

and consequently leads to fast growing calli which in turn affected further induction of embryos. Also in medium without AC, the white colour of medium was changed to yellowish brown. This may in consistence with the hypothesis that phenolic oxidation or brown exudates accumulation can be substantially decreased in the presence of AC (Teixeira *et al.* 1994). When medium was enriched with AC, embryogenic character was retained and higher number of embryos was produced with the inclusion of 0.1%. Similarly AC showed a significant effect on embryo development from long term cultures (Motoike *et al.* 2001). Our studies revealed that by further increase of AC in the medium resulted in reduced embryogenesis. It was observed that addition of higher levels of AC (0.2-0.3%) decreased embryogenesis probably due to the excess adsorption of growth regulators and medium components by charcoal limiting embryogenesis. For long term cultures, in medium without charcoal a further reduction in embryogenesis was observed. In contrast, in the presence of AC, a proportional increase in embryogenesis was obtained from 3 year old cultures suggesting that by continuous culture on charcoal containing medium helped balancing the auxin / cytokinin ratio which in turn helped retention of embryogenesis. However, according to Motoike *et al.* (2001) during long term maintenance of cultures, charcoal may remove ingredients from culture medium that are essential for maintenance of proembryogenic mass (Motoike *et al.* 2001) and this is in contrast to our observation.

5. 5. 3. Long term maintenance by secondary embryos

From our studies, it was also observed that the process of secondary somatic embryogenesis was continued up to 1 year by generating embryos from root pole of the newly formed embryos. In most cases, embryogenesis occured as clusters. However, the secondary embryogenesis frequency was differed depending on the developmental stage of embryos. Compared to all stages, immature cotyledonary embryos induced more secondary embryos. A similar kind of pattern of secondary embryogenesis was reported by Nair and Gupta (2006). According to them, there is a significant effect on developmental stage of explanted somatic embryo on secondary embryogenesis. However, in contrast to the results of the present study, Nair and Gupta (2006) obtained a higher percentage of secondary embryogenesis from globular and heart stage embryos while cotyledonary embryos showed a poor response to induce secondary embryogenesis. Our results are in consistence with this report that less secondary embryos were developed from mature cotyledonary and germinated embryos. The reduction in secondary embryogenic potential in later stages may attribute to the fact that the developing root tissues gradually replace the embryogenic cells.

5. 6. Effect of light

Embryogenesis is a series of interlocking process where the alteration of one in turn affects many others. Because of this, it is difficult to identify the relative contribution of each factor or which one is the more important than others. Among the most defined factors affecting the whole process of somatic embryogenesis, environmental factors such as light and dark have also seemed to play important roles in every stages of somatic embryogenesis. From the present study, it is indicated that even though callus induction occurred under both conditions; only dark incubation favoured both induction and proliferation of calli. By transferring the callus to embryo induction medium, callus turn to brownish colour and then produces friable highly embryogenic calli with mucilagenous coating. This period was very critical during which the calli acquired embryogenic competence and was occurened only in dark period. Therefore, in *Hevea*, upto acquisition of embryogenic competence, callus cultures needed continuous darkness. However, light incubation favoured embryo maturation and plant development. Similar to our observation, in most of the reports in other species also, absolute darkness was needed for embryogenesis (Michler and Bauer 1991; Fiore *et al.* 1997; Saji and Sujatha 1998). Induction of somatic embryogenesis is also reported under dark and low light conditions (Wang and Wei 2004; Ganeshan *et al.* 2003; Nuutila *et al.* 2002). According to Prakash and Gurumurthi (2005), in *Eucalyptus*, the highest frequency of somatic embryogenesis was observed under low light condition (16h Light / 8h Dark) while under dark condition the frequency was reduced to very low percentage. Contrast to this, light completely suppressed somatic embryogenesis in *Manihot glaziovi* (Joseph *et al.* 2000). The exact role of darkness is not completely understood, but it is likely that incubation of plant tissue in darkness preserves light sensitive endogenous plant growth regulators and other compounds (Hartmann *et al.* 1997).

5. 7. Hardening and SEM studies

5. 7. 1. Hardening

Somatic embryogenesis protocols usually involved several steps each of which may be very critical in deciding the success of the system. Although, the hardening is the last step, it is a very crucial step prior to transplanting of plants to the soil. However, transition from test tube to soil is often very difficult because the *in vitro* produced plants are not well adapted to *in vivo* conditions (Pierik 1996). In *Hevea* also, during somatic embryogenesis, abnormality was seen and both normal and abnormal plants were developed.

In the present study, the results clearly indicated that non-sterile condition was unsuitable for *Hevea,* similar in *leucaena* (Dhawan and Bhojwani 1987). In *Hevea,* fungal contamination was also very severe under this condition. Out of the three sterile potting mixtures used, highest survival was found in sand: soil: cowdung mixture. IBA pretreated plantlets also showed high survivability. The high survival may attribute to the well developed root system induced by IBA or the presence of lateral roots induced by IBA. During the initial days of transplantation, regardless of three potting mixtures, the plants remained healthy without any notable change. Thereafter, the leaves started yellowing in colour and finally get wilted. However, the abnormal plants dehydrated very quickly after transplantation and become somewhat wilted. Under *in vitro* hardening procedure, fungal contamination was more which in turn induced senescence of leaves and eventual death of plants. In contrast, *in vitro* hardening method was found to be very effective for leucaena plants and 85% survival was obtained by this procedure (Dhawan and Bhojwani 1987).

In the present study, a high RH was maintained for an initial period of two weeks. Such an environment was created by covering the plants with polythene bags. However, during this period, due to high RH, the chance for fungal contamination was prominent although sterile potting mixtures were used. To avoid this, 2 hrs removal of polythene bag in morning hr was practiced and also plants were irrigated with ½ strength MS. Although the regenerated plants were successfully acclimatized (72%) in the hardening process, only 60% was survived after 2 months of transfer. A yellowing of leaves was often observed culminating in the death of some plants. Such behaviour has been described in other species also (Steinmacher *et al.* 2007) and could be related to the photosynthetic apparatus of the plantlets (Rival *et al.* 1997) and or inadequate plant nutrition under these culture conditions. Acclimatization losses are also probably due to poor rooting and or a poor management of the *in vitro* environment (Rival *et al.* 1997), however, this observation may not always be relevant (Steinmacher *et al.* 2007).

5. 7. 2. SEM study

Epicuticular wax is having many functions and presence of epicuticular wax helps in reducing cuticular transpiration and stomatal transpiration (Mercy *et al.* 2005). Several reports suggests that epicuticular wax present in the leaves of tissue culture plants is considered as a major factor responsible for the low survival of *in vitro* derived plants (Sutter 1988). The importance of epicuticular wax in somatic plants is highly valued and therefore needs to be studied through SEM studies.

During somatic embryogenesis, both normal and abnormal plantlets were produced. These plants showed variation in morphological characters and based on these characters, plants could be distinguished as weak and healthy plants. Morphologically the weak plants appeared with thin elongated pale green leaves or with stunted shoot with close internodes. On the other hand, healthy plant had dark green leaves arranged on the stem with internodes spaces. Though epicuticular wax was present in all samples tested, the deposition was poor in weak plants. This may be attributed to one reason for high mortality rate during hardening. Presence of epicuticular wax was less on healthy plants before hardening, however, the pattern was nearly same to that of control plant during and after hardening. Low survival rates of tissue culture plants have been attributed to high rate of cuticular transpiration and epicuticular wax is considered to be a major factor responsible for excessive water loss (Sutter 1988). Results of our study clearly revealed scanty distribution and poor development of epicuticular wax particularly in weak plants which in turn lead to the very poor survival rate. However, for healthy plants, wax deposition was continous and in a stage development and well developed during the hardening process which plausibly helped the plants during hardening and resulted in enhanced rate of successful transplanting. Scant deposition of epicuticular wax on the leaves of plants grown *in vitro* has been ascribed to high humidity inside the cultural vessel (Ziv 1986). According to Ziv (1986) it was observed that leaves of plants cultured on low agar or liquid medium lacked surface wax. Environmental conditions including artificial support medium, low light regimes, high relative humidity imposed during in vitro culturing induced abnormalities. All these factors directly or inditrectly involved in survivability of plants during hardening. These aspects require more direct experimental avidence.

5. 8. Histological studies

During *in vitro* culture, the somatic cells or tissues acquire embryogenic potential after a dedifferentiation process (Fambrini *et al.* 1997). During this transition, cells have to activate their cell division cycle, reorganize their physiology, metabolism and gene expression patterns (Feher *et al.* 2003) and thereby drastic alterations may be occurre in the cell. This transition of somatic to embryogenic cell is the first and crucial step in any somatic embryogenesis system but it is also the least understood. Studies using light and electron microscopy have provided detailed descriptions of the morphological and cellular changes

that characterize embryogenic competence (Namasivayam 2007). Based on histological observations on various plant system including pearl millet and cork oak, the embryogenic cells that form somatic embryos are characterized generally as small, isodiametric in shape, have large and densely staining nuclei and nucleoli and are densely cytoplasmic (Namasivayam 2007). In *Hevea*, relatively few studies have been reported on the histology of somatic embryogenesis (Carron *et al.* 1992; Michaux- Ferriere *et al.* 1992) and the authors were focused only on the origin and ontogeny of somatic embryos. However, no information is available on the histological aspects of embryogenic nature of callus, particularly for the clone RRII 105 and therefore in the present study we made an attempt to characterize the histological features of embryogenic calli.

In the present study, embryogenic calli consisted of actively dividing small cells with prominent nuclei. The embryogenic cells were surrounded by a thicknened outer wall. According to Verdeil *et al.* (2001), cell wall thicknening was the existence of an outer most fibrillar matrix i.e. callose deposition. Callose deposition was thought to be a pre-requisite for somatic embryogenesis in *Cichorium intybus* (Dubois *et al.* 1991). Histological study performed in *C. japonica* pointed out that increase in cell wall thickness of embryogenic cells was due to the deposition of callose and it does not deposit in non embryogenic calli. Therefore, changes in the cell wall composition may be considered one of the first symptoms of the acquisition of embryogenic competence (Pedroso and Pais 1995). In coconut, according to Verdeil *et al.* (2001) acquisition of embryogenic potential can be achieved by increasing 2,4-D level which may act as a stress signal which trigger the cells to undergo major genome reprogramming and cytological changes similar to those occurring *in vivo* gametogenesis in plants.

Histological analyses in *Hevea* detected the presence of a large amount of stained black blusih starch grains dispersed through out the cells of embryogenic calli, in contrast with those of non embryogenic calli. In A. *polyneuron,* embryogenic cells contained large amount of starch grains with an average of 15-18 grains per cell (Ribas *et al.* 2000). According to Barciela and Vieitez (1993) starch may have provided energy for the onset of division and formation of embryogenic cells and embryos. Our observations showed that starch accumulation is considered as a histochemical marker of the embryogenic cells. This was in agreement with the finding of many authors where starch is generally considered to be an indicator of the development towards somatic embryos (Verdeil *et al.* 2001; Sane *et al.* 2006). However, with the findings of Barciela and Vieitez (1993) starch accumulation

cannot be considered as a histochemical marker for embryogenic process and reported that a large deposits of starch was also seen in non embryogenic tissues. Our results also showed the localization of total lipids by the presence of orange droplets and were uniformly distributed throughout the cells of embryogenic calli, however, lacking or declining in non embryogenic cells. This observation is in agreement with the observations made in oil palm by Schwendiman *et al.* (1988) who noted the early accumulation of lipid reserves that could be a good indicator of the acquisition of the embryogenic potential in tissues and these storage lipids form small droplets which combined to large form droplets in each cell. In the current study, although there is protein bodies, dense accumulation was observed in embryogenic calli and decreased or very weak accumulation was seen in non embryogenic calli. In date palm, Sane *et al.* (2006) observed that embryogenesis is accompanied by significant accumulation of proteins. Additionally, in oil palm, storage proteins were accumulated more precociously during the development of somatic embryos (Morcillo *et al.* 1998). According to Merkle *et al.* (1995) accumulation of proteins were considered as a potential marker to evaluate vigor and quality of somatic embryo development. In agreement with the studies of Lee *et al.* (1997), the present study also revealed significant differences between embryogenic and non embryogenic cells.

5. 9. Cytological and molecular analysis of somatic plants

5. 9. 1. Ploidy level

In general, a problem with anther explants is that the regenerants shows a considerable level of ploidy variation and frequently results in mixoploids. Ploidy level will vary depending upon the origin of callus from which the plantlets were differentiated. However, the results of our study proved that by using immature anther as explants, no ploidy variation will occur since the explants at the diploid stage were cultured. Further in our study, callus was induced from the anther wall. Similar observations were made by Fraser and Harvey (1986) who used anthers as explants for somatic embryo induction and plantlets were produced and reported that callus was produced from anther wall. In grape, according to Rajasekaran and Mullins (1983), anther somatic tissues gave rise to embryogenic cultures but, not gametic cells, since any haploids and homozygous diploids were not seen among the regenerants. However, the origin of embryogenic cells in primary explants in grape is obscure. In *Hevea*, Arokiaraj (1994; 1996) used mature anther derived calli as target tissue for genetic transformation studies and genetically modified rubber plants were obtained and

so far no phenotypic variation with respect to ploidy level was reported. However, for Chinese clones of *Hevea*, the cytological observations of root tips of regenerated plants using mature anther at the late uninucleate stage, a mixture of aneuploids and haploids but no dipolids were obtained (Chen *et al.* 1979).

5. 9. 2. Analysis of genetic stability by RAPD

An important aspect of any *in vitro* propagation protocols is the genomic stability of regenerants. When the *in vitro* system is used for genetic transformation, genetic instability is also not desirable. Regeneration via somatic embryogenesis in general, direct embryogenesis is expected to produce less number of variation. However, in many cases, plantlets regenerated from *in vitro* culture might exhibit somaclonal variation as a consequence of epigenetic influence or induced by tissue culture conditions (Larkin and Scowcroft 1981). Our study was therefore aimed to determine the genetic stability or variability of somatic plants using RAPD markers. The results of our study indicated that by somatic embryogenesis no variation was occurred. Similarly, our study is not in agreement with the hypothesis of Vasil and Vasil (1980) who reported the species, explant type, the donor genotype, the composition of culture medium, conditions of the physical culture and the duration between successive subcultures affecting the frequency of somaclonal variants. The utility of RAPD as a means of molecular analysis of *in vitro* regenerated plants has been well documented by many others (Rout *et al.* 1998; Goto *et al.* 1998; Lattoo *et al.* 2006; Ray *et al.* 2006). RAPD technique is sensitive to changes in PCR conditions resulting in changes to some of the amplified fragments. Polymorphism detected by RAPD marker could also be due to a change in primer binding site only and the sequence between the primer binding sites may also have the same effect.

In current study, the RAPD banding patterns of 10 randomly selected somatic plants with budgrated control plants were compared. All the 13 primers tested in this study revealed no detectable variation and the amplified banding patterns produced were monomorphic in 10 regenerated plantlets suggesting the genomic stability among somatic plants. Generally, regeneration through somatic embryogenesis is expected to produce less number of variants. Our results are in agreement with this hypothesis where no detectable variation has been noticed. Our results are also in agreement with that of Tang (2001) in which 21 random primers were used to analyse 21 regenerated plantlets of loblolly pine. In this case, all the markers generated were monomorphic across all 21 plants showing genetic homogeneity. Similar kind of observation was reported by Goto *et al.* (1998) in *Pinus* where plantlets derived from callus via organogenesis

were also shown to be homogenous. Thakur *et al.* (1999) and Shoyama *et al.* (1997) also observed no somaclonal variation in *Quercus serrata* somatic seedlings as well as in *Panax* somatic embryo derived plants. According to Shoyama *et al.* (1997), somatic plantlets were regenerated via callus phase, however, the amplification products were monomorphic for all plants suggesting that somatic embryogenesis can be used as for clonal micropropagation of this plant. In several *Citrus* species, somatic embryo derived plants have been reported to be genetically stable with respect to morphology, cytology and physiology. Also genetic stability is reported for willow (*Salix* sp.) and date palm (*Phoenix dactylifera*) plants regenerated through somatic embryogenesis (Akhar *et al.* 2000). However, in spruce, Heinze and Schmidt (1995) concluded that a low frequency of genetic instability was present in the population of somatic derived plantlets. In contradictory to this, RAPD polymorphism has been reported in many plants derived from callus via organogenesis. Plants derived from leaf base callus of tumeric showed polymorphism with some primers (Salvi *et al.* 2001) and 16.5% polymorphism was detected. In tomato plants, regenerated from callus cultures by organogenesis, produced polymorphic bands and in this study, out of the 10 primers producing scorable bands, 6 primers produced polymorphism for 11 plants (Soniya *et al.* 2001). Since even single base change at the primer annealing site itself is manifested as appearance or disappearance of RAPD bands, it could be suggested that tissue culture conditions have induced varied amount of genetic changes at different regenerated plants. However, in our study no such variation could be seen with all tested primers. Also we compared the results of RAPD profile of somatic plants with monoclonal seedlings. All tested 5 primers revealed polymorphism with seedlings. With 5 primers, 70 scorable bands including 22 polymorphic bands were obtained. These polymorphic RAPD bands showed varied degree of genetic difference. Some of the polymorphic bands were not seen in control plant as well as in somatic plants and also in some of the seedlings. However, the degree of polymorphism was more with A 12 and A20 compared to B7, B20 and D18. On the other hand, although the number of primers was low to confirm the genetic stability of somatic plants, with the same primer, polymorphism was seen in seedlings. Absence of polymorphisms in the RAPD profile of somatic plantlets indicated the genetic stability of *Hevea* plants derived *in vitro* through this protocol. The genetic stability of *Hevea* plantlets observed in our study indicates good genetic fidelity and no somaclonal variation in the plantlets induced via somatic embryogenesis. However, with the seedlings polymorphism was seen using the same primers. Such variation may derive due to cross pollination of *Hevea*.

5. 9. 3. Cytogenetic stability of long term cultures

Plant cell behaviour is the result of a complex process of genetic programming and is sensitive to hormonal changes especially under stressful conditions causing irregular cells with abnormal chromosomes (Zhao *et al.* 2005). It is well documented that *in vitro* culture conditions induce a genomic stress that might resulted in chromosome breakage (Al Zahim *et al.* 1999). Several studies on regenerated plants have shown frequent occurrence of chromosome breakage in plant tissue culture and that break points are often associated with late replicating hetero chromatin regions (Radic *et al.* 2005). The chromosomal instability observed in tissue culture generated plants is either induced by media components, culture age, explant tissue and even plant genotype (Psechke and Phillips 1992). However, during *in vitro* culture, plant growth regulators applied is also an important factor associated with chromosome alteration. Although plant growth regulators are required for cell division, it was shown that exogenously added plant growth regulators, especially auxin and cytokinin, can induce chromosomal mutations in plant cell culture material (Radic *et al.* 2005). According to the observation of Lee and Phillips (1988), frequency of mutation in regenerated plants increased with age of culture. Similarly, Zhao *et al.* (2005) observed abnormal chromosome number and chromosome fragments on micropropagated one year old rhubarb (R. *rhaponticum* L.) plants. Results of our study were in disagreement with these observations. From our results it could be observed that that long term cultures induced no instability in normal chromosome number, although the number of samples which have been analyzed is still low. This suggests that culture age, subculture interval and added plant growth regulators showed no effect on cytogenetic stability from long term cultures. This is in agreement with the findings of Radic *et al.* (2005) who reported that culture age and added plant growth regulators showed no effect on cytogenetic stability from long term cultures of *C. ragusina.*

5. 9. 4. Genetic stability of long term cultures by RAPD

Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequences of plants. Several authors have applied the RAPD technique to investigate genetic variability and found it very efficient and reliable (Goto *et al.* 1998; Al Zahim *et al.* 1999; Latto *et al.* 2006). In the current study, we compared RAPD banding pattern of long term callus cultureswith short term callus cultures as well as budgragted control plants. The results obtained by RAPD analysis revealed no genomic variation within long term cultures. In contrast, somaclonal variations were reported in long term derived plants of garlic (Al Zahim *et*

al. 1999). According to him, RAPD analysis of garlic plants produced a total of 7903 bands of which 50 were polymorphic with an average frequency of 0.63%. Similarly, in rice callus cells (Yang *et al.* 1999), amplification profile of one month cultured calli was significantly different from the original explant suggesting that somaclonal variation might be induced in the initial stage of culture. However, when the cultures were maintained for 4 months, many polymorphism was observed compared to one month old calli where only fewer polymorphism was seen suggesting that somaclonal variation increases with culture age. This result is in disagreement with our observation. On the other hand, Goto *et al.* (1998) reported genetic stability from long term (10 year old) micropropagated shoots of *Pinus thunbergii* Parl. using RAPD markers. In this report, eventhough morphological variations were observed among shoots, no somaclonal variation could be detected in 36 micropropagated shoots.

Generally, chromosome based variation resulted from changes in chromosome number, chromosome rearrangement, breakage and lagging is believed to be the fundamental cause of somaclonal variation (Zhao *et al.* 2005). Consequently, chromosomal aberrations could occur and changes in chromosome structure may be responsible for genetic variation resulting morphological variations (Zhao *et al.* 2005). For a species any form of chromosomal rearrangements can lead to many changes in the genome at structural or possibly at functional levels which may play a critical role in generating several forms of somaclonal variation. However, from our results, cytological investigations are in conformity with results obtained with RAPD analysis and therefore conclude that long term cultures were used as a target tissue for producing uniform plantlets and thereby as a micropropagation technique as well as a technique for genetic transformation.

5. 10. Biochemical studies during somatic embryogenesis

5. 10.1. Protein changes associated with somatic embryogenesis

The ability of somatic plant cells in culture to regenerate entire plants by somatic embryogenesis is a remarkable biological phenomenon. Since morphological differentiation or developments are often associated with a continous synthesis and degradation of enzymes and structural proteins (Scandalios 1974), correlating the changes in their profile with respect to specific morphogenetic developments would be of very significant. Several biochemical variables have shown to discriminate between embryogenic and non embryogenic tissues. One biochemical approach to learn about embryogenic and non embryogenic calli as well as to the different stages of somatic embryogenesis could be the analysis of proteins and isozymes that

differentially appear during the course of development. Hence the present work was attemped to correlate the morphological differences between embryogenic and non embryogenic calli with proteins and isozymes expressed in them and expressed during different developmental stages as well as among different types of embryos.

Proteins are valuable indicators of differentiation that could be useful as biochemical markers to identify specific stages of somatic embryos (Yuff *et al.*1994). In the present study, during different developmental stages of somatic embryogenesis, the number of proteins and its concentrations were more in embryogenic calli as well as in embryos. In embryogenic calli, low molecular weight proteins (14 kDa to 29 kDa) expressed in higher intensity than high molecular weight proteins. Similarly, there was a relative abundance of low molecular weight proteins (14.7 kDa to 43 kDa) in embryogenic calli of *Santalum album* L (Suma and Balasundaran 2004). In the current study, as development proceeded to embryo stage, a relative abundance of both low and high molecular weight proteins were observed. In embryo stage, protein pattern reveals 12 distinctive polypeptides. Among these bands, few polypeptides with high molecular weight (above 97 kDa) were found only in embryo stage. These polypeptides were not seen in other developmental stages. These results indicated that these polypeptides may be synthesized only during embryo formation or may correlate with embryogenic ability or to the induction of embryogenesis. Embryogenic and non embryogenic calli significantly varied in their protein profiles. Non embryogenic calli was characterized by the absence of bands and this difference was clearly detected in electrophoretic profiles. Similar observations were made during coffee somatic embryogenesis where differences between embryogenic and non embryogenic calli with respect to proteins are reported (Yuff *et al.* 1994), however, electrophoretic profile of non embryogenic calli revealed seven distinctive polypetides same as in embryogenic calli.

The present study also describes the protein pattern among 3 different types of somatic embryos. Although, all 3 types of embryos revealed similarity in protein profiles, wide variations with respect to expression of proteins were observed. Among the stages, globular embryos exhibited a much higher expression. On subsequent stages, the expression level remained more or less same or decreasing. These results were in accordance with Catarina *et al.* (2003) who reported that globular stage embryoids had significantly accumulated higher levels of total proteins however, gradually decreased with maturation of embryos. Similarly, in embryogenic cultures of *Laris – leptoeuropeae,* variations in the protein levels were observed and cultures showed a high protein levels in the 2nd week and decreasing significantly in the 5th week (Gutmann *et al.* 1996). In O. *catharinensis,* embryogenic cultures showed high frequencies of embryos in 1-2 weeks old cultures

and high protein levels. The decrease of proteins levels in embryoids were associated to their maturation which mainly consisted of differentiation and growth supported by cell enlargement. According to Chanprame *et al.* (1998), during maturation soybean somatic embryos decreased their protein content by 25%. In contradiction, during somatic embryogenesis, coffee somatic embryos, at globular, heart and torpedo stages, showed changes in some proteins that are progressively accumulated throughout the development (Yuff *et al.* 1994).

5. 10. 2. Isozyme studies

Isozymes are easily detectable and their variation is often associated with genetic differences and developmental stages (Scandalios 1974). It has been shown earlier that isozyme response vary with tissue organization during development and differentiation. Peroxidase and esterase have been proved as useful markers for the detection of somatic embryogenesis (Alves *et al.* 1994) and embryogenic potential (Martinelli *et al.* 1993). Peroxidase attributed vital roles in regulation of cell growth, differentiation and embryogenesis. Although the precise function of esterase has yet to be elucidated, this enzyme enhance pectin gelling playing a promotive role in embryogenesis.

In the Prx zymogram, the embryogenic calli were associated with the synthesis of specific isoperoxidase and increased in peroxidase acitivity and found 5 bands that may be useful to discriminate biochemically between embryogenic and non embryogenic calli. Interestingly, although same bands were observed in non embryogenic callus, the activity of peroxidase was very negligible compared to embryogenic calli. These results are in consistence with Asokan *et al.* (2001) where non embryogenic calli showed marked differences. In sweet potato also, peroxidase showed difference in embryogenic and non embryogenic calli (Alves *et al.* 1994). In our investigation, at different developmental stages of somatic embryogenesis, increased peroxidase activity was observed on embryogenic calli, a state just before the induction of embryos as well as in embryos. However, when embryos reached to plantlets peroxidase activity was gradually declined. These results are also in agreement with Asokan *et al.* (2001) who reported that the most enzyme activity, with four bands, were exhibited by embryogenic calli derived from integumental tissues of *Hevea*. Accordingly, embryos also had a similar pattern of expression. In sweet potato, a very similar pattern of Prx was found both in embryogenic calli and globular embryos (Alves *et al.* 1994). Devi and Radha (1997) reported the appearance of peroxidase bands in the embryogenic calli of *Vigna radiata* which are useful as biochemical marker for somatic embryogenesis. Different developmental stages during ontogeny of somatic

embryogenesis in sandal revealed specific differences in the banding profile of peroxidase and there was an increase in the activity of peroxidase in friable embryogenic calli. However, in the developing embryos the peroxidase activity was found to be low (Suma and Balasundaran 2004). In the present study, initially callus displayed one distinct broad band in the fastest migration zone with a fainter narrow band in the slower migration zone. Among 3 types of embryos, peroxidase displayed uniform activity. It has been proposed that peroxidase activity is controlled by auxins especially 2, 4-D (Suma and Balasundaran 2004). In our study, except band 5, no band is notably increased in intensity during callus formation on 2, 4-D containing medium. Hence in the present study, we concluded that the differenes in Prx isozyme might be indicative of the developmental stage of the tissue. Similar correlations between specific developmental events and peroxidase patterns have been reported by (Suma and Balasundaran 2004) in sandal. The presence of several peroxidases in plants represents a variety of metabolic reactions including metabolism of auxins, lignification, wound healing, pathogen defence etc and therefore it may be considered as a suitable marker for biochemical studies.

In the present study, Est expression was confined to a distinct single band in embryos and plantlets. This band with maximum intensity observed in the fast migration zone was absent in embryogenic calli. These results confirmed that Est activity were also correlated with embryogenesis and plantlet regeneration. In accordance with Suma and Balasundaran (2004) esterase activity was stimulated in later stages of development with two additional bands at embryo induction, embryo maturation and matured embryos. Similarly, in barley, the esterase activity was triggered in the later stages of development (Everett *et al.* 1985). The results of our study reported that in the extracts of globular, torpedo and cotyledonary embryos, the appearance of bands were on the same zone of activity, however, maximum activity was observed on later stages. In *Hevea*, during somatic embryogenesis of integumantal tissue, both embryogenic calli as well as embryos exhibited maximum activity for esterase (Asokan *et al.* 2001). Callus indicated no bands or activity. Since callus symbolize late stage of dedifferentiation when structural gene expression might have been very minimal or absent. Non embryogenic calli showed some activity with a low intensity in slow migration zone than in fatser zone. However, there was a marked difference in isozyme pattern between embryogenic and non embryogenic calli. In integumental callus of *Hevea*, embryogenic callus exhibited specific bands for Est, though activity was less compared to non embryogenic callus (Asokan *et al.* 2001) where no bands were detected.

Chapter-VI

SUMMARY AND CONCLUSION

Hevea brasiliensis (Muell.Arg.), the Brazilian rubber tree, is the only commercially cultivated species as a source of natural rubber, regarded as nature's most versatile raw material. With the ever growing demand of natural rubber on one hand and the limitations of classical breeding on the other, efforts on research and developments are directed towards rubber breeding programmes to generate improved clones through biotechnology. Therefore biotechnology holds great potential for genetic manipulation thereby complementing conventional methods of *Hevea* breeding. Plant regeneration via somatic embryogenesis is the key to such genetic manipulation approaches. Development of an *in vitro* regeneration system via somatic embryogenesis is therefore placed immense value for *Hevea*. The successful exploitation of somatic embryogenesis demands high frequency embryo induction and plant regeneration.

The present study describes a plant regeneration system via somatic embryogenesis. The suitability of utilizing immature anther as explants were explored and proved as an ideal explant for somatic embryogenesis. For reducing explant browning and obtaining maximum callus, ascorbic acid solution was essential during dissection of anthers. Experiments on investigating the effectiveness of two basal media showed that modified MS medium was more effective than MS. Of the four auxins, 2, 4-D, NAA, IAA and IBA tested for callus induction, 2, 4-D was found as the potent auxin. Among the various combinations tried, optimal concentration of 2, 4-D and KIN for obtaining maximum callus induction was 2.0 mg/l 2, 4-D and 0.5 mg/l KIN. Depending upon 2, 4-D concentrations, three types of calli were obtained *viz*, Type I, Type II and Type III. On subsequent culturing of three types of callus for embryo induction, only Type II callus was successful. All tested auxins in combination with KIN induced embryos, however, NAA showed a positive response on embryo induction. Maximum number of embryos were produced on medium supplemented with 0.2 mg/l NAA and 0.7 mg/l KIN.

The present study aimed at improving the responsiveness of the tissue to various factors, have been proven successful in improving the embryogenic process. Before inoculation on solid medium, explants pretreated in liquid medium reduced the duration of callus induction. Immature anthers pretreated in liquid medium for 10 d followed by 25 d culturing on solid callus induction medium was found to be the most suitable. Results revealed that short duration pretreatments in liquid increased the production of embryogenic callus, but, prolonged

pretreatment adversely affected the embryogenic calli formation. After primary callus induction, when maintained in 2, 4-D containing callus induction medium, influenced embryogenic capacity of callus. Results indicated that the optimal time of callus transfer for embryogenesis is 50^{th} d (35 d + 15 d) of explant inoculation. On the other hand, by increasing the time of callus maintenance in 2, 4-D containing medium, embryogenic calli formation was decreased. The effect of polyamine showed that spermine does not enhance embryogenesis. However, spermidine was found to slightly improve the embryogenesis when used at a concentration of 0.5 mg/l. In brief, polyamines had no significant stimulatory effect on embryogenesis. Among various amino acids, inclusion of alanine and arginine showed no effect on embryo enhancement. With asparagine, all tested concentrations allowed embryo differentiation similar to that of control. By the addition of glutamine, embryo induction was increased and maximum response on embryo induction was obtained at higher concentration of glutamine (200mg/l). Similarly, casein hydrolysate also improved embryogenesis and maximum number of embryos was produced when medium was enriched with 400 mg/l casein hydrolysate.

Efforts were also directed towards the optimization and enhancement of embryo germination and plant regeneration. On hormone free medium, mature embryos were converted into plantlets and 27% of embryos were germinated into full plantlets. However, by the inclusion of cytokinins combined with GA_3, the somatic embryo germination and plantiet development was significantly enhanced. By the inclusion of BA and ZEA, maximum response was obtained at 0.5 and 2.0 mg/ respectively. Of the four cytokinins tested, the response of TDZ at 0.25 mg /l concentration was found to be more effective for embryo germination as well as plant regeneration followed by BA and ZEA. Response with KIN was found to be low compared with other cytokinins.

For long term maintenance of embryogenic cultures, a subculture interval of 50 d was found to be ideal. When embryogenic callus aggregates, derived from primary somatic embryogenesis, were used as target tissue, the embryogenic potential could be maintained up to 3 years by serial subculture of 50 d intervel. When proline was included in embryo induction medium, embryo induction frequency was increased with maximum being at 100 mg/l. Similarly, AC was found to be essential for long term maintenance of cultures. Embryogenesis was reached maximum at 0.1% AC. Embryogenic lines could also be maintained up to 1 year by inducing secondary embryos from primary embryos. Among different developmental stages of embryos, secondary embryos were higher in immature cotyledon stage embryos.

Explants incubated both under dark and light conditions induced callus, however, further proliferation of calli was found to be affected by light. The studies indicated that *Hevea* needed continuous darkness till the acquisition of embryogenic competence or embryo induction. Further embryo development and maturation was favoured by light. Similarly light is a critical factor for plant regeneration though few plantlets could be regenerated under dark.

Results of the experiments on hardening indicated that non sterile potting mixture was unsuitable for *Hevea*. Of the three sterile potting mixtures tried, highest survival was obtained with sand: soil: cowdung mixture. IBA pretreated plantlets survived well with a higher survival rate. SEM studies revealed that the amount of wax varied with the source of leaf. Leaves from weak plants at the time of hardening deposited low epicuticular wax. With healthy plants, epicuticular wax was started to appear during pre hardening and deposition was continued to increase during and after hardening.

Histological examinations indicated that embryogenic calli consisted of small cells with prominent nuclei, a thickened outer wall. In contrast, non embryogenic cells were characterized by the large size containing prominent nuclei and thin cell wall. Histochemical characterization revealed that embryogenic calli contained few starch grains at early stage and at late stage; almost all cells were densely accumulated with starch. At early stage, lipids were seen in small droplets and the number of droplets was more at late stage. Protein bodies appear to be significantly more abundant in late stage than in early stage. In contrast, non embryogenic calli were poorly filled with starch grains and both lipid content and protein bodies were less in number or quantity.

Cytological studies with root tips of tested plants showed that all the plants having a chromosome number, 2n= 36. This confirms the diploid nature of regenerants by utilizing immature anther. With all tested primers, somatic plants showed no detectable variation. All the amplification products generated were monomorphic in all somatic plants as well as mother plant showing the genomic stability. However, with the same primers, monoclonal seedlings showed detectable variation. Results of cytogenetic studies revealed no alteration in chromosome number from 3 year old callus cultures. RAPD analysis of long term embryogenic callus cultures displayed same banding pattern. With all primers, genetic similarity was observed with short term and long term callus cultures as well as mother plant.

During different developmental stages of somatic embryogenesis, different protein profiles were observed. More protein bands were observed in embryogenic calli as well as in embryos. Compared to embryogenic calli, embryos showed a relative abundance of both low and high molecular weight proteins. Embryogenic calli and non embryogenic calli significantly

varied in their protein profiles. No clear protein bands were observed in non embryogenic calli. The protein electrophoretic analysis of somatic embryos at different stages of development revealed that the overall pattern of proteins was similar in all 3 embryos, however, varied in their relative expression. When embryos proceeded from globular stage to next stages of development, proteins were expressed in a much lower intensity.

The isozyme analysis at the five sequential stages of embryogenesis revealed remarkably distinct zymogram profiles for peroxidase and esterase. Increased peroxidase activity was seen on embryogenic calli as well as in embryos. In contrast, non embryogenic calli showed very faint activity. Three types of embryos exhibited same banding pattern with an increase in peroxidase activity. With esterase enzyme, the isozyme pattern varied with different developmental stages. Embryos and plantlets had a very similar esterase pattern. Non embryogenic calli was distinguished from embryogenic calli by the absence or a very weak esterase activity. Among the 3 types of embryos, globular embryos showed less activity and when reached to later stage such as torpedo and cotyledon embryos showed maximum activity.

In conclusion, the present study demonstrated that immature anther is an ideal explant for inducing somatic embryogenesis thereby exploring the system in crop improvement programmes. Various factors affecting the frequency of somatic embryo induction could be identified and the plant regeneration frequency of up to 82% could be achieved. Embryogenic callus cultures derived from primary somatic embryogenesis could be maintained with embryogenic competence up to 3 years without any chromosomal abnormalities ensuring the year round availability of target tissue for genetic transformation. Molecular analysis of somatic plants and long term callus cultures reveals its genomic stability thereby providing the system as an alternative micropropagation system. Results of histochemical and biochemical characterization help us for the timely identification of embryogenic callus for further subculture which could enhance the efficiency of the regeneration system as well as to reduce the time span required for the completion of regeneration pathway.

For a recalcitrant crop like rubber tree, transformation techniques provide an attractive tool for crop improvement; however, the key to transformation appears to be the development of *in vitro* methods. Therefore, besides transformation, a more important goal is the development of a suitable high frequency plant regeneration system via somatic embryogenesis. The ability of rubber trees to respond well in tissue culture, particularly plant regeneration via somatic embryogenesis has allowed the application of various powerful biotechnology tools for genetic improvement. In future, there is a necessity to concentrate

efforts for the development of more transgenic rubber plants with new genes encoding resistance to biotic and abiotic stresses. Engineering of *Hevea* with rubber biosynthesis genes may contribute revolutions in rubber industry. Similarly, improvement of wood quality and volume by the generation of timber clones is other promising future objectives. Besides their usefulness as potential valuable breeding material, somatic embryogenesis opens the perspective of exploitation of *Hevea* transgenic plants for the production of recombinant proteins. Further prospects will focus on the production of plants via somatic embryogenesis utilizing bioreactor and synthetic seed technology. Nevertheless special attention has to be paid to the use of automation which could enhance the use of long term somatic embryogenesis for micro-propagation by reducing the cost and time. Therefore, the protocol developed in the present study would serve as a useful tool for desirable gene integration aiming at the genetic improvement of *Hevea* in the future.

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