INFLUENCE OF JUVENILITY ON SOMATIC EMBRYOGENESIS AND PHASE CHANGE RELATED GENE EXPRESSION IN *HEVEA BRASILIENSIS*

Thesis

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By

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Declaration

I hereby declare that this thesis entitled **'Influence of juvenility on somatic embryogenesis and phase change related gene expression in *Hevea brasiliensis*'** submitted to the Mahatma Gandhi University, Kottayam, for the award of the degree of Doctor of Philosophy in Botany, is an authentic record of the research work carried out by me, under the supervision of Dr. A. Thulaseedharan, Deputy Director (Biotechnology), at The Rubber Research Institute of India, Kottayam. The work presented in this thesis has not been submitted earlier for any other degree or diploma at any university.

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CERTIFICATE

This is to certify that the thesis entitled **'Influence of juvenility on somatic embryogenesis and phase change related gene expression in *Hevea brasiliensis*'**, is an authentic record of the original research work carried out by **Smt. Kala. R. G.,** 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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PREFACE

Somatic embryogenesis as the name implies is the development of embryos from somatic cells deploying the phenomenon of cellular totipotency. Somatic embryogenesis has potentially rich biotechnological applications in crops mainly in micropropagation, transgenic plant production and artificial seed synthesis.

Genetic improvement of *Hevea* through conventional methods is very elaborate and time consuming as in many other perennial species due to narrow genetic base, non-synchronous flowering, low fruit set, long gestation period and heterozygous nature. *In vitro* culture of *Hevea* was initiated earlier in different laboratories with a view to develop protocols for micropropagation and for genetic improvement through transgenic approaches. Protocols for plant regeneration through somatic embryogenesis from different explants such as inner integument, immature anthers and immature inflorescence have been reported in *Hevea.* All the protocols developed earlier for somatic embryogenesis in *Hevea* were from floral/fruit derived explants which were seasonal. Development of alternate systems for plant regeneration through somatic embryogenesis using easily available explants would be of much use in *Hevea* crop improvement through micropropagation and genetic transformation. The present study was undertaken with the objective of developing a protocol for the induction of somatic embryogenesis and plant regeneration from leaf explants of *Hevea,* identification of the embryogenic competence of explants collected from source plants of different physiological maturity, identification of genes related to juvenile-mature phase change in *Hevea* and to explore the feasibility of the developed system in *Hevea* crop improvement through *Agrobacterium* mediated genetic transformation.

This thesis contains six chapters. In the first chapter the topic is being introduced along with the objectives of the study. A brief description of the crop along with micropropagation techniques and a review of work done in these areas are included in the second chapter. The third chapter deals with the materials and the methodologies followed in the study. Results of the experiments are detailed in the fourth chapter and discussions regarding these are included in the fifth chapter. In the sixth chapter the results are summarized and a conclusion has been drawn about the future prospects of the work.

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ABSTRACT

Plant cell possesses remarkable developmental plasticity, example of which is somatic embryogenesis, during which differentiated plant cells regain totipotency and develop into embryos. Somatic embryogenesis, the development of embryos from somatic tissue is a valuable tool for the micropropagation of plant species as well as in genetic manipulation experiments. This regeneration system is generally more difficult with woody plant species. Success of *in vitro* tree regeneration greatly depends on selection of the appropriate explant, age of the source plant, culture medium and environmental conditions employed. *In vitro* propagation of mature trees is generally more difficult than that in juvenile plants. *In vitro* culture of *H. brasiliensis* was initiated in different laboratories with a view to develop protocols for micropropagation and genetic improvement through transgenic approaches.

All the protocols developed earlier for somatic embryogenesis and plant regeneration were from floral/fruit derived explants. Since flowering in *Hevea* is seasonal and adverse environmental conditions during the season may hinder normal flowering, availability of viable explant is unpredictable. Development of alternate systems for plant regeneration through somatic embryogenesis using explants which are easily available throughout the year would be useful in *H. brasiliensis* crop improvement through micro propagation and genetic transformation. Leaf tissues have been reported to be an amenable explant for tissue culture. There are several reports in other crops, where leaf tissue has been mentioned as an ideal explant for micro propagation and a potential target tissue for *Agrobacterium* infection in genetic transformation experiments.

In the present work, an attempt has been made to develop a protocol for callus induction, somatic embryogenesis and plant regeneration from leaf explants of *Hevea.* The leaf source, ideal growth stage of leaf, culture conditions and media requirements in each step of the somatic embryogenesis and plant regeneration pathway were optimized. Leaf cultures initiated with the medium mature leaves collected from glass house grown bud grafted plants could produce about 73% contamination free, viable cultures when surface sterilized with 0.15% (w/v) $HgCl_2$ for two minutes. Modified MS medium with addition of 1200 mg/l calcium nitrate, L-cysteine HCl (50 mg/l), casein hydrolysate (1.0 gm/l), B_5 vitamins, sucrose (20g/l) and containing phytohormones 2,4-

D (5.4 μM), BA (4.4 μM) and NAA (1.08 μM) was identified as the most suitable medium for callus induction from leaf explants of bud grafted plants of *Hevea.* Embryo induction was obtained in modified MS medium $Ca(NO_3)_2.4H_20$ (250 mg/l) containing B_5 vitamins and phytohormones, BA (2.2 μM), GA_3 (2.9μM), Kin (1.25 μM), ABA (0.75 μM) and NAA (0.54μM). Amino acids added to the medium were arginine (40 mg/l), glutamine (300 mg/l), proline (200 mg/l) and L-cysteine (20 mg/l). The medium also contained organic supplements such as coconut water (5 %), casein hydrolysate (300 mg/l), PEG (5.0 g/l), sucrose (60 g/l) and 0.2% activated charcoal. The medium was solidified with 5.0 g/l phytagel. Maturation and apex induction of embryos was obtained when cultures were dark incubated in WPM containing sucrose (60 g/l), amino acids such as glutamic acid (150 mg/l), arginine (40 mg/l) and glycine (10 mg/l), organic supplements and phytohormones BA (2.2 μM), (Kin 1.38 μM), IBA (0.49 μM) and GA_3 (5.3 μM). Plant regeneration was obtained in hormone free MS medium. Sixty percent of the plantlets transferred to sterile sand could withstand transplanting shock and were free of fungal attack after two weeks. During the second transplant, 30% of the plants continued to survive in the glasshouse

Leaf explants from clonal materials are available throughout the year from source plants of different maturity. It is necessary to compare the embryogenic capacity of the leaf explants collected from different sources to identify the one that is most amenable to *in vitro* culture and optimize the culture conditions suitable for *in vitro* plant regeneration. Embryogenic competence of the explant in terms of embryogenic callus initiation and further embryo induction was highly dependent on the source plant and was found to be reduced with maturity of the source plant. Response of callus in terms of time and frequency of embryogenic callus initiation was found to vary when proliferated callus obtained from leaves collected from different source plants were cultured. The callus developed from *in vitro* derived somatic plants initiated embryogenic callus with a minimum time of three months with a higher frequency (68 %). In callus developed from *in vitro* derived seedlings also the response was almost similar, and a frequency of 72% was obtained. In callus obtained from leaves of bud grafted plants, both the rate (35 %) and time (5 months) taken for initiating embryogenic callus varied with the explant. Mature trees showed no embryogenic potential.

The somatic embryogenesis protocols developed earlier for plant regeneration in *H. brasiliensis* from floral/fruit derived explants have been used for crop improvement through transgenic approaches. The feasibility of using the plant regeneration system from leaf explants for *Agrobacterium* mediated genetic transformation was explored so that the system developed could be used in future for crop improvement through transgenic approaches. Proliferated leaf callus was also proved to be an amenable explant for *Agrobacterium* mediated genetic transformation. It was observed that addition of 10.0 mg/l silver nitrate in the infection, co-cultivation and selection medium significantly suppressed bacterial overgrowth and improved the texture of callus in newly emerged lines. Improvement in the transformation frequency by 5-10% was obtained by the inclusion of either of the antioxidant such as 50 mg/l lipoic acid or 100 mg/l L– cysteine in the infection, co-cultivation and selection medium and addition of the surfactant 300 mg/l pluronic F68 in the infection medium. The protocol developed for somatic embryogenesis and plant regeneration from leaf explants in *H. brasiliensis* was also proved to be useful for crop improvement through transgenic approaches.

Juvenile- mature phase change is associated with changes in gene expression. *In vitro* regeneration is usually achieved by culturing tissues that are in a more juvenile, potentially regenerative state than most other tissues of the tree. *H. brasiliensis* tissues collected from mature trees are found to be highly recalcitrant to *in vitro* culture. In several other crops, comparison of cDNA of juvenile and mature trees indicated differences in gene expression. Differential gene expression in juvenile and mature tissues in turn influences the *in vitro* culture response. Chlorophyll a/b binding protein gene could be amplified from the isolated genomic and cDNA of the *Hevea* clone RRII 105. Amplified product was cloned and sequenced. The sequence showed 91% sequence homology to the Cab protein gene from *Ricinus communis*. No introns were observed in the coding region. Differences were observed in gene expression pattern in juvenile and mature tissues through RT-PCR and northern analysis.

Key Words

Callus induction, Genetic Transformation, Gene expression, *Hevea brasiliensis*, *In vitro* culture, Juvenility, Leaf explants, Plant regeneration, Somatic embryogenesis.

Introduction

Chapter I

INTRODUCTION

The improvement of tree crops through conventional breeding techniques has been limited due to inherent problems such as long life cycle with extended juvenile period, floral morphology, existing hybridization barrier, sterility, apomixis and long term inbreeding depression. Totipotency of a living plant cell is well known that the nucleus of every living somatic cell contains genetic information necessary to direct the development of the complete plant. The attempt by Haberlandt (1902) to establish plant tissue culture systems provided support for a better understanding of the totipotency of plant cells. Somatic embryogenesis (SE) is a process in which a bipolar structure resembling a zygotic embryo, develops from a non zygotic cell without vascular connections with the original tissue. It is an ideal system for investigation of the whole process of differentiation of plants, as well as the mechanisms of expression of totipotency in plant cells. Since the first observation of somatic embryo formation in *Daucus carota* cell suspensions by Steward *et al.*, (1958), the potential for somatic embryogenesis has been shown in a wide range of plant species. Somatic embryogenesis is a promising method for the establishment of protocols for rapid multiplication of new and elite genotypes, synthetic seed production, *in vitro* selection approaches for various biotic and abiotic stresses and for genetic manipulation.

1.1. The Rubber tree - *Hevea brasiliensis*

Hevea brasiliensis (Wild.ex Adr.de Juss.) Muell Arg. is the most important source of natural rubber - a product of vital importance. The natural rubber producing tree *H. brasiliensis*, is a species indigenous to the tropical rain forests of Central and South America and is one of the most recently domesticated crop species in the world. *H. brasiliensis* remains as the only cultivated species as a commercial source of natural rubber because of its abundance in the latex, high quality and convenience of harvesting. The rubber tree (*H. brasiliensis*) a member of the family

Euphorbiaceae, is a native of Brazil and was introduced to South Eastern countries during 1876 through Kew gardens from the seeds brought from the Raio Tapajo's region of the upper Amazon region of Brazil by Sir Henry Wickham (Dijkman,1951). At present more than 10 million hectares in about 40 countries are devoted to rubber cultivation.

1.2. Habitat and Biology

Rubber is predominantly grown in the tropics where an equatorial monsoon climate prevails such as a rainfall of 2000 mm or more evenly distributed with a maximum temperature of 29-34^{0}C, 80% atmospheric humidity and bright sunshine amounting to 2000 hrs. per annum (Watson,1989; Rao and Vijayakumar, 1992). *H. brasiliensis* is a sturdy perennial tree which attains a height of about 30m (Fig.1). Yield potential of the tree is high during the winter season. The tree is deciduous with annual leaf fall. Wintering of the tree occurs during December-January followed by refoliation and flowering. Natural rubber is produced in highly specialized laticifers, which is a specifically differentiated organ localized in the vascular tissue and is present in all parts of the tree *H. brasiliensis.* Natural rubber is a biopolymer consisting of isoprene units $(C_5H_8)n$ linked together in a 1,4 *cis*- configuration. Rubber is harvested from the tree as latex by controlled wounding of the bark in the main trunk of the tree, a process termed tapping. By tapping, rubber particles can be collected, together with other cellular components in latex. The yield potential should be over 2500 kg/ha/year for high yielding clones.

1.3. Economic importance

Nature has not provided any other industrial raw material of plant origin as flexible as natural rubber. For reasons of high yield and low impurities, *H. brasiliensis* now accounts for over 99% of the worlds natural rubber (NR) produced and used as base material for manufacturing an incredible variety of products for numerous applications in diverse fields. Due to higher strength, elasticity, low heat build up and better resistance to wear, 70% of natural rubber is used for the manufacture of automobile tires (Nayanakantha and Seneviratnae 2007). Natural

Fig. 1: Rubber plantation (Insert: Trunk of a single mature tree showing latex collection)

rubber is now used in over 40,000 products including medical devices, surgical gloves, tires and various engineering and consumer products. Rubber is an important plant not only for world economic strategies but also for proper living of human kind. The more the social developments, the requirement and utilization of products made of rubber are increasing every day. The main producers of natural rubber in the world are Thailand, Indonesia, Malaysia, India, Vietnam, China, Ivory Coast and Srilanka. Recently rubber wood has become a second product of rubber cropping; it represents about 15% of the total income of the farmers and generated a profitable industry mainly in Malaysia, Thailand and India. The global demand for natural rubber is steadily increasing and hence the production of rubber needs to be increased to meet the demand.

1.4. Breeding and propagation

Earlier rubber plantations were established with unselected seedlings, which resulted in considerable heterogeneity. Conventional methods of genetic improvement include introduction, selection and hybridization. Genetic improvement of *H. brasiliensis* is very elaborate and time consuming as in many other perennial species. The major limitations are the very narrow genetic base, non-synchronous flowering, low fruit set, long gestation period, heterozygous nature and absence of fully reliable early selection parameters. Since *H. brasiliensis* is cross pollinated, the seedlings are highly heterozygous. The difficulty of using cuttings as planting material was the low rhizogenic potential of the selected material. The rhizogenic capacity of *H. brasiliensis* is fugacious and soon gets exhausted during the development of the plant. The current propagation method of grafting on to unselected seedlings maintains intraclonal heterogeneity for vigor and productivity and hence a great improvement may be expected using micro propagation *in vitro*. For the production of true to type individuals of selected genotypes, there is a need for the development of protocols for *in vitro* propagation of clonal materials. In *H. brasiliensis* increased growth and vigor have already been reported for plants regenerated through tissue culture (Carron *et al.*, 1995, 2000). Even a very small

increment per tapping will be a great attribute to a tree crop like *H. brasiliensis* with an economic life span of 30 years.

1.5. Micropropagation

Among the major perennial crops, *H. brasiliensis* species being commercially valuable has a great potential to benefit from biotechnological tools, such as micro propagation and genetic transformation. Research to develop somatic embryogenesis as an *in vitro* propagation method for *H. brasiliensis* began in the 1970's. Wang *et al.,* (1980) reported the successful production of the first somatic embryogenesis derived plantlets from anther explants of *H. brasiliensis*. Since then, efficient protocols for plant regeneration through somatic embryogenesis from a range of explants such as inner integument, immature anthers and immature inflorescence have been reported in *H. brasiliensis.* However, *H. brasiliensis* clones show considerable variability in *in vitro* response of the explants such as life of the calli and its embryogenic capacity (Blanc *et al.,* 1999). Recently there is a growing interest in developing somatic embryogenesis systems for *H. brasiliensis* as a useful and efficient method for clonal propagation of selected material and genetic transformation.

Rubber is a deciduous tree in which refoliation and flowering follow wintering. Since flowering in *H. brasiliensis* is seasonal and adverse environments during the season may hinder normal flowering, explant availability and quality is unpredictable. In all the protocols developed earlier for somatic embryogenesis in *H. brasiliensis*, floral/fruit derived explants were used. Moreover, culture initiation from floral explants is also laborious and time consuming. Leaf tissues have been reported to be amenable explants for tissue culture. In several other crops leaf tissue has been mentioned as a potential target tissue for micro propagation and for *Agrobacterium* infection in genetic transformation experiments. Development of plant regeneration systems through somatic embryogenesis using explants other than floral/seed organs which are available throughout the year would significantly contribute in *H. brasiliensis* crop improvement through micro propagation and genetic transformation.

1.6. Phase change and *in vitro* culture

Most trees have marked phase changes that result in a decline *in vitro* response such as in their potential for somatic embryogenesis or micropropagation. Responses that show marked differences between juvenile and adult stages are induction of organogenesis and embryogenesis in tissue culture, with juvenile tissues such as embryonic stages and young seedlings being the most responsive (Greenwood 1995). In *H. brasiliensis* also, the frequency and time taken for embryogenic callus initiation was found to be highly influenced by the explant source Lardet et *al.,* (2009). Studies have shown that, in addition to the use of appropriate *in vitro* conditions, the choice of explant is important when attempting organogenesis or embryogensis in tissues from mature trees. *In vitro* regeneration is sometimes achieved by isolating and culturing tissues that are in a more juvenile, potentially regenerative state than most other tissues of the tree. Embryogenic capacity of the cultured explant is dependent on the physiological age of the source plant and was found to reduce with maturity of the source plant. Interaction and balance of media constituents also determines the efficiency of a system along with the cultured tissue. The pattern of developmental response of cultured tissue and the requirement of auxins and other plant growth regulators for the initiation of somatic embryogenesis is largely determined by the nature and the developmental stage of the explant (Litz and Gray, 1995). By altering conditions of the source material *ex vitro*, or by changing *in vitro* conditions, rejuvenation and increased propagation can sometimes be accomplished. Development of an amenable plant regeneration pathway is always dependent on the physiological maturity of the explant used for initiating the culture. The most juvenile tissues were found to show maximum embryogenic competence in a minimum time frame. The embryogenic capacity of the explant collected from different sources should be studied to identify the one most amenable to *in vitro* culture.

Age dependant differential gene expression in explants collected from tree species were also reported. In both larch and English ivy, a comparison of cDNA libraries made from RNA extracted from juvenile and mature foliage indicates that

there are some differences in gene expression between juvenile and mature shoots (Hutchison *et al.* 1990; Woo *et al.,* 1994). Age-dependent changes in gene expression profiles were studied in vegetative *Pinus radiata* buds by means of differential display.

1.7.Transgenic approaches

H. brasiliensis being a tree crop, conventional agriculture relying on selection tion and traditional breeding programmes will not be able to deploy new varieties with modified traits quickly. Application of genetic transformation as a biotechnological tool for crop improvement is an essential element of short and long term strategies to exploit plant genomics for overcoming yield impediments faced by conventional breeding (Martino-Catt and Sachs 2008). The major requirements for the development of transgenic plants include the availability of efficient transformation protocols for the introduction of genes into crop plants, selection of transgenic tissues, reliable and reproducible *in vitro* plant regeneration system through somatic embryogenesis, molecular and genetic characterization of transgenic plants for stable and efficient gene expression (Sharma *et al.,* 2005). The main challenge with genetic transformation of tree species is achievement of high transformation efficiency for desired clones or cultivars and efficient plant regeneration (Arokiaraj *et al.,* 2009). A protocol has been developed earlier for *Agrobacterium* mediated genetic transformation and plant regeneration in *H. brasiliensis.* Transgenic plants incorporated with MnSOD gene were regenerated from anther callus of *H. brasiliensis* (Jayashree *et al.,* 2003). Efficiency of the system with respect to transformation frequency and transgenic tissue recovery without bacterial overgrowth is found to vary with each new infection. A critical step in *Agrobacterium* mediated transformation and transgenic tissue regeneration in *H. brasiliensis.* is the establishment of optimal conditions for T-DNA delivery into infected tissue and tissue recovery without bacterial overgrowth. The leaf is a favored explant for most transformation experiments in many plants since it is easy to handle and *Agrobacterium* readily penetrates the cut ends aiding regeneration from the infected tissues. Genetic transformation using a plant regeneration system from leaf explants

would be very useful in future for the genetic improvement of *H. brasiliensis* through transformation technology. The feasibility of using proliferated fresh leaf callus as an ideal explant for *Agrobacterium* mediated genetic transformation and improve rate of gene transfer by using compounds that enhance transformation efficiency could be explored.

Objectives

The present study was undertaken with the following objectives:

1. Development of a protocol for the induction of somatic embryogenesis and plant regeneration from leaf explants of *H. brasiliensis* for which extensive experiments will be done to identify the leaf source, ideal stage of leaf, optimize culture conditions and media requirements in each step of the somatic embryogenesis pathway such as callus induction, embryogenic callus induction, embryo induction, embryo maturation and plant regeneration.
2. Identification of suitable source and stage of leaf explants for better *in vitro* response and assess the embryogenic competence of explants collected from plants of different physiological maturity.
3. Identify genetic factors related to juvenile-mature phase change in *H. brasiliensis* and characterize genes differentially expressed in juvenile and mature tissues which in turn make the tissue recalcitrant to *in vitro* culture.
4. Study the feasibility of using the developed plant regeneration system for crop improvement through transgenic approaches.

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1. Conventional breeding for crop improvement in *Hevea brasiliensis*

Crop improvement programmes through conventional breeding in *H. brasiliensis* has limitations due to narrow genetic base of the cultivated clones, long juvenile period, highly heterozygous nature of the seed propagated plants, poor seed set etc. Since considerable yield variability was observed in seedling population the technique of bud grafting was perfected to facilitate fixation of desired characters. Primary clones were developed through ortet selection, where ortet is the original tree from which members of clones have descended. Major objectives of rubber tree breeding is to develop potential clones with high rubber yield combined with secondary characters such as high initial vigor, smooth and thick bark with good latex vessel system, good bark renewal, good growth after initiation of latex harvest and tolerance to major diseases and wind (Annamma *et al.,* 1990; Varghese *et al.,*1992). Recently importance has also been given to develop clones resistant to abiotic stress such as drought, high temperature, cold etc. (Thulaseedharan *et al.,* 2000). Hand pollination between selected parents, evaluation of F1 hybrids, selection of promising recombinants and multiplication by bud grafting are still the most important methods of conventional breeding (Varghese and Mydin, 2000). In India, the clone RRII 105 produced by the hybridization of Tjir 1 and GL1 which is having several desirable characters, has been released by Rubber Research Institute of India in 1980. Recently few other clones of the RRII 400 series obtained by hybridization of RRII 105 and RRIC 100 (a Srilankan clone) have been released by Rubber Research Institute of India and are reported to have a yield increment of 40% over RRII 105.

Since *Hevea* is open pollinated and highly heterozygous, commercial propagation is by bud grafting. Elite clones of *H. brasiliensis* are commercially propagated by bud grafting uniform scion buds from selected clones on to seedling root

stocks. But, root stocks which are derived from cross pollinated seeds are heterozygous and hence lead to undesirable stock-scion interactions causing intra-clonal variations in the field performance (Combe, 1975; Seneviratnae and Flegmann, 1996). Improvement of *H. brasiliensis* is being carried out for considerable increase in yields and stress tolerance through conventional breeding.

2.2. *In vitro* approaches in *Hevea brasiliensis*

Plant biotechnology currently comprises a range of activities, such as micropropagation and other *in vitro* culture, genome analysis, molecular breeding through marker assisted selection (MAS) and gene cloning, DNA recombination and gene transfer etc. Plant tissue culture is the art of growing plant cells, tissue or organs on artificial media by isolating them from the mother plant (George, 1993). Plant tissue culture is based on the cell doctrine that states a cell is capable of autonomy and is potentially totipotent. Plant cell and tissue culture are used for clonal propagation, production of disease free plants, haploid production, triploid production, *in vitro* pollination and fertilization, embryo rescue, somatic hybridization and cybridisation, somaclonal and gametoclonal variant selection, germplasm conservation, secondary metabolite production and genetic transformation. Although application of biotechnology in trees and ornamental woody plants is just in its infancy, micropropagation is rapidly becoming a standard tool for tree improvement. In addition, emerging success and practical application have become visible in genetic transformation. These initial achievements have already proven that biotechnology will make inestimable impact on tree improvement. In 1902, the German botanist Gottlieb Haberlandt developed the concept of *in vitro* cell culture. He isolated single cells from palisade tissue of leaves, pith parenchyma, epidermis of various plants and cultured on Knop's salt solution. Following this, many workers such as Gautheret (1939) cultured cambial tissue of carrot root for prolonged periods of time. Through tissue culture techniques, new plants can be obtained through direct or indirect morphogenesis and somatic embryogenesis. Direct morphogenesis is production of shoots from explants without passing through callus phase while in indirect morphogenesis shoot induction occurs through a callus phase. Plant cell possesses remarkable developmental plasticity

example of which is somatic embryogenesis, during which differentiated plant cells regain totipotency and develop into embryos (Feher, 2008). The present goals of biotechnological research in *H. brasiliensis* in different countries include development of efficient plant regeneration systems through somatic embryogenesis, characterization of genes controlling important agronomic traits understanding the molecular mechanism of latex biosynthesis, tolerance to diseases and abiotic stresses, characterization of lacticiferous specific promoters, removal of latex allergens and development of transgenic plants for better agronomic qualities and production of recombinant proteins (Thulaseedharan *et al.*, 2004).

2.3. Micropropagation through shoot tip culture

Micropropagation uses the principle of cellular totipotency and this involves the *in vitro* multiplication of plants on a precisely defined growth medium incorporating specific growth regulators (Giles and Morgan, 1987). Although, the basic nutritional requirements of *in vitro* cultured plant cells are similar, the nutritional composition varies depending on type of cells, tissues, organs and plant species. Even genotypes or cultivars of the same species show difference in nutritional requirements. A nutrient medium is defined by it's composition of mineral salts, carbon source, vitamins, plant growth regulators and other organic supplements. Though several media have been developed the Murashige & Skoog, (1962) (MS) medium is very widely used in plant tissue culture systems. Organic additives and the type and concentration of growth regulators that are added to the basic media are generally the factors that determine the response of plant tissue in culture. However other additives such as activated charcoal, polyamines and exogenous physical factors such as temperature, light intensity and quality are also important in determining the response of tissues in culture (Mathias and Boyd, 1986). One of the main uses of plant tissue culture is for micro propagation of many commercially useful trees which take a long time to grow under the field condition. *In vitro* vegetative propagation will lead to the production of copies of mother trees which are faithful from roots to leaves; it will avoid having to interpose the variability of stock produced from seed and ageing of scions. It will then be possible to select the root part of plants with regard both to the selectivity for

different types of soil and resistance to diseases. Micro propagation can help to speed up the process considerably.

Since conventional method of propagation may lead to undesirable stock scion interactions, clonal propagation of *H. brasiliensis* by tissue culture which can produce uniform individual plants is a relevant technique to a greater extent. A reproducible regeneration system for each genotype of *H. brasiliensis* through tissue culture is essential for crop improvement programmes. The first known work on *in vitro* culture of *H. brasiliensis* was carried out by Bouychou (1953) of the Institute Francais Cautchouc, with the aim of using calli to obtain convenient material for the study of the laticiferous system. The Rubber Research Institute of Malaysia pioneered tissue culture research in the 1960s. Research was taken up again by Chua (1966) of the Rubber Research Institute of Malaysia and later by Wilson (Wilson and Street 1974, 1975), with the backing of the Malaysian Rubber Producers' Research Association. Over the past two decades, considerable work has been made on *in vitro* techniques for multiplication and improvement of *H. brasiliensis.* Most of the *in vitro* culture work in *H. brasiliensis* is directed towards micropropagation through shoot tip culture, nodal culture, somatic embryogenesis and genetic transformation (Nayanakantha & Senivirtnae, 2007).

Paranjothy & Gandhimathy (1976) had attempted shoot tip culture from 2-4 weeks old aseptic seedlings. They could introduce rooting among some of seedling derived cultures, but failed to do so with clonal materials. Micro propagation was investigated later by Carron and Enjalric (1982) and led to the production of several hundreds of plantlets by micro cuttings from 1-3 year old green house grown seedlings. Carron and Enjalric (1983) also reported that the propagation of elite *H. brasiliensis* stock material from stem cutting was a failure due to inadequate rooting system, necessary for tree stability. Thereafter plantlets with shoot and root development were successfully obtained from seedlings by different investigators (Gunatillege and Samaranayake, 1988; Carron *et al.,* 1989; Sompong and Muanghaewngam, 1992; Seneviratnae and Flegmann, 1996).

There are several problems associated with using explants derived from elite clones of mature *H. brasiliensis* trees. Work was done at the Rubber Research Institute of India with elite *H. brasiliensis* clones using shoot tip explants derived from mature trees (Sinha *et al.,* 1985; Sobhana *et al.,* 1986). Shoots have been regenerated from axillary bud explants of a few *H. brasiliensis* clones by Sinha *et al.,* (1985), but failed to obtain rooting. Asokan *et al.,* (1988) has successfully produced self rooted clonal plants of *H. brasiliensis* by shoot tip culture but there still existed problems of root formation since explants derived from elite clones of mature *H. brasiliensis* are highly recalcitrant. The major problem is the failure of producing adequate root system with tap root quality necessary for tree stability (Carron and Enjalric; 1983). Another problem is the presence of bacteria and fungal contamination in the field grown mature plant derived explants. Effective sterilization techniques for obtaining contamination free initial explants were developed by Enjalric *et al*; (1987); Asokan *et al*; (1988).The effect of different fungicides and antibiotics to control microbial contamination in *H. brasiliensis* cultures were also examined (Kala *et al.,* 2004). Physiological juvenility of the explants found to have significant role in micro propagation. Senevirtnae and Flegmann (1996) reported multiple shoot production, further elongation and rooting from nodal explants of juvenile origin and elongated shoots produced roots. Senevirtnae and Wijesekara (1997); demonstrated that axillary bud development can be accelerated by application of cytokinins. Mendanha *et al.,* (1998) described shoot development from the axillary buds in MS medium containing phytohormones. Carron *et al.,* (2000) have demonstrated the rooting capacity of *in vitro* propagated plantlets *H. brasiliensis.* The explants derived from elite clones of mature *H. brasiliensis* trees are highly recalcitrant. Micropropagation with nodal and shoot tip explants derived from seedlings are possible but with mature clonal explants the response was poor. Only limited reports are available on successful micropropagation using clonal materials of *H. brasiliensis.* Moreover, explants of mature origin posed problems both of establishment in culture and recalcitrance to proliferate *in vitro* (Seneviratnae, 1991). Apart from these *H. brasiliensis* being a crop predominantly grown under tropical climate, presence of bacterial and systemic fungal contamination in explant taken from

field grown mature plants is also a major limitation in viable culture initiation. Since rooting of clonal materials is fugacious and only a single plant could be produced from a single explant this is undesirable in clonal propagation. Rejuvenated explants by micro grafting (Perrin *et al.,* 1994) and buds of nodal explants taken from dormant branches were found to exhibit better *in vitro* response (Senevirtnae and Wijesekara 1997; Lardet *et al.,* 1998). Conditions for an efficient and reproducible system for *in-vitro* micro grafting for the induction of explant rejuvenation as well as for the rescue of important difficult to root plant materials were standardized by Kala *et al.,* (2002). Though there are several reports on *H. brasiliensis* micropropagation using different explants mostly derived from seedlings (Thulaseedharan *et al.,* 2000) an efficient protocol for large scale propagation of *H. brasiliensis* has yet to be developed.

Several workers have attempted in *vitro* organogenesis from various explants but the success rate was very low. Initial attempt was made to develop callus and suspension cultures in *H. brasiliensis* by Wilson and Street (1974). Paranjothy and Gandhimathi (1975) described a method for full plantlet regeneration from dehusked seeds or freshly fallen seeds. Cotyledon, epicotyl or hypocotyl explants produced compact callus with roots but failed to regenerate plants (Paranjothy and Gandhimathy 1975). Carron and Enjalric (1982) demonstrated callus induction from the leaf explants but no organogenesis was noticed. Perrin *et al.,* (1997) compared the ability for *in vitro* axillary shoot organogenesis and rhizogenesis between mature and rejuvenated clones of *H. brasiliensis.* Mendenha *et al.,* (1998) reported abundant callus initiation from the leaf explants however, no regeneration was noticed when they were subcultured onto embryo induction medium.

2.4. Somatic embryogenesis

Embryogenesis is a complex process that is regulated by various factors including phytohormones, proteins and transcription factors. Many chemical substances act in gene expression as signals, and the correct expression is required for normal and rapid development of the embryos. Somatic embryogenesis is defined as a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic

cell without vascular connection with the original tissue (Arnold *et al.,* 2002). Somatic embryogenesis is a multi step regeneration process starting with formation of dedifferentiated tissue called callus which is then redifferentiated to form pro embryogenic masses followed by formation of somatic embryos, maturation and plant regeneration. The number of tissue culture systems achieving somatic embryogenesis in plants which were considered as formerly recalcitrant species is increasing. The embryogenic capability of somatic plant cells is a general feature. The potential use of somatic embryogenesis in developmental studies, micro propagation and crop improvement has already been recognized in several angiosperms (Ammirato, 1987; Das *et al.,* 1995; Romano *et al.,* 2002). However, reports on somatic embryogenesis are relatively limited for woody species, since most of them are highly recalcitrant to *in vitro* culture. The appropriate condition for expression of this trait varies and is mainly determined by it's given physiological state which in turn is determined by genetic and developmental conditions and environmental cues (Feher, 2006). Since the first observation of somatic embryo formation in *Daucus carota* cell suspension by Steward *et al.,* (1958) the potential for somatic embryogenesis has been shown in a wide range of plant species. Somatic embryogenesis can probably be achieved from all plant species provided that the appropriate explant, culture media and environmental conditions are employed. The use of somatic embryogenesis in developmental studies, crop improvement and genetic transformation has been widely recognized and the number of species and explants displaying this potential is constantly increasing.

Experiments on somatic embryogenesis of *H. brasiliensis* started as early as the 1970s. Since then number of studies have been reported. Differentiation of embryoids from anther wall derived callus was achieved by Paranjothy (1974) and Carron and Enjalric (1982). Subsequently, shoot development was also achieved (Paranjothy and Ghandhimathi 1975; Paranjothy and Rohani 1978). The first successful somatic embryogenesis in *H. brasiliensis* was reported by Wang *et al.,* (1980) and Wan *et al.,* (1981) using anther walls, and then by Carron *et al.,* (1985) using the internal integument of seeds, mother tissue that allow propagation of a known genotype. Wang *et al.,* (1980) reported for the first time, the establishment in the field, of *in vitro*

cultured *H. brasiliensis* plants of anther-wall derived callus. However, according to Wan Abdul Rahaman *et al.,* (1981), clonal differences had been observed in the frequency of embryogenesis of anther-wall derived callus and therefore the reported technique had not been applicable to all clones of *H. brasiliensis*. Recently there has been an increasing interest into the induction of somatic embryogenesis especially for use in genetic transformation. In order to identify the suitable explant source, a variety of explants such as leaf, tender shoots, integument tissues of immature fruit, immature anther, immature inflorescence etc. were tried. A plant regeneration protocol via somatic embryogenesis was developed using immature anthers (Jayasree *et al.,* 1999) and immature inflorescence (Sushamakumari *et al.,* 2000) in *H. brasiliensis* clone RRII 105, the most popular Indian clone. Optimum callus induction was obtained in modified MS medium supplemented with 2.0 mg/l 2,4-D and 0.5 mg/l kin. Somatic embryo induction was found to be better with 0.7 mg/l kin and 0.2 mg/l NAA. Further development of the embryos into plantlets was achieved on a hormone free medium.

Successful embryo formation and plant regeneration have been reported by few researchers in different countries using limited genotypes of *H. brasiliensis*. However, the frequency of somatic embryo induction was found to be very low and non-synchronous its germination remained very difficult and thus *H. brasiliensis* embryogenic systems needed further investigation (Carron *et al.,* 1995; Linossier *et al.,* 1997). Extensive experiments were carried out by many researchers to enhance the frequency of embryo induction and plant regeneration in *H. brasiliensis.* Studies were conducted to optimize cultural conditions, nutritional and hormonal requirements during somatic embryogenesis. The culture medium has a determinant influence on both the initiation of somatic embryogenesis and the quality of embryo development. Embryogenic potential is affected by polyamines (Hadrami *et al.,*1989) hormone balance (Michaux-Ferriere,1992), water status of the medium and explant, mineral and carbohydrate nutrition, (Etienne *et al.,* 1991), interaction of growth regulators, sucrose and calcium on callus friability (Montoro *et al.,* 1993) role of sucrose and ABA on embryo induction (Veisseiere *et al.,* 1994 a, b; Cailloux *et al.,* 1996; Linossier *et al.,* 1997) and carbohydrate types (Blanc *et al.,* 2000). According to Montoro *et al.,* (1993)

the structure of calli and their morphogenetic capacities are not strictly specific to the genotype but it depends on genotype and medium interaction. These studies revealed the necessity to optimize the culture conditions in order to achieve somatic embryogenesis in each genotype of *H. brasiliensis*. According to Veisseire *et al.*, (1994b) combinations of growth regulators such as ABA and cytokinins improve maturation and germination of *H. brasiliensis* somatic embryos.

The water relation between the embryo and its environment *in vitro* play a determinant regulatory role in embryo development and in particular in its maturation (Adams and Rinne, 1980). According to Etienne *et al.*, (1993a) dessication, medium osmolarity and addition of ABA improve germinability of *Hevea* somatic embryos by setting up a maturation phase. The effect of different carbohydrates was tested on early embryogenesis of *H. brasiliensis* by Blanc *et al.*, (1999) and found that somatic embryo production was significantly higher with maltose. Further development of the embryos into plantlets was achieved on a hormone free medium. A culture procedure using temporary immersion in a liquid medium was tested for somatic embryogenesis of *H. brasiliensis* (Etienne *et al.*, 1997). Temporary immersion resulted in substantially more consistent, synchronized somatic embryo development, reducing the number of abnormal embryos by half and stimulating germination.

Sushamakumari *et al.*, (2000) developed a technique for somatic embryogenesis and plant regeneration using immature inflorescence as explants. They also studied the role of sucrose and ABA on embryo induction. A higher sucrose level was found to be beneficial for plant regeneration. Culture conditions and other nutritional requirements for improving the efficiency of somatic embryo induction and germination were investigated by Kumari Jayasree *et al.*, (2001). A study on the gibberellic acid requirement on embryo induction and maturation revealed that incorporation of GA_3 up to 2.0 mg/l increased the embryo induction frequency. Germination percentage was also significantly enhanced by higher concentrations, however, further plant development was affected by increasing GA_3 levels (Kumari Jayasree and Thulaseedharan, 2001). The hormone type, concentration and their ratio are also found

to have influence on callus induction, somatic embryogenesis and germination when explants from different sources were used (Montorro *et al.,* 1993).

Silver nitrate has been proved to be a very potent inhibitor of ethylene action and is widely used in tissue culture of many plant species. Few properties of silver nitrate such as easy availability, solubility in water, specificity and stability make it very useful for various applications in exploiting plant growth regulation and morphogenesis *in vivo* and *in vitro* (Kumar *et al.,* 2009). In *Zea mays,* embryogenic callus initiation rate was improved when immature embryos were cultured on a modified Murashige & Skoog medium containing various concentrations of silver nitrate (5,10 and 20 mg/l). Regeneration ability of calli initiated and maintained in presence of silver nitrate was enhanced (Vain *et al.,* 1989). Loblolly pine (*Pinus taeda* L.) culture initiation was improved by the addition of abscisic acid (3.7 µM), silver nitrate (20 µM), and guanosine 30,50 cyclic monophosphate, 8-bromo-, sodium salt (10 µM) to the medium and by raising cytokinin levels in the presence of 50 mg/l activated charcoal (Pullman *et al.,* 2003). Alkhateeb (2006) reported enhancement in embryo induction in date palm at 5% PEG 8000 in presence of 60 g/l sucrose. In melon, somatic embryogenesis was enhanced by the addition of 0.5 µM ABA along with 200 mM sucrose (Nakagawa *et al.,* 2001). The abnormal development features observed in somatic embryos of most of the plants studied are probably caused by an altered endogenous hormonal balance in the cultured tissue. Prevost *et al.,* (1985) showed that both a decrease of 3,4-D and BA supply in the solid culture medium and the addition of ABA stimulated embryo induction. Somatic embryogenesis was achieved in callus cultures derived from immature cotyledonary explants of *Hardwickia binata* Roxb; on semisolid modified Murashige and Skoog's medium containing potassium nitrate supplemented with kinetin and NAA (Das *et al.,* 1995). Maturation of somatic embryos achieved in half strength MS basal medium supplemented with IBA and sucrose.

A high frequency of secondary embryogenesis was induced from isolated early cotyledonary- stage somatic embryos of *H. brasiliensis* (Cailloux *et al.,* 1996)

Repetitive embryogenesis was also induced from primary somatic embryos derived from integument tissue. Secondary embryogenesis has also been developed in *H. brasiliensis* trees for improving the efficiency of former somatic embryogenesis technology, which was based on the use of budded-tree integuments as primary explants, mainly by reducing subculture number and duration (Lardet *et al.,* 2008). However, despite these improvements, the success rate of the secondary embryogenesis technology based on explants from budded-tree integuments remained unexpectedly low, unpredictable and highly dependent on the composition of the medium (Lardet *et al.,* 2008). Repetitive embrogenesis was also induced from primary somatic embryos derived from integumental tissue. Somatic embryos cultured on B-5 medium supplemented with 0.5 mg/l NAA, 2.0 mg/l KIN, 0.5 mg/l IAA and 4.0 mg/l 2,4-D enhanced repetitive embryogenesis and 5% sucrose was found to be optimum (Asokan *et al.,* 2002). Embryogenic cultures had been maintained for over three years for retaining the embryo induction and plant regeneration potential (Kumari Jayasree and Thulaseedharan, 2004). Somatic embryos cultured on B-5 medium supplemented with NAA, kin, IAA and 2,4-D enhanced repetitive embryogenesis (Asokan *et al.,* 2002). However, there is sufficient progress at research level to suggest that tissue culture of *H. brasiliensis* can and should be further developed.

Several *in vitro* plants have been raised through somatic embryogenesis from immature anther as well as immature inflorescence of the *Hevea* clone RRII-105 (Thulaseedharan, 2002) and they confirmed that those plants were morphologically as well as genetically uniform. During 1979-1989, Shiji *et al.,* (1990) produced 1700 plants out of 52,896 embryoids by inoculating 31,584 anthers in tubes. They successfully transplanted and established 539 plants of 13 clones. However, great variation among the clones for their induction frequency was reported. In spite of many reports on progress achieved in developing micro propagation systems through somatic embryogenesis from different explants of *H. brasiliensis* like anthers and inner integuments, only limited success have been achieved in large scale plant production due to recalcitrant nature of *H. brasiliensis* to *in vitro* culture (Thulaseedharan *et al.,* 2004).

2.5. Protoplast culture

Rohani and Paranjyothi (1980) made an attempt to isolate protoplasts from different tissues of *Hevea* and reported that pith from young shoots and suspension cultures of anther derived calli were the most promising sources for obtaining viable protoplasts. Wilson and Power (1989) obtained protoplasts from stem tissues of *Hevea* and they were successful in cell wall regeneration and divisions, but rapid degeneration of the protoplasts was recorded. Harris *et al.,* (1988) isolated protoplasts from cell suspensions derived from anther calli while Cazaux and d'Auzac (1994) used embryogenic calli for protoplast isolation and obtained microcalli from *Hevea* protoplasts. Sushamakumari *et al.,* (1999) successfully isolated protoplasts from two month old embryogenic cell suspensions derived from immature inflorescence. Further, cell division and microcalli formation were also obtained. Subsequently, Sushamakumari *et al.,* (2000) regenerated plants from protoplast derived callus via somatic embryogenesis.

2.6. Somatic embryogenesis from leaves

In all the protocols developed earlier for somatic embryogenesis in *H. brasiliensis,* floral/fruit derived explants were used. Since flowering in *H. brasiliensis* is seasonal and adverse environments during the season may hinder normal flowering, explant availability and quality is unpredictable. Moreover, culture initiation from floral explants is also time consuming. Therefore, development of alternate systems through somatic embryogenesis using explant sources other than floral/seed organs, which are available throughout the year would be useful in utilizing more of the possibilities of this system in genetic modification of *H. brasiliensis*. In several plant species reports are available on using leaf as an explant for somatic embryogenesis. In most of the reports, leaf tissue has been mentioned as a potential target tissue for *Agrobacterium* infection (De Block, 1988) and plant regeneration. There are reports on several crops where leaf tissue has been mentioned as a potential target tissue for micro propagation and for *Agrobacterium* infection in genetic transformation experiments (De Block, 1988). Both direct and indirect somatic embryogenesis from leaves have

been reported in several crops (Hulme *et al.,* 1992; Hammat, 1993; Pedroso and Pais, 1993; San-Jose and Vietez, 1993; Ignacimuthu *et al.,* 1999; Jayasree *et al.,* 2001; Huang *et al.,* 2002). Work on plant regeneration from *H. brasiliensis* leaves was initiated earlier, but only callus formation has been obtained (Carron & Enjalric, 1982; Mendanha *et al.,* 1998). For the feasibility of genetic manipulation studies in efficient plant regeneration system is needed. Somatic embryogenesis is an important technique for clonal micro propagation of elite plants and also a powerful tool for the development of transgenic plants. Stable transformation of plants becomes more likely if more number of embryos can be originated from single transformed cells.

Direct embryo formation in leaves of *Camellia japonica L.* were established (Pedroso and Pais, 1993). An auxin treatment followed by incubation in darkness on diluted modified basal medium induced direct morphogenesis. The number of subcultures, subculture interval and leaf age affected *in vitro* leaf response. The cells from a cultured leaf respond differentially to the same culture conditions by forming embryos and roots and non-morphogenic as well as organogenic callus. According to leaf regions there exist a difference in morphogenetic competence. Direct embryo formation occurred only in the marginal leaf regions and direct root formation only occurred in a well-defined region of the mid rib where as callus was formed on the leaf bases. Direct plant regeneration was achieved from leaf explants of *Plumbago rosea* and *Plumbago zeylanica* on MS medium supplemented with BA, IAA, adenine sulfate and sucrose (Das and Rout, 2002). From leaf explants of *Phalaenopsis amabitis* var. Formosa somatic embryos were formed directly from epidermal cells when cultured on half strength modified MS medium supplemented with thidiazuron (TDZ) (Chen et al; 2006). Direct somatic embryogenesis was successfully achieved from immature leaves of cassava (*Manihot esculenta* Crantz) cultured on medium containing 2,4-D or NAA (Guohua and Qushang, 2000). Changing the duration of the induction or changing plant growth regulators resulted in differences in regeneration of somatic embryos or adventitious shoots. Direct somatic embryogenesis from *in vitro* cultured leaf segments of *Piper colubrinum* was reported by Yusuf *et al.,* (2001). Somatic embryo were initiated on MS basal medium containing benzyl adenine and kinetin and multiplied

profusely through secondary embryogenesis on the same medium. Somatic embryos were converted into plantlets on MS medium supplemented with benzyl adenine and kinetin and plantlets developed on half-strength MS medium containing IBA. Organogenesis was obtained from leaf discs of apple in a medium containing BAP and 2,4-D (James *et al.,* 1984). Direct somatic embryogenesis and plant regeneration from leaf explants of golden pothos was reported by Zhang *et al.,* (2005).

Plants were regenerated through somatic embryogenesis from mature leaf explants of *Eryngium foetidium* (Ignacimuthu *et al.,* 1999). Leaf explants were cultured on Linsmaier and Skoog medium with combination of 2,4-D and BAP. Somatic embryos were induced from embryo forming callus cultures on MS medium supplemented with 2,4-D, BAP and gibberrelic acid. Conversion of these somatic embryos into plantlets occurred on MS medium supplemented with GA_3 and BAP. Somatic embryogenesis from leaf explants of potato was reported by Jayasree *et al.,* (2001). A protocol was developed for the induction, maturation and germination of somatic embryo from leaf tissues of Jojoba (Hamama *et al.,* 2001). Explants cultured on half MS medium containing 2,4-D, BAP and cytokinins resulted in formation of embryogenic cultures and somatic embryos. Somatic embryo maturation, germination and plantlet formation were achieved using NAA or IBA in combination with BA. Plant regeneration from leaf derived callus in *Citrus grandis* was reported (Tao *et al.,* 2002). Different embryogenic lines were established and maintained by repetitive embryogenesis in multiplication medium containing BA and NAA. Experiments were performed to determine the influence of proliferation medium on the maintenance of embryogenic competence and on repetitive embryogenesis in *Castenia sativa* Mill. Somatic embryos were derived from leaf explants (Corredoira *et al.,* 2003). Somatic embryo proliferation was carried out by both direct secondary embryogenesis and by culture of nodular callus tissues originated from cotyledons of somatic embryos. Carbon source and concentration had a marked influence on maturation and subsequent germination ability of somatic embryos. The effects of plant growth regulators on somatic embryogenesis were studied in leaf cultures of *Coffea canephora* (Hatanaka *et al.,* 1991). The maximum number of somatic embryos was obtained on media that

contained only cytokinins as a plant growth regulator. All of the auxins tested inhibited the formation of embryos.

Regeneration from leaf segments was found to be highly dependent on genotype and leaf age. Of the various medium factors tested, the ammonium nitrate level had the most significant effect on callus and shoot formation from leaf tissue. A low level of ammonium nitrate proved beneficial to the induction of regeneration in all genotypes. Immature leaf explants of *Sorghum bicolor* were stimulated *in vitro* to form roots, shoots and embryos (Wernicke *et al.,* 1980, 1982) The effect of different vitamins and inorganic micronutrients on callus growth and induction and proliferation of somatic embryos from leaves of *Capsicum annum* L.(chilli pepper) was investigated by Kutzious *et al.,* (2001). The somatic embryogenesis in pepper leaves is favored by the addition of nicotinic acid to the medium and increase of copper concentration. Leaf explants of populus were induced to form embryogenic callus in darkness on MS medium with 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.05 mg/l benzyladenine (BA), and 30 g/l sucrose. In addition to explant origin, the *in vitro* culture conditions have a major effect on the success of somatic embryogenesis from mature tree tissues. Growth regulators have a determining influence on the capacity for somatic embryogenesis of tree species (Dunstan *et al.,* 1995). The regulation of plant aging by phytohormones has been recognized for a long time (Haffner *et al.,* 1991), and endogenous cytokinins appear to play a major role in the aging process in *H. brasiliensis* (Perrin *et al.,* 1997). Growth regulator pretreatment improves somatic embryogenesis from leaves of *Cucurbita pepo* (squash) and *Cucumis melo* (melon) (Kintzious *et al.,* 2001). Leaf explants were pretreated with different concentrations of 2,4-D and kinetin. Initial pretreatment of squash explants and melon explants with kinetin significantly promoted the formation of somatic embryos. Landi *et al.,* (2006) studied different types of organogenic and callus formation responses of leaves of different *Fragaria* species. Genotypes in response to MS medium supplemented with TDZ alone and in combination with IBA and 2,4-D, BSA. TDZ along with IBA promote the highest shoot regeneration efficiencies from leaves of nearly all of the

genotypes, while TDZ / BA and TDZ / 2,4-D combination promoted high regeneration efficiency for only some of the genotypes.

2.7. Juvenile –mature phase changes and somatic embryogenesis

Both micro propagation and somatic embryogenesis are influenced by the physiological phase changes and the culture environment. Most trees have marked physiological phase changes from juvenile to mature, that result in a decline in their potential for somatic embryogenesis or micro propagation. Phase change is a poorly understood phenomenon, particularly the reversal of phase change, from mature to juvenile, as occurs in the sexual process. By altering conditions of the source material *ex vitro*, or by changing *in vitro* conditions encountered by the explant, rejuvenation and increased propagation can sometimes be accomplished. All woody plants exhibit phase changes. These include morphological alterations, as exemplified by phyllotaxy or leaf form (Gupta *et al.,* 1991), and physiological phase changes, eg., differences in anthocyanin production (Murray *et al.,* 1994b). Other responses that show marked differences between juvenile and adult stages include inducibility of organogenesis and embryogenesis in tissue culture, with juvenile tissues such as embryonic stages and young seedlings being the most responsive (Greenwood, 1995). Loss of organogenesis and embryogenesis, whether progressive or sudden, reflects transition to the mature phase. A higher organogenic responsiveness on a wider range of culture media for physiologically juvenile tissues compared to mature ones was observed in other species (Monteuuis 1987, 2004), indicating that it may be a general phenomenon.

Juvenility prevails during the initial stages of the development of a new organism mainly from sexual reproduction (Lavee *et al.,* 1996). The length of the juvenile phase in woody plants is variable, and can be quite lengthy. There are progressive changes occur during juvenile period that involve morphological, anatomical, physiological and developmental differences (Hackett and Murray, 1996). These include leaf shape, thickness and epidermal characteristics, phyllotaxis, thorniness, shoot orientation, shoot growth vigor, anthocyanin pigmentation, photosynthetic characteristics, disease and insect resistance and competence to form

adventitious buds and roots and somatic embryos (Hackett and Murray, 1993). However, these characteristics may change at different rates from species to species (Greenwood *et al.,* 1989; Steele *et al.,* 1989). I*n vitro* propagation of mature trees is generally more difficult than their juvenile counterparts. With mature trees of a few species, it has been possible to induce somatic embryogenesis in cultures derived from commonly used explants such as leaves and roots (Bonga *et al.,* 1992). Juvenility of plant materials is a key factor in micro propagation because the regeneration ability of woody plants decreases with maturity (Huang *et al.,* 2000). Bonga *et al.,* (1992) have reviewed the factors influencing rejuvenation in trees using methods of enhancing micro propagation through manipulations that involve application of osmotic, temperature or hormonal stress. Both micro propagation and somatic embryogenesis are influenced by phase change and the culture environment. The term maturation includes the transition from the juvenile to the mature phase, while the term aging includes loss of vigor associated with increased complexity of the plant. Maturation occurs in seedling development; whereas plants propagated vegetatively from sexually mature plants, unless rejuvenated, only undergo aging (Hackett, 1985). Micropropagation of some species is enhanced by the use of *in-vitro*-derived material. Ruaud *et al.,* (1992) showed that seedlings and young plants derived from somatic embryos were more juvenile than their similarly aged zygotic counterparts. Accordingly in *Picea abies* (L.) Karst., for instance, *in-vitro* cultured somatic embryos showed higher ability for somatic embryogenesis than their similarly aged or older zygotic counterparts. Furthermore, somatic embryogenesis was more readily induced from mature plants derived by somatic embryogenesis than from plants of seedling origin (Pacques *et al.,* 1997). Previous studies have shown that physiologic aging negatively affects the micro propagation capacity of *H. brasiliensis* from microcuttings (Lardet *et al.,* 1990, Perrin *et al.,* 1994). This phase change phenomenon has been observed in many studies and is associated with a noticeable decline in the potential for micro propagation or somatic embryogenesis of most arborescent species (Bonga 1982; Hackett, 1985; Monteuuis, 1989; von Aderkas and Bonga, 2000). Explants with a high ability for somatic embryogenesis generally originate from reproductive organs (e.g., anthers, inner integument of the seed and zygotic embryos), young seedlings (Dunstan

et al., 1995) and somatic embryogenesis derived plantlets. In *Larix decidua* Mill., somatic embryogenesis from cotyledons and needles has been successful only for tissues obtained from somatic embryogenesis derived plantlets or from immature and mature zygotic embryos (Lelu *et al.,* 1994). According to Wendt *et al.,* (2002) in Parana pine the genotype of the mother tree and the developmental explant stage affected the embryogenic callus induction frequency.

2.8. Differential gene expression during juvenile –mature phase changes

Difference in the expression of phase dependent characteristics within an individual is referred to as epigenetic variation and is ultimately due to differential expression of genes (Goldberg *et al.,* 1987). Till now, which factors are involved in the process of the phase changes from juvenility to maturity stages and the mechanisms responsible for the exchange of the phases are still unknown (Lavee *et al.,* 1996). In fact, biotechnological research approaches hold a great promise and are beginning to give new insights into possible mechanisms. Control of the differential gene expression in a relatively small percentage of the cells in the plant body can result in rather large differences in phenotype. For example, red stems and leaf petioles in juvenile ivy plants as compared to green stems and petioles in mature plants is the result of differential expression of anthocyanin accumulation. Maturation is clearly associated with changes in gene expression. Genes encoding elements of the photosynthetic apparatus such as the chlorophyll a/b binding protein have been found to be differentially expressed in juvenile and mature plants. Greenwood *et al.,* (1989) reported greater expression of chlorophyll a/b binding protein gene in *Larix laricina* in developing juvenile foliage than mature foliage. In *Hedera helix* Woo *et al.,* (1994) reported an auxin decreased expression of a proline rich protein gene in juvenile petioles but not in mature petioles. In both larch and English ivy, the expression of sequences for the chlorophyll a/b binding protein (cab) gene decreases with maturation (Hutchison *et al.,* 1990, Woo *et al,.* 1994). Genes encoding elements of the photosynthetic apparatus would be differentially expressed between juvenile and mature plants. In *Hedera helix* Murray *et al.,* (1994b) reported that the gene for dihydroflavanol reductase (DFR) is expressed in petioles of juvenile foliage but not in mature foliage.

Higher photosynthesis in juvenile trees appears to be associated with higher biochemical potential for photosynthesis rather than to stomatal conductance, which is in line with other studies on temperate species (Day *et al.,* 2001; Niinemets, 2002). In *Hedera helix* Murray *et al.,* (1994b) reported that the gene for dihydroflavanol reductase is expressed in petioles of juvenile foliage but not in mature foliage. The lack of anthocyanin in mature petioles is due to the absence of DFR in the petioles, which in turn is due to lack of expression of the DFR gene (Murray and Hackett, 1991; Murray *et al.,* 1994b). In addition, the proline-rich protein (PRP) gene is expressed more strongly in mature petioles than in juvenile petioles, as indicated by a five- to tenfold increase in the concentrations of PRP mRNA (Woo *et al.,* 1994). Because the expression of the PRP gene occurs in cells associated with root regeneration in the petiole, Murray *et al.,* (1994*a*) propose that elevated expression of this gene may inhibit root meristem regeneration.

2.9. Transgenic approaches

The natural capacity of the gram negative soil bacterium *Agrobacterium tumefaciens* to introduce a segment of oncogenic DNA present in the Ti plasmid makes it an efficient vector system in genetic transformation. T-DNA integrated into the plant genome encodes auxin and cytokinin synthesis enzymes essential for plant growth and development. Other genes in the T-DNA, determines enzymes aiding in opine synthesis which are tumour specific compounds. Since these can be easily detected in plant tissues, they have been used as markers for genetic transformation. Deletion of the oncogene from the T- DNA region does not interfere its transfer to plant cells (Smith and Townsend, 1907). Since these regions are tumorous and cannot be regenerated into plants, disarmed strains of *Agrobacterium* are used for plant genetic transformation. Plant cells transformed with such *Agrobacterium* strains regenerate into normal fertile plantlets. By linking genes of interest to the disarmed T region by recombination or cloning them between the border repeats present in an independent replicon, they can be co transferred to the plant cells. To select the transgenic plants, selective markers which confer resistance to antibiotics or herbicides have been developed. Transformation is an important topic in plant biology and transgenic plants have become a major focus in plant research and breeding programmes. A. *tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-

inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester *et al.*, 1984). Usually the efficiency of T-DNA transfer varies with plant species, cultivars and target tissues. Different protocols of plant regeneration are tried, from putatively transgenic lines emerged, when different target tissues such as leaves, roots, hypocotyls, anthers etc. are used, depending on plant species for transformation. Several factors such as difference in tissue culture regime, conditions of source tissue and strains of *Agrobacterium* used contribute to variations in efficiency of infection, copy number of T- DNA in transgenic plant cells.

Application of genetic transformation as a biotechnological tool for crop improvement is an essential element of short and long term strategies to exploit plant genomics for overcoming yield impediments faced by producers (Martino-Catt and Sachs, 2008). *Agrobacterium* mediated genetic transformation was reported in the 1980's (Block *et al.*, 1984). Evidences of transgenic tobacco conferring insect tolerance by expressing genes encoding for insecticidal proteins from *Bacillus thuringiensis* (Bt) were developed (Vaeck *et al.*, 1987). Since then great progress have been achieved in understanding *Agrobacterium* mediated genetic transformation as a practical and common method for introducing specific DNA fragments into the plant genome and several transgenic plants harboring agronomically important genes were regenerated. Success in generation of transgenic plants depends on two major factors: regeneration capacity of explants and transgene integration. Whereas the former one depends on totipotency, the ability of a cell to differentiate and give rise to a new plantlet, the latter depends on multiple factors, including transgene delivery into the host cell cytoplasm, transport through cytoplasm and nucleus, and integration into the host genome (van Attikum and Hooyakaas, 2003; Citovsky *et al.*, 2007). Lack of success in regeneration of transgenic plants from certain plant species could be explained either by poor regeneration of explants and poor acceptance of transgenes or, frequently, by both. It can be hypothesized that successful transgenesis occurs when regeneration capacity of a cell co-insides with its ability to accept a transgene.

Hevea brasiliensis being a tree crop, conventional agriculture relying on selection and traditional breeding programmes will not be able to deploy new varieties with modified traits quickly. Due to the simplicity of the tansformation system and precise integration of transgenes, *Agrobacterium* Ti plasmid based vectors continue to offer the best system for plant transformation (Veluthambi *et al.,* 2003). The major requirements for the development of transgenic plants include the availability of reliable and reproducible *in vitro* regeneration systems, preparation of gene constructs and efficient transformation protocols for the introduction of genes into the crop plants, isolation of the transgenic tissues and transgenic plant regeneration, molecular and genetic characterization of transgenic plants for stable and efficient gene expression and evaluation of the transgenic plants for their effectiveness in alleviating the biotic and abiotic stresses without being an environmental biohazard (Sharma *et al.,* 2005). The main challenge with genetic transformation of tree species is achieving high transformation efficiency for desired clones or cultivars (Arokiaraj *et al.,* 2003). A critical step in *Agrobacterium* mediated transformation and transgenic tissue regeneration in *Hevea* is the establishment of optimal conditions for T-DNA delivery into infected tissue and tissue recovery without bacterial overgrowth. The transfer of T-DNA and its integration into the plant genome is influenced by plant genotype, explants, vector–plasmids, bacterial strains, tissue damage and suppression and elimination of *Agrobacterium* infection after co-cultivation.

Transgenic plants incorporated with MnSOD gene were regenerated from anther calli of *H. brasiliensis* (Jayashree *et al.,* 2003; Sobha *et al.,* 2003) and leaf derived embryogenic callus has also given good response as a suitable target tissue for *Agrobacterium* infection (Kala *et al.,* 2006). Although a standardized transformation protocol is available, the efficiency of the system with respect to transformation frequency and transgenic tissue recovery without bacterial overgrowth is found to vary with each new infection. A critical step in *Agrobacterium* mediated transformation and transgenic tissue regeneration in *H. brasiliensis* is the establishment of optimal conditions for T-DNA delivery into infected tissue and tissue recovery without bacterial overgrowth. Various parameters that are found to influence the transfer of T-

DNA and its integration into the plant genome such as texture of target tissue used for infection, time of infection, inoculum density, acetosyringone concentration, components of the infection, co-culture and selection medium were already studied (Subramaniam *et al.,* 2009). Apart from plant genotype, *Agrobacterium* strains, plasmid vectors, virulence gene inducing compounds and tissue specific factors are also important for improving transformation efficiency of plant species (Opabode, 2006). The optimization of such factors had led to the improvement of gene transfer procedure in many plant species (Chabaud *et al.,* 1988; De Bondt *et al.,* 1994). 6-14% increase in transformation efficiency was obtained in *H.brasiliensis* by preculture of target tissue in medium containing calcium nitrate and increased levels of acetosyringone (Sobha *et al.,* 2010). Silver nitrate has been found to suppress over growth of *Agrobacterium* facilitating plant cell recovery that resulted in increased transformation efficiency (Zhao *et al.*. 2001). Successful use of antioxidants such as ascorbate, cysteine, dithiothreitol (DTT) and glutathione has helped in solving the problems of tissue browning and necrosis in tissue culture. Rate of T-DNA transfer and genomic integration was found to be increased through the inclusion of thiol compounds such as L-cysteine, DTT and sodium thiosulphate in the infection and cocultivation medium resulting in an increased production of transgenic lines (Enrique-Obregon, 1998; Olhoft *et al.,* 2003). Olhoft *et al.,*(2001) and Liu and Pijut (2008) suggest that L-cysteine improves transformation efficiency by reducing plant defense response to pathogen attack as a result of plant wounding and environmental stresses throughout the co-cultivation period. L-cysteine, therefore reduced plant cell death, enzymatic browning of wounded tissues and increased bacterial susceptibility which in turn improved transformation efficiency. α- Lipoic acid reduces browning of *Agrobacterium* transformed cells or tissues and subsequently the death of the cells or tissues. α-Lipoic acid increases the survivability of *Agrobacterium* transformed cells or tissues, resulting in an increased transient expression level and plant transformation efficiency. It reduces escapes through promoting the differentiation, proliferation and regeneration of the transformed cells or tissues and overcoming the growth of non-transformed cells or tissues under selective pressure (Dan *et al.,* 2009). Addition of surfactants such as Silwet L77 (0.07%) and Pluronic F68 (0.03%) in the inoculation medium enhanced T –

DNA delivery in wheat (Cheng *et al.,* 1997) and dramatically increased transient GUS expression in sorghum (Henrique *et al.*, 2004).

Conventional agriculture relying on selection and traditional breeding programmes is incapable of deploying new plants with improved or modified traits quickly. New systems are essential for delivery of next generation plants with novel traits to exploit the potential of crop based and bio products industry to meet social and environmental needs. Development of leaf based plant regeneration system would be useful for incorporation of desired genes for crop improvement in *Hevea* circumventing the difficulties of explant availability and culture initiation. Optimization of the protocol by identifying the ideal explant source and conditions would speed up genetic modification in *Hevea.*

Materials and Methods

Chapter III

MATERIALS AND METHODS

In the present study, experiments were carried out to optimize a plant regeneration system through somatic embryogenesis from leaf explants of *Hevea brasiliensis*. Attempts were made to standardize the ideal stage of explants and nutritional and hormonal requirements for callus induction, somatic embryogenesis and plant regeneration. The influence of source plant juvenility on the embryogenic competence of explants was studied. The feasibility of using the developed system for genetic transformation was also experimented. Attempts were made to identify the genes differentially expressed during juvenile - mature phase changes.

3.a. Somatic embryogenesis from leaf explants of bud grafted plants

3.a.1. Explant

For the initial optimization experiments, leaf explants collected from newly sprouted shoots of green budded *Hevea* plants of clone RRII 105 were used. Green budded *Hevea* plants were produced by grafting green buds collected from newly sprouted flushes of plants maintained in the bud wood nursery, on to six month old seedlings maintained in the seedling nursery of Rubber Research Institute of India (Fig.2). One month after budding, the shoot above the bud union was removed and the stumps were planted in poly bags filled with garden soil and maintained in the glass house. Leaves from primary shoots of the bud grafted plants were used for experiments on development of the somatic embryogenesis system.

3.a.1. Culture initiation

3.a.1.1. Effect of sterilant

Leaves were collected along with the petioles from the glass house with the petioles dipped in water in a 500 ml beaker kept in an ice box and immediately brought to the laboratory. The leaves were washed in running tap water. After removing the petioles they were rinsed in sterile distilled water. Further the leaves were sterilized in both mercuric chloride and sodium hypochlorite. Five different

Fig.2. Bud grafted plant of *Hevea brasiliensis* (one month after bud sprouting)

concentrations of mercuric chloride ($HgCl_2$. 0.1, 0.15, 0.2, 0.25 and 0.3 %) and sodium hypochlorite (NaOCl - 0.1, 0.2, 0.3, 0.4 and 0.5%) were tried for surface sterilization. Ten leaves of three different growth stages, such as immature leaves with reddish brown soft laminae, medium mature with light green shiny appearance and soft laminae and mature leaves with dark green thick laminae (Fig.3 A-F)) were used for surface sterilization. The leaves were soaked for two minutes in 150 ml of the sterilant along with two drops of Tween 20 in a one litre beaker in an agitated condition. After draining out the sterilant, they were rinsed 4-5 times in sterile distilled water and blotted on sterile filter paper. The leaflets were immersed in sterile ascorbic acid solution (100 mg/l) in glass petri dishes, removed the margins and cut into two halves through the midrib. Further they were cut into 1x1cm pieces and cultured in semi solid medium. The leaves were cultured in Murashige & Skoog (1962) (MS) and Woody Plant (WPM) (Lloyd and Mc Cown, 1980) basal medium. Culture contamination and tissue damage were scored from the fifth day onwards and the efficacies of the two sterilants were assessed one month after culture initiation.

3.a.2. Callus induction

3.a.2.1. Effect of basal medium

In initial experiments, the response of four different basal media and two phytohormone combinations on callus induction in leaf explants of medium maturity, collected from new flushes of glass house grown bud grafted plants were studied. Two different basal media such as MS and WPM as such and with modifications were tried for callus induction. The basal medium was modified by changing the concentration of major salts and addition of calcium nitrate. Concentration of major salts in the different basal media are given in table.1.

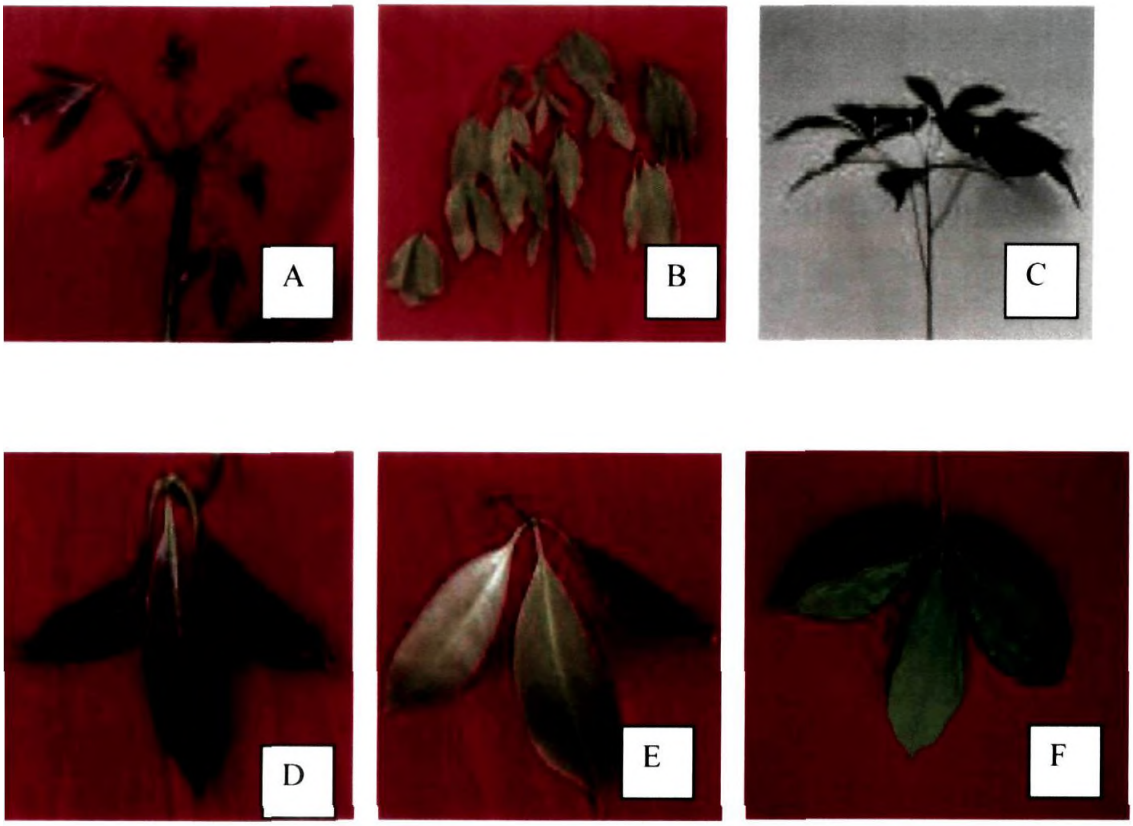


Fig.3. (A - F). Different growth stages of *Hevea* shoot and leaves .
A. Immature shoot.
B. Medium mature shoot.
C. Mature shoot.
D. Immature leaf.
E. Medium mature leaf.
F. Mature leaf

Table.1. Composition of major salts of different basal media tried for callus induction

Major salts	MS(mg/l) **(MI)**	modified MS (mg/l) **(M2)**	WPM(mg/l) **(M3)**	modified WPM (mg/l) **(M4)**
NH_4NO_3	1650	600	400	400
KNO_3	1900	900	--	--
$CaCl_2$	333	333	132	132
$MgSO_4$	181	360	370	370
KH_2PO_4	170	270	170	270
$Ca(NO_3)_2.4H_20$	--	850	560	850
K_2SO_4	--	--	990	450

The media were supplemented with MS minor salts, NaFeEDTA (36.7 mg/l), B_5 vitamins (Gamborg *et al.*, 1968), myoinositol (100 mg/l), L-Cysteine hydrochloride (50 mg/l), casein hydrolysate (1.0 g/l) and sucrose (20 g/l). Phytohormones were also added directly before autoclaving. The basal media were supplemented with two phytohormone combinations, found suitable for callus induction in preliminary experiments, are given below:

1. No hormones - T1
2. BA- 4.4 µM, 2,4-D- 4.5 µM and NAA-1.08 µM - T2
3. Kin- 4.6 µM, 2,4-D- 4.5 µM and NAA- 1.08 µM - T3.

Stock solutions of the phytohormones were prepared after dissolving in respective solvents (70% ethanol, 1N KOH/IN HCL) and the required volume was added before adjusting the pH of the medium. Unless otherwise mentioned, pH of media was adjusted to 5.7 with 1N KOH. All media were solidified with 0.25% phytagel and prepared in culture tubes. The cultures were incubated at 27 ± 2°C in the dark. For all standardization experiments on callus induction and proliferation, media were prepared in culture tubes. Optimized medium were prepared in Petri plates for initiating more number of cultures. Frequency of callus induction is the number of explants in which callus was induced from the total number of explants collected and

represented in percentage. Rate of callus induction represents the amount of callus produced from the responded explant.

3.a.2.2. Effect of phytohormones on callus induction

After identifying the suitable basal medium and phytohormone combination mentioned in section 3.a.2.1. for callus induction, experiments were also done to identify the optimum concentration of the phytohormones. Different concentrations of BA (3.5, 4.4, 5.2 μM) and 2, 4-D (0-7.2 μM) was tried in the optimized medium. Total 10 replicate cultures were kept for each treatment and the experiment was repeated four times. The frequency of callus formation was determined 40 days after culture initiation.

3.a.2.3. Effect of calcium nitrate on callus induction

Calcium nitrate was found to be an essential constituent in the basal medium for callus induction from leaf explants. The initial medium containing 850 mg/l calcium nitrate which was identified as the suitable concentration, gave 55% callus induction. Different concentrations were tried so as to identify the concentration giving improved callus induction with good callus texture. Calcium nitrate at different concentrations (0.55, 0.85, 1.0, 1.2 and 1.5 g/l) were supplemented in the basal medium previously identified to be favoring callus induction.

3.a.2.4. Effect of explant stage on callus induction

Response of cultures in terms of callus induction frequency and the time taken for callus induction, in cultures initiated with leaf explants of different maturity were examined, so as to identify the ideal developmental stage of the leaf giving good callus induction. Three different stages of the explants such as immature, medium mature and mature leaves were cultured in the optimized callus induction medium. Immature leaves were sterilized in 0.1%mercuric chloride for two minutes which gave 45% contamination free viable cultures during sterilization experiments (section 3.a.1.1). Leaves of medium maturation and mature leaves were sterilized with 0.15% mercuric

chloride for two minutes. Contamination free viable cultures were used for studying the callus induction frequency on explants collected from source plants of different maturity.

3.a.3. Callus proliferation

Callus formed in culture was carefully excised from the explant surface after 40 days and sub cultured for proliferation. Modification of the callus induction medium was attempted to get enhanced frequency of callus proliferation and improve the proliferation rate and texture. Approximately 50 mg callus was transferred to each culture tube containing the proliferation medium. Frequency of proliferation was visually scored based on the number of culture tubes in which the callus proliferated. Rate of callus proliferation was visually scored taking into consideration the cultures showing 30% increase in callus formation compared with the callus cultured initially.

3.a.3.1. Effect of 2, 4-D on callus proliferation

Initial experiments on callus induction showed that texture of the callus is influenced by the 2,4-D concentration in the medium. In order to optimize the 2,4-D requirements for improving callus proliferation and texture, three different concentrations of 2,4-D (1.08, 2.7 and 3.6 μM) were experimented in the optimized callus induction medium. The phytohormones were added directly to the callus induction medium before autoclaving.

3.a.3.2. Effect of calcium nitrate and sucrose on callus proliferation

Different concentrations of calcium nitrate, ranging from (250-1200 mg/l) along with different levels of sucrose (20, 30, 40 and 50 g/l) were tried in the callus induction medium containing optimum concentration of 2,4-D (2.7 μM) identified to be suitable for callus proliferation.

3.a.3.3. Effect of silver nitrate on callus friability

Three different concentrations of silver nitrate (10, 20 and 30 mg/l) was added to the optimized proliferation medium during the second subculture of callus

proliferation. Silver nitrate stock solution (1.0 mg/ml) was prepared in water and the required quantity was added after filter sterilization to the autoclaved medium. The effect of silver nitrate in improving callus friability and making the calli more embryogenic was examined. Medium containing silver nitrate was stored in dark.

3.a.4. Somatic embryogenesis

3.a.4.1. Embryogenic callus initiation

Proliferated calli were transferred for embryogenic callus initiation to sterile medium prepared in disposable petri dishes (90 x15 mm). For preparing petri plates containing sterile media, the media were autoclaved in conical flasks at 120 ^{0}C for 15 minutes, cooled to 47-50^{0}C and poured into sterile petri plates in the laminar flow hood after addition of filter sterilized phytohormones. After medium solidification, the plates were sealed with parafilm. The callus clumps were kept in the initial medium for about 60 days till the callus changes colour from yellow to brown. These were again subcultured thrice in fresh media with 60 days interval for emergence of embryogenic callus. All cultures were maintained in the dark and observed once in a month for embryogenic callus initiation. The frequency of embryogenic callus formation was recorded at the end of the third subculture and the proliferated embryogenic calli were sub-cultured for embryo induction.

For other explants in *Hevea brasiliensis* low salt basal medium is preferred for embryogenic callus formation and embryogenesis (Jayasree *et al.*, 2003, Sobha *et al.*, 2009). Hence M2 basal medium containing half strength major elements was used for embryogenic callus initiation. Constituents found beneficial for somatic embryogenesis in other explants of *Hevea* were also added to the medium. Accordingly, the medium was supplemented with MS minor salts, NaFeEDTA (36.7 mg/l), myoinositol (100 mg/l), adenine sulphate (50 mg/l), B_5 vitamins, amino acids, organic supplements and sucrose (50 g/l). Amino acids added to the medium were glutamine (500 mg/l), proline (200 mg/l), arginine (40 mg/l), and L-cysteine (20 mg/l). The organic supplements used were casein hydrolysate (400 mg/l) and coconut water (5 %). Concentration of phytohormones used in callus induction medium was changed

so as to increase the cytokinin/auxin ratio. BA (8.8 μM), Kin (1.25 μM) and NAA (1.08 μM) were used. GA_3 was also proved to be useful in initiating embryogenesis with other explants of *Hevea.* Accordingly GA_3 (4.4 μM) was also supplemented to the medium. Medium was fortified with activated charcoal (0.2%) and solidified with 0.3% phytagel.

After identifying the response of cultures in initiating embryogenic callus in the initial medium tried, necessary modifications were made in original medium to improve the frequency of embryogenic callus formation. The effect of basal medium, different phytohormones, phytagel and silver nitrate were evaluated. 20 callus clumps, each weighing approximately 100 mg, with 10 groups per plate, were tried for each treatment. The experiment was repeated thrice. Rate of embryogenic callus initiation was scored based on the number of callus clumps producing embryogenic callus.

3.a.4.1.2. Effect of calcium nitrate on embryogenic callus initiation

The basal medium tried was evaluated in terms of the rate and time taken for embryogenic callus formation and subsequent embryogenesis. A factorial experiment was done to find the combined effect of calcium and sucrose on embryogenic callus initiation. Since calcium nitrate was found to have pronounced effect in callus induction and proliferation, different concentrations of $Ca(NO_3)_2 .4H_20$ ranging from (0-1200 mg/l) were tried in the basal medium optimized for embryogenic callus formation.

3a.4.1.3. Effect of phytohormones on embryogenic callus initiation

Since BA was found to be more effective for callus induction in leaf explants, a factorial experiment was carried out with different concentration of BA along with GA_3 which is reported to stimulate embryogenesis with other explants of *Hevea.* The optimal concentration of these phytohormones favoring embryogenic callus induction was identified. BA ranging from (0 - 8.8 μM) along with GA_3 ranging from (0 – 4.4 μM) were tried. Along with these, the medium was also supplemented with fixed

concentrations of other phytohormones such as Kin (1.25 μM) and NAA (1.08 μM). The hormones were filter sterilized and added to the autoclaved medium.

3. a.4.1.4. Effect of phytagel on embryogenic callus initiation

Physical restriction of water availability was given by varying the level of phytagel to induce embryogenic callus. Phytagel (M/S Sigma, Aldrich, USA) at different concentrations (3.0, 4.0, 5.0, 6.0 and 8.0g/l) were used in the optimized medium for solidification. After adjusting the pH of the medium to 5.7 phytagel was added to the medium and autoclaved.

3.a.4.1.5. Effect of silver nitrate on embryogenic callus initiation

In experiments on callus proliferation, silver nitrate has been found to have influence in changing the callus texture. Hence different concentrations of silver nitrate (10, 20, 30 mg/l) were added filter sterilized to the autoclaved medium, optimized for embryogenic callus formation. Modified MS basal medium containing 800 mg/l $CaNO_3.4H_2O$ supplemented with the optimized level of phytohormones was used for embryogenic callus formation (section 3.a.4.1.3). The medium was also supplemented with myo inositol (100 mg/l), adenine sulphate (50 mg/l), B_5 vitamins, amino acids, organic supplements, and sucrose (60 g/l). The organic supplements used were casein hydrolysate and coconut water. The medium was also fortified with activated charcoal (0.2%) and solidified with phytagel (0.5%). Phytohormones were added after filter sterilization to the autoclaved medium.

3.a.4.2. Somatic embryo induction

Embryogenic callus were separated from the primary callus clumps, proliferated and experimented for embryo induction. The embryogenic calli were subcultured for proliferation and embryo induction in the same basal medium used for embryogenic callus formation avoiding silver nitrate. Phtyohormones tried initially were BA (8.8 μM), GA_3 (4.6 μM), 2,4-D (0.45 μM) and NAA (0.54 μM). Amino acids supplemented in the medium were proline (100 mg/l), arginine (40 mg/l), L- cysteine (20 mg/l) and serine (20 mg/l). Medium containing activated charcoal (0.2%) and

medium devoid of it were tried for embryo induction. Approximately 100 mg of embryogenic calli were cultured as a single group and about ten such groups were kept in each Petri dish containing the embryo induction medium. The calli were incubated in the dark as in callus induction phase. After studying the response of this medium for embryo induction, experiments were carried out to optimize the medium for improving the frequency of embryo induction. The effect of different phytohormones, amino acids, poly ethylene glycol and phytagel were evaluated. Embryo induction frequency was visually scored based on the number of groups forming embryos. Rate of embryo induction, calculated from the number of embryos produced per group, was also scored visually.

3a.4.2.1. Effect of phytohormones on somatic embryo induction

Concentration of the phytohormones such as BA and GA_3 used in the initial medium were optimized for maximum embryo induction. A factorial experiment was carried out with different concentrations of BA (0-8.8μM) and GA_3 (0 – 5.8 μM). Along with these, the medium was also supplemented with Kin (2.3 μM), 2,4-D (0.45 μM) and NAA 0.54 μM) at fixed concentration.

3.a.4.2.2. Effect of amino acids on somatic embryo induction

Three different combinations of the amino acids such as glutamine, proline, arginine and L- cysteine which were found to influence somatic embryogenesis were tried in the medium with the optimized level of phytohormones.

The combinations tried were

A1. Glutamine (300 mg/l), proline (100 mg/l), arginine (40 mg/l), L- cysteine (50 mg/l)

A2. Glutamine (300 mg/l), arginine (40 mg/l), L- cysteine (50 mg/l)

A3. Proline (100 mg/l), arginine (40 mg/l), L- cysteine (50 mg/l),

The amino acids were added directly to the medium before autoclaving.

3.a.4.2.3. Effect of Polyethylene glycol and Abscisic acid on somatic embryo induction

Polyethylene glycol (PEG) (MW 8000) at different concentrations (0 - 10.0 g/l) was incorporated in the medium to improve rate of embryo induction by providing medium stress. PEG was added directly to the medium before autoclaving. Different concentrations of ABA (0-1.9 μM) were also tried along with PEG to find the optimum concentration. ABA was initially dissolved in IN KOH solution and a stock solution (0.1 mg/ml) was prepared in water. ABA solution was added after filter sterilization to the autoclaved medium.

3.a.4.2.4. Effect of gelling agents on somatic embryo induction

Both white medium and medium fortified with activated charcoal (0.2%) were tried for embryo induction. The effect of gelling agents such as bacto agar (15.0-25.0 g/l), phytagel (4.0-10.0 g/l) and agar-agar (10.0-20.0 g/l) (M/S Sigma Aldrich, USA) on the rate of embryo induction were tried. The cultures were incubated in the dark for embryogenesis. Data was recorded after 50 days.

3.a.5. Embryo maturation

Callus in contact with the surface of the medium containing pro-embryos and somatic embryos at the globular stage were aseptically removed from culture after 50 days and transferred onto fresh medium for further development. Experiments on maturation and apex induction of the somatic embryos were carried out in both MS and WP medium containing two phytohormone combinations.

(1.) BA (1.3 μM) GA_3 (7.2 μM), IBA (0.49 μM), TDZ (2.1 μM)

(2.) BA (1.3 μM), GA_3 (7.2 μM), IBA (0.49 μM), Kin (1.38 μM)

Basal medium was supplemented with NaFeEDTA 36.7 mg/l, myo inositol (100 mg/l), B_5 vitamins, amino acids such as glutamic acid (150 mg/l), arginine (40 mg/l) and (glycine 10 mg/l), organic supplements such as coconut water 10%, casein hydrolysate (300 mg/l) and malt extract (100 mg/l) and sucrose (30 g/l). Rate of embryo maturation was calculated based on the number of embryos showing

germination. The effect of phytohormones, sucrose, organic supplement and coconut water on somatic embryo maturation was also studied.

3.a.5.1. Effect of phytohormones on embryo maturation

An optimum phytohormone combination is required for embryo maturation. After identifying the suitable basal medium, the concentration of BA and GA_3 favoring embryo maturation were optimized. A factorial experiment was carried out with different concentrations of phytohormones BA (0 - 8.8 μM) and GA_3 (0 - 8.8 μM) to find their combined effect on maturation of somatic embryos. The medium was also supplemented with TDZ (2.1 μM) at fixed concentration.

3.a.5.2. Effect of organic supplements on embryo maturation

The effect of different concentrations of coconut water (0-20%) along with fixed concentrations of other organic supplements such as casein hydrolysate (300 mg/l) and malt extract (100 mg/l) on embryo maturation were studied. These were added directly to the medium before pH adjustment and medium sterilization. The optimum concentration of coconut water was identified and then used in combination with malt extract and casein hydrolysate.

The pH of all media was adjusted to 5.7 prior to autoclaving at 121°C for 10 minutes. Media were solidified with 0.2% phytagel. Medium were prepared in culture tubes containing 10 ml medium and were either dark incubated or kept in light at 25 ± 2 °C under cool white fluorescent lamps. Rate of embryo maturation was calculated based on the number of embryos showing germination.

3.a.5.3. Effect of sucrose on embryo maturation

Embryo maturation which includes their enlargement and germination are usually influenced by the carbohydrate source of the medium. After optimizing the basal medium and phytohormone concentration, different concentrations of sucrose (30 - 80 g/l) were also experimented to identify the concentration suitable for embryo maturation

3.a.6. Plant regeneration

Apex induced mature embryos were cultured for plant regeneration. MS and WP basal medium containing MS minor, NaFeEDTA (36.7 mg/l), MS vitamins, myoinositol (100 mg/l), coconut water (5%) and sucrose (30 g/l) were used for plant regeneration. Basal medium containing phytohormones and devoid of phytohormones were also used. The medium was also supplemented with 0.3% activated charcoal and solidified with 0.2% phytagel.

3.a.6.1. Effect of gelling agent on plant regeneration

After identifying the basal medium suitable for plant regeneration the effect of different gelling agents was studied. Different concentrations of two gelling agents such as phytagel (0.2, 0.25, 0.3, 0.35 and 0.4%) and bacto agar (0.7, 0.75, 0.8, 0.85 and 0.9%) were used for medium solidification.

The pH of all media was adjusted to 5.7 prior to autoclaving at 121°C for 10 minutes. All cultures were raised in culture tubes containing 20 ml medium and incubated at 25 ± 2° C under cool white fluorescent lamps. Experiments were repeated thrice with twenty replications. Data was recorded after one month.

3.a.7. Secondary embryogenesis

During plant regeneration from the apex induced mature somatic embryos, few plantlets after about 3 weeks growth developed abnormalities which prevented their further development. About 20% of these regenerating plantlets showed profuse secondary embryo formation from the hypocotyl region. Embryo formation was observed when the plants started showing hindrance in continued growth, either by multiple shoot formation, cessation of root growth or leaf senescence. Embryogenesis was initiated from the hypocotyl region of the plantlet as a very small lump which expanded showing speedy growth, comprising several small embryos (Fig. 15 A& B). Each plantlet was found to produce nearly 30- 50 secondary embryos. Within two weeks the lump enlarges showing the presence of a large number of healthy embryos

(Fig.15 C). These embryos were detached from the mother plant and sub cultured for maturation and apex induction. Plant regeneration from these secondary embryos was also attempted.

3.a.7.1. Secondary embryo maturation

For maturation and apex induction, the secondary embryos were sub cultured in the apex induction medium mentioned in section (3.a.5.3). Simultaneously, maturation and apex induction was also attempted in MS and modified MS basal media containing the respective major salts along with MS minor, organic supplements such as 10% coconut water, 100 mg/l malt extract, 500 mg/l casein hydrolysate and phytohormones. Concentration of major salts of the different basal media used for maturation of secondary embryos is given in table.2. Concentration of the phytohormones GA_3, BA, Kin and IBA optimized earlier were used for further experiments on embryo maturation (section 3.a.5.1). Accordingly phytohormones tried were GA_3 (5.3 µM) and BA (2.2 µM) in presence of Kin (1.38 µM) and IBA (0.49 µM). The medium also contained MS minor salts, myoinositol (100 mg/l), B5 vitamins and sucrose (60 g/l). Charcoal (0.3%) was also added to the medium and was solidified with 0.2% phytagel. 10 embryos were tried in each replication and experiment was repeated thrice.

Table 2. Different basal media tried for maturation and apex induction of secondary embryos

Major elements	MS (mg/l)	WPM (mg/l)	Modified MS (mg/l)
NH_4NO_3	1650	400	500
KNO_3	1900	--	900
$CaCl_2$	333	132	333
$MgSO_4$	180	370	260
KH_2PO_4	170	270	270
K_2SO_4	--	990	--
$Ca(NO_3)_2 4H_2O$	--	560	--

3.a.7.2. Plant regeneration from secondary embryos

Apex induced mature embryos obtained after three weeks culture in maturation medium were transferred for plant regeneration in MS and WPM containing hormones or devoid of them. The medium also contained NaFeEDTA (36.7mg/l), myoinositol (100 mg/l), coconut water (5%) and sucrose 30 g/l. Phytohormones tried were BA (1.3 μM), GA_3 (0.87 μM) and IBA (0.49 μM). Apex induced embryos were cultured individually for plant regeneration and kept under light. pH of all the media were adjusted to 5.6 with 1N KOH. 20 ml media were added to each culture tube. The media were autoclaved at 120 ^{0}C for 10 minutes.

3.a.8. Plant Hardening

Plantlets after complete development and leaf maturation were carefully removed from the culture tubes and washed gently in running tap water to remove adhering medium. Dead tissues if any, near the cotyledons were also removed. The plantlets were blotted to remove the adhering water particles by keeping for 2 minutes in tissue paper, and transferred to small cups (5 cm diameter x 8 cm height) containing sterile sand and kept in a growth chamber. The plants were covered with transparent polythene covers to maintain humidity during the initial one week of transfer. Humidity was gradually reduced by providing holes in the cover after two days of transfer. After one month, plantlets that survived, showing continued growth were transferred to small poly bags (30 x15 cm) containing potting mixture (mixture of sand + soil +soilrite) and kept in the glass house. After two months the plantlets with two – three whorls of leaves were transferred to big polybags (90 x 30 cm) containing garden soil and kept in the glass house. The plants were watered with ½ X Hoagland's solution (Hoagland and Arnold, 1950) once in a fortnight. After emergence of 2-3 whorls of leaves, the plants were transferred to the shade house and watered once in three days.

3.a.9. Media preparation

All stock solutions were prepared following the standard procedures. Accordingly stock solutions of major nutrients (10 x) and minor nutrients (100 x)

were prepared and stored refrigerated. Stock solutions of vitamins and amino acids (100 x) were also prepared and stored in refrigerator. Phytohormones stocks (0.1mg/ml) were dissolved in respective solvents such as 70% ethanol / 1N KOH / 1N HCl, volume made up in a standard flask and stored in refrigerator. Coconut water was collected from tender coconuts, boiled to 80^0C, cooled and filtered before use. After adjusting the pH, required quantity of the gelling agent was added. For preparing medium in culture tubes, gelling agent was melted by constant stirring in a stirring hot plate. Activated charcoal if present was then added and 10/20 ml aliquots were dispensed into culture tubes (25x15 cm). The tubes were covered tightly with cotton plugs and autoclaved for 10- 15 minutes. For preparing petri dishes (90 x15 mm) containing sterile media, the media containing activated charcoal and gelling agent were autoclaved in conical flasks at 120 ^{0}C for 15 minutes. Phytohormones or other media supplements to be added after filter sterilization were added after the medium was cooled to 47-50^0C, stirred well and poured into sterile petri dishes in the laminar flow hood. After medium solidification, the plates were sealed with parafilm.

b.Effect of source plant juvenility on somatic embryogenesis

3.b.1. Explant source and culture initiation

Leaf explants were collected from different sources namely newly sprouted shoots of six month old glass house grown bud grafted *Hevea* plants, axenic plantlets derived through somatic embryogenesis from leaf explants and 15 year old field grown mature trees of clone RRII 105 and also seedlings derived from *in vitro* cultured zygotic embryos. Zygotic embryos were dissected out from mature seeds and surface sterilized with 0.2% mercuric chloride for three minutes. These were cultured in MS medium for germination.

Leaves of medium maturity were collected from *in vitro* derived plantlets such as those regenerated through somatic embryogenesis and *in vitro* germinated seedlings. The leaves were carefully taken out of the culture tube, the petioles were removed and the lamina was cut into pieces (1x1 cm) by immersing them in ascorbic acid solution (100 mg/l). Explants of the optimal stage, collected from *ex vitro* sources

such as glass house grown bud grafted plants and mature trees were surface sterilized with 0.15% (w/v) $HgCl_2$ for two minutes as described earlier (section1.1). The leaves were then cultured with their adaxial side in contact with the surface of the semisolid callus induction medium.

3.b.2. Callus induction

Optimized callus induction medium was used for culture of the explants (Section 3.a.2.3). Modified MS medium with addition of calcium nitrate (1200 mg/l), casein hydrolysate (1.0 gm/l), B5 vitamins, sucrose (20 g/l) and containing phytohormones 2,4-D (5.4 μM), BA (4.4 μM) and NAA (1.08 μM) was used for callus induction (section 3.a.2.2). Response of cultures in terms of the rate and time of callus induction in cultures initiated with leaf explants collected from source plants of different maturity were studied.

3.b.3. Embryogenic callus initiation

Proliferated callus induced in leaves collected from different sources such as budded plants grown in poly bags in the glass house, field grown mature trees and *in vitro* sources seedlings germinated *in vitro* and *in vitro* somatic embryo derived plants were cultured for embryogenic callus induction. The optimal medium identified for embryogenic calli formation was used for culture (section 3.a.4.1.4.). Modified MS medium containing ($CaNO_3$ – 800 mg/l) and supplemented with B_5 vitamins, silver nitrate (10 mg/l) and phytohormones, BA (2.2 μM), GA_3 (2.9 μM), Kin (1.25 μM) and NAA (1.08 μM) was used. Amino acids present in the medium were glutamine (500 mg/l), proline (200 mg/l), L-cysteine (100 mg/l) and arginine (40 mg/l). The medium also contained organic supplements such as coconut water (5.0 %) and casein hydrolysate (300 mg/l), sucrose (60 mg/l), 0.2% activated charcoal and was solidified with 5.0 g/l phytagel (Section 3.a.4.1.4). Calli were subcultured every 50 days into fresh medium. The medium used for embryogenic callus initiation were prepared in Petri dishes. The embryogenic competence of the callus derived from different leaf sources was measured by recording the rate and the time taken for embryogenic callus formation. The response of callus obtained from leaves of different source plants for

embryogenesis was observed in the medium. Necessary modifications were then made in the basal medium to improve the embryogenic competence of the callus.

3.b.3.1. Effect of basal medium

Basal medium was modified by optimizing the concentration of calcium nitrate and sucrose, aiding maximum embryogenic callus initiation. A factorial experiment was carried out with different concentrations of Ca $(NO_3)_2 \cdot 4H_20$ (200-1000 mg/l) and sucrose (50-100 g/l) using proliferated callus obtained from leaves of source plant that gave maximum in vitro response during initial culture.

3.b.4. Somatic embryo induction and plant regeneration

Embryogenic callus formed was subcultured for proliferation and kept in embryo induction medium. Embryo induction medium used was the one standardized earlier. Modified MS medium with B_5 vitamins, amino acids, organic supplements and phytohormones as BA (2.2 µM), GA_3 (1.45 µM), in presence of Kin (1.25 µM), ABA (0.75 µM) and NAA (1.08 µM) produced embryos. The embryo induction medium also contained the amino acids, glutamine (300 mg/l), proline (200 mg/l), L-cysteine (50 mg/l), and arginine (40 mg/l) along with organic supplements, coconut water 5%, 400 mg/l casein hydrolysate and 50 mg/l malt extract. Maturation and apex induction of embryos were tried in medium standardized earlier (section 3.A.4.2.4). WPM containing organic supplements and phytohormones GA_3 (5.3 µM) and BA (2.2 µM) in presence of Kin (1.38 µM) and IBA (0.49 µM) was used. Organic supplements such as coconut water (5 %), casein hydrolysate (400 mg /l) and malt extract (100 mg/l) that favored embryo maturation were also supplemented in the medium. The medium also contained activated charcoal (0.2%) and was solidified with 0.2% phytagel. Embryos were separated and plant regeneration attempted in medium standardized earlier (section 3.a.5.3).

c. *Agrobacterium* mediated genetic transformation

The suitability of using the plant regeneration system through somatic embryogenesis developed from leaf explants of *Hevea,* as target tissue for

Agrobacterium infection was experimented. Proliferated fresh callus were used as the target tissue for *Agrobacterium* infection. Genetic transformation was carried out using the protocol developed earlier with callus derived from anther explants (Jayashree *et al.*, 2003). Various aspects of transformation were examined to improve the efficiency of the system. Experiments were carried out to control overgrowth of the bacteria and increase the efficiency of transformation by modifying the infection, cocultivation and selection medium.

Callus was induced in leaf explants collected from six month old glass house grown bud-grafted plants of *Hevea brasiliensis.* To get sufficient target tissue for transformation, the calli formed were separated from the explant and proliferated. Proliferated friable callus obtained from leaf explants, were used as target tissue for *Agrobacterium* infection. *Agrobacterium tumefaciens* strains EHA 101 harboring the construct PDU 97.0612 carrying a gene encoding isopentenyltransferase (*ipt)* from the Ti plasmid of *Agrobacterium tumefaciens* was used for genetic transformation. The binary vectors contains β-glucuronidase (GUS) gene as the reporter gene and *npt*II gene for plant antibiotic selection (*Fig.* 4).

3.c.1. Preparation of bacterial culture

50 µl *Agrobacterium* suspension from glycerol stock was spread over semisolid AELB (Luria-Bertani) medium containing 50 mg/l kanamycin and 20 mg/l gentamycin and incubated at 28^0C for two days. *Agrobacterium* single colony was selected with sterile loop and suspended in 10 ml sterile liquid AELB medium containing the antibiotics 50 mg/l kanamycin and 20 mg/l gentamycin. The cultures were incubated at 28^0C in a shaker with 200 rpm for 24 hrs. to reach an optical density of 0.5 units at 420 nm. Volume of bacterial suspension required for 10 ml infection medium was calculated and pelleted by centrifugation at 5000 rpm for 10 minutes and resuspended in the infection medium so as to get a bacterial density of $5x10^8$ cells/ml. MS medium containing 100 µm actosyringone and 1.0 mM each of proline and betaine hydrochloride was used as the infection medium. The pH of the medium was adjusted to 5.2 with 1N KOH and filter sterilized.

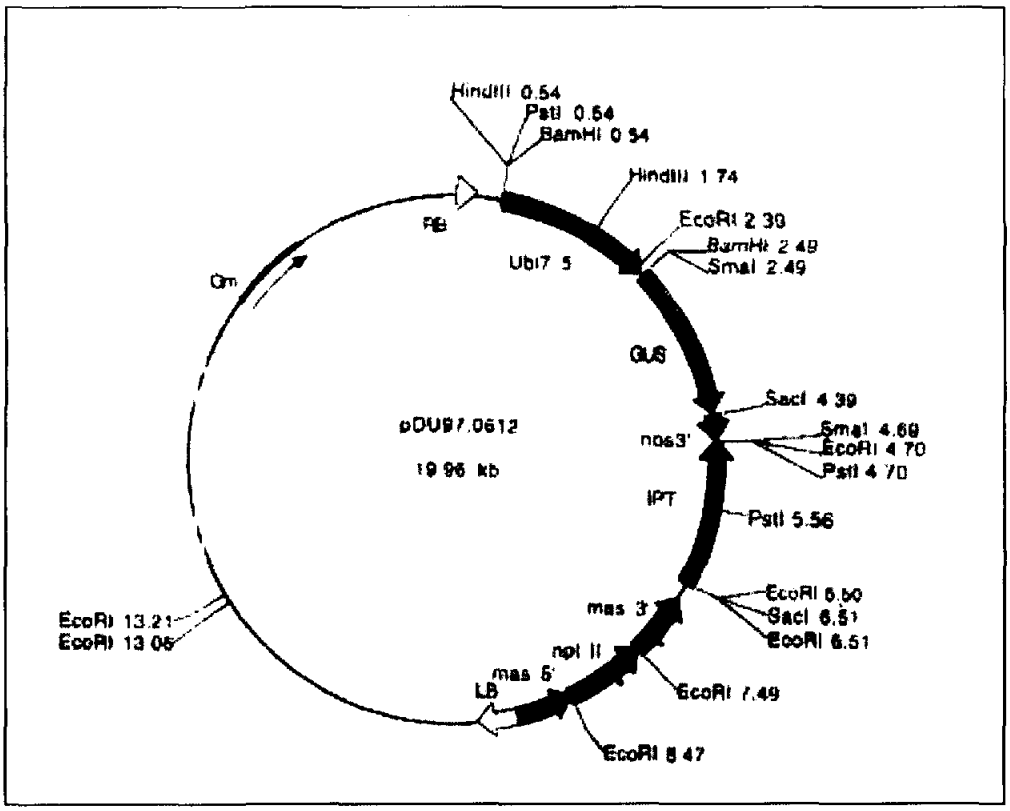


Fig.4. Detailed map of binary plasmid vector (pDU97.0612) containing isopentenyl transferase gene

3.c.2. *Agrobacterium* infection and co-cultivation

Infection of the bacterial culture with the target tissue was done after four hours incubation in an incubater shaker at 28^0C. 100 mg of proliferated friable *H. brasiliensis* calli derived from leaf explants were transferred to a small sterile Petri dish and air dried for 15 min in the laminar flow hood. Bacterial solution (5ml) was poured over the callus, and infection was done for 15 minutes. The callus in the bacterial solution was injured with a sterile scalpel blade to facilitate entry of the bacteria. After infection the bacterial solution was drained out and the infected tissues were blotted dry by transferring them to sterile filter papers. The tissues were then transferred to co-cultivation medium. For co-cultivation, the infected tissues were spread over filter paper kept on the surface of semisolid co-cultivation medium. The infected tissues were incubated for 72 hrs. in darkness at 28^0C (Sobha *et al.*, 2003; Jayasree *et al.*, 2003). The tissues were then first transferred to selection medium which was the same proliferation medium containing only selection antibiotic carbenicillin (400 mg/l) to control bacterial overgrowth. After two weeks the tissues were subcultured to medium which contained both selection antibiotics carbenicillin (400 mg/l) and kanamycin (300 mg/l). Transformation efficiency was scored based on the number of lines that emerged from the infected tissues. After the second subculture, carbenicillin was avoided from the selection medium, for subculture of the infected tissues devoid of over growth. The newly emerged lines were further subcultured for proliferation after one month in selection medium containing kanamycin (200 mg/l). About 30 callus clumps were cultured in petriplates (90 x 15 mm) containing the selection medium.

3.c.3. Influence of media components

Experiments were carried out to control overgrowth of bacteria by modifying the infection, co cultivation and selection medium using freshly proliferated friable leaf callus as the target tissue for *Agrobacterium* infection. Effect of silver nitrate in controlling bacterial over growth and improving the texture of callus in newly emerged lines was studied. Simultaneously the effect of surfactant pluronic F68 and

antioxidants such as L- cysteine and α – lipoic acid in improving the transformation efficiency were also studied. To determine the optimum concentrations of these, these were included at fixed concentration in the infection and co-cultivation medium and infected tissues were subcultured in selection medium containing different concentrations of each compound. The effect of each compound on percentage of proliferating lines with transient GUS expression was evaluated. Each experiment was repeated thrice with four replications.

3.c.3.1 Effect of silver nitrate

The effect of silver nitrate in controlling bacterial overgrowth and improving transformation efficiency by changing the callus texture of regenerated tissue was studied. Silver nitrate (10 mg/l) at fixed concentrations was supplemented in the infection and co-cultivation medium. Selection medium was supplemented with different concentration of silver nitrate (0-30 mg/l) to find its effect on controlling bacterial growth and improvement in callus texture. The pH of the infection medium was adjusted to 5.2 with 1N KOH and the whole solution was filter sterilized before tissue infection. The pH of the co-cultivation and selection medium was adjusted to 5.7 with 1N KOH before autoclaving at 121° C for 10 min. Silver nitrate was added filter sterilized along with phytohormones and antibiotics to the autoclaved co-cultivation and selection medium.

3.c.3.2. Effect of surfactant Pluronic F-68

Infection medium was modified by addition of silver nitrate (10 mg/l) so as to improve the transformation efficiency by controlling overgrowth of the bacteria. To the modified infection medium, different concentrations of the surfactant Pluronic F-68 (0-500 mg/l) was added. After addition of surfactant the whole solution was filter sterilized and used for tissue infection.

3.c.3.3. Effect of L-cysteine and α-Lipoic acid

The infection, co-cultivation and selection medium containing the optimized concentration of silver nitrate and pluronic F68 was used to study the effect of

antioxidants. The effect of antioxidants L-cysteine (100 mg/l) in the infection and co-cultivation medium and (0-300 mg/l) in the election medium was studied. Similarly the effect of α-Lipoic acid (50 mg/l) in the infection, co-cultivation medium (0-100 mg/l) and in the selection medium in improving transformation frequency and transgenic tissue regeneration was studied. After identifying optimal concentration of antioxidants, infection medium containing silver nitrate, Pluronic F68 and L-cysteine/ α-Lipoic acid was filter sterilized and used for tissue infection. L-cysteine/ α-Lipoic acid was added directly to the co-cultivation and selection medium before autoclaving while silver nitrate, phytohormones and antibiotics were added filter sterilized to the medium.

3.c.3.4. Somatic embryogenesis

The proliferated transgenic calli were subcultured in selection medium containing kanamycin (300 mg/l) and carbenicillin (400 mg/l). Tissues without overgrowth were further subcultured for embryogenic callus initiation and subsequent embryogenesis in selection medium containing kanamycin (200 mg/l) as the selection antibiotic. Embryogenic callus initiation and embryo induction was tried from proliferated callus in medium standardized earlier which was modified MS basal medium (Ca $(NO_3)_2 \cdot 4H_20$-300 mg/l) containing Gamborg B5 vitamins, amino acids, organic supplements such as coconut water (5%), casein hydrolysate (300 mg/), sucrose 80 g/l and phytohormones BA (2.2 μM), GA_3 (2.9 μM), Kin (1.25 μM), and NAA (0.54 μM). Amino acids present in the medium were glutamine (500 mg/l), proline (100 mg/l), L- cysteine HCl (50 mg/l) and arginine (40 mg/l).

3.c.3.5 GUS Histochemical assay

GUS activity assays were performed following the method reported by Jefferson (1987). Newly emerged lines from the infected tissues were immersed in X-Gluc solution (2 mM X-Gluc, 100 mM $NaH2PO_4$, 0.5 mM potassium ferricyanide and 50 mM ferro cyanide). The tissues were incubated at room temperature. Transient GUS expression (Tissue blue colouration) frequency was examined visually.

3.c.3.6. DNA isolation

DNA was isolated from transgenic as well as control callus of the *Hevea* clone RRII 105. DNA extraction was done with a modified CTAB procedure of Doyle *et al.*, (1990). One gram callus was ground to a very fine powder in liquid nitrogen using a mortar and pestle. The ground tissue was homogenized with 10 ml 2x CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl (pH-8.0), 1% Polyvenylpolypyrrolidone and 0.1% β-mercapto ethanol) The sample was then kept at 60°C for 30 min in a 30 ml centrifuge tube with intermittent shaking by gentle inversion. Centrifuged at 8000 rpm for 10 min, pellet was discarded and the supernatant transferred to a new tube. Equal vol. of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed by gentle inversion. The sample was then spun at 10,000 rpm for 10 min. and the aqueous phase was transferred to a new tube. The organic phase containing the denatured proteins was discarded. RNA in the sample was degraded by incubating at 37°C for 1 h after the addition of 5µl of DNase free RNase (10mg/ml, Sigma).Equal vol of chloroform: isoamyl alcohol was added to the sample, mixed gently and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a fresh tube and the organic phase containing lipids and carbohydrates were discarded. To the sample equal volume of chloroform : isoamyl alcohol was added, mixed gently and centrifuged at 10,000 rpm for 10 min. Aqueous phase was transferred to a fresh tube and the organic phase was discarded. To the sample 0.6 vol. ice-cold isopropyl alcohol was added and mixed by gentle inversion to precipitate the DNA. The tube was kept in ice for 20 min and the precipitated DNA was pelleted by centrifuging at 8000 rpm for 10 min at 4°C.The DNA was washed in 70 % ethanol and once in absolute ethanol. The pellet was air-dried and suspended in TE buffer. The quality and quantity of genomic DNA was checked in a UV spectrophotometer (Beckman, USA). The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280). A ratio between 1.7 – 1.8 indicates good quality DNA without protein contamination. DNA quantification was done according to the following formula.

1 O.D. at 260 nm = 50 ng of DNA

The O.D. of each DNA sample at 260 nm was measured and quantified accordingly.

3.c.3.7. Agarose Gel Electrophoresis.

The quality of isolated genomic DNA sample was checked by agarose gel electrophoresis. Agarose gel was prepared by suspending dry agarose powder at a concentration of 0.8 % in 0.5 X TBE (pH 8) buffer in a conical flask and mixed by swirling, boiled in a microwave oven until all the agarose was melted to form a clear solution. The melted agarose was cooled to 50°C and ethidium bromide was added to give a final concentration of 5 µg/ml. This was poured into a suitable clean gel casting tray containing a comb of appropriate size to form wells, and allowed to cool to form a rigid gel. After the gel has set, the comb was carefully removed and placed the gel into an electrophoresis tank filled with running buffer. 2 µl of loading buffer (0.25 % bromophenol blue and 30% glycerol in TE buffer) was added to 2 µl of DNA. After thorough mixing the samples were loaded into the resultant wells. Gel was subjected to a constant electric field of 50 V and electrophoresis was done until the bromophenol blue dye front has migrated to the bottom of the gel. The molecular marker used was Lamda DNA double digested with EcoRI and HindIII restriction enzymes. The gel was visualized in a UV transilluminator. DNA bands showed up in the gel as bands of fluorescence and photographed under UV light using Kodak EDAS 290 gel documentation system.

3.c.3.8. PCR Amplification of ipt gene from transgenic lines

PCR amplification was carried out using 20 ng of genomic DNA with isopentenyltransferase gene specific primers. Since *ipt* is a bacterial gene responsible for cytokinin biosynthesis, *ipt* primers were designed from the coding region sequence of isopentenyl transferase gene sequence of *Agrobacterium tumefaciens.*

Forward primer: 5' CTTGCACAGGAAAGACGTCG3'

Reverse primer: 5' CGTAAGCGGCTGCG3'

Amplifications were carried out in 20 µl reactions, which containing 1.0 µl Template DNA (50 ng), 2.0 µl Reaction buffer (Tris-HCl, pH- 9-10, KCl- 50 mM,

$MgCl_2$- 15 mM), 2.0 µl dNTP mix(100 µM), 1.0 µl of each forward and reverse primer (250nM each) and 0.16 µl *Taq* DNA polymerase (0.5 U). Volume of the reaction mix was made up to 20 µl with sterile distilled water.The reaction mix was overlaid with a drop of mineral oil and amplification was carried out in MJ Research PTC 200 peltier thermal cycler. The PCR conditions were as follows:

Step I-	Initial denaturation-	2 min-	94°C
Step II-	Denaturation-	1 min	94°C
	Annealing-	1 min.-	(55°C)
	Extension-	2 min.-	72 °C
Step III-	Repeat step II-	(36 cycles)	
Step IV-	Final elongation-	10 min.-72°C	

The PCR products were analysed in 1.5 % agarose gels. Gel images were captured using 'EDAS 290' (Electrophoresis Documentation and Analysis System- Kodak, USA).

3.d. Characterization of Phase change related genes and their expression in *Hevea brasiliensis*

3.d.1. Identification of phase change related genes from genomic DNA

3.d.1.1. DNA isolation

DNA was isolated from young, uninfected leaves collected from bud grafted plants of *Hevea* clone RRII 105 growing in the glass house of Rubber Research Institute of India following the protocol mentioned in section 3.c.3.6. The quality and quantity of genomic DNA was checked as mentioned in the sections, 3.c.3.7 and 3.c.3.8.

3.d.1.2. Design of gene specific primers

Based on previously published nucleotide sequences of phase change related genes, gene specific oligonucleotide primers were designed. The nucleotide sequences of a particular gene reported from other related species were downloaded from the NCBI site and the conserved sequences were identified after alignment of the sequences using the online Bioinformatic tool Clustal W

(http://www.ebi.ac.uk/Tools/msa/clustalw2/). Forward and reverse primers were designed from the conserved region. The genes, their primer sequences designed and Tm values are shown below

1. **Chlorophyll a/b binding protein gene:** Greater expression shown in developing juvenile foliage than in mature foliage.

Forward primer- 5' CTA CTT GGG TCCATT CTC 3' Tm- 54.0

Reverse primer- 5' GCC TGA ACA AAG AAT CC 3' Tm- 55.3

2.Glossy 15 gene: Developmental gene that plays a central role in regulating epidermal cell traits and leaf identity and has a primary role in maintaining juvenile phase.

Forward primer- 5' CTG TCT CTC ATT GGC AGG 3' Tm- 59.5

Reverse primer- 5' CAT GAG CTG GCC GAT CCT G 3' Tm- 69.3

3. Dihydro flavonol reductase: Catalyses reaction in anthocyanin biosynthetic pathway. Activity limited in mature phase.

Forward primer- 5' GAT TTG CCA AAC GCG AAG ACG C 3' Tm- 73.3

Reverse primer- 5' GCA CAT ACT GTC CTT GTC 3' Tm- 53.4

4. AAA-ATPase: Expression increases with tree aging

Forward primer- 5' CCT CAC TAA ATC GGT CAT AG 3' Tm-56.2

Reverse primer- 5' CAT TAT GCT GAG TGA TAT C 3' Tm- 49.8

5. QRCPE: Expression related with ontogenic age of shoots

Forward primer- 5' CTT TCC TTC TTC TTG GTC TC 3' Tm-57.1

Reverse primer- 5' CCG TGT CCG TGT CCG TG 3' Tm- 68.0

6. SQUINT: Regulates vegetative phase change.

Forward primer- 5' GAT GGG TAG GTC AAA GTG 3' Tm-55.1

Reverse primer- 5' CCT ATA CGA ACA TTT TTG C 3' Tm- 52.9.

3. d.1.3. PCR amplification of the genomic sequence coding for phase change related genes from genomic DNA

Extensive experiments were carried out to amplify the genes mentioned above. Genomic DNA was amplified initially using the specific primers to detect their

presence in *Hevea* clone RRII 105. PCR amplification could be obtained with primers designed for Cab protein gene from the genomic DNA isolated from the *Hevea* callus. PCR conditions such as the annealing temperatures and number of cycles were optimized so as to get good amplification from a 20 ng template. The concentration of the PCR components were the same as mentioned in section 3.c.3.8. For PCR amplification, an annealing temperature of 55^0C and 36 number of cycles has been adopted.

The PCR products were analysed in 1.5 % agarose gels. Gel images were captured using 'EDAS 290' (Electrophoresis Documentation and Analysis System- Kodak, USA).

3.d.1.4. PCR amplification of the genomic sequence coding for Cab gene from genomic DNA

Since the initial PCR amplifications were found to give positive amplification with the Cab gene specific primer, the further experiments ware focused on the characterization of Cab gene from *Hevea*. PCR amplification of genomic DNA using the gene specific primers mentioned above were carried out in 20 µl reactions using 20 ng of genomic DNA following the protocol mentioned in section 3.c.3.8. The PCR product was analysed in 1.5 % agarose gels. Gel image was captured using 'EDAS 290' (Electrophoresis Documentation and Analysis System- Kodak, USA).

3.d.2. Cloning and Characterization of PCR amplified Sequences Coding for Cab gene from the genomic DNA.

3.d.2.1. Elution of amplified products from agarose gels

The samples were run in 1.5 % low melting point agarose gels and the DNA bands were cut out from the lane after viewing the gel over long wavelength UV light quickly so as to avoid nicks. The gel slices were taken in a 1.5 ml micro-centrifuge tube and kept at 65°C for 10 min or till the agarose melt completely. Elution of DNA

bands from agarose gels had been performed using GFX gel band purification system (M/S Amersham Biosciences, USA) according to the manufacturer's instructions.

3.d.2.2. Ligation of the PCR products to the cloning vector

Strata clone™ PCR cloning kit (M/S Stratagene,USA) was used for the cloning of purified PCR products. Strataclone vector was ligated to the insert according to the manufacturer's instruction.

The ligation mix was prepared as follows

StrataClone™ Cloning Buffer -3 µl

PCR product- 2 µl

StrataClone™ Vector Mix- 1 µl

Mixed gently by repeated pipetting, and then incubated the ligation reaction at room temperature for 5 minutes. When the incubation was complete, the reaction was kept on ice. The ligation mix was frozen till the time of transformation.

3.d.2.3. Transformation of Competent Cells

The strataclone – solo pack competent cells provided along with the cloning kit was transformed using the ligated strataclone vector.

1. Thawed one tube of Strata Clone solo pack competent cells on ice for each ligation reaction.
2. Added 1 µl of the cloning reaction mixture to the tube of thawed competent cells. Mixed gently.
3. Incubate the transformation mixture on ice for 20 minutes. During the incubation period, pre-warm SOC medium.
4. Heat-shock was given to the transformation mixture at 42°C for 45 seconds.
5. Incubated the transformation mixture on ice for 2 minutes.
6. Added 250 µl of pre-warmed SOC medium to the transformation reaction mixture. Allowed the competent cells to recover for 1 hour at 37°C with agitation by keeping the tube of cells on the shaker horizontally for better aeration.

SOC medium (100ml)

2.0g Bacto®-tryptone

0.5g Bacto®-yeast extract1ml 1M NaCl

0.25ml 1M KCl

1ml 2M Mg2+ stock, filtersterilized (20.33g MgCl2 • 6H2O+ 24.65g MgSO4 • 7H2O. Add distilled water to 100ml. Filter sterilize)

1ml 2M glucose, filter-sterilized

Added Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stirred to dissolve. Autoclaved and cooled to room temperature. Added 2M Mg2+ stock and 2M glucose, each to a final concentration of 20mM. Volume made upto100ml with sterile, distilled water. The final pH should be 7.0.

LB–Ampicillin Agar (per Liter)

10 g of NaCl

10 g of tryptone

5 g of yeast extract

Adjust pH to 7.0 with 5 N NaOH

Add deionized H_2O to a final volume of 1 liter

20 g of agar liter of LB agar, autoclaved

Cool to 55°C and Pour into petri dishes

Add 10 ml of 10-mg/ml filter-sterilized ampicillin

2% X-Gal (per 10 ml)

0.2 g of 5-bromo-4-chloro-3-inodlyl-β-Dgalactopyranoside (X-Gal)
10 ml of dimethylformamide (DMF)
Store at –20°C
Spread 40 µl per LB-agar plate

7. Plated 5 µl of the transformation mixture on the LB– ampicillin–X-gal plates. Incubate the plates and incubated the plates overnight at 37°C.

3.d.2.4. Analyzing the positive transformants by Colony PCR.

Blue and white selection has been done for the identification of the colonies containing the recombinant plasmids with the cloned insert. White colonies were selected for colony PCR. The concentration of the PCR components are as described earlier except with template for which a portion of separate single colonies have been used.

The PCR conditions:

Step I-	Initial denaturation	10 min.-	94°C
Step II-	Denaturation- Annealing- Extension-	1min.- 1 min.- 1 min.-	92°C (50-60°C) 72 °C
Step III-	Repeat step II-	(28 cycles)	
Step IV-	Final elongation-	10 min.-72°C	

After PCR the amplicons were analysed on 1.5% agarose gel.

3.d.2.5. Isolation of recombinant plasmids from positive colonies:

Recombinant plasmids were isolated from the selected colonies using the Perfect Prep Plasmid isolation kit (M/S eppendorff, USA). The presence of the insert in the plasmids was later confirmed through PCR reaction of the recombinant plasmids.

3.d.2.6. Sequencing:

The nucleotide sequencing of the insert in the recombinant plasmids were done by the commercial firm M/S Macrogen, Korea using the M13 forward and M13 reverse primer.

3.d.2.7 Sequence analysis:

The sequence obtained was later compared with the cab gene sequences of the other reports from different plants with the BLAST analysis programme of NCBI (National Center for Biological Information), USA (Altschul *et al.,* 1990).

3.d.3. Isolation of the full length Cab gene from genomic DNA.

3.d.3.1. PCR Amplification of the full length *Cab* gene from genomic DNA.

The initial cloning and characterization of the partial sequence coding for the Cab gene was showing 91 % sequence homology with the Cab gene report from *Ricinus communis*. So attempts were carried out to get the full length Cab gene sequence from *Hevea brasiliensis*. For that Forward and reverse primers for full length amplification of the Cab gene was designed based on the mRNA sequence reported from *Ricinus communis* (Genbank accession no: XM_002524570). The forward and the reverse primers contained the start and the stop codons respectively.

Primer sequence for full length amplification of the Cab gene

Forward primer 5'- CAA ATG GCT ACC TCT ACA ATG G - 3'
Start codon

Reverse primer - 5' – CTC ACT TTC CGG GGA CAA AG - 3'
Stop codon

The synthesised primers (Sigma Aldrich) were dissolved in sterile double distilled water to get a concentration of 100 pmols/µl. The primer stock solutions were stored in –20°C. PCR analysis of genomic DNA was carried out with primers designed for full length amplification of the Cab gene in the MJ Research PTC 200 Peltier Thermal Cycler .PCR conditions were the same used in section 3.d.1.2.3. Analysis of the amplified product has been done by agarose gel electrophoresis (1.5 % agarose gel). The amplified band was later eluted from the gel using GFX gel band purification system (M/S Amersham Biosciences, USA). Cloning of the amplified product has been done in the Strataclone vector (M/S Stratagene, USA). The ligated vector with

the insert has been transformed to the solo pack chemically competent E.coli cells (M/S Stratagene, USA). Colony PCR has been done and recombinant plasmid has been isolated from the positively transformed clones. Sequencing of the insert in the recombinant plasmid has been done with M13 Forward and M13 reverse primers at M/ S Macrogen, Korea.

3.d.4. Differential expression of Cab gene.

3.d.4.1. Isolation of RNA from leaves from juvenile and mature plants

RNA was extracted from leaves of bud grafted plants (grown in glass house), seedlings, mature trees and somatic embryogenesis derived plants (*invitro* grown) of clone RRII 105 grown in glass house through a modified protocol of Kush *et al.*, (1990). RNA isolation protocol is as follows:

An RNase inhibitor di ethyl pyrocarbonate (DEPC) was used to remove the RNase contamination from all the glasswares and reagents. DEPC treated water was prepared by adding 1ml DEPC to one litre of double distilled water and stirring overnight. All glass wares were thoroughly washed and rinsed in DEPC treated water, autoclaved and baked at 160^0C overnight. All the reagents required were prepared in DEPC treated water. DEPC was inactivated by autoclaving at 121^0C for 20 minutes. The RNA isolation protocol involved the following steps.

i) The leaves collected from different sources mentioned above were washed with running tap water and cleaned with DEPC treated sterile distilled water.

ii) After removing the veins and mid rib one gram of leaf tissue from each source was powdered finely in liquid nitrogen using a mortar and pestle.

iii) Added 200 mg polyvinyl pyrrolidone (PVP) and thaw the powder in 15 ml extraction buffer (100 mM Tris-HCl (pH-8.0), 200 mM NaCl, 10 mM EDTA and 1.5 % SDS. freshly added 1% β mercapto ethanol).

It was then treated with an equal volume of extraction buffer saturated phenol, mixed gently and centrifuged at 10,000 rpm for 10 min.

iv) The aqueous layer was recovered and mixed with an equal volume of choloform

v) Centrifuged at 10,000 rpm for 10 minutes and the organic layer was discarded.

vi) RNA in the aqueous layer was precipitated by adding 1/3 vol of 8 M LiCl. The precipitation continued overnight in –20°C.

vii) RNA was pelleted by centrifugation at 10,000 rpm for 10 min. at 4°C.

viii) The pellet was washed with 2 ml of 2 M LiCl twice, then centrifuged at 8000 rpm for 5 minutes.

ix) The pellet was air dried and suspended in 200 µl of DEPC treated H_2O.

x) RNA was further purified and concentrated by precipitation with 2.5 vol ethanol in presence of 0.1 vol, 3 M sodium acetate (pH- 5.2).

xi) The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C.

xii) The pellet was washed twice in 70% alcohol, air dried and re-suspended in sterile H_2O.

xiii) The quantity of RNA was checked using UV spectrophotometer and its quality and DNA contamination, if any, was checked in 1% agarose gels.

xiv) The isolated RNA samples were stored in 3 vol of 100% ethanol at –70°C.

3.d.4.2. First strand cDNA synthesis

First strand cDNA was synthesised from the isolated leaf RNA of the different sources by reverse transcription reaction with oligo-(dT) primers using the 'Improm-II™ Reverse Transcription System' (Promega, USA) as follows:

i) 1 µl of total RNA (1 µg) was combined with 1 µl oligo-(dT) primers (0.5 µg). The reaction was made up to 5 µl by the addition of nuclease free water.

ii) The tube was incubated for 10 min at 70°C in a pre-heated block and immediately chilled in ice at 4^0C for 5 min. and kept in ice

iii) The tube was then spin down for 10 sec in a micro-centrifuge to collect the condensate and maintain the original volume. This RNA-primer combination was kept on ice until the reverse transcription reaction mix gets ready.

iv) The RT-reaction mix was combined in a 1.5 ml tube on ice. 4 µl of reaction buffer supplied by the manufacturer along with 2.4 µl $MgCl_2$ (1.8 mM), 5 µl dNTP mix (0.5 mM of each dNTP), 0.5 µl of RNase inhibitor and 1 µl reverse transcriptase. It was made up to 15 µl with nuclease free water.

v) The RNA-primer mix (5 µl) was added to the reaction mix to form the final volume of 20 µl.

vi) Annealing was done by incubating the reaction at 25°C for 8 min.

vii) Primer extension was carried out at 42°C for 2 h in a heated block.

viii) The reaction was stopped by inactivating the reverse transcriptase by keeping the tube at 70°C for 15 min.

ix) The synthesised first strand cDNA was stored at –20°C for subsequent PCR amplification.

3.d.4.3. PCR amplification of Cab gene from cDNA

PCR amplification was performed with one µl of cDNA (100 ng) as templates in 20 µl reactions. The PCR was performed as described in section 3.c.3.7 wit 28 number of cycles.

The cloning, sequencing and sequence analysis of the sequence were carried out as described in earlier sections .

3.d..5. RT-PCR assay for differential expression

RNA isolated from leaves of different source plants such as glass house grown bud grafted plants, mature trees, somatic embryogenesis derived plants of clone RRII 105 and seedlings. RNA samples were tested for the presence of genomic DNA contamination by using extracted RNA directly as a PCR template, prior to cDNA synthesis, under the same PCR conditions. First strand cDNA was synthesised from total RNA by reverse transcription with oligo-(dT) primers using the 'Improm II reverse transcription system' (Promega, USA) according to manufactures protocols (Section 3.d.1.2.). PCR was carried out with primers for Cab gene amplification (section 3.d.1.4.). One µl of the first strand cDNA was used to co-amplify the β-actin

transcripts in a 20 µl reaction. The PCR reaction products were separated on a 1.5% agarose gel, visualized with Ethidium Bromide staining under UV light and the image was captured using EDAS 290.

3.d.6. Gene Expression Studies through Northern Hybridization

3.d.6.1. Plant materials used

RNA was isolated from leaf samples collected from seedlings, bud grafted plants,, *in vitro* developed somatic plants and mature trees of *Hevea* clone (RRII 105).

3.d.6.2. Electrophoresis of RNA

Agarose (1%) was melted in 31 ml of H_2O and cooled to 60°C. In a fume hood, 10 ml of 5X formaldehyde gel running buffer (FGRB) and 9 ml of formamide was added to give a final concentration of 1X and 2.2 M respectively. The gel was allowed to set for at least 30 min at room temperature.

5X Formaldehyde gel-running buffer (FRGB) 0.1 M MOPS (pH- 7.0), 40 mM sodium acetate, 5 mM EDTA (pH-8.0)

Around 20 µg of RNA (9 µl) was incubated for 15 min at 65°C along with 4 µl FGRB, 7 µl formaldehyde and 20 µl formamide. After a brief spin, 4 µl of formaldehyde gel loading buffer was added to the sample.

3.d.6.3. RNA blotting

Before transfer to the membrane, the gel was washed thrice in DEPC treated H_2O to remove the formaldehyde. The nylon membrane (Hybond N^+, Amersham, UK) was cut in to the size of the gel and was presoaked in 10 X SSC. DNA was transferred from the treated gel to nylon membrane (Hybond N^+, Amersham, UK) through capillary blotting method (Sambrook and Russell, 2001).

A tray was filled to a height of 5 cm with 10X SSC. A suitable platform with dimensions slightly bigger than the gel was placed in the tray.

i) The surface of the platform was covered with Whatman No. 3 filter paper presoaked in 10X SSC in such a way that the ends of the paper are immersed in the SSC. Three sheets of Whatman No.1 filter paper trimmed to the same dimensions of the gel and presoaked in 10X SSC was placed on top of the platform. Air bubbles trapped any were removed by rolling the surface with a glass rod.

ii) The gel was placed carefully on top of this and then a Hybond N+ nylon membrane, presoaked in 10X SSC was placed on top of the gel. Any air bubbles were removed by gently rolling a glass rod on the surface.

iii) Two sheets of pre-soaked Whatman No.1 filter papers were placed on top of this assembly. Three more sheets of clean dry filter paper were stacked on it over which ordinary filter papers cut to the gel dimensions were stacked to a height of 10 cm.

iv) Over this, a suitable weight of around 200-300 g was placed in such a way that the weight should not crush the gel but should be sufficient to keep the papers tight.

v) The transfer was allowed to proceed overnight for a period of 12-16 h.

vi) After transfer, the assembly was disassembled and the nylon membrane was briefly washed in 5X SSC and air-dried.

vii) The membrane was fixed using a UV cross linker (Hoefer, USA) at 12000 J/cm^2.The membrane was wrapped in Saran and stored between ordinary filter papers in the refrigerator till use.

3.d.6.4. Hybridisation and washing

3.d.6.4.1. Preparation of labeled probes

The cab gene probe was radiolabeled using 'Multiprime DNA labeling system' from Amersham, (UK) following manufacturers instructions. It utilizes random hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The procedure was as follows:

i) About 50 ng of template cDNA was diluted to 5 µl with dist. H_2O and boiled for 5 min to denature.

ii) Chilled immediately on ice for 5 min and centrifuged briefly.

iii) Added 2.5 µl of buffer, which contains all the dNTPs except dCTP.

iv) Then 2.5 µl of random primer solution was added.

v) To this 2.5 µl of α-^{32}P labeled dCTP (sp. activity ~ 3000 Ci/mMol or 10 µ Ci/µl) was added.

vi) Then 11.5 µl of nuclease free autoclaved water was added.

vii) Finally 1 µl of the enzyme (Klenow fragment of DNA polymerase I) was added and mixed gently by pipetting up and down.

viii) Spun for few seconds and incubated at 37°C for 30 min.

ix) The reaction was stopped by adding 0.5 µl of 0.5 M EDTA and the probe was diluted to 100 µl with distilled water.

The labeled probe was purified by passing through a Sephadex G-50 column as follows.

i) Sephadex G-50 was added to dist. water to form a slurry (10 g of dry powder yields around 160 ml of slurry).

ii) Glass wool was placed at the bottom of a 1 ml column and 1 ml of the slurry was added without trapping of air bubbles.

iii) The column was spun at 3000 rpm for 3 min in a swinging bucket rotor.

iv) More slurry was added until the Sephadex tightly packed up to 1 ml level.

v) The column was equilibrated first with STE buffer and then with dist. water.

vi) The labeled probe was then passed through the column and purified.

vii) The column purified probe was denatured by boiling at 100°C for 3 min and immediately chilled in ice. It was stored in the freezer till use.

3.d.6.4.2. Hybridisation.

Hybridisation of the labeled probe to the nylon membrane was performed according to Sambrook *et al.*, (1989).

i) The blotted membrane was placed in a hybridisation tube and appropriate amount of pre-hybridisation solution (6 X SSC, 5 X Denhardt's reagent, 0.5 % SDS) (0.2 ml/cm^2 of the blot – 25 ml for the 13x10 cm membrane) was added.

ii) The pre-hybridisation was carried out at 65°C for 1 h in a hybridisation oven (Amersham, UK) with rotary movement at very low speed.

iii) The pre-hybridisation solution was poured out and hybridisation solution (pre-hybridisation solution containing denatured probe DNA labeled with α-^{32}P) was poured into the tube and then incubated with slow rotation for 12 – 16 h at 65°C.

3.d.6.4.3. Washing of the blot and autoradiography

After hybridisation, the membrane was washed twice at room temperature for 5 and 15 min respectively with solution I (2 X SSC, 0.1 % SDS). Then the blot was subjected to two high stringent washes at 65°C for 30 min each with solution II (0.1 X SSC, 0.5 % SDS).

The membrane was then floated briefly in 0.1 X SSC at room temperature, air-dried, wrapped in a cling film and placed and subjected to autoradiography with the phosphor image analyzer FLA 5000 (M/S Fujifilm, Japan). The blot was exposed to the image plate BAS IP (MS 2025) (M/S Fujifilm, Japan) and kept sealed in the BAS cassette overnight. Next day, the image plate was removed and read in the phosphor image analyzer with laser beam (625 nm). The relative abundance of Cab protein gene mRNAs was determined by visualising the net intensity of band in northern blots.

Results

CHAPTER IV

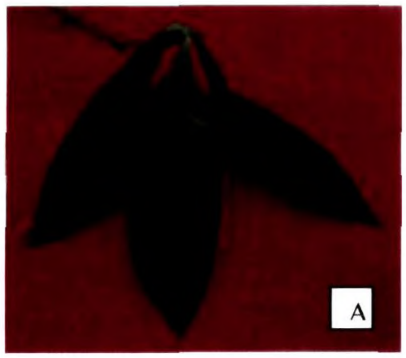
RESULTS

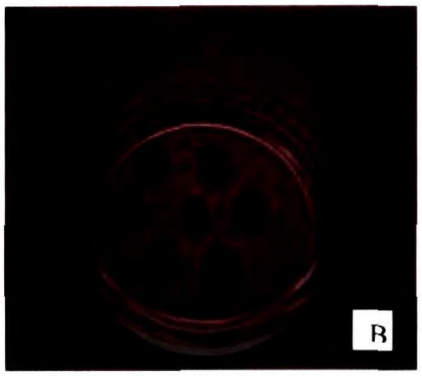
A plant regeneration system through somatic embryogenesis from leaf explants of *Hevea brasiliensis* was developed as a result of experiments done to optimize culture conditions and media requirements in each step of the protocol. Since leaf explants are available from source plants of different physiological maturity, the effect of juvenility on *in vitro* response of leaf explants was studied and the explant source that shows maximum embryogenic competence was also identified. Chlorophyll a/b binding protein gene was identified to be differentially expressed in juvenile and mature plants. The plant regeneration system developed from leaf explants was also proved to be suitable for *Agrobacterium* mediated genetic transformation.

3.a. Somatic embryogenesis from leaf explants of bud grafted plants.

3.a.1.1. Effect of sterilants

Culture contamination and explant viability showed varied response when mercuric chloride and sodium hypochlorite were used for surface sterilization. Out of the two sterilants used for surface sterilization, $HgCl_2$ was found to be better. Higher concentrations of NAOCl (0.5%) could give about 50% contamination free cultures, but the explants gradually changed to yellow and dried up. Among the different concentration of $HgCl_2$ tried 0.15% was found to be the most suitable, with leaves of medium maturation. At this concentration 73% contamination free cultures could be obtained and the explants were found to be viable with greenish appearance after two weeks of culture (Table.3.). Although, the rate of contamination free cultures could be increased with higher concentration of the sterilant, explant damage was more (Fig.5.C.). At concentration below 0.2% contamination rate with mature explants was more and at increased concentrations explant damage was observed. With immature leaves at concentrations above 0.1% contamination free cultures could be obtained at a higher rate but explant damage was also more. Leaf cultures initiated with the medium mature leaves





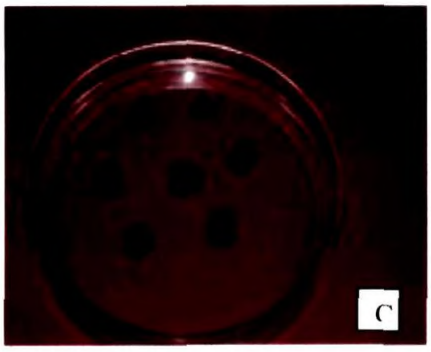


Fig. 5. (A-C). Intact leaf and culture initiation

A. Medium mature (Ideal stage for culture initiation) intact leaf

B. Freshly initiated culture after surface sterilization

C. Culture containing damaged explants due to sterilization (after two weeks in culture)

(Fig.5.A&B.) collected from glass house grown bud grafted plants could produce about 73 % contamination free, viable cultures when surface sterilized with 0.15% (w/v) $HgCl_2$ for two minutes.

Table. 3 Effect of $HgCl_2$ and NaOCl on recovery of axenic cultures and explant viability.

Sterilant	Con. (%)	Contamination free culture (%)			Tissue response		
		Immature	Medium mature	Mature	Immature	Medium mature	Mature
$HgCl_2$	0.10	46.6	40	20	G	G	G
	0.15	76.6	73.3	25	B	G	G
	0.20	76.6	76.6	40	B	Y	G
	0.25	83.3	76.6	60	B	Y	Y
	0.30	83.3	70	66.6	B	Y	B
NaOCl	0.10	30	25	23.3	G	G	G
	0.20	33.3	26.6	23.3	G	Y	Y
	0.30	33.3	25	25	B	Y	Y
	0.40	46.6	43.3	30	B	B	Y
	0.50	46.6	46.6	30	B	B	Y

CD-5.3 Values given are mean of 10 cultures repeated thrice. Tissue response: B- Black and wrinkled, G-Green viable, Y-Yellow damaged

3.a.2 Callus induction

3.a.2.1. Effect of basal medium

When callus induction was attempted in the four basal medium (MI, M2, M3 and M4) (Table.1) (section 3.a.2.1), callus initiation was observed in leaf sections cultured in all the basal media containing calcium nitrate. Callus formation was observed from the cut end of the veins. Though callus induction could be obtained in both MS and WP medium, rate of callus induction was more in modified MS medium (M2). Maximum callus induction (40%) was observed in modified MS basal medium containing phytohormones. Modified WPM medium also gave a callus induction frequency of 30%. It was observed that along with the

presence of 850 mg/l calcium nitrate and lower concentration of ammonium and potassium nitrates was suitable for inducing callus in leaf explants. Hence M2 medium was identified as the suitable one for callus induction. In this medium, concentration of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) in the original MS medium was reduced. The concentration of NH_4NO_3 was reduced to 600 mg/ and KNO_3 to 900 mg/l and the concentration of KH_2PO_4 was increased to 270 mg/l. The medium was also supplemented with 850 mg/l $Ca(NO_3)_2.4H_20$.

Out of the three phytohormone treatments (T1, T2 and T3) tried, callus induction was observed only in media containing phytohormones (T2 andT3). Among the two phytohormone combinations tried (T2 and T3), callus induction rate was more in medium containing T2 (4.4μM BA, 4.5 μM 2,4-D and 1.08 μM NAA). It can be inferred from Table.4 that when the two cytokinins, BA and kin were used for callus induction, the callus induction frequency was more in the medium containing BA as the cytokinin. Medium containing 4.4 μM BA could induce callus with twice the frequency as that in medium containing 4.6 μM Kin. Callus induced was compact and pale yellow. Callus induction could be obtained at with 40 % frequency within 4 weeks of incubation in modified MS medium with addition of 850 mg/l calcium nitrate, 1.0 gm/l casein hydrolysate, B_5 vitamins, 20 g/l sucrose and containing phytohormones 4.4 μM BA, 4.5 μM 2,4-D and 1.08 μM NAA (M2 + T2 combination).

Table. 4. Effect of different basal medium and phytohormone combinations on callus induction

Medium	Frequency of Callus induction		
	T1	T2	T3
MI	—	—	—
M2	—	++++	++
M3	—	+	+
M4	—	+++	++

T1- without phytohormones, T2-(BA- 4.4 μM, 2,4-D- 4.5 μM and NAA- 1.08 μM), T3 - (Kin-4.6 μM, 2,4-D- 4.5 μM and NAA- 1.08 μM).

Callus rating: No callus = -- ; 1-10 % = + ; 10-20 %= ++; 20-30 % = +++; 30-40 % = ++++;40-50 % = +++++

3.a.2. 2. Effect of phytohormones on callus induction.

Table. 5 . Effect of 2,4-D and BA in presence of 1.08 μM NAA on callus induction frequency and texture

BA μM	2,4-D μM	Callus texture	Callus induction %
3.52	0.0	--	--
	1.8	Watery, soft, white creamy	20
	3.6	Friable, little watery, soft	35
	5.4	Friable, little watery	50
	7.2	Compact, pale yellow, hard	40
4.4	1.8	Watery, soft, white creamy	30
	3.6	Friable, soft, pale yellow	50
	5.4	Compact, pale yellow	55
	7.2	Pale yellow, hard	40
5.28	1.8	Soft, turning green	40
	3.6	Friable, soft, pale yellow	45
	5.4	Compact, pale yellow	40
	7.2	Compact, hard, pale yellow	40

Values are taken from 10 replicated cultures repeated four times

In the factorial experiment done to find the optimum concentration of phytohormones, different concentrations of BA (3.52, 4.4 and 5.28 μM) in combination with different levels of 2,4-D (1.8, 2.6. 5.4 and 7.2 μM) were used. It was observed that 5.4 μM 2,4-D and 4.4 μM BA in presence of 1.08 μM NAA was found to be optimum. In this combination, frequency of callus induction was increased to 55% and texture of the callus was also improved (Table.5). No callus induction was observed in medium without the auxin 2,4-D. Variation in frequency of callus formation and differences in callus type were noticed when different concentration of 2,4-D were used. The callus induction frequency gradually increased with increasing 2,4-D concentration upto5.4 μM. Increasing 2,4-D concentration above (5.4 μM) made the calli very hard. Both green compact calli with nodules and yellowish white friable calli were formed in media with different 2,4-D concentrations. Frequency of callus induction was around 55%. Results also show that medium containing BA (4.4 μM) and 2,4-D (5.4 μM) in presence of low concentration of NAA (1.08 μM) and 20 mg/l sucrose was

most effective for good callus induction in leaf explants. Presence of NAA helped in improving the quality of the callus. Callus induced in darkness was friable and yellowish while in presence of light the callus became green and hard. The green compact calli on subculture expanded and became spongy without further development.

3.a.2. 3. Effect of calcium nitrate on callus induction

When different concentrations of calcium nitrate were supplemented in the optimized medium (section 3.a.2.3.) giving good callus induction, change in callus induction frequency as well as callus texture was observed (Table.6). There was significant difference in the frequency of callus induction at different concentrations of calcium nitrate. It was observed that increasing the level of calcium nitrate helped in increasing frequency of callus induction. A callus induction frequency of 60% was obtained when 1200 mg/l calcium nitrate was supplemented in the medium. At this concentration the rate of callus induction was also maximum (40-60%) and this trend was continued with increasing concentration of calcium nitrate. The frequency could be increased by 10% when the concentration of calcium nitrate was increased from 850 mg/l to 1200 mg/l. At this concentration, the texture of the callus was changed to more friable. Increasing the $Ca(NO_3)_2.4H_20$ concentration above 1200 mg/l made the callus soft and watery and hence was not found good.

Table. 6. Effect of different concentrations of calcium nitrate on callus induction in M2 medium with phtyohormones

Calcium nitrate (mg/l)	Callus induction frequency (mean %)	Rate of callus induction
550	50.00	++
850	55.00	+++
1000	58.33	+++
1200	60.00	++++
1500	58.33	+++
CD	**1 .41**	

Values given are the mean % of 20 cultures repeated thrice. Callus rating: + = 0-20 %; ++ = 20-40 %; +++ = 40-60 %; ++++= 60-80 %

From the above mentioned optimization experiments for callus induction from medium mature leaf explants, modified MS medium with addition of 1200 mg/l calcium nitrate, L-cysteine HCl (50 mg/l), casein hydrolysate (1.0 gm/l), B5 vitamins, sucrose (20g/l) and containing phytohormones 5.4 μM 2,4-D,4.4 μM BA and 1.08 μM NAA was identified as the most suitable medium for callus induction from medium mature leaf explants collected from bud grafted plants of *Hevea.* In the optimized callus induction medium, callus induction could be obtained (60%) within 4 weeks of incubation (Fig.6.A&B). The callus obtained was compact, pale yellow and nodular (Fig.6.C). Composition of the basal medium optimized for callus induction is given in table.7.

Table.7 . Composition of callus induction medium optimized with medium mature leaf explants

Media Constituents	Modified MS(mg/l)
Major elements	
NH_4NO_3	600
KNO_3	900
$CaCl_2$	333
$MgSO_4$	181
KH_2PO_4	270
$Ca(NO_3)_2.4H_20$	1200
Minor	MS
NaFeEDTA	36.7
Myoinositol	100
Vitamin B5	B_5
L-Cysteine hydrochloride	50
Caseinhydrolysate	1.0 g/l
Sucrose	20 g/l
Agar (Phytagel)	0.25%

3.a.2.4 Effect of explant stage on callus induction

When leaves of three different stages such as immature, medium mature and mature were cultured for callus induction in the optimized callus induction medium, it was observed that callusing frequency of the explants depended on stage of the leaf. Immature leaves when cultured, became wrinkled and gradually turned black in the culture medium. Callus induction frequency in these was very low (10%). On using mature leaves, the sections remained intact for more than

three weeks and then turned brown without any callus formation. In leaves of medium maturation having a shiny appearance, the leaves swelled after two weeks of culture initiation and callus initiation occurred from the cut ends within four weeks. When mature leaves were cultured, no callus induction occurred. Hence leaf explants of medium maturation were identified as the optimal stage for callus induction, when explants were collected from six month old bud grafted plants grown in the glass house (Fig.5.A).

3.a.3. Callus proliferation

When calli formed on the surface and cut ends of the explants were cultured for proliferation in the optimized fresh callus induction medium, callus proliferation was very low. Most of the callus clumps remained intact. Texture of the callus was also not found to be improved. Since the callus induction medium did not give a positive response in callus proliferation, modification of the medium was attempted by changing the concentration of phytohormones and calcium and addition of silver nitrate to increase the frequency and rate of callus proliferation so as to obtain friable proliferated callus.

3.a.3.1. Effect of 2,4-D on callus proliferation

Three different levels of 2,4-D (1.8, 2.7 and 3.6 μM) were supplemented in the optimized callus induction medium. When the leaf callus was sub cultured in this medium, it was observed that callus proliferation was obtained with maximum frequency in medium that contained reduced level of 2,4-D (2.70 μM) than the callus induction medium. Callus proliferated at a faster rate (40-50%) in medium that contained 1.8 μM and 2.7 μM 2,4-D. Reducing auxin concentration modified the callus texture (Table .8). Friable but loose callus was obtained in medium containing 1.8 μM 2,4-D. When the concentration was increased to 3.6 μM callus friability and proliferation rate was found to be reduced and callus became hard.

Table. 8. Effect of different concentrations of 2,4-D on callus proliferation

2,4-D µM	Frequency of callus proliferation %	Rate of callus proliferation	Callus texture
1.80	40.0	+++	Friable,loose
2.70	46.6	++++	Friable,compact
3.60	40.0	++	Hard

Callus proliferation rate: 10-20 % = +, 20-30 % = + +, 30-40 % = + + + 40-50 % = + + + +

3.a.3.2. Effect of calcium nitrate and sucrose on callus friability

In the factorial experiment done to identify the suitable concentration of calcium and sucrose favoring callus proliferation and friability, it was observed that a 10% increase in callus proliferation rate was obtained when the callus induction medium containing 20g/l sucrose and 1200 mg/l calcium nitrate was modified to 40 g/l sucrose and 800 mg/l calcium nitrate (Table.9). Callus proliferated in this medium showed an improvement in callus texture. Rate of callus proliferation was found to be increased with reduced concentration of calcium nitrate than in the callus induction medium (Table. 9). When the concentration of 2,4-D was reduced to 2.70 µM keeping the concentration of calcium nitrate and sucrose the same as in callus induction medium, callus proliferation was obtained at a rate of 26%. By increasing the concentration of sucrose from 20 g/l to 40 g/l, 18% increase in callus proliferation was obtained. At all the concentrations of calcium nitrate tried, increasing the level of sucrose up to 40 mg/l showed an increasing trend in frequency of callus proliferation. Significant difference in callus proliferation rate was observed in medium containing 30 g/l and 40 g/l sucrose. The combined effect of calcium and sucrose showed pronounced effect on callus proliferation and friability (Fig.6. D).

Table. 9. Effect of calcium nitrate and sucrose on callus proliferation

Sucrose (g/l)	Calcium nitrate (mg/l)					
	200	400	600	800	1000	1200
20	20.00	25.00	25.00	26.66	24.33	43.30
30	26.33	38.33	36.11	45.00	38.33	38.11
40	35.00	51.11	53.33	**55.00**	46.66	45.00
50	41.66	46.00	48.33	48.33	45.11	38.66

CD 1.86 Values given are mean % of 20 cultures repeated thrice

3.a.3.3. Effect of silver nitrate on callus friability

Though three different levels of silver nitrate (10, 20 and 30 mg/l) were tried in the proliferation medium during the second subculture, a concentration of 20 mg/l helped in improving the texture of the callus. Presence of silver nitrate made the calli yellow and friable. Addition of silver nitrate (20 mg/l) to the proliferation medium during the second and third subculture also made the calli more embryogenic.

To summarize the results of the optimization experiments on callus proliferation, it was observed that callus induction medium with reduced calcium nitrate (800 mg/l) and 2,4-D (1.8 µM) along with increased sucrose (40 g/l) than in the optimized callus induction medium (section 3.a.3.2.) was found good for proliferation. Proliferation of the callus could be obtained after three weeks in this medium (Fig.6.E). Proliferated callus had a loose and friable texture. With 2-3 subcultures in proliferation medium, the texture of the callus was further improved. Addition of 20 mg/l silver nitrate in the proliferation medium during the second and third subcultures made the calli yellow and friable.

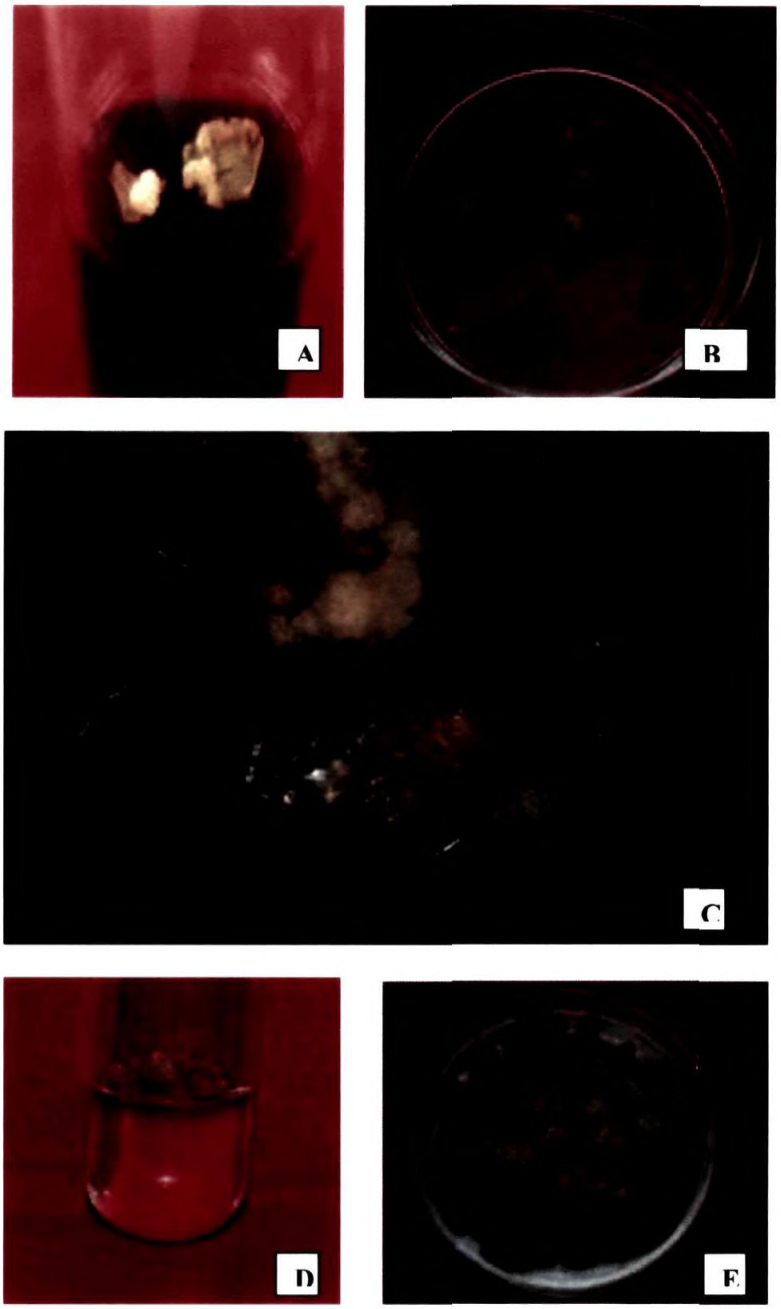


Fig.6. (A - E) Different stages of callus induction from leaf explant

A&B. Explant showing callus initiation
C. Microscopic view of callus in the explant
D&E. Callus proliferation

3.a.4. Somatic Embryogenesis

3.a.4.1. Embryogenic callus initiation

When the proliferated callus was cultured for embryogenic callus formation, the original callus clumps gradually turned black in the medium (Fig.7.A). The callus clumps that had regeneration capacity produced yellow friable embryogenic callus from one or more than one region of the cultured callus (Fig.7. B&C). Embryogenic callus formation was obtained from the proliferated leaf callus after five months in M2 medium where the concentration of major salts were reduced to half the original concentration (½ X). The medium was also supplemented with B_5 vitamins, sucrose (60 g/l) and phytohormones, BA (4.4 µM), GA_3 (4.4 µM), Kin (1.25 µM), and NAA (1.08 µM). Embryogenic callus emerged as a small lump which proliferated further (Fig.7. D). In some callus clumps, embryogenic callus initiation occurred from more than one region. In some cultures simultaneous embryo induction was also observed (Fig.7.E &F). The time and frequency of embryogenic callus formation varied with the callus. Frequency of embryogenic callus initiation was found to be low (20%) in the initial medium used. Hence the effect of different parameters that would help to initiate embryogenic callus formation was studied.

3.a.4.1.1. Effect of calcium nitrate on embryogenic callus initiation

Increasing the concentration of both sucrose and calcium nitrate, than that in the initial medium used for embryogenic callus initiation, with ½ X major, favored embryogenic callus induction. In the experiment done to find the effect of calcium in initiating embryogenic callus, it was found that $Ca(NO_3)_2.4H_20$ at a concentration of 800 mg/l was found to be optimum for embryogenic callus initiation at a sucrose concentration 60 g/l. The concentration of calcium nitrate that was identified to be suitable for callus proliferation was found to be optimal for embryogenic callus induction. The concentration of $Ca(NO_3)_2.4H_20$ (600 mg/l) used earlier has to be increased to 800 mg/l. Embryogenic callus formation could be obtained with improved frequency (30%) from the proliferated leaf callus in modified MS medium ($Ca(NO_3)_2.4H_20$ - 800 mg/l) containing B5 vitamins, amino acids, sucrose (60 g/l) and phytohormones, BA (4.4 µM), GA_3 (4.4 µM), Kin (1.25 µM), and NAA (1.08 µM) (Table.10). The medium also contained organic

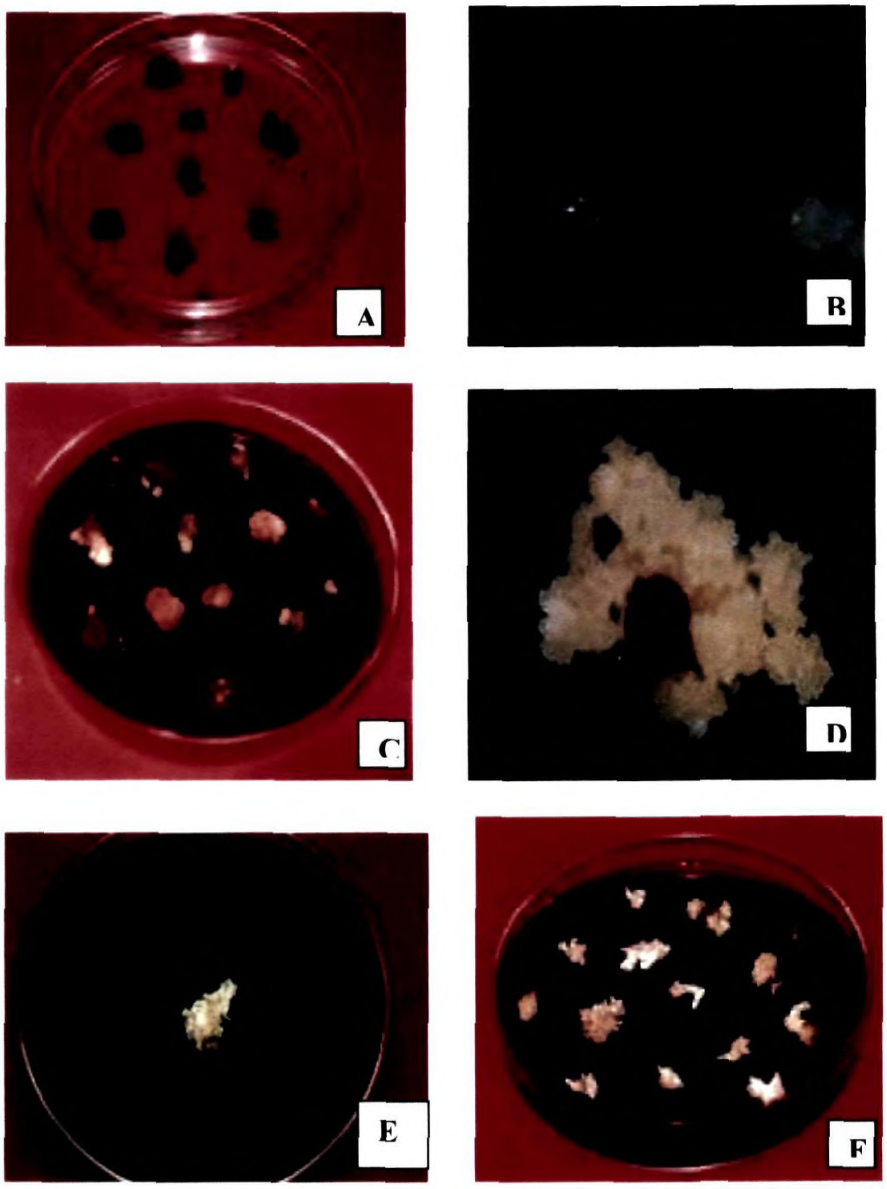


Fig.7. (A - F) different stages of embryogenic callus initiation with simultaneous embryo induction

A. Callus clumps cultured for embryogeic callus initiation
B. Microscopic view of embryogenic calli initiation;
C - D. Embryogenic callus initiation
E - F. Embryogenic callus initiation with simultaneous embryo induction.

supplements, 0.2% activated charcoal and was solidified with 3.0 g /l phytagel. From Table.10 it can be inferred that with increase in concentration of calcium nitrate alone, keeping sucrose concentration fixed, an increase in embryogenic callus formation was noticed. At the optimum concentration of ($Ca(NO_3)_2.4H_20$ - 800 mg/l) about 9 % increase could be obtained in the frequency of embryogenic callus formation.

Table .10. Effect of different concentrations of $Ca(NO_3)_2.4H_20$ on embryogenic callus initiation

$Ca(NO_3)_2.4H_20$ mg/l	**Embryogenic callus initiation (mean %)**
0	5.67
200	24.40
400	25.00
600	26.66
800	**28.90**
1000	28.90
1200	25.00
CD	**1.2**

Each value is the mean% of 20replicate samples repeated thrice

3.a.4.1.2 Effect of phytohormones on embryogenic callus initiation

In the factorial experiment done to find the effect of phytohormones BA and GA_3 on embryogenic callus formation, it was observed that a concentration of 2.2 µM BA and 2.9 µM GA_3 was optimal in presence of Kin (1.25 µM), and NAA (1.08 µM) (Table.11). Frequency of embryogenic callus initiation could be increased by 5% when the optimum concentration of the phytohormones was provided in the medium. It was observed that GA_3 at lower concentration could give a higher rate of embryogenic callus formation at 2.2 µM BA. BA at concentrations of 2.2 µM and 4.4 µM gave embryogenic callus induction frequency of 35%. Since no difference was observed in the rate of embryogenic

callus induction in the two BA concentrations 2.2 μM and 4.4 μM, 2.2 μM was selected as the optimum.

Table.11. Effect of different conentrations of BA and GA_3 on embryogenic callus initiation

BA (μM)	GA_3 (μM)				
	0	**1.4**	**2.9**	**3.5**	**4.4**
0	11.11	15.00	18.33	18.33	21.11
0.5	15.00	15.00	20.00	26.66	23.33
1.1	18.33	24.40	21.11	25.00	30.83
2.2	21.11	23.33	**35**.00	28.55	25.11
4.4	18.33	30.83	35.00	31.11	30.83
6.6	20.00	28.75	25.00	21.11	33.30
8.8	26.66	28.11	28.55	25.00	25.00

CD 1.36

Values given are mean % of 20 replicate samples repeated thrice

3.a.4.1.3. Effect of phytagel on embryogenic callus initiation

When different concentrations of phytagel (3.0, 4.0, 5.0, 6.0, 8.0 g/l) were supplemented in the optimized medium for providing water stress it was observed that 0.5% phytagel is the optimum concentration for inducing embryogenic callus (Table. 12). Frequency of embryogenic callus formation could be increased by 3% at 0.5% phytagel. At this concentration the rate of embryogenic callus formation was also found to be more. An embryogenic callus initiation frequency of 38 % was obtained in the optimized phytagel concentration of 5.0 gm/l. At this concentration, the rate of embryo induction obtained was 40%. At concentrations above this, the medium was found to be dry which inhibited tissue initiation. At lower concentrations the tissue remained intact and time taken for embryogenic callus formation was more. In the optimized medium for embryogenic callus formation somatic embryo induction was also noticed in few cultures. The frequency of cultures forming somatic embryos was found to be increased with increasing concentration of phytagel and was maximum at 0.5% phytagel. The medium which contained 800 mg/l $Ca(NO_3)_2.4H_20$ along with 60 g/l sucrose was

found to be ideal for embryogenic tissue initiation when solidified with 0.5% phytagel.

Table. 12. Effect of phytagel on embryogenic callus initiation and embryo induction

Phytagel (g/l)	Embryogenic callus (mean %)	Embryo induction (mean %)
3.0	34.44	20.00
4.0	35.00	33.33
5.0	38.33	**40.00**
6.0	34.44	40.66
CD	**2.41**	**5.02**

Each value is the mean % of 30 replicate cultures repeated thrice

3.a.4.1.4. Effect of silver nitrate on embryogenic callus initiation

In the experiment done to find the effect of silver nitrate on embryogenic callus initiation, it was observed that in the medium containing 10 mg/l silver nitrate, texture of the proliferating callus became more friable. In the optimized medium without silver nitrate, the frequency of embryogenic callus formation was 38%. When different concentration of silver nitrate was supplemented in the optimized medium, frequency of embryogenic callus formation was increased. At 5.0 and 10.0 mg/l concentrations of silver nitrate, significant difference in frequency of embryogenic callus formation was noted. At the optimum concentration of silver nitrate identified (10 mg/l), 5% improvement could be obtained in the frequency of embryogenic callus formation compared to medium without silver nitrate. No significant difference was observed in the frequency of embryogenic callus formation at higher concentration. After optimizing the concentration of media components aiding embryogenic callus initiation, embryogenic callus formation could be obtained with improved frequency (43%) from the proliferated leaf callus (Table 13).

Table.13. Effect of silver nitrate on embryogenic callus formation

Silver nitrate (mg/l)	Embryogenic callus (mean %)
0	38.33
5	40.00
10	43.33
20	43.33
CD	**1.41**

Each value is the mean % of 20 replicate samples repeated thrice

To summarize, embryogenic calli originated from more than one region of each primary callus clump as a small yellow lump which then proliferated within a month to form a mass of friable golden yellow callus. After optimizing the concentration of media components aiding embryogenic callus initiation, embryogenic callus formation could be obtained with improved frequency (43%) from the proliferated leaf callus (Table 13). Modified MS medium ($Ca(NO_3)_2$. $4H_20$ -800 mg/l) containing B_5 vitamins, 10 mg/l silver nitrate and phytohormones, BA (2.2 µM), GA_3 (2.9 µM), Kin (1.25µM) and NAA (1.08 µM) was found to be the optimal medium for callus proliferation.. Amino acids added to the medium were arginine (40 mg/l), glutamine (500 mg/l), proline (200 mg/l) and L-cysteine (100 mg/l). The medium also contained organic supplements such as coconut water (5 %), casein hydrolysate (300 mg/l), sucrose (60 g/l), 0.2% activated charcoal and was solidified with 5.0 g/l phytagel. Theprimary callus clump could be further subcultured in fresh medium from which emergence of embryogenic calli was observed for about 6 months.

3.a.4.2. Somatic embryo induction

Simultaneous with the proliferation of embryogenic calli (Fig.8. A) 40% embryo induction was also observed (Fig.8. B& C and D). The embryos became

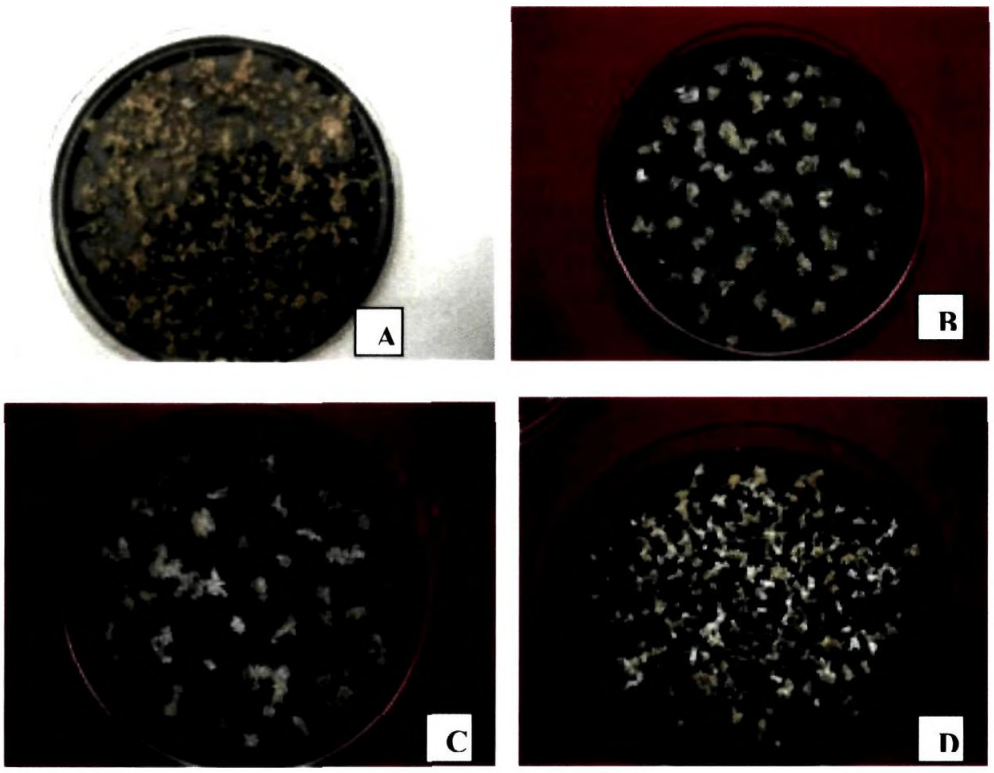


Fig.8. (A - C) Initial stages in Embryo induction

A- Proliferated embryogenic Callus

B- C Embryogenic callus showing proembryo formation

D Visible white embryos

visible after one month (Fig.8.D). The embryogenic calli that proliferated in the embryogenic callus induction medium was of soft friable texture. Further embryo induction from this proliferated calli was found to be low in the same medium and the callus had a tendency to proliferate more. Hence experiments were done to improve the rate of embryo induction by optimizing the nutritional and hormonal content of the medium optimized for embryogenic callis initiation (section 3.a.4.1.4).Since callus proliferated in the embryogenic callus initiation medium was soft, in the medium used for further embryo induction, the concentration of (Ca $(NO_3)_2.4H_20$ was reduced to 250 mg/l to make the calli more friable for embryo induction. The medium was also supplemented with B_5 vitamins, amino acids, organic supplements such as coconut water (5%), malt extract (50 mg/l), casein hydrolysate (300 mg/l) and sucrose (60 gm/l). Phytohormones added along with the medium were BA (4.4 µM), GA_3 (2.9 µM), Kin (1.25µM), 2,4-D (0.45µM) and NAA (0. 54 µM). The medium also contained the amino acids proline (200 mg/l), L-cysteine HCl (20 mg/l), serine (20 mg/l) and arginine (40 mg/l). In this medium the callus became more friable and embryo induction was achieved at a rate of 52% (Fig.9. A&B).

3.a.4.2.1. Effect of phytohormones on somatic embryo induction

Table.14. Effect of BA and GA_3 on somatic embryogenesis

BA (µM)	GA_3 (µM)				
	0	**1.45**	**2.9**	**4.35**	**5.8**
0	20.0	21.11	25.00	26.66	30.83
1.1	23.33	45.00	48.33	55.00	45.00
2.2	25.00	50.00	**58.33**	50.00	55.11
4.4	30.00	48.33	55.11	48.33	55.66
6.6	25.00	55.33	56.66	55.66	50.00
8.8	26.66	38.83	46.11	35.50	35.00

CD 5.3 Values given are mean% rate of embryo induction in 20 callus groups each comprised of 100 mg callus. Experiment was repeated thrice.

An increase of 6 % embryo induction frequency was obtained in this medium where the concentration of BA and GA_3 was optimum. In the factorial experiment, it was observed that a concentration of 2.2 μM BA and 2.9 μM GA_3 along with other phytohormones gave an embryo induction frequency of 58%. At lower concentrations of both the hormones, callus friability could be increased which increased the rate of embryo induction. At higher concentration of BA in presence of 2,4-D, callus proliferation was more, reducing the rate of embryo induction. It can be seen from the table.14 that embryo induction could be obtained with a maximum frequency of 30% when the concentration GA_3 was gradually increased in medium containing 1.25 μM Kin and without BA. The frequency could be increased to 58% when 2.2 μM BA and 2.9 μM GA_3 was also provided in the medium. No significant difference in frequency of embryo induction was observed with higher concentration of BA.

3.a.4.2.2. Effect of Polyethylene Glycol and Absiscic Acid on somatic embryo induction

When the optimized medium (section 3.a.4.2.1.) was supplemented with phyto hormones such as 4.4 μM BA, 2.9 μM GA_3, 1.25μM Kin, 0.45 μM 2,4-D and 1.08 μM NAA was used for further embryogenesis, it was observed that proliferation of the callus was also high. Addition of polyethylene glycol provides osmotic stress to the medium. The effect of addition of ABA and PEG was studied avoiding 2,4-D from the medium to reduce callus proliferation and improve the frequency and rate of embryo induction. When polyethylene glycol (MW 8000) at different concentrations (0-10.0 g/l) was tried along with ABA (0-1.9 μM), it was observed that these had a great influence in embryo induction. PEG at a concentration of 5.0 g/l and ABA at a concentration of 0.75μM gave an embryo induction frequency of 62 % (Table 15). Callus proliferation was considerably reduced with all the calli on the medium surface getting converted into pro embryos. At an increased concentration of 1.1μM ABA also no significant difference was observed on rate of embryo induction. On increasing the concentration further, the calli became white and embryo induction was found to be reduced. Similarly increasing the PEG concentration gradually with increasing

level of ABA rate of embryo induction also increased, but at higher concentrations above 5.0 g/l no significant increase in rate of embryo induction was observed.

Table. 15. Effect of ABA and PEG on embryo induction

ABA (μM)	PEG (g/l)				
	0	**3.0**	**5.0**	**8.0**	**10.0**
0	55.00	55.50	55.00	55.00	52.50
0.38	52.50	55.00	57.50	57.50	52.50
0.75	52.50	60.00	**62.50**	57.50	57.50
1.1	57.50	60.00	62.50	60.00	45.00
1.5	50.00	57.50	57.50	55.00	47.50
1.9	55.00	55.00	55.00	57.50	52.50

CD 1.83 mean % of embryo induction in 20 replicate samples of 100 mg callus Experiments were repeated four times.

3.a.4.2.3. Effect of amino acids on somatic embryo induction

When three different combination of amino acids (A1, A2 and A3) (section 3.A.4.2.2.) was supplemented in the embryo induction medium, it was observed that the combination A1 which included glutamine (300 mg/l) and proline (100 mg/l) arginine (40 mg/l), L- cysteine (50 mg/l) was more favorable (Table. 16). Supplementing the optimized medium with the amino acid combination A1 helped in increasing the rate of embryo induction. ie. The number of embryos produced in a responding culture was increased. No difference was observed in the frequency of embryo induction in the three combinations ie. the number of cultures producing embryos remained the same. The amino acid combination supplemented with both the amino acids such as glutamine and proline was found good for embryo induction. In medium supplemented with this amino acid (A1) combination rate of embryo induction (number of embryos per 100 mg callus) was above 60%.

Table 16. Effect of amino acid mixtures on embryogenesis

Amino acid mixture	Rate of embryo induction
A1	++++
A2	++
A3	++

Rate of embryo induction was calculated by visual scoring of number of globular embryos produced per 100mg of callus.
Embryo rating: + = 10-20%; ++ = 20-40%;+++ = 40-60%;++++ = 60-80%.

3.a.4.2.4. Effect of charcoal and gelling agent on somatic embryo induction

Table. 17. Effect of different gelling agents on embryo induction

Gelling agent	Concentration (%)	Embryo induction frequency (%)
Phytagel	0.30	56.50
	0.40	61.66
	0.50	**62.30**
	0.60	61.66
	0.70	59.00
Agar-agar	0.80	56.50
	1.5	53.00
	1.75	**56.66**
	2.00	55.00
	2.25	55.00
	2.50	56.33
CD		**1.68**

Values given are mean % of 20 replicate samples repeated thrice

From the preliminary experiments done with three gelling agents for solidifying the embryo induction medium, it was observed that bacto agar did not show any significant positive impact on embryo induction. Hence this was not used for further experiments. When the medium was solidified with different concentration of phytagel and agar- agar the following observations were made.

An embryo induction frequency of about 62 % was obtained when the medium was solidified with 0.5% phytagel. Though agar- agar at a concentration of above 1.75% also responded in a similar way phytagel was found more suitable and used for further experiments (Table.17). The embryos formed in medium solidified with agar- agar were small compared to embryos obtained in medium solidified with phytagel. Though 5.0 g/l phytagel favored embryo induction, repeated subculture in medium containing increased phytagel made the embryogenic calli dry and opaque. On subculture of the callus to medium with reduced phytagel (3.0 g/l) the calli could be recovered to form vigorously growing embryogenic mass (Fig.9.A-B). Embryo induction was also observed in medium devoid of activated charcoal (Fig.9.D). In medium devoid of activated charcoal embryo induction took was observed only after three months but the embryos induced were larger in size. Though embryogenic callus formation and embryo induction was observed in this medium, presence of charcoal was found to be more favorable.

After optimizing the different factors influencing embryo induction, further embryo induction was carried out in modified MS basal medium ($Ca(NO_3)_2.4H_20$ -250 mg/l) containing B_5 vitamins, and phytohormones, BA (2.2 μM), GA_3 (2.9μM), Kin (1.25 μM), ABA (0.75 μM) and NAA (0.54 μM). Amino acids added to the medium were arginine (40 mg/l), glutamine (300 mg/l), proline (200 mg/l) and L-cysteine (50 mg/l). The medium also contained organic supplements such as coconut water (5 %), and casein hydrolysate (300 mg/l), PEG (5.0 g/l), sucrose (60 g/l), 0.2% activated charcoal and was solidified with 5.0 g/l phytagel

Intermittent subculture of the embryogenic calli in hormone free medium after prolonged exposure to phytohormones was also found good. The callus changed its texture and became more efficient in embryo induction, giving an increase of 15 - 20% in rate of embryogenesis, producing normal healthy embryos. Subculture of the callus to fresh medium at 50 days interval helped in callus proliferation and subsequent embryo induction (Fig.9.C&D). Somatic embryos started developing from the calli after a month and gradually matured to the cotyledonary stage in the same media. From the initial translucent proembryos at the globular stage, the embryos gradually became white and cotyledonary. Between 30 - 45 days, globular and heart shaped embryos were clearly visible.

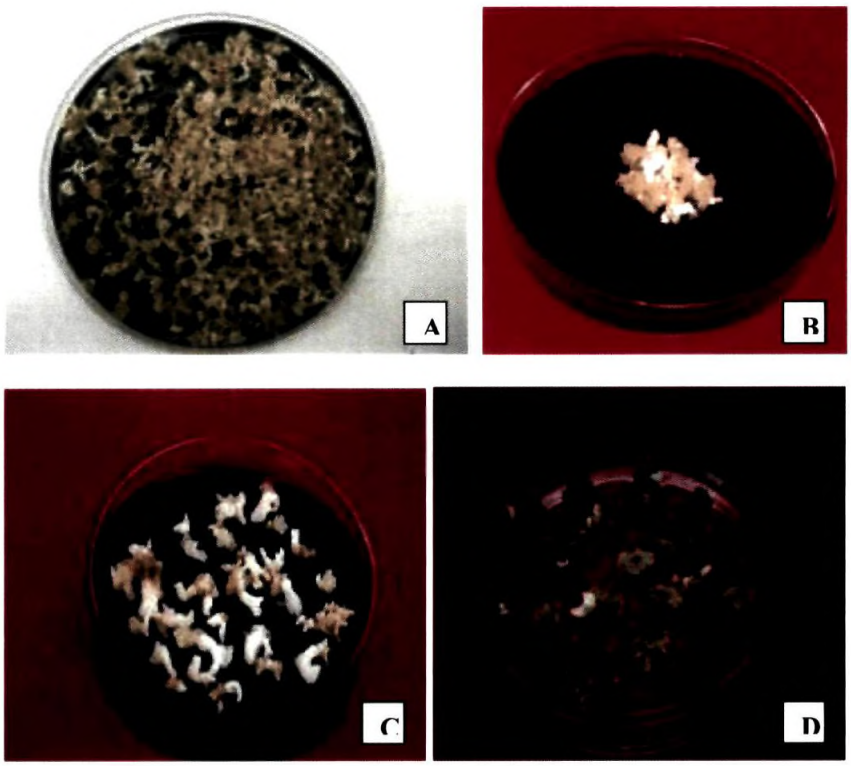


Fig. 9. (A - D) Different stages of embryogenesis

A-B. Development of embryos
C. Cotyledonary embryos
D. Embryo induction in medium devoid of charcoal

3.a.5. Embryo maturation

Callus in contact with the surface of the medium containing pro embryos and somatic embryos at the globular stage were aseptically removed from culture and transferred onto fresh medium for further development of embryos. Among the two basal medium tried for embryo maturation such as MS and WP medium, enlargement in size and germination related changes occurred in a faster rate in WPM than in MS medium. The medium also contained other additives such as NaFeEDTA (36.7 mg/l), myo inositol (100 mg/l), B_5 vitamins, amino acids such as glutamic acid (150 mg/l), arginine (40 mg/l), glycine (10 mg/l), coconut water (10%), casein hydrolysate (300 mg/l), malt extract (100 mg/l) and sucrose (30 gm/l). Phytohormones supplemented in the medium were BA (1.3 μM) GA_3 (4.4 μM) and TDZ (2.1 μM). In WPM, the embryos enlarged in size within two weeks of culture with simultaneous germination (Fig.10. A). Rate of embryo maturation was higher in WPM accounting for about 50-60 % whereas in MS medium it was 40% (Fig.10. B). Embryo maturation and germination could be obtained with a similar frequency (55 %) when two phytohormone combinations such as (1.) BA (1.3 μM) GA_3 (7.2 μM), IBA (0.49 μM), TDZ (2.1 μM) and (2.) BA (1.3 μM), GA_3 (7.2 μM), IBA (0.49 μM), Kin (1.38 μM) were supplemented in the medium. On comparing the nature of embryos produced in phytohormone combination (2) where TDZ was replaced with Kinetin, the embryos were larger in size. Embryos cultured for maturation enlarged and during start of germination a gradual transition occurred from white to yellowish pink and then to green colour. (Fig.10.C&D). Further experiments for improving embryo maturation were carried out in this medium. Accordingly the effect of changing the concentration of phytohormones, organic supplements and sucrose were studied.

3.a.5.1. Effect of phytohormones on embryo maturation

WP basal medium containing phytohormone combination BA (1.3 μM), GA_3 (7.2 μM), IBA (0.49 μM) and Kin (1.38 μM) was identified suitable for embryo maturation. Different concentrations of phytohormones BA (0 - 8.8 μM), GA_3 (0 – 5.8 μM) along with Kin (2.3 μM) and IBA (0.49 μM) were tried in the embryo maturation medium. From Table. 18. it can be inferred that GA_3 (5.3 μM) and BA (2.2 μM) in presence of Kin (1.38 μM) and IBA (0.49 μM) gave an

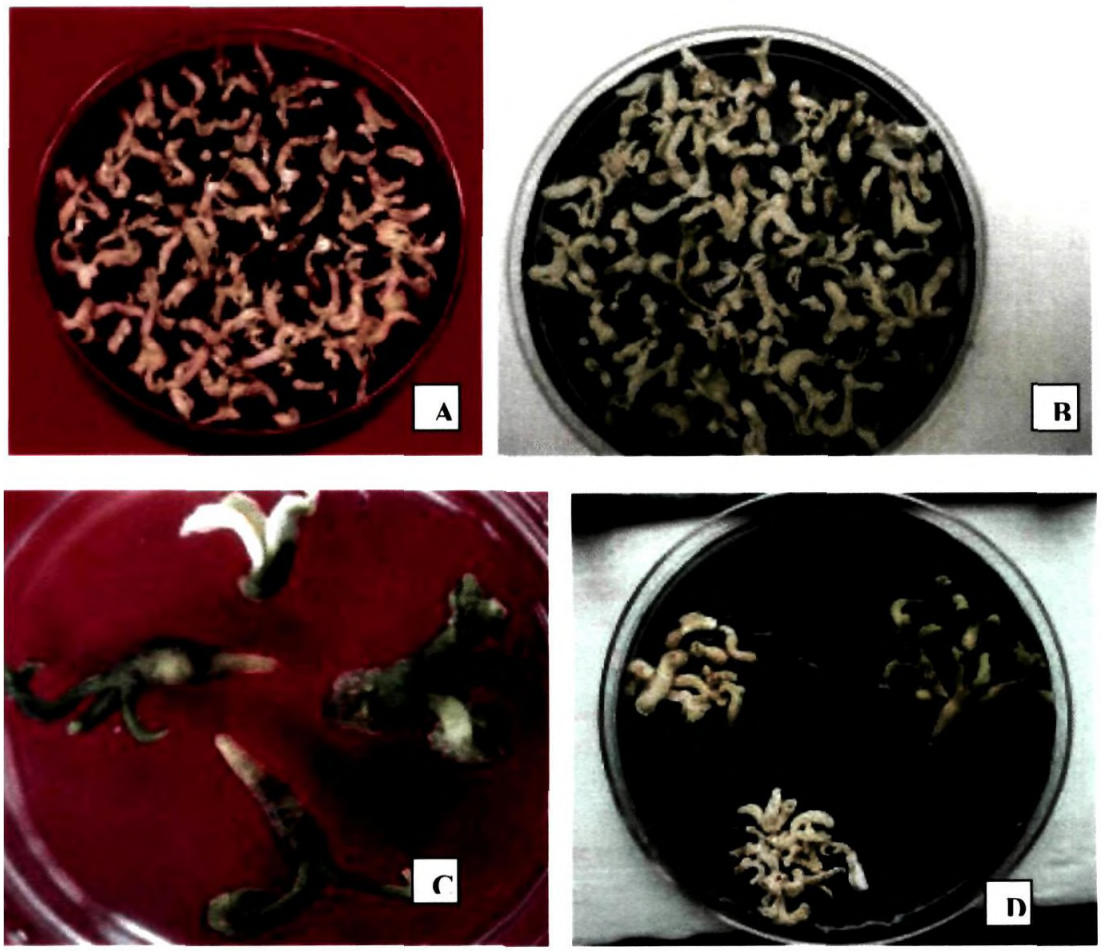


Fig.10. (A - D) Embryo maturation & germination

A. Initiation of maturation
B. Mature embryos showing initial stages of germination
C. Apex induced embryos
D. Different stages of embryo maturation and germination

embryo maturation frequency of 62%. The optimum concentration of both BA was found to be increased for effective embryo germination. Significant difference was observed in rate of embryo maturation in different concentration of the phytohormones tried. The embryos enlarged in the medium and apical meristem induction was obtained after three weeks.

Table. 18. Effect of different concentrations of BA and GA_3 on embryo maturation

GA_3 (µM)	BA(µM)				
	0	**1.1**	**2.2**	**4.4**	**8.8**
0	13.33	18.66	20.66	31.00	26.66
1.45	23.66	48.00	53.00	56.00	58.00
2.9	29.33	49.00	58.33	58.33	58.00
4.4	38.00	53.33	56.33	58.00	56.33
5.3	43.00	60.33	**62.66**	62.00	58.00
5.8	43.00	48.33	60.33	58.66	48.33
8.8	38.00	53.66	40.33	48.00	58.00

CD-5.41 Values are mean % of 20 replications repeated thrice

3.a.5.2. Effect of Organic supplements on embryo maturation

The medium found suitable for embryo maturation (section 3.a.5) was supplemented with the organic supplements such as 300 mg/l casein hydrolysate, 100 mg/l malt extract and 5% coconut water. Since organic supplements in the medium stimulated maturation and germination of embryos, the effect of different concentrations of coconut water (0-20%) were studied. It was observed from table.19, that addition of coconut water, improved the rate of embryo maturation.

Table 19. Effect of different concentrations of coconut water on embryo maturation.

Coconut water %	Maturation rate
0	60.00
5	**65.00**
10	**65.00**
15	62.00
20	60.50
CD	1.68

Values given are mean % of 10 replications repeated thrice

When 5% coconut water added in the medium, a 5% improvement in embryo maturation could be obtained. An embryo maturation frequency of 65% was obtained in medium by addition of both 5% and 10% coconut water. Increasing the level of coconut water above 10% did not increase in maturation rate or any deleterious effect on embryo germination. No significant difference in the rate of embryo maturation was noticed when higher concentrations were given.

3.a.5.3. Effect of sucrose on embryo maturation

The medium found suitable for embryo maturation (section 3.a.5) also contained 30 g/l sucrose as the carbohydrate source. When experiments were carried out to optimize the concentration of sucrose for embryo maturation, by supplementing the medium with different concentrations of sucrose, (30 - 80 g/l) it was observed that 60 g/l sucrose favored embryo maturation. When the concentration of sucrose was increased from 30-60 g/l embryo maturation rate also increased. An embryo maturation rate of 77.5 % was obtained when 60 g/l sucrose was supplemented in the medium (Table. 20). Embryo maturation could be considerably enhanced by increasing sucrose concentration. After two weeks culture in this medium the embryos enlarged, showed colour changes from yellowish pink to light green with apex induction. Significant difference was observed in rate of embryo maturation by gradual increase in sucrose concentration from 30 g/l to 60 g/l. At higher concentration of above 60 g/l no significant difference was observed in the rate of embryo maturation.

Table. 20. Effect of different concentration of sucrose on embryo maturation

Sucrose g/l	Embryo maturation %
30	65.00
40	67. 50
50	70. 00
60	**77. 50**
70	75.00
80	75.00
CD	**2.31**

Values given are mean % of replicate cultures ,experiment repeated thrice

Embryos cultured individually and maintained in the dark enlarged showing maturation and apex induction. Maturation and apex induction of embryos was obtained when cultures were dark incubated in WPM containing MS minor salts, 36.7 mg/l NaFeEDTA, 100 mg/l myoinositol, B_5 vitamins, amino acids such as glutamic acid (150 mg/l), arginine (40 mg/l) and glycine (10 mg/l), organic supplements, sucrose (60 g/l), and phytohormones BA (2.2 µM), (Kin 1.38 µM), IBA (0.49 µM) and GA_3 (5.3 µM). Organic supplements in the medium such as coconut water, casein hydrolysate and malt extract favored embryo maturation. Presence of activated charcoal was essential for embryo maturation. The optimized medium was also supplemented with organic supplements such as CW (5 %), malt extract (100 mg/l) and casein hydrolysate (300 mg/l) The embryos enlarged and changed colour gradually becoming pink ant then green with the apical meristem showing continued growth Apex induced embryos were formed in this medium within one month culture.

3.a.6. Plant regeneration

Apex induced embryos at the cotyledonary stage were transferred to light for embryo germination. Among the different media tried for plant regeneration, MS major salts containing MS minor, MS vitamins, myo inositol (100 mg/l), organic supplements such as coconut water (5 %), casein hydrolysate (300 mg /l),

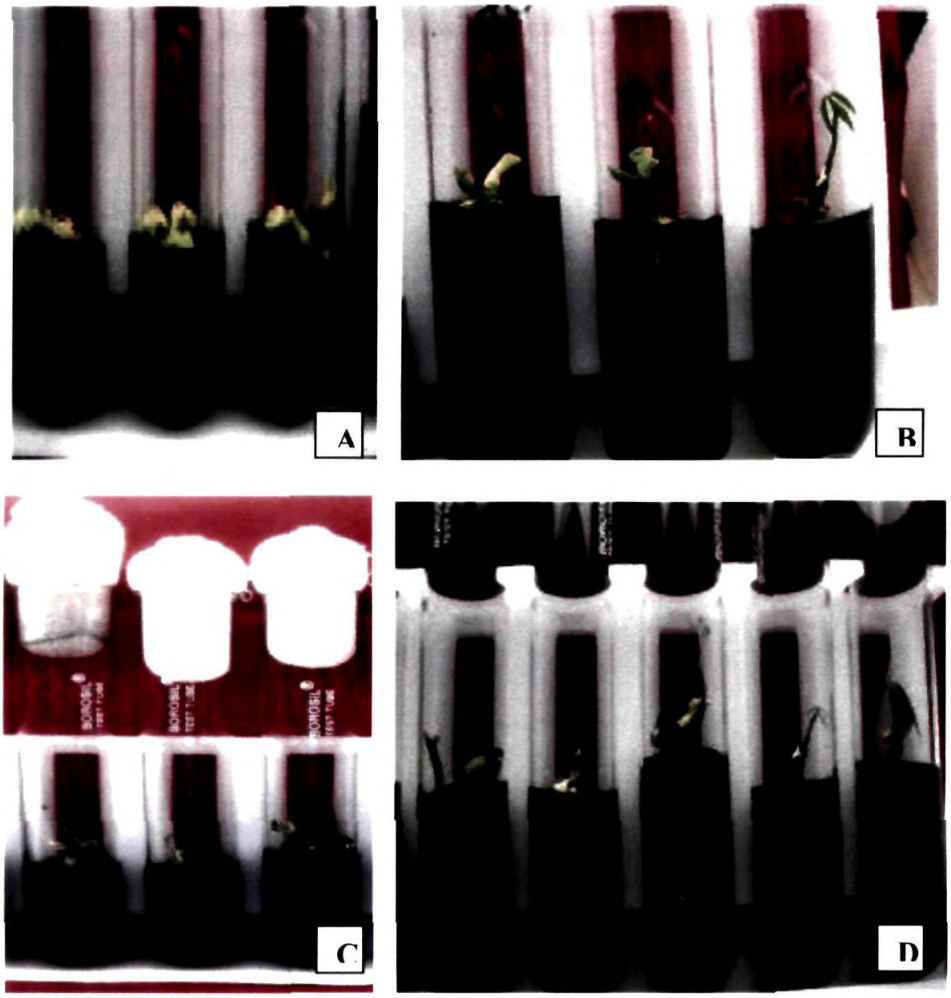


Fig.11. (A - D) Early stages of plant regeneration

A - Apex induced embryos
B, C & D – Regenerating plantlets

malt extract (100 mg/l) and sucrose (30 g/l) was found good for germination of embryos (Fig.11.A&B). Plant regeneration (43%) could be obtained both in hormone free MS medium as well as in MS medium containing phytohormones such as 2.2 μM BA and 2.9 μM GA_3 along with 0.49 μM IBA and solidified with 0.2% phytagel (Fig.11. C&D). No significant difference was observed in the frequency of plant regeneration in the two media. In medium fortified with phytohormones, plant regeneration was faster but in some, the leaves were narrow. Though germination took more time in hormone free medium, shoot development occurred with good leaves which helped in further survival of the plants (Fig.11.C). Hence for further experiments, hormone free MS medium was used. Normal embryos could develop into complete plantlets within four weeks in this medium .

3.a.6.1. Effect of gelling agent on plant regeneration

Experiments on plant regeneration were carried out initially in hormone free MS media (section 3.a.6) solidified with 0.2% phytagel. When the plant regeneration media were solidified with both phytagel and bacto agar, plant regeneration occurred at different rates. Between the two types of gelling agents used, plant regeneration was found to be favored by 7.5g/l bacto agar (Fig. 12.A-C). When the media were solidified with phytagel, a concentration of 0.35% gave a plant regeneration frequency of 48% and by solidifying the medium with bacto agar, a concentration of 0.75% gave a plant regeneration frequency of 55%. Bacto agar helped more root proliferation and in turn overall growth of the plantlets (Fig. 12.D&E). Fully developed plantlets were obtained within 3-4 weeks of culture in this medium (Fig.13). Bacto agar needed a higher concentration for media solidification than phytagel. For both the gelling agents lower concentrations favored plant regeneration. Increasing the concentration above the optimum identified did not make significant difference in plant regeneration.

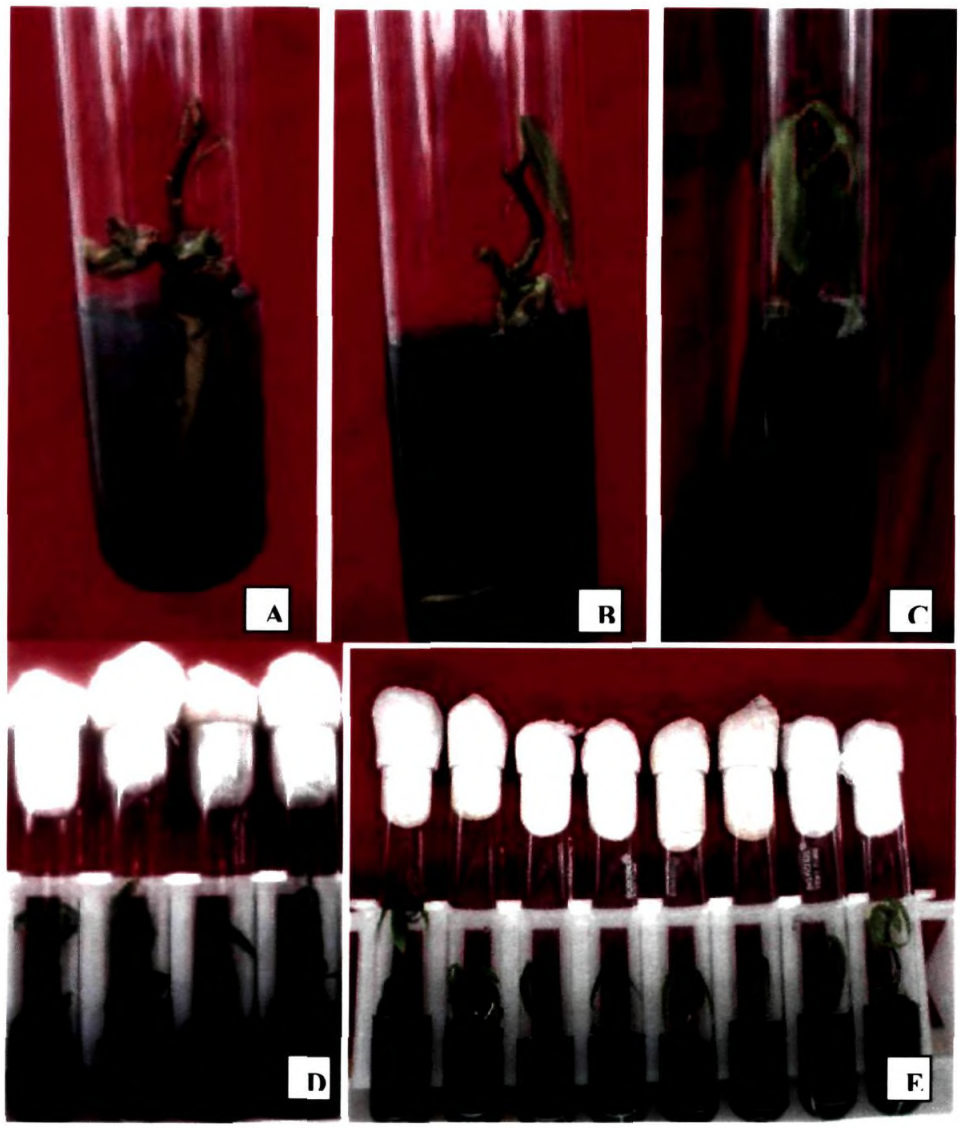


Fig.12. (A-E) Different stages of plant development

A-C - Closer view of regenerating plantlets

D & E - Fully developed plantlets

Fig.13. Fully developed plantlets ready for hardening

Table 21: Effect of gelling agent on plant regeneration

Gelling agent	Concentration (%)	Plant regeneration %) (MS medium)
Phytagel	0.20	43.0
	0.25	46.0
	0.30	46.0
	0.35	**48.5**
	0.40	45.5
Bacto agar	0.70	53.0
	0.75	**55.0**
	0.80	**55.0**
	0.85	53.0
	0.90	53.0
CD		**2.48**

Values given are mean % of 10 embryos repeated four times

Plantlets obtained had well developed shoots, fully expanded leaves and good root system. In the developed system for plant regeneration from leaf explants, it took 12-14 months from culture initiation to plant regeneration (Fig. 14). Healthy plantlets survived in minimal salt medium, while feeble ones which developed in more complex media could not survive while hardening. Since plants regenerated in medium with minimal nutrients showed higher survival rates during further growth and hardening, hormone free MS medium containing organic supplements was used for further plant regeneration.

3.a.7. Secondary embryogenesis

3.a.7.1 Secondary embryo maturation

Secondary embryos originated from hypocotyls region of regenerating plants as a small lump comprised of several embryos (Fig.15.A-C). When different media such as the earlier optimized maturation medium (section 3.a.5), MS and modified MS medium were used, the rate of embryo maturation was found to be lower

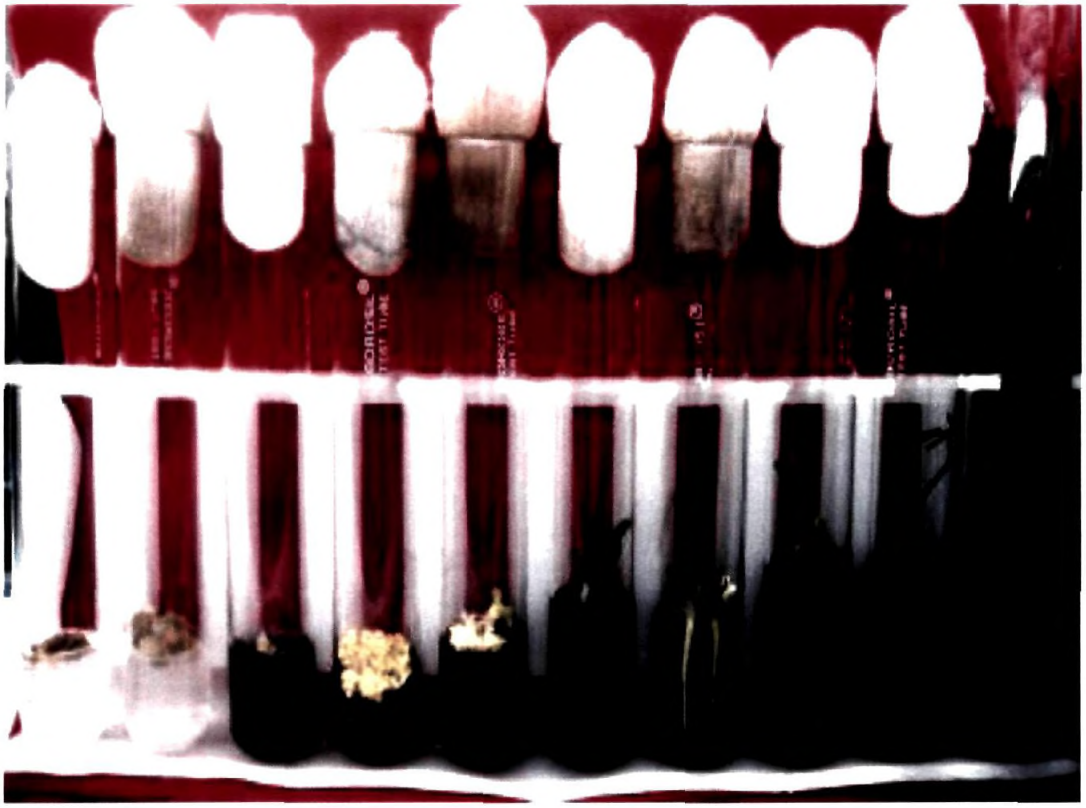


Fig.14. The sequence of somatic embryogenesis and plant regeneration from leaf explants

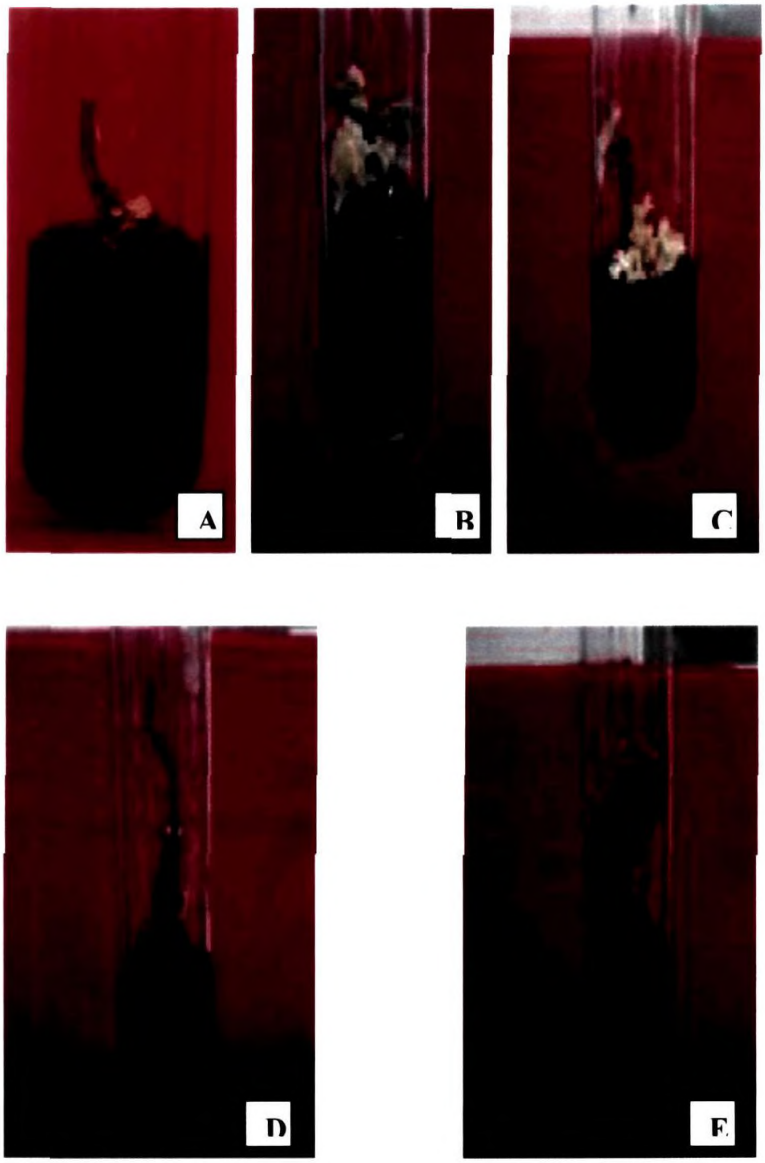


Fig.15. (A - E) Secondary embryogenesis

A- Initiation of secondary embryos from the abnormal somatic plants
B & C- Maturation of seedling embryos
D & E- Plant Regeneration from secondary embryos

(40%). Embryo maturation rate was almost similar in all the three media used. The embryos showed abnormal enlargement of the cotyledons with callusing and apex induction was less. Apex induced secondary embryos were bigger and seemed to be more healthy. Germination of the secondary embryos was less and they showed more tendency towards callus induction.

3.a.7.2. Plant regeneration from secondary embryos

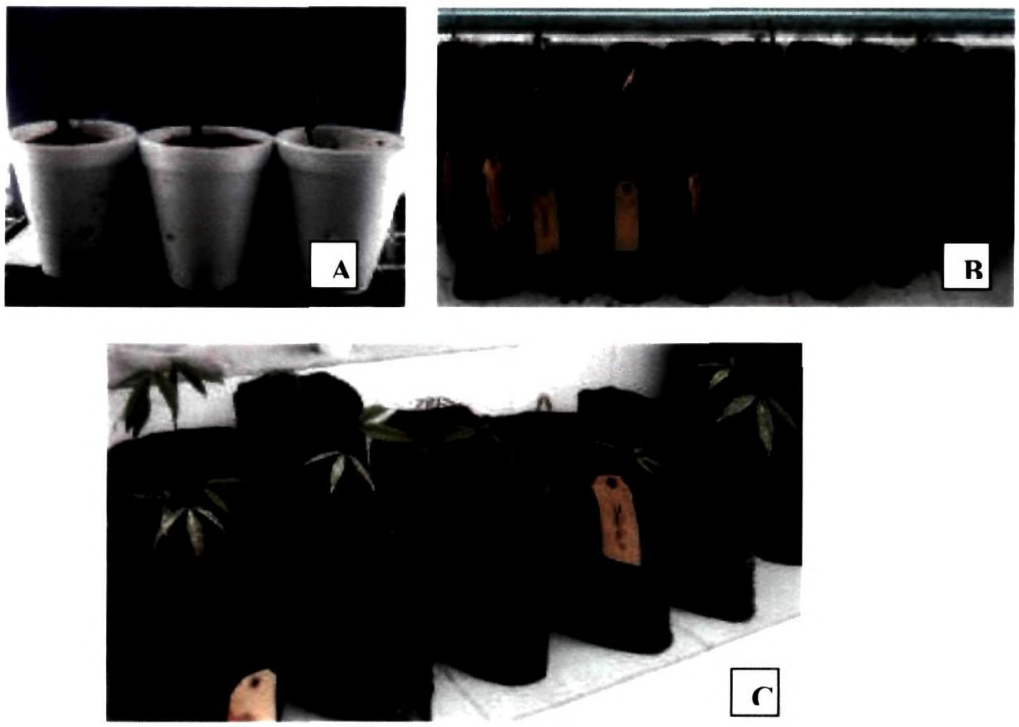
Plant regeneration from mature germinated secondary embryos was tried in MS and WPM with and devoid of hormones. When apex induced secondary embryos when sub cultured for plant regeneration, it was found that only 10-15% could regenerate into complete plants. Plant regeneration could be obtained by four weeks culture (Fig.15. C&D). The plants obtained were larger in size than compared with primary embryos. When different media were tried for plant regeneration such as MS and WP medium with and without hormones, the plant regeneration rate varied from 11-15% between treatments.

Table.22. Frequency of plant regeneration from secondary embryos.

Treatments	(Mean %)
M1 (MS)	15.00
M2 (MS-H)	13.33
M3 (WPM)	11.66
M4 (WPM-H)	13.33
CD	1.9

Values given are mean% of 20 embryos repeated three times

Maximum regeneration rate (15%) of the secondary embryos was observed in MS (M1) medium devoid of hormones. Since the regenerating plantlets were healthy, they showed good growth in MS media devoid of hormones, comparable to normal regenerating embryos. The plantlets obtained were double in size than the ones obtained from normal embryos and showed normal development (Fig.15 b). Full plant development from these embryos could be obtained within 3 weeks and could be successfully transferred to polybags.



g.16 (A - C) Different stages of plant hardening

A. Plantlets initially transferred to small cups
B. Plantlets transferred to poly bags
C. Hardened plants in polybags maintained in glass house

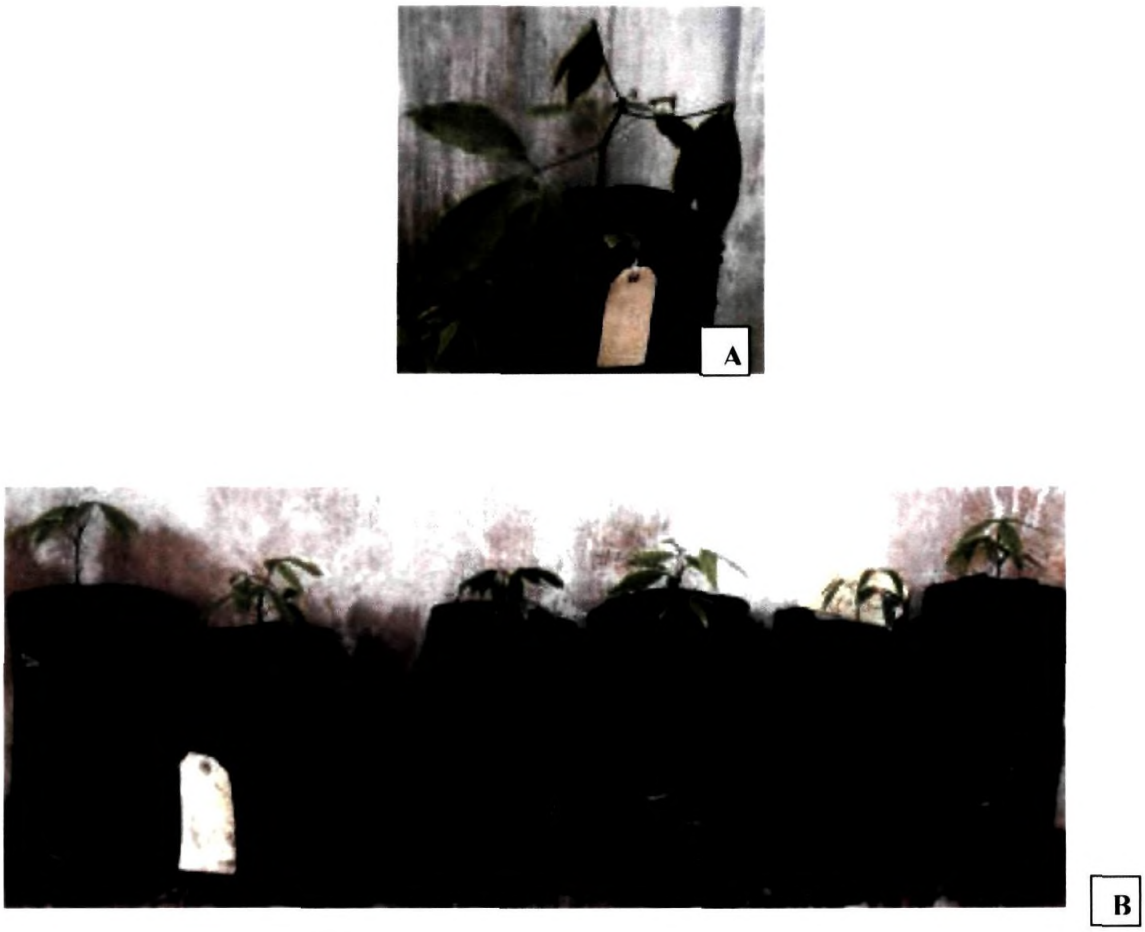


Fig. 17. A &B. Hardened plants in shade house

3.a.8. Plant hardening

Sixty percent of the plantlets transferred to sterile sand could withstand transplanting shock and were free of fungal attack after two weeks. Fifty to sixty percent survival of the plants was observed in the growth chamber (temp. 28 ^{0}C and 80 % RH) during the initial transfer in smallcups (Fig.16.A). The plants showed continued growth with formation of new leaves after two weeks with daily watering. These plants were transferred to small poy bags (30x15cm) containing potting mixture (sand + soil + soilrite in the ratio 1:1:1) after 10-15 days and watered once in three days (Fig 16.B&C). The plants were supplemented with ½ X Hoagland's solution (Hoagland and Arnon,1950) once in a fortnight. These plants were maintained in the glass house for three months. During the second transplant 30% of the plants continued to survive in the glasshouse. After the formation of two - three whorls of leaves the plants were transferred to big poly bags (30x 60 cm) (Fig.16.D). Survival rate of 20% was observed when the plants were transferred to big polybags containing garden soil and transferred to shade house. These plants were kept in the shade house for five months (Fig.17.A&B). Some of the survived plants gradually developed problems of fungal attack and sudden wilting in the shade house. The plants that showed continued growth in the shade house could be established in soil.

3.b. Effect of source plant juvenility on somatic embryogenesis

3.b.1. Sources of leaf explants

Leaf explants were collected from different source plants such as *in vitro* cultured seedlings, *in vitro* somatic embryogenesis derived plantlets, glass house grown bud-grafted plants and mature trees. Aseptic seedlings could be obtained by culture of zygotic embryos (Fig.18A-C) in MS medium. Contamination free viable cultures initiated with leaf explants (medium mature) collected from *ex vitro* sources such as glass house grown bud-grafted plants and mature trees were used for the experiments. From seedlings and somatic plants grown *in vitro,* the leaves (medium mature) were cultured directly (Fig.18. D) for callus induction. These were used for experiments on comparison of *in vitro* culture response of leaves.

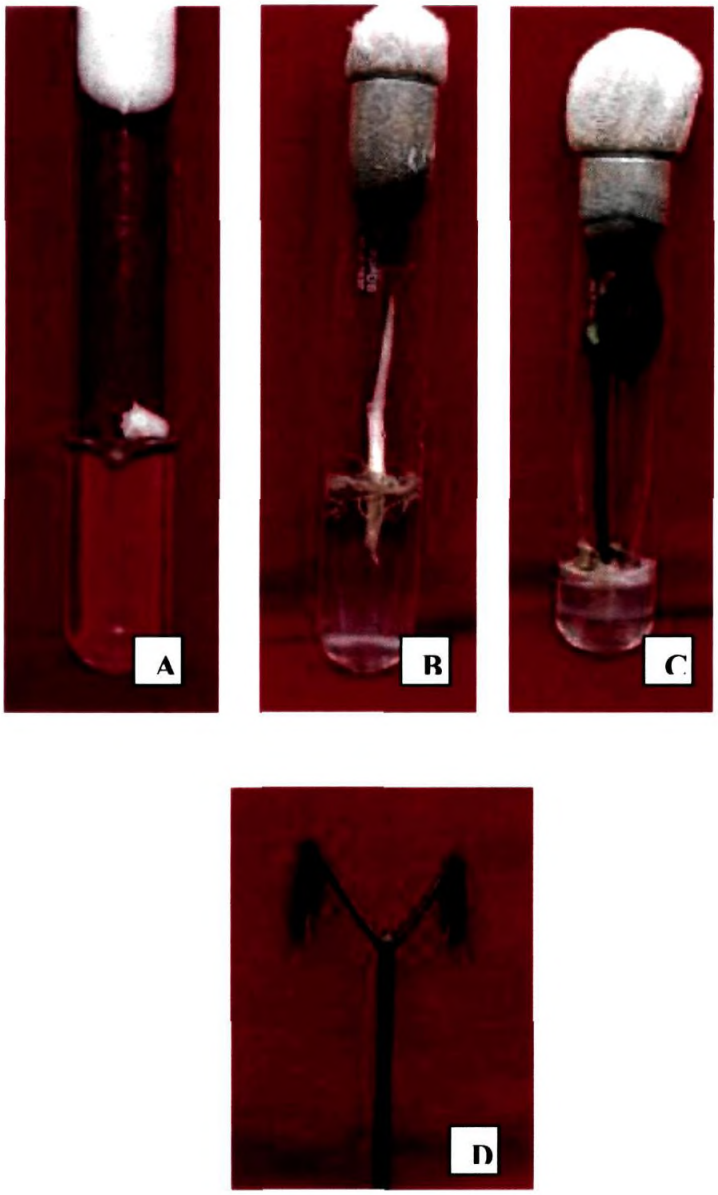


Fig.18. (A - D) Different stages of *In vitro* raised seedlings

A- Cultured zygotic embryo
B- Germinating zygotic embryo
C- *In vitro* raised seedlings
D- Optimal stage of leaf for *in vitro* culture

3.b.2. Callus induction and proliferation

When callus induction was attempted from leaves of optimal stage (medium maturation) collected from different explant sources, in the standardized callus induction medium, the following observations could be made. Leaves collected from different sources could induce callus with varied time and frequency. In the case of leaves collected from *ex vitro* sources such as glass house grown budded plants and mature trees, callus induction could be obtained within 40-50 days. In leaves collected from *in vitro* seedling cultures, and *in vitro* somatic embryogenesis derived plantlets, callus induction could be obtained within 20-30 days. Table.21. shows the rate of callus induction in leaf explants collected from different sources after 50 days of culture. In leaves collected from *in vitro* somatic embryogenesis plantlets and *in vitro* raised seedlings, rate of callus induction was around 85%. In leaves collected from bud grafted plants the rate was 60 % and in mature trees rate of callus induction was 41%. Texture of the callus was similar in cultures initiated from leaves of all sources when cultured in the same medium. Proliferated callus had a friable texture with brownish yellow appearance.

Table.23. Time taken and frequency of callus induction in leaves from different sources

Source of leaf explants	Time taken (Days) for callus induction	Callus induction frequency Mean (%)
In vitro seedlings	20-30	86.23 ± 2.93
In vitro somatic plants	20-30	84.16 ± 2.89
Bud grafted plants	40-50	60.66 ± 3.82
Mature trees	40-50	41.45 ± 1.44

Values given are the mean % of 20 replicated cultures repeated thrice

3.b.3. Embryogenic callus initiation

Variation was observed in the rate and time taken for embryogenic callus initiation when proliferated calli obtained from different explant sources were cultured in the embryogenic callus induction in medium standardized earlier (section 3.a.2.2) with bud grafted plants. Embryogenic callus initiation was earlier

obtained from proliferated embryogenic callus in modified MS basal medium containing ($Ca(NO_3)_2 \cdot 4H_20$-800 mg/l) and supplemented with B_5 vitamins, amino acids, organic supplements such as coconut water (5%), casein hydrolysate (300 mg/l), sucrose (60 gm/l) and phytohormones BA (4.4 μM), GA_3 (2.9 μM), Kin (2.3 μM), 2,4-D (1.8 μM) and NAA (1.08 μM). The medium was solidified with 0.5% phytagel. Embryogenic callus formation with simultaneous embryo induction was obtained in few of the cultures. Varied response in terms of time and frequency of embryogenic callus initiation was observed when proliferated callus obtained from leaves collected from different source plants were cultured. In the callus developed from *in vitro* derived somatic plants, embryogenic callus initiation was observed within three months with a higher frequency (55 %) and in *in vitro* raised seedlings it was 58%. In callus obtained from leaves of bud grafted plants, the rate was 43% and time taken was about five months. Embryogenic calli originated from more than one region of each callus clump as a small yellow lump and proliferated to form a mass of friable golden yellow callus. The callus derived from mature tree leaves showed no embryogenic potential.

3.b.3.1. Effect of basal medium

For embryogenic callus induction in proliferated callus obtained from leaf explants of different source plants, a factorial experiment was carried out using different concentrations of calcium nitrate (0-1200 mg/l) and sucrose (50-100 g/l). The proliferated callus developed from *in vitro* derived somatic plants that initiated embryogenic callus with a minimum time was used for the experiment. Modification of the earlier optimized medium for faster embryogenic callus initiation showed that $Ca(NO_3)_2 \cdot 4H_20$ (500 mg/l) and sucrose (80 g/l) could efficiently induce embryogenic callus from the proliferated friable callus obtained from somatic embryogenesis derived *in vitro* plants (Table. 24). A phytagel concentration of 0.5 % was used in the medium. In the standardized medium, the callus developed from *in vitro* derived somatic plants initiated embryogenic callus with a minimum time of two months with a higher frequency (68 %). Increasing the concentration of calcium nitrate from 200 to 800 mg/l gradually increased the rate of embryogenic callus induction. At concentrations above 800 mg/l the rate

was not significantly increased and the calli became soft. Similarly at higher concentration of sucrose the tissues remained intact.

Table 24. Effect of $Ca(NO_3)_2 \cdot 4H_20$ and sucrose on initiation of embryogenic cal

Sucrose (mM)	$Ca(NO_3)_2 \cdot 4H_20$ (mM) Rate of Embryogenic callus initiation (Mean)							
	200	**400**	**500**	**600**	**700**	**800**	**900**	**100**
50	27.99	30.00	33.99	39.00	44.01	35.01	41.01	39.0
60	30.00	25.54	45.99	45.99	48.99	18.67	43.50	44.0
70	39.00	42.00	60.00	57.99	57.99	56.00	48.99	45.
80	41.01	56.01	**67.78**	64.98	64.98	57.99	56.01	48.9
90	44.01	48.99	67.98	57.99	64.96	57.99	57.99	41.0
100	42.00	44.01	57.99	56.01	64.98	48.99	48.99	50.1

CD (Ca)= 1.2; CD (Suc.) = 1.30 ;CD (Ca X Suc.) = 3.44
Each value is the mean % of 30 replicates. Experiment was repeated thrice

In the optimized medium also, response of callus in terms of time an frequency of in embryogenic callus initiation was found to vary, when proliferate callus obtained from leaves collected from different source plants were cultured. Th callus developed from *in vitro* derived somatic plants initiated embryogenic callus wit a minimum time of two months with a higher frequency (68 %). In callus develope from *in vitro* derived seedlings also the response was almost similar, and a frequency o 72% was obtained. In callus obtained from leaves of bud grafted plants embryogeni callus induction was obtained at a rate of 33 % (Table. 25). An increase of 10% wa noted in the rate of embryogenic callus emergence with a considerable reduction in time Here the combined effect of calcium and sucrose by varying the osmolarity of th medium along with medium desiccation provided by higher levels of phytagel migh have aided faster embryogenic tissue initiation. The proliferated calli turned brown i this medium and embryogenic calli emerged as a small yellow clump which the proliferated and induced embryos. The callus derived from mature tree leaves did nc show any embryogenic potential.

Table.25. Embryogenic callus initiation rate in media containing 0.5 % phytagel.

Source of leaf explants	Time taken (months)	Embryogenic callus Initiation. Mean (%)
In vitro seedlings	3	72.23± 3.93
In vitro somatic plants	3	68.33 ± 4.64
Budded plants	5	33.33 ± 5.77
Mature trees	---	0

Values given are the mean % of 20 replicated samples repeated thrice

3.b.4. Somatic embryo induction

Further embryo induction was obtained from the proliferated embryogenic calli when the cultures were dark incubated in modified MS basal medium ($Ca(NO_3)_2 \cdot 4H_20$- 250 mg/l) and containing B5 vitamins, amino acids, organic supplements such as coconut water (5%), casein hydrolysate (300 mg/l), 60 g/l sucrose and phytohormones BA (2.2 µM), GA_3 (2.9 µM), Kin (1.25 µM), and NAA (0.54 µM). The rate of embryo induction from the proliferated embryogenic callus was similar irrespective of the source of the leaf, whether *ex vitro* or *in vitro* derived (60-70 %). Rate of embryo induction from the embryogenic callus was similar irrespective of the explant source.

3.b.5. Embryo maturation and plant regeneration

Embryo maturation and plant regeneration occurred in medium standardized earlier. It was found that after three weeks culture in WPM, embryos enlarged and apex induction also occurred. Sucrose (60 gm/l) along with organic supplements and phytohormones, BA (2.2 µM), Kin (1.38 µM), IBA (0.49 µM) and GA_3 (5.3 µM) favored maturation of embryos. Embryo germination was obtained when mature apex induced embryos were transferred to culture tubes containing hormone free MS medium with 0.3% charcoal and solidified with 0.2% phytagel. Full plantlet development occurred within one month in this medium. No difference was observed in embryo maturation and plant regeneration in

explants collected from different sources except the seedling derived plants where 90% of the plants could withstand hardening.

3.c. *Agrobacterium* mediated Genetic Transformation

3.c.1. *Agrobacterium* infection and co-cultivation

Agrobacterium mediated genetic transformation was carried out using proliferated friable callus obtained from leaf explants as target tissue (Fig.18). *Agrobacterium tumefaciens* strains EHA 101 harboring the construct PDU 97.0612 carrying a gene encoding isopentenyltransferase *(ipt)* from the Ti plasmid of *Agrobacterium tumefaciens* was used for infection. When the earlier developed protocol with other explants (Jayashree *et al.*, 2003) was followed for genetic transformation of proliferated leaf callus with *ipt* gene, a transformation frequency of 9% was obtained. Here the infected tissues were cultured in selection medium containing kanamycin (300 mg/l) and carbenicillin (400mg/l). Further experiments on optimization of parameters for controlling bacterial overgrowth and improving the transformation efficiency were carried out using the strain EHA 101 with the binary vector PDU 97.0612 carrying the *ipt* gene.

3.c.2. Influence of media components

3.c.2.1. Effect of silver nitrate

Bacterial overgrowth in the *Agrobacterium* infected tissue was common causing hindrance to transgenic tissue regeneration during most of the earlier experiments. In the present study, when different levels of silver nitrate was supplemented in the infection, co-cultivation and selection medium it was found that overgrowth of bacteria could be controlled. Tissues were infected in infection medium containing silver nitrate (10 mg/l) and transferred for co-cultivation. The cocultivation medium was also supplemented with silver nitrate (10 mg/l). After 72 hours of cocultivation the tissue was subcultured in selection medium containing selection antibiotic carbenicillin (400 mg/l). When different concentration of silver nitrate (0-30 mg/l) was supplemented in the selection medium, it was observed that with increasing concentration of silver nitrate the bacterial over growth was reduced significantly. In the control, when no silver nitrate was provided, 50% of the cultures showed bacterial overgrowth. In

medium containing 20 mg/l silver nitrate, only 20% bacterial overgrowth was observed while increasing the silver nitrate concentration to 30 mg/l did not give significant difference. The tissue recovered free of bacterial overgrowth were subcultured every three weeks in fresh medium. In the present study, it was observed that in tissues where *Agrobacterium* infection was done in presence of silver nitrate (10 mg/l) in the infection and co-cultivation medium and subcultured in the selection medium containing 10 mg/l silver nitrate, bacterial overgrowth could be controlled up to 80% (Table. 26). Maximum control was obtained at silver nitrate concentration was 20 mg/l. Control of bacterial overgrowth was significant at silver nitrate concentrations starting from 5.0 mg/l onwards. At concentrations above this significant difference was noted in control of overgrowth (Fig.19. A.). In the present study when silver nitrate was supplemented in the medium, the texture of the putatively transgenic callus lines that emerged from the infected tissue was found to be improved, with most of them friable, yellow and showing good proliferation. In the control experiments, the texture of the callus in the putatively transgenic lines that emerged from the infected tissues also varied, with most of the calli being hard and showed no proliferation.

Table.26. Effect of silver nitrate in controlling bacterial over growth

Silver nitrate (mg/l) (selection medium)	Overgrowth (Mean %)
0	50.0 (3.87)
5	30.0 (2.59)
10	22.0 (1.72)
15	22.5 (1.72)
20	20.2 (1.57)
30	20.4 (1.57)
CD	0.3

Data from 30replicate samples ,experiment repeated thrice

Values in paranthesis indicate transformed values

Surfactants may enhance T-DNA delivery by aiding *Agrobacterium* attachment or by elimination of substances that inhibit it. In the present study it was observed that addition of the surfactant, pluronic F68 (300 mg/l) to the modified infection medium helped in 2% increase in the transformation efficiency (Table. 27).A transformation frequency of 11.1 % was obtained when 300 mg/ l pluronic F-68 was added to the *Agrobacterium* infection medium. At higher concentrations of the surfactant significant difference was not observed indicating that concentrations above the optimum identified did not have a pronounced effect on increasing transformation efficiency.

Table. 27. Effect of Pluronic F-68 on transformation efficiency

Pluronic F68 (mg/l) (selection medium)	**% transformation efficiency**
0	9.1 (1.64)
100	10.0 (1.72)
200	10.2 (1.97)
300	**11.1 (2.28)**
400	11.1 (2.28)
500	10.8 (1.97)
CD	0.39

Data from 30replicate samples ,experiment repeated thrice

Values in paranthesis indicate transformed values

3.c.2.3. Effect of L-cysteine and α-Lipoic acid

Inclusion of the antioxidant L– cysteine (100 mg/l) in the infection, cocultivation and selection medium containing silver nitrate helped in improving the transformation frequency by 5 %. Presence of L-cysteine along with silver nitrate helped in reducing overgrowth and improving quality of callus in newly formed transgenic lines, along with improvement in transformation frequency. It was observed that presence of 100 mg/l cysteine in the infection, cocultivation, and selection medium in presence of Pluronic F68 and $AgNO_3$ increased the transformation frequency from 11 to 16%. From table. 28 it can be seen that L–cysteine concentrations of above 250 mg/l negatively influenced transformation efficiency. The transgenic calli obtained from the infected tissues was of improved

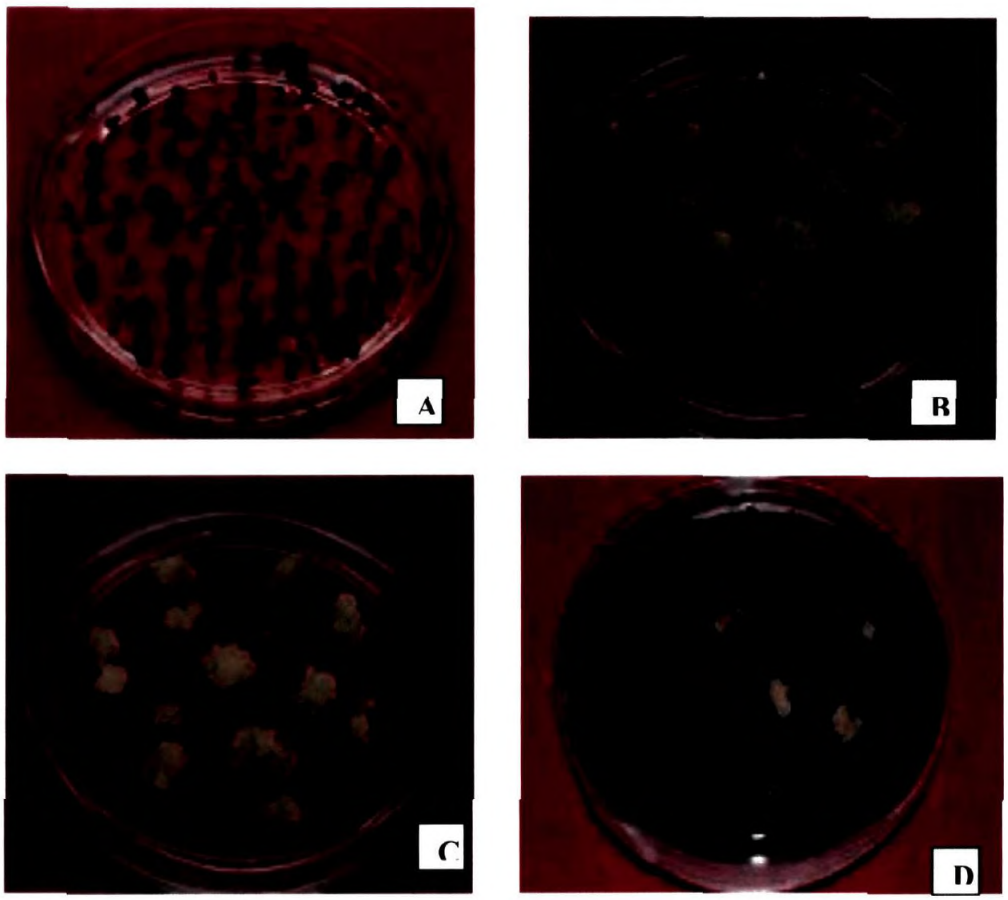


Fig.19. (A – D) Different stages of transgenic cell line formation in the selection medium

A. *Agrobacterium* infected callus in selection medium
B. Development of transgenic cell lines
C. Proliferation of cell lines
D. Embryogenic callus formation

texture than the control. The callus was yellow and friable and showed good proliferation.

Table.28. Effect of different concentrations of L-cysteine on transformation efficiency

L-cysteine (mg/l) (selection medium)	Transformation efficiency
0	11.1 (1.64)
50	13.3 (1.99)
100	**15.6 (2.23)**
200	15.6 (2.23)
250	14.6 (2.12)
300	13.5 (2.06)
CD	**0.29**

Data from 30 replicate samples,experiment repeated thrice
Values in paranthesis indicate transformed values.

Addition of another antioxidant α-Lipoic acid (50 mg/l) in the infection and cocultivation medium and subculture of the infected tissues in selection medium containing α-Lipoic acid (50 mg/l), the transformation frequency was improved by 5 %. From the table.29 it can be inferred that, addition of α-Lipoic acid to the selection medium containing silver nitrate resulted in an increase in transformation frequency from 11to16%. Maximum transformation efficiency was obtained when 50 mg/l α-Lipoic acid was supplemented in the selection medium. Here also increasing the concentration above the optimum did not result in a significant increase in transformation efficiency. Thus by addition of either L-cysteine (100 mg/l) or α-Lipoic acid (50 mg/l) to the infection, cocultivation and selection medium improve transformation efficiency (Fig.19 B).

Proliferated leaf callus was proved to be an amenable example for *Agrobacterium* mediated genetic transformation (Fig.19.A-D).

Table .29. Effect of α- lipoic acid on transformation efficiency

α-lipoicacid (mg/l) (selection medium)	Transformation efficiency (%)
0	11.1 (1.64)
25	13.3 (2.69)
50	**16.4 (3.87)**
75	15.5 (3.64)
100	14.8 (3.74)
CD	**1.53**

*Data from four replicate experiments using 30 callus clumps per petri plate.

Values in paranthesis indicate transformed values

3.c.3. *Somatic embryogenesis*

Proliferation of the putatively transgenic callus, embryogenic callus initiation and further embryo induction were obtained in medium standardized earlier for leaf explants (section 3.a.3.2) (Fig.19.C&D). Kanamycin (200 mg/l) was also added to all the medium used for subculture of transgenic callus. Callus proliferation could be obtained in modified MS (Murashige and Skoog, 1962) medium with the addition of calcium nitrate (800 mg/l) and containing Gamborg B_5 vitamins, sucrose (40 gm/l) and growth regulators 2,4-D (1.8 μM), BA (4.4 μM) and NAA (1.08 μM). Embryogenic callus initiation and embryo induction was obtained from proliferated callus in modified MS basal medium ($Ca(NO_3)_2 \cdot 4H_20$- 300 mg/l) containing B5 vitamins, amino acids, organic supplements such as coconut water (5%), casein hydrolysate (300 mg/l), sucrose (80 g/L) and phytohormones BA (4.4 μM), GA_3 (2.9 μM), Kin (2.3 μM), 2,4-D (1.8 μM) and NAA (1.08 μM) (Fig.20. A&B). Amino acids present in the medium were glutamine (500 mg/l), proline (100 mg/l), L-cysteine (50 mg/l), and arginine (40 mg/l). The medium was solidified with 0.5% phytagel. Rate of embryo induction from the transformed callus was low (40%) compared to untransformed controls (60%). Embryos obtained were small compared to those induced in controls (Fig.20. A- D). Maturation of the embryos could also be obtained in the medium standardized earlier (Section 3.a.5.3.). Plant regeneration from these embryos are being attempted.

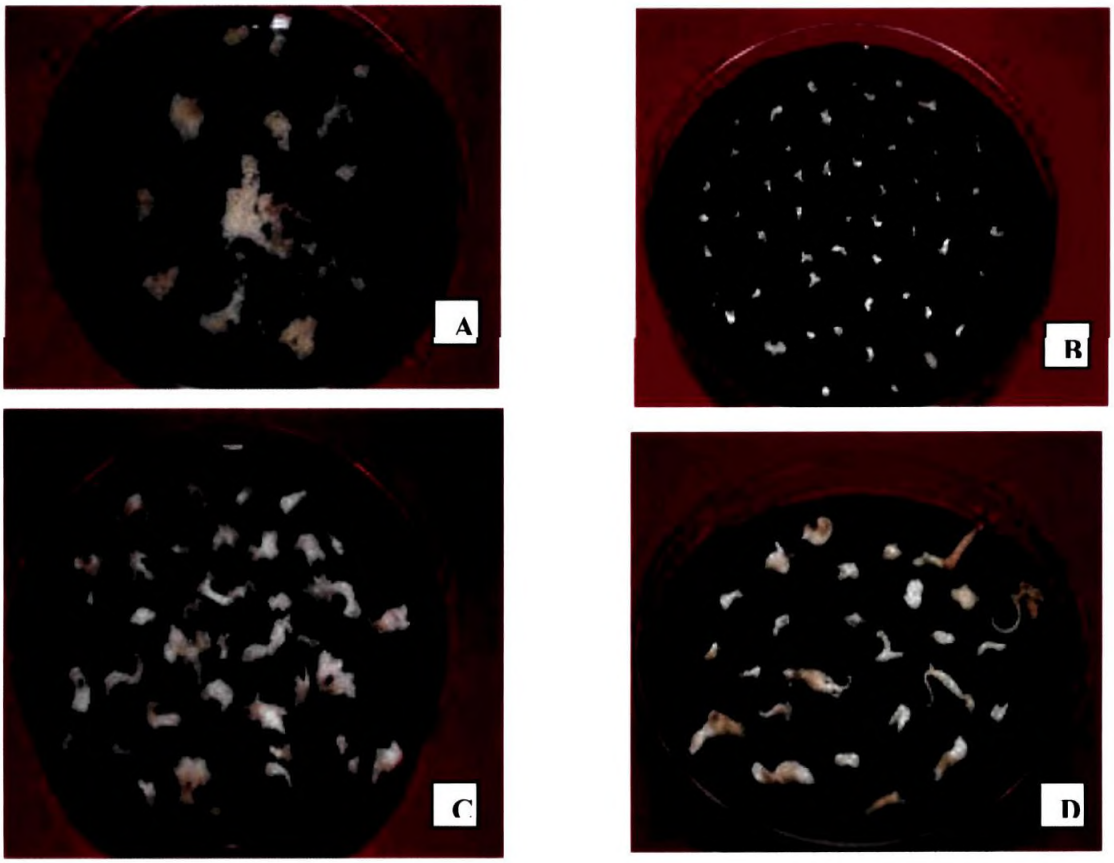


Fig.20. (A –D) Different stages of embryogenesis of transgenic tissue

A. Embryogenic callus proliferation
B. & C. Embryo induction
D. Embryo maturation

3.c.4. Molecular confirmation of *ipt* gene in transgenic tissues

3.c.4.1. GUS Histochemical assay

Approximately 2 mg callus from each new line was used for GUS histochemical staining. β- glucuronidase catalyses the hydrolysis of the substrate liberating indoxyl and indolyl groups which gets dimerized to form insoluble indigo causing blue colouration. When putatively transgenic callus which are antibiotic resistant and showed proliferation in selection medium was tested for GUS activity it was observed that the newly emerged lines stained blue colour. (Fig.21.C). When the antibiotic resistant lines were randomly selected and subjected to GUS assay, all the samples were found to be GUS positive.

3.c.4.2. PCR Amplification of *ipt* gene

Good quality DNA could be isolated from leaves of *Hevea brasiliensis* clone RRII 105. The genomic DNA isolated from the normal and transgenic callus was used for PCR amplification using *ipt* gene specific primers. In an agarose gel the clear band was observed without shearing of DNA and RNA contamination (Fig.21.A). The RNase treatment helped to get RNA free DNA. 2μl (20ng) was used as template for PCR analysis.

When PCR amplification was carried out with gene specific primers for isopentenyltransferase gene an amplified band of approximately 700 bp was obtained. Amplification of the gene was obtained only in the transgenic callus showing gene integration. No amplification could be obtained in the primer controls and in untransformed callus (Fig.21.B).

Efficient transformation systems using easily available explants are indispensable for genetic modification of agronomically important plants. Proliferated leaf callus was proved to be an amenable example for *Agrobacterium* mediated genetic transformation in *Hevea.* It was observed that addition of silver nitrate in the infection (10.0 mg/l), cocultivation (10.0 mg/l) and selection (20.0 mg/l) medium significantly suppressed bacterial overgrowth and improved the texture of callus in newly emerged lines. Improvement in the transformation frequency by 7% was obtained by the inclusion of either of the thiol compounds

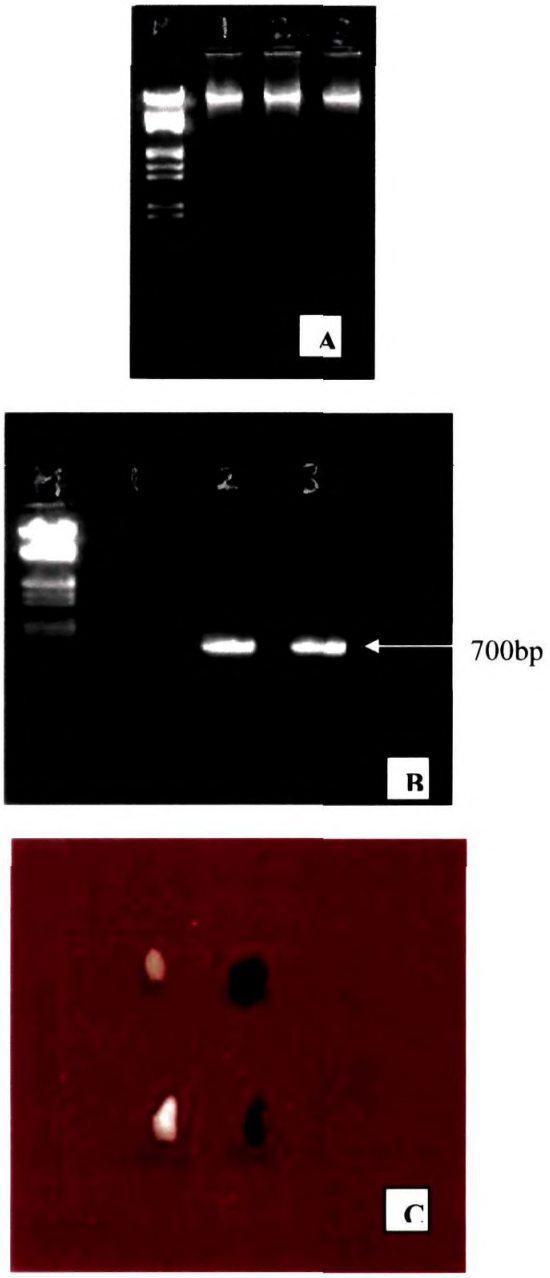


Fig. 21. (A-C). Molecular confirmation of *ipt* gene in transgenic tissue.

A. Isolation of DNA from callus (Lanes, M- Marker; 1- DNA from non transgenic control; 2-3- DNA from transgenic callus)
B. PCR amplification of 700 bp *ipt* gene ((Lanes, M- Marker; 1- no amplification in non transgenic control; 2-3- amplification from transgenic callus.
C. GUS staining in control & transgenic callus and embryo

lipoic acid (50 mg/l or L– cysteine (100 mg/l) in the infection, cocultivation and selection medium and addition of the surfactant pluronic F68 (300 mg/l) in the infection medium. The putatively transgenic lines that showed continued growth in the selection medium were also proved to be GUS positive and gave PCR amplification with *ipt* gene specific primers. The infection, co-culture, selection medium components and *Agrobacterium* strains significantly influence T-DNA delivery, integration and stable transformation.

3.d. Characterization of phase change related genes and their expression

3.d.1. Identification of phase change related genes from *Hevea*

3.d.1.1. Isolation of genomic DNA

Good quality DNA could be isolated from leaves of *H. brasiliensis,* Clone RRII 105 (Fig.22.A). Since, RNase treatment has been given while isolation, the DNA isolated was found to be free from RNA. The genomic DNA isolated from the leaves was used for PCR amplification using the primers designed for selected gene.

3.d.1.3. PCR amplification of phase change related genes

PCR amplification was carried out using the primers for Chlorophyll a/b binding protein gene (Cab gene), Glossy 15 gene, Dihydro flavonol reductase, AAA-ATPase, QRCPE and SQUINT genes mentioned in section (3.d.1.3.). With the primer for Cab gene, a specific amplification of a single band was obtained. The amplified product was about 0.5 kb (Fig.22. B & 23.A). Although, with Glossy 15 gene specific primers, a band of 1.3 kb was amplified, while sequencing it was found as a non specific amplification and therefore results are not shown. With the primers for other genes no specific amplification was obtained. Therefore, the 0.5 kb band obtained with Cab gene alone was selected for further cloning and expression studies.

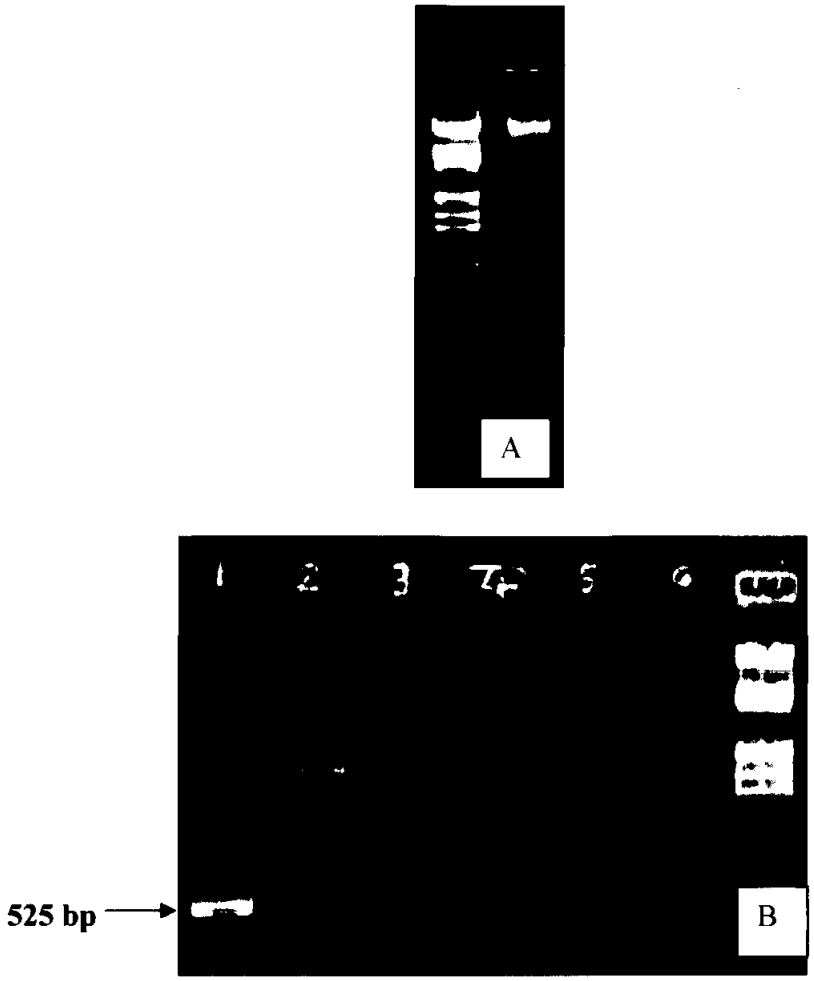
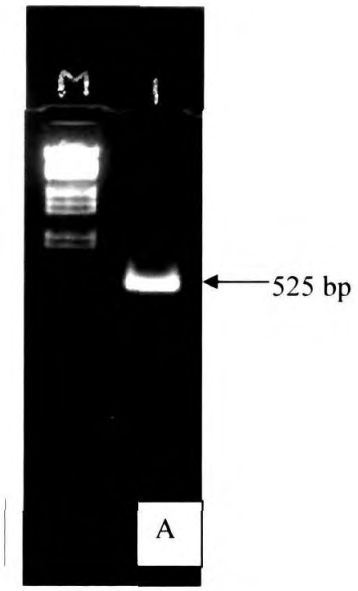


Fig.22. (A- C) Amplification of phase change related genes from genomic DNA

A. Genomic DNA (Lane 1-Marker, 2- DNA)

B. PCR amplification of Genomic DNA (1- Cab, 2- Glossy 15, 3- DFR, 4 -AAA-ATPase, 5-SQUINT, 6- QRCPE)



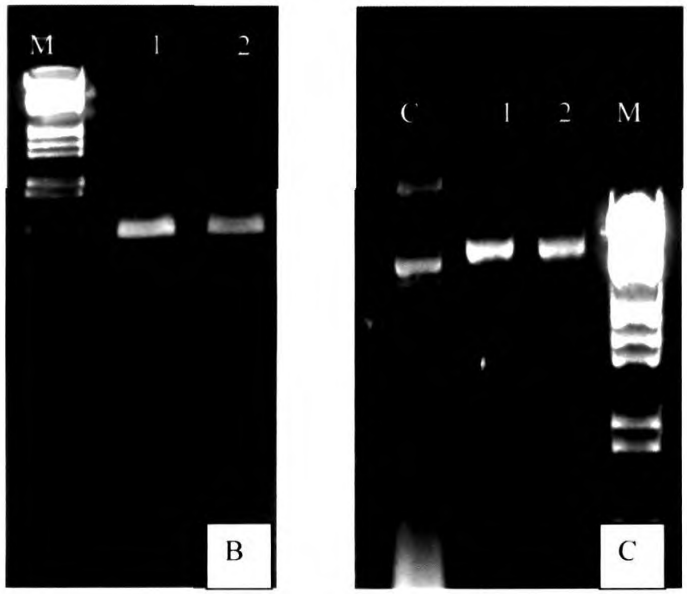


Fig.23.(A- C) PCR amplification and cloning of the 525 bp Cab gene from genomic DNA

A. PCR amplification of Cab gene. M-Marker,1-Cab gene

B. Colony PCR of the 0.5 kb cab gene insert from the transformed colonies

C. Isolation of recombinant plasmids; C-Control, 1&2 recombinant plasmids, M-Marker

3. d.2. Cloning and characterization of the PCR products

The 0.5 kb amplified product obtained with Cab gene specific primers was cloned in StrataClone^{TM} vector (M/S Strataclone, USA). Colony PCR of the white colonies identified were also showed the amplification of the same 0.5 kb fragment. Plasmids were isolated from the positive colonies. Positive transformants were identified through colony PCR (Fig. 23.B).The isolated *plasmids from the PCR positive colonies were sequenced after the PCR* confirmation of the plasmids. (Fig.23.D). The sequencing has been done using M13 forward primer the gene sequence has been shown in Fig.:24.

Fig. 24. Nucleotide sequence of the 0.5 kb Cab gene amplified.

5'
GTACTTGGGTCCATTCTCTGGTGAGCCCCCATCCTACTTGACCGGTGA
GTTCCCTGGTGACTATGGCTGGGACACTGCTGGTCTCTCTGCTGACCCA
GAAACCTTTGCCAAGAACCGTGAGCTCGAAGTGATCCACTGCAGATGG
GCCATGCTTGGAGCCCTTGGGTGCGTCTTCCCCGAGCTCTTGGCCCGCA
ACGGAGTCAAGTTCGGCGAGGCAGTGTGGTTCAAGGCAGGAGCCCAG
ATCTTCAGCGAGGGTGGTCTTGACTACTTGGGTAACCCAAGCTTGATCC
ACGCACAAAGCATCTTGGCCATCTGGGCCGTCCAGGTAGTGTTGATGG
GTGCCGTTGAAGGTTACAGAATTGCCGGTGGGCCGCTCGGTGAGGTCA
CAGACCCAATCTACCCAGGTGGAAGCTTTGACCCATTGGGCTTGGCTG
ATGACCCAGAAGCATTTGCTGAGCTGAAGGTGAAGGAGATCAAGAAC
GGCAGATTGGCTATGTTCTCCATGTTC**GGATTCTTTGTTCAGGC** 3'
Forward and reverse primers

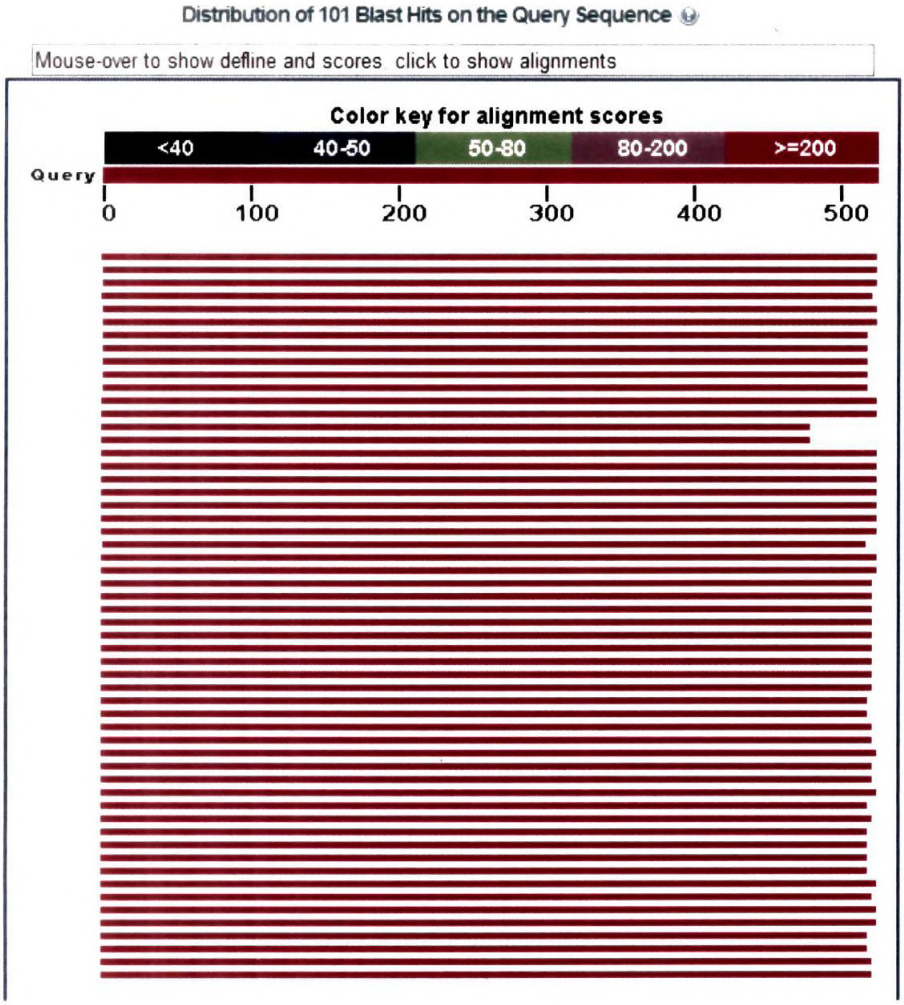
The sequence analysis showed that the isolated gene fragment is having 525 base pairs after deleting the vector sequences (Fig.24). The sequence comparison using BLAST N analysis of the isolated sequence with the reported cDNA sequence of cab gene showed 91% sequence homology in the 525 base pair region with *Ricinus communis* (NCBI accession No: M60274). BLAST N analysis also revealed sequence homology with cab mRNA sequence from other species like *Manihot esculenta* (88%) and *Oryza sativa* (79%) (Fig.25. A&B).

Fig. 25. A. Blastn analysis of 525 bp Cab gene sequences producing significant alignment

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XM_002524570.1	Ricinus communis chlorophyll A/B binding protein, putative, mRNA	732	732	100%	0.0	91%	G
XM_002307689.1	Populus trichocarpa light-harvesting complex II protein Lhcb1 (Lhcb1-3), mRNA	719	719	99%	0.0	91%	UG
EF146739.1	Populus trichocarpa clone WS01213_B15 unknown mRNA	719	719	99%	0.0	91%	UG
XM_002519678.1	Ricinus communis chlorophyll A/B binding protein, putative, mRNA	715	715	99%	0.0	91%	G
XM_002306891.1	Populus trichocarpa light-harvesting complex II protein Lhcb1 (Lhcb1-2), mRNA	713	713	99%	0.0	91%	UG
EF147785.1	Populus trichocarpa clone WS0125_A05 unknown mRNA	713	713	99%	0.0	91%	UG
EF148774.1	Populus trichocarpa x Populus deltoides clone WS0137_M02 unknown mRNA	708	708	98%	0.0	91%	
EF148596.1	Populus trichocarpa x Populus deltoides clone WS0134_I20 unknown mRNA	708	708	98%	0.0	91%	
EF148766.1	Populus trichocarpa x Populus deltoides clone WS0137_J16 unknown mRNA	702	702	98%	0.0	91%	

Maximum sequence homology was obtained with *Ricinus* communis belonging to the same family, Euphorbiaceae. On comparison with cDNA sequence of *Ricinus communis*, no introns were observed in the amplified region. The genomic sequence has been registered in NCBI with the accession no:HM803119.1.

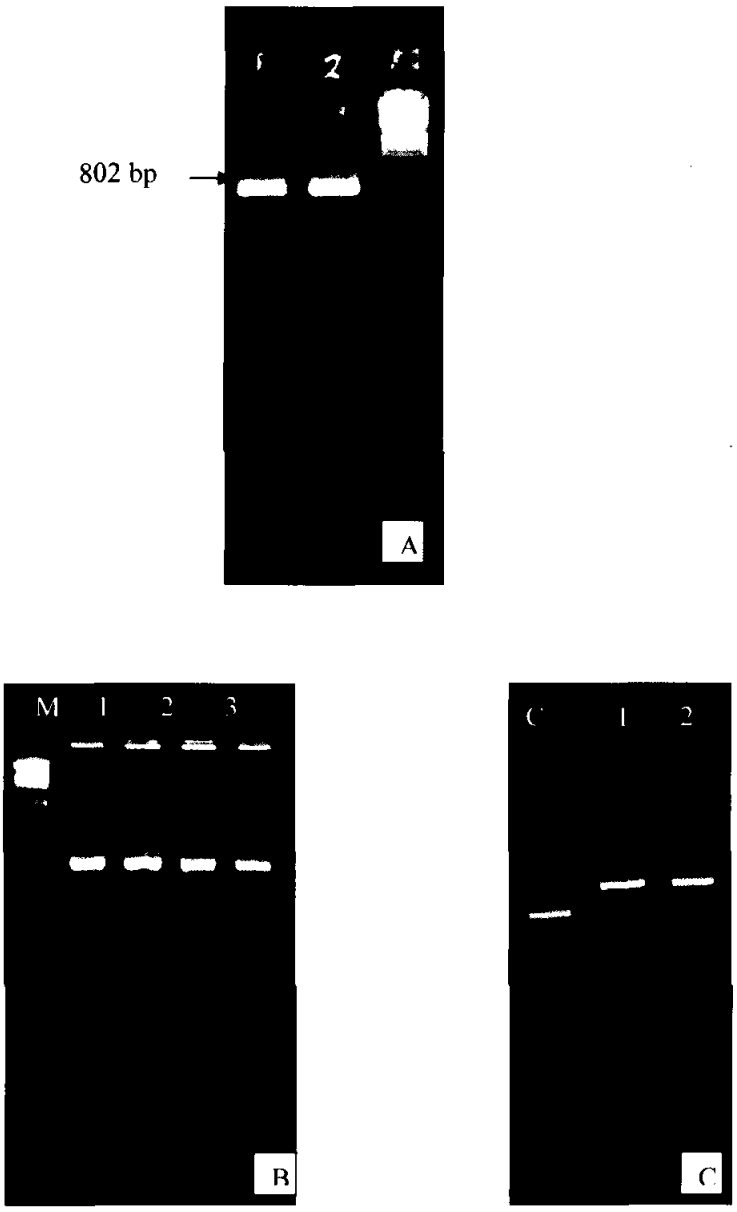
Fig. 25.B. Blastn sequence alignment score of 525 bp Cab



3.d.3. PCR Amplification of full length Cab gene from genomic DNA

Since the sequence showed 91% (maximum) homology with *Ricinus communis*, an attempt has been made to amplify the full coding region of the sequence by designing primers from cDNA sequence of Cab gene reported in that species (Accession No.XM_002524570.1).

Using genomic DNA isolated earlier, specific amplification could be obtained using the primer designed for full length Cab protein gene after optimization of PCR conditions (Fig.26.A). PCR amplified product had a molecular weight of approximately 0.8 kb.



g. 26. (A-C) Full length Cab gene amplification

A. Cab 802 bp cab gene amplification, 1&2 Cab amplification, M-Marker
B. Colony PCR of transformed colonies
C. Isolation of recombinant plasmids; C- Control, 1&2- recombinant plasmids

PCR amplified fragment was cloned (Fig.26.C&D) and sequenced. The nucleotide sequenced revealed the presence of 802 base pairs (Fig.27). The sequence on comparison with the reported cDNA sequence of cab gene from *Ricinus communis* (Accession. No: XM002524570.1) using CLUSTAL W (Thompson *et al.*, 1994) showed 90 % sequence homology.

Fig. 27. The nucleotide sequence of the full length coding region of Cab gene from *Hevea basiliensis*

5'

CAAATGGCTACCTCTACAATGGCCCTCTCCTCCCCCTCCTTCGCCGGC
AAGGCGGTGAAACTCACCCCATCTGCCCCTGGGCTCATGGGCAATGCC
CGTGTCTCAATGAGGAAATCTGTTGGCAAGCCTGTTTCATCTGGAAGCC
CATGGTATGGTCCAGACCGTGTTAAGTACTTGGGTCCATTCTCTGGTGA
GCCCCCATCCTACTTGACCGGTGAGTTCCCTGGTGACTATGGCTGGGAC
ACTGCTGGTCTCTCTGCTGACCCAGAAACCTTTGCCAAGAACCGTGAGC
TCGAAGTGATCCACTGCAGATGGGCCATGCTTGGAGCCCTTGGGTGCG
TCTTCCCCGAGCTCTTGGCCCGCAACGGAGTCAAGTTCGGCGAGGCAG
TGTGGTTCAAGGCAGGAGCCCAGATCTTCAGCGAGGGTGGTCTTGACT
ACTTGGGTAACCCAAGCTTGATCCACGCACAAAGCATCTTGGCCATCT
GGGCCGTCCAGGTAGTGTTGATGGGTGCCGTTGAAGGTTACAGAATTG
CCGGTGGGCCGCTCGGTGAGGTCACAGACCCAATCTACCCAGGTGGAA
GCTTTGACCCATTGGGCTTGGCTGATGACCCAGAAGCATTTGCTGAGCT
GAAGGTGAAGGAGATCAAGAACGGCAGATTGGCTATGTTCTCCATGTT
CGGATTCTTTGTTCAGGCCATTGTGACAGGAAAGGGACCATTGGAGAA
CTTGGCTGACCACCTTGCTGACCCTGTCAACAACAATGCCTGGGCTTAC
GCCACAAA**CTTTGTCCCCGGAAAGTGAG 3'.**

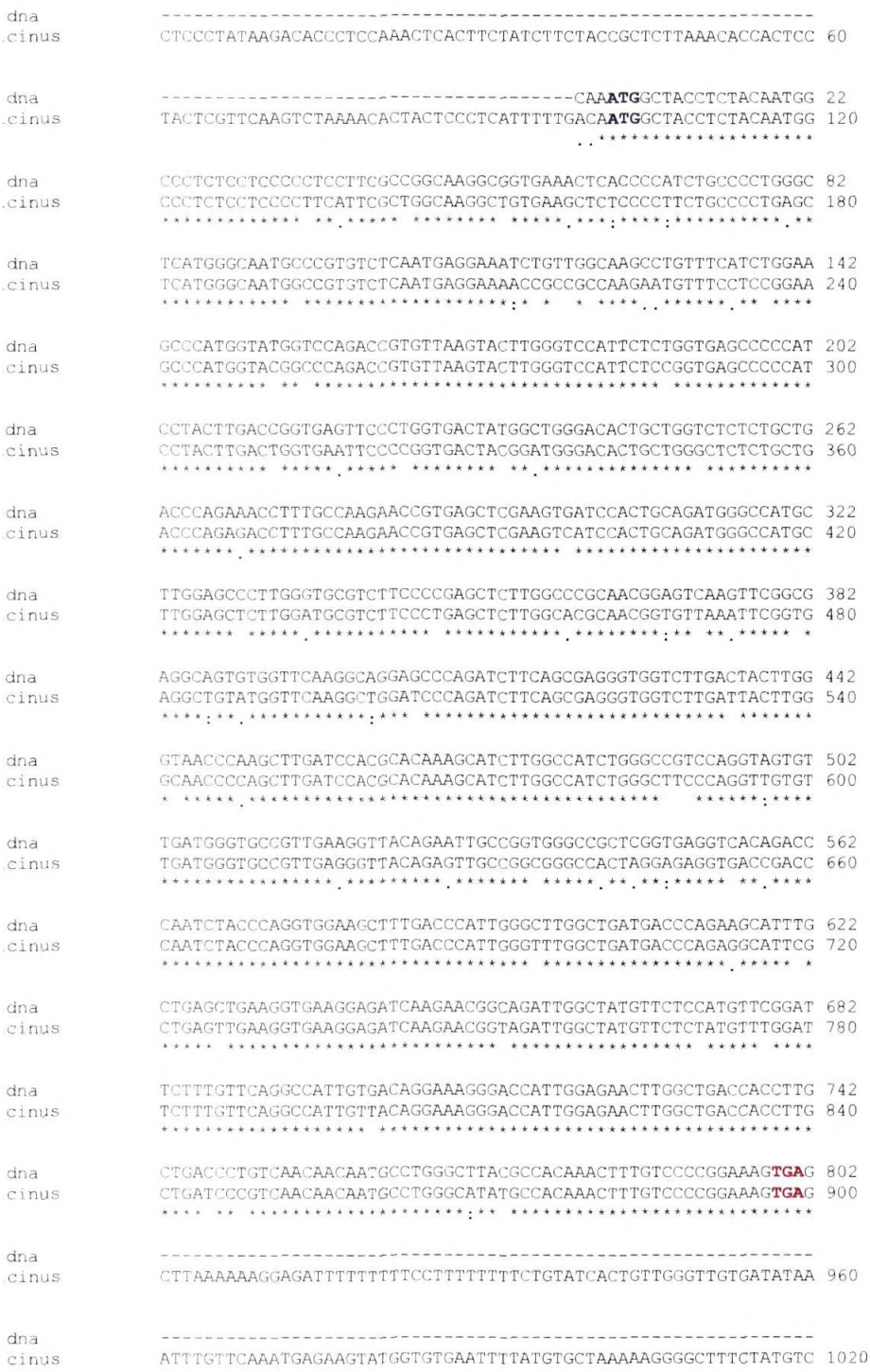
Start codon

Stop codon

Forward and reverse primers

The amplified sequence contained 802 bps. The region amplified in the present study contains the full protein coding sequence. The start codon 'ATG' starts from the 4th position and TGA the stop codon starts at the 799th position. The number of coding DNA sequence contains 798 nucleotides including the stop codon. On comparison with the other reported mRNA sequences from other species, the the 802 bp full length coding region isolated also contains no introns. The sequence showed higher homology (91.1 %) to the mRNA sequence reported from *Ricinus communis (Acc. No:* XM_002524570.1) (Fig. 28). The phylogenetic tree analysis showed the close relationship of the Cab gene of *Hevea* with *Ricinus comminis* (Euphorbiaceae) and *Populus trichocarpa* apart from the other eukaryotic plants like *Solanum*, *Gossypium*, *Tobacco* etc (Fig. 29). The predicted aminoacid sequence using the Expasy prot- param tool showed that the gene codes

ig 28. CLUSTALW multiple sequence alignment of the isolated genomic DNA sequence of Chlorophyll a/b gene with *Ricinus comminis.*

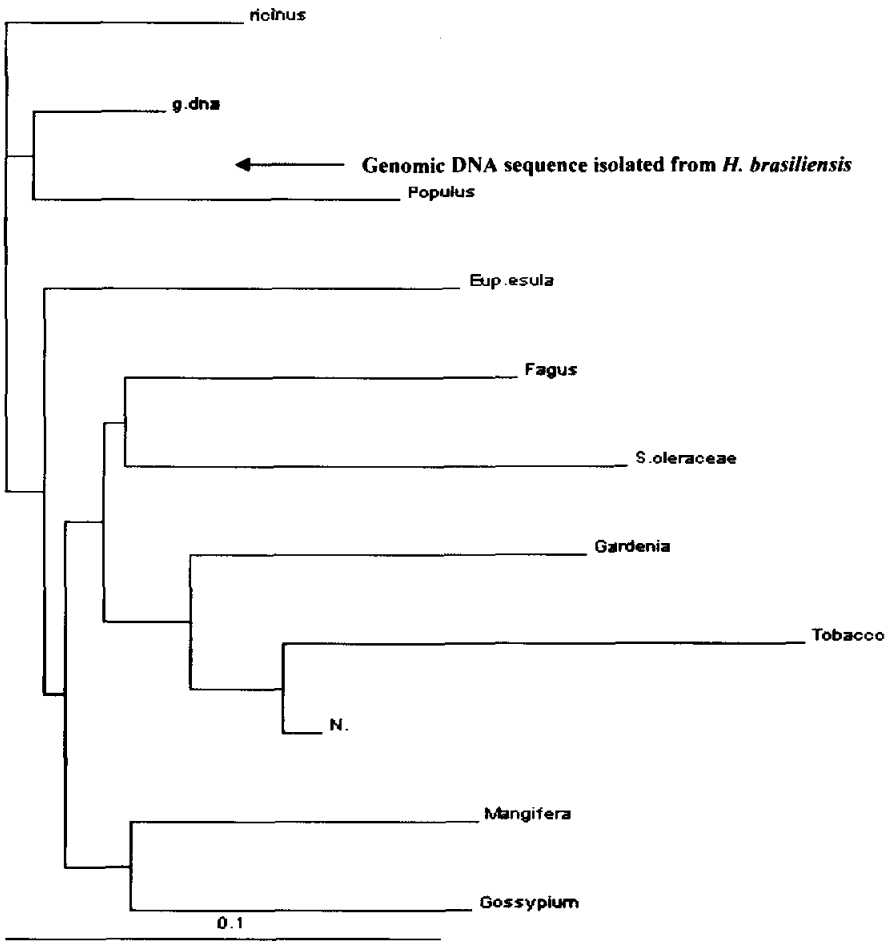


```
g.dna           ------------------------------------------------------------
ricinus         ATTATCTTATGAATAACACTTGCACTGGACAACCAACATTTGAGAGAAAACGCCGCGACA 1080

g.dna           ------------------------------------------------------------
ricinus         GTAAAATGACAATTAGTGGCAGTGCAACTAATATCAAATTAAAATTAGCATGAAACTTGT 1140

g.dna           ----------------------------------------------------
ricinus         GAGATCAGAAAGAGTAAAATGCGCGTTCTAAAGCACCGCTTGTGCCCTGCAA 1192
```

Start codon
Stop codon

Re.

Fig: 29 Phylogenetic tree analysis of the isolated full length genomic DNA sequence of *H. brasiliensis* with the other reported sequences from the eukaryotes.



Ricinus : 0.05469,
Genomic DNA from *Hevea* : 0.03006,
Populus :0.08465 : 0.00628,
Eup.esula : 0.09579,
Fagus : 0.09021,
S.oleraceae : 0.11591:0.00494,
Gardenia : 0.0913,
Tobacco : 0.12005, N.: 0.00888: 0.0213: 0.02021: 0.00876,
Mangifera : 0.08004,
Gossypium :0.0786: 0.01517 :0.0047: 0.00891.

for a protein with 265 amino acids. The molecular weight of the predicted protein is 28.1 kDa. The native protein is with an isolelectric pH of 5.45. The details of the deduced amino acids and other details are given in Fig. 30.

Fig. 30. The deduced amino acid sequence and other details of the predicted protein of the 802 bp Cab gene isolated.

MATSTMALSSPSFAGKAVKLTPSAPGLMGNARVSMRKSVGKPVSSGSPWYGPDRVKYLGP
FSGEPPSYLTGEFPGDYGWDTAGLSADPETFAKNRELEVIHCRWAMLGALGCVFPELLAR
NGVKFGEAVWFKAGAQIFSEGGLDYLGNPSLIHAQSILAIWAVQVVLMGAVEGYRIAGGP
LGEVTDPIYPGGSFDPLGLADDPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLE
NLADHLADPVNNNAWAYATNFVPGK-
265 amino acids.
Molecular weight: 28108.1
Theoretical pI: 5.45
Total number of negatively charged residues (Asp + Glu): 25
Total number of positively charged residues (Arg + Lys): 21

3.d.4. Expression analysis of Cab gene from juvenile and mature plants

3.d.4.2. RNA Isolation and first strand cDNA synthesis

RNA was isolated from medium mature leaves of different sources such seedlings, *in vitro* somatic plants, six month old bud grafted plants and mature trees of clone RRII 105. The RNA was checked for DNA contamination and quality by agarose gel electrophoresis. Good quality RNA with minimal DNA contamination could be obtained (Fig.31.A). Before any downstream applications, the RNA was subjected to *DNase* treatment to completely remove the DNA contamination any.

First strand cDNA synthesis was synthesised from the isolated RNA by reverse transcription reaction with oligo-(dT) primers using the 'Improm-II^{TM} Reverse Transcription System' (Promega, USA).

3.d.4.3. PCR amplification, cloning and characterization of cDNA encoding Cab gene

The Cab protein cDNA was amplified using the primer pairs used to amplify the 525 bp genomic sequence using cDNA developed from the medium mature leaves of bud grafted plants. In this experiment also a 0.5 kb single band was amplified (Fig.31.B). After cloning and sequencing, it was observed that, the cDNA also contains a 525 bps as observed in the genomic DNA. On alignment

with the 802 bp genomic sequence characaterized earlier, it showed 100 % homology (Fig:.32).

3.d.5. RT-PCR assay for differential Cab gene expression

Cab gene amplification could be obtained from cDNA synthesized from RNA isolated from leaves of seedlings, somatic plants, six month old bud grafted plants and mature trees using the following primer sequences. Differential expression of the gene was also observed when RT-PCR was carried out with cDNA synthesized from seedlings, somatic plants, six month old bud grafted plants and mature trees (Fig.31.C). Expression of the gene was found to be more in seedlings and *in vitro* derived plants which are juvenile and showing better *in vitro* culture response. With maturity of the plant expression of the gene was found to be reduced with the leaves from the mature trees showing the minimum expression. The β-actin gene was found to be constitutively expressed in all the samples (Fig.31.D) which shows that equal amount of RNA has been loaded in all the wells.

3.d.6. Gene Expression Studies through Northern Hybridization

When northern analysis was carried out using the RNA isolated from leaves of different sources such as seedlings, somatic plants, six month old bud grafted plants and mature trees using the 525 bp (Fig.33.A&B). Cab cDNA as the probe, bands with different intensity was obtained although equal amount of RNA was loaded which indicates differential expression of the gene in different samples. The *in vitro* plants raised through somatic embryogenesis showed the maximum expression and the mature tree leaves showed the minimum expression as evident from the blots. In all the lanes equal amount of RNA was loaded. When northern analysis was conducted with leaves of three different stages such as immature, medium mature and mature leaves from the six month old bud grafted plants, maximum expression was observed in the medium mature leaves with immature and mature leaves showing slightly less activity (Fig.33.C&D).

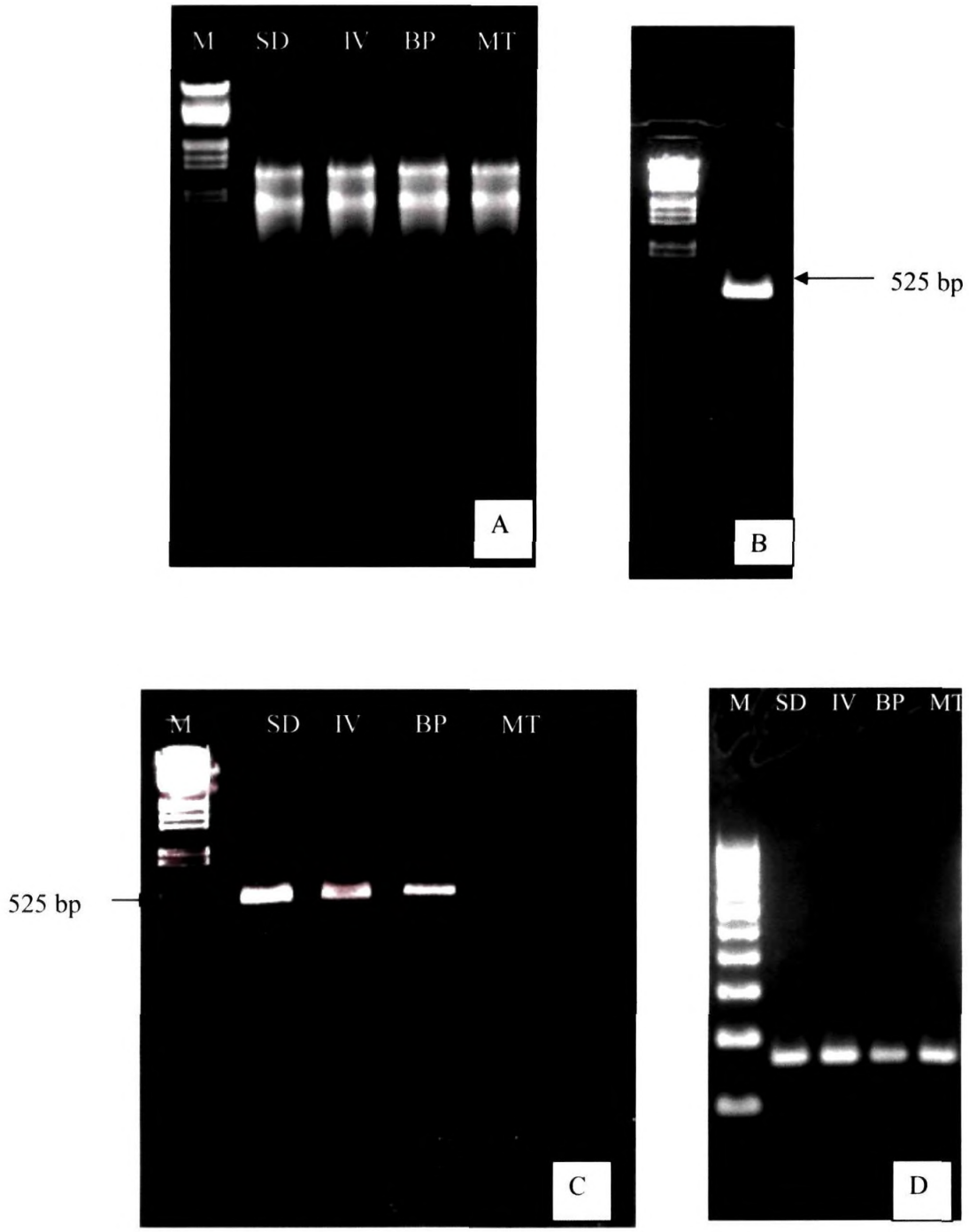


Fig.31 (A-D).Differential expression Cab gene

A. RNA of different samples (Lane 1-Marker, 2- Seedling, 3-Bud grafted plant, 4-Mature tree)

B. B. Cab gene amplification from cDNA.

C. RT-PCR showing differential Cab gene expression (Lane 1-Marker, 2- Seedling, 3-Bud grafted plant, 4-Mature tree)

D. β-actin amplification in different samples (Lane 1-Marker, 2-Seedling, 3-Bud grafted plant, 4-Mature tree)

Fig 32. CLUSTALW multiple sequence alignment of cdna and genomic dna sequences of Chlorophyll a/b gene.

```
cdna            ----------~----------
g.              CAAATGGCTACCTCTACAATGGCCCTCTCCTCCCCCTCCTTCGCCGGCAAGGCGGTGAAA 60

cdna            ----------~----------
g.              CTCACCCCATCTGCCCCTGGGCTCATGGGCAATGCCCGTGTCTCAATGAGGAAATCTGTT 120

cdna            ----------~----------GTACTTGGGT 10          TGGGT10
g.              GGCAAGCCTGTTTCATCTGGAAGCCCATGGTATGGTCCAGACCGTGTTAAGTACTTGGGT 180
                                                **********

cdna            CCATTCTCTGGTGAGCCCCCATCCTACTTGACCGGTGAGTTCCCTGGTGACTATGGCTGG 70
g.              CCATTCTCTGGTGAGCCCCCATCCTACTTGACCGGTGAGTTCCCTGGTGACTATGGCTGG 240
                ************************************************************

cdna            GACACTGCTGGTCTCTCTGCTGACCCAGAAACCTTTGCCAAGAACCGTGAGCTCGAAGTG 130
g.              GACACTGCTGGTCTCTCTGCTGACCCAGAAACCTTTGCCAAGAACCGTGAGCTCGAAGTG 300
                ************************************************************

cdna            ATCCACTGCAGATGGGCCATGCTTGGAGCCCTTGGGTGCGTCTTCCCCGAGCTCTTGGCC 190
g.              ATCCACTGCAGATGGGCCATGCTTGGAGCCCTTGGGTGCGTCTTCCCCGAGCTCTTGGCC 360
                ************************************************************

cdna            CGCAACGGAGTCAAGTTCGGCGAGGCAGTGTGGTTCAAGGCAGGAGCCCAGATCTTCAGC 250
g.              CGCAACGGAGTCAAGTTCGGCGAGGCAGTGTGGTTCAAGGCAGGAGCCCAGATCTTCAGC 420
                ************************************************************

cdna            GAGGGTGGTCTTGACTACTTGGGTAACCCAAGCTTGATCCACGCACAAAGCATCTTGGCC 310
g.              GAGGGTGGTCTTGACTACTTGGGTAACCCAAGCTTGATCCACGCACAAAGCATCTTGGCC 480
                ************************************************************

cdna            ATCTGGGCCGTCCAGGTAGTGTTGATGGGTGCCGTTGAAGGTTACAGAATTGCCGGTGGG 370
g.              ATCTGGGCCGTCCAGGTAGTGTTGATGGGTGCCGTTGAAGGTTACAGAATTGCCGGTGGG 540
                ************************************************************

cdna            CCGCTCGGTGAGGTCACAGACCCAATCTACCCAGGTGGAAGCTTTGACCCATTGGGCTTG 430
g.              CCGCTCGGTGAGGTCACAGACCCAATCTACCCAGGTGGAAGCTTTGACCCATTGGGCTTG 600
                ************************************************************

cdna            GCTGATGACCCAGAAGCATTTGCTGAGCTGAAGGTGAAGGAGATCAAGAACGGCAGATTG 490
g.              GCTGATGACCCAGAAGCATTTGCTGAGCTGAAGGTGAAGGAGATCAAGAACGGCAGATTG 660
                ************************************************************

cdna            GCTATGTTCTCCATGTTCGGATTCTTTGTTCAGGC---------- 525
g.              GCTATGTTCTCCATGTTCGGATTCTTTGTTCAGGCCATTGTGACAGGAAAGGGACCATTG 720
                **********************************

cdna            ----------~----------
g.              GAGAACTTGGCTGACCACCTTGCTGACCCTGTCAACAACAATGCCTGGGCTTACGCCACA 780

cdna            ----------~
g.              AACTTTGTCCCCGGAAAGTGAG 802
```

Start codon
Stop codon

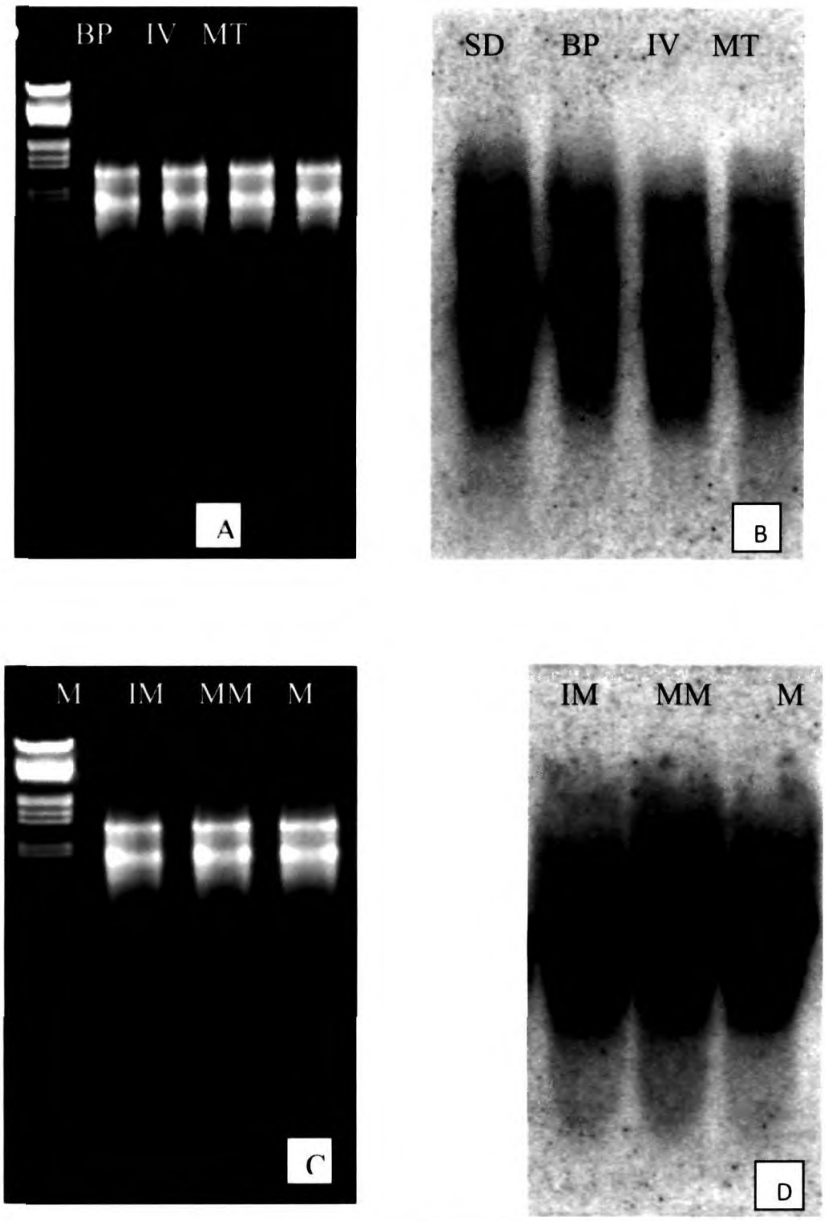


Fig.33. (A-D) Northern blotting for differential Cab gene expression

A. RNA. (M-Marker, SD-Seedling, BP- Bud grafted plant, IV –*in vitro* plant, MT- Mature tree)

B. Northern blot showing differential Cab gene expression in leaves of source plants of different maturity

C. RNA from leaves of different stages of budgrafted plants (M-Marker, IN- Immature, MM-Medium mature, M-Mature leaf)

D. Northern blot ahowing differential Cab gene expression in leaves of different maturity.

Discussion

CHAPTER V

DISCUSSION

Biotechnology may be defined as the manipulation of biological systems via modern technologies to solve practical problems in agriculture, medicine and industry. Biotechnology offers unique potential benefit and opportunities to accelerate tree improvement through clonal propagation and genetic manipulation. Regeneration systems are generally more difficult with woody plant species. Recently there is a growing interest in developing somatic embryogenesis systems for *Hevea* as a useful and efficient method for clonal propagation of selected material and genetic transformation. Somatic embryogenesis of *Hevea brasiliensis* (Muell. Arg.) has potential as a method for mass clonal propagation of mature selected genotypes (Carron *et al.*, 1995), and for genetic engineering (Montorro *et al.*, 2000).

In the present study, the optimization of media requirements to improve somatic embryogenesis and plant regeneration from leaf explants collected from bud grafted plants of *H. brasiliensis* (clone RRII 105) was achieved. Compared to floral explants, the leaf explants are easily available and culture initiation is easy. The effect of media constituents especially the basal medium, phytohormones, and gelling agent on somatic embryogenesis and plant regeneration in *H. brasiliensis* has been studied. Juvenility of plant materials is a key factor in micropropagation because the regeneration ability of woody plants decreases as they approach maturity. It was observed that physiological age of the source plant from where explants were collected determines its embryogenic capacity. The embryogenic potential of callus was found to reduce with maturity of the source plant. Obvious difference was noted in embryogenic competence of leaf explants collected from source plants of different physiological maturity as shown by varied response in embryogenic tissue initiation in *Hevea brasiliensis*. Rate of embryogenesis from the proliferated embryogenic calli formed was similar irrespective of the explant source. The leaf is a favored explant for

most transformation experiments in other plants since it is easy to handle and *Agrobacterium* readily penetrates the cut ends aiding direct or indirect regeneration from the infected tissues. The callus derived from leaf explants could also be used as an ideal target tissue for *Agrobacterium* mediated genetic transformation. Genetic transformation using the leaf based regeneration system would be very useful in future for the genetic improvement of *Hevea.* Differential Cab gene expression was also observed in leaves of juvenile and mature origin that may have an influence on tissue recalcitrance during *in vitro* culture.

6.a. Somatic embryogenesis from leaf explants of bud grafted plants.

6.a.1 Culture initiation

The trifoliate leaves have immature laminae with reddish or bronze colour. As they grow older they become light green with a shining appearance. The mature laminae are dark green on their upper surfaces and a paler, glaucous green below. Leaf cultures initiated with the optimal stage of the explants collected from glass house could be recovered with minimum culture contamination when surface sterilized with 0.15% mercuric chloride for 2 minutes. In the present study, the ideal sterilant and optimum concentration necessary for getting contamination free cultures with minimum tissue damage was identified. Sterilization with sodium hypochlorite could also produce contamination free cultures when 0.4% NaOCl was used. These cultures gradually changed to yellow showing the prolonged deleterious effect of the sterilant on the explant. Hence in further experiments mercuric chloride was used as the surface sterilant. Mercuric chloride and sodium hypochlorite are extensively used for explant sterilization in plant tissue culture. Jayasree *et al.,* (1999) has reported the successful use of 0.5% NaOCl for sterilization of immature anther explants. Sushamakumari *et al.,* (2000) reported the use of 0.1 % mercuric chloride for surface sterilization of immature inflorescence explants of *Hevea.* Sterilization with mercuric chloride helped in initiating sterile and viable cultures. Seneviratnae *et al.,* (1998) and Kala *et al.,* (2004) reported the use of 0.2% and 0.25% mercuric chloride respectively for the sterilization of shoot tip explants of *Hevea.* This concentration is slightly

higher than the report in the present study due to the fragile nature of the explant used in this study. Leaf cultures initiated with the optimal stage of the explants collected from glass house could be recovered with minimum culture contamination when surface sterilized with 0.15% w/v for 2 minutes. During rainy months, depending on the environmental conditions, the concentration of the sterilant had to be varied from 0.15- 0.2% and time of sterilization also was varied from 2-3 minutes so as to get contamination free cultures. Optimal stage of leaf responding to *in vitro* culture was identified from leaves of three different growth stages such as immature, medium mature and mature leaves. Leaf explants collected from glass house grown budgrafted *Hevea* plants of clone RRII 105 were used for experiments on development of the somatic embryogenesis system.

6.a.2 Callus induction

MS basal medium with modifications was found suitable for callus induction in leaf explants of *Hevea.* Leaves of medium maturation were identified to give maximum culture response. Mendanha *et al.,* 1998 used newly expanded leaves of rubber trees for initiating callus but no regeneration occurred. In the present study it was observed that newly expanded leaves were so fragile that they could not withstand sterilization when leaves were collected from *ex vitro* sources. In leaves collected from *in vitro* cultures, both immature and medium mature leaves responded. MS basal medium with or without modifications have been widely used for callus induction from different explants in tissue culture of *Hevea* (Jayasree *et al.*,1999; Sushamakumari *et al.,* 2000). In *Hevea,* a low level of inorganic nitrogen always favored somatic embryogenesis (Jayasree *et al.*,1999; Sushamakumari *et al.,* 2000). Mendanha *et al.,* 1998 reported the induction of callus induction in leaf explants in MS basal medium. In the present study also, MS medium with a lower level of ammonium nitrate and potassium nitrate favored callus induction from leaf sections. Along with this addition of 1200 mg/l calcium nitrate was also found suitable for callus induction in leaf explants. Here the presence of elevated levels of calcium favoring callus induction in presence of 2,4-D might be explained by the change in cell wall elasticity mediated by the calcium mediated signally pathway. The effect of

nutrient factors and growth regulators on somatic embryogenesis have been intensively studied on somatic embryogenesis in many important crops (Zimmerman, 1993; Pedroso and Pais, 1995). Even then these factors need to be optimized with respect to the tissue and type of explant. Generally inorganic forms of nitrogen is being used in most plant tissue culture media (Dodds and Roberts, 1995). Work on the carrot system has shown that Ca^{2+} enhances embryogenic frequency (Jansen *et al.*, 1990), and its deprivation arrests somatic embryo formation (Overvoorde and Grimes 1994). These observations are suggestive of an intermediary role for Ca^{2+} during plant embryogenesis. The role of calmodulin (CaM) as a Ca modulator during somatic embryogenesis has also been investigated (Overvoorde and Grimes, 1994). Most media in plant tissue culture contain sucrose as carbon and energy source. In this study, lower levels of sucrose (20 g/l) favored callus induction. Concentration of sucrose affects the different stages of somatic embryogenesis. In the case of *Oncidium* where higher embryogenic response was obtained in medium containing lower levels of sucrose (10-20 g/l) (Su *et al.*, 2002) in the present work also 20 g/l sucrose favored callus induction.

In *Hevea,* medium requirements for plant regeneration with different explants was found to vary. Jayasree *et al.*, (1999) has reported that kinetin was more favorable for callus induction for somatic embryogenesis from immature anther explants of *Hevea.* In the present study, BA was found more suitable for callus induction.This may be due to the physiological state of the two explants. Culture parameters were found to efficiently modify callus texture and induce callus friability. Reducing the auxin /cytokinin balance during callus induction, the concentration of sucrose and calcium in the proliferation medium also modifies callus texture. Presence of increased level of 2,4-D was found suitable for callus induction in leaf sections. Higher concentration of this phytohormone usually makes the callus hard and prevents proliferation. During subculture of fresh callus it is good to reduce the concentration of 2,4-D. Callus proliferation was also found to be influenced by the concentration of sucrose in the medium. Usually higher levels are found to help proliferation of callus. Nature of friability and compactness of anther derived callus of *Hevea* differed

depending upon 2,4-D concentration. Similar inference has also been made by Jayasree *et al*, 1999 where the texture of callus induced in immature anther explants of *Hevea* varied with 2.4-D concentration. Friable but loose or watery callus was obtained when media contained low concentrations of 2,4-D. However, friability was reduced with an increase in 2,4-D concentration. The percentage of callus proliferation increased when the concentration of 2,4-D increased to 3.6 µM , but callus was non embryogenic in nature. Callus friability may be obtained by interaction between calcium and growth regulator contents. Higher concentrations led to friable calli whose ability to produce embryos then depended on low calcium concentrations in the embryogenesis medium.

Despite other media components, phytohormones have a crucial role in induction of callus in explants. The ideal phytohormones in the optimal concentration and combination need to be supplemented in the medium for good callus induction. Though callus induction was observed in all combinations of the hormones tried, 5.4 µM 2,4-D, 4.4 µM BA and 1.08 µM NAA was found to be the ideal concentration identified to produce good quality callus (Kala *et al.*,2005). Mendanha *et al.*, (1998) could induce callus in leaf explants of *Hevea* was obtained in a phytohormones combination of kin, BA, IAA and NAA. Earlier Etienne *et al.*, (1993) has observed that callusing frequency and quality of callus was found to be dependent on concentration and combination of growth regulators tried. 3,4-D and 2,4-D has been mostly used by earlier workers in other explants of *Hevea* such as anther and inner integument for callus induction and in some instances NAA was also used (Carron *et al.*, 1998; Etienne *et al.*, 1993a, Jayasree *et al.*, 1999). With immature anthers, 2, 4-D (9.0 µM) in callus induction medium along with different growth regulator combinations induced callus (Jayasree *et al.*, 1999). Variation in frequency of callus formation and differences in callus type were noticed in different media tried. Frequency of callus induction and quality of calli was found to be dependent on concentration and combination of growth regulators used (Montorro *et al.*, 1993). Sushamakumari *et al.*, (2000) has also reported callus induction in immature inflorescence explants of *Hevea* when cultures were dark incubated in medium

containing 2,4-D (4.5 μM), NAA (2.7 μM) and Kin (2.3 μM). Mendanha *et al.,* (1998) has reported callus induction from leaf explants and axillary buds of rubber trees on MS basal medium containing 58 mM sucrose and phytohormones BA, 2,4-D, NAA and IBA. Our results also show that medium containing BA (4.4 μM) and 2,4-D (5.4 μM) in presence of low concentration of NAA (1.08 μM) and 58 mM sucrose was most effective for inducing good quality callus in leaf explants irrespective of the source of the leaf. Presence of NAA helped in improving the quality of the callus and its embryogenic competence. 3,4-D has been mostly used in other explants of *Hevea* such as anther and inner integument for callus induction and in some instances NAA was also used (Carron *et al.,* 1998; Etienne *et al.,* 1993).

Montorro *et al.,* (1993) has reported that culture parameters were found to efficiently modify callus texture and induce callus friability. According to him, reducing the auxin /cytokinin balance during callus induction, the concentration of sucrose and calcium in the proliferation medium also modifies callus texture. Similar observations were also made in this study where callus proliferation and texture could be improved by reducing auxin and calcium concentrations in the medium and increasing sucrose levels. Montorro *et al.,* (1993) has also observed that reducing auxin /cytokinin balance modifies callus texture. Jayasree *et al.,* (1999) reported that the nature of friability and compactness of anther derived callus of *Hevea* differed depending upon 2,4-D concentration resulting in 3 types of callus: friable; less friable; and compact. Friable but loose or watery callus was obtained when media contained low concentrations of 2,4-D. However, friability was reduced with an increase in 2,4-D concentration. The percentage of callus proliferation increased when the concentration of 2,4-D increased to 3.6 μM, but callus was non embryogenic in nature. Similarly, friable callus and compact callus was observed on MH medium with 3,4-D/Kn by Montorro *et al.,* (1993).

In the present work, it was observed that increased levels of sucrose (40 g/l) than in the callus induction medium (20 mg/l) favored for callus proliferation. According to Montorro *et al.,* (1995) callus friability is often associated with excess water content in the tissue and changes with the water status of the callus. Callus

friability may be obtained by interaction between calcium and growth regulator contents. Higher concentrations led to friable calli whose ability to produce embryos then depended on low calcium concentrations in the embryogenesis medium (Montorro *et al.,* 1995). Lardet *et al.,* (2007) has reported that decreasing the exogenous calcium concentration in preculture medium enhanced callus growth and proliferation. Blanc *et al.,* (2002) has observed a positive effect of increased levels of sucrose on callus proliferation. Sucrose level in the MH medium had a marked effect on the callus texture (Montorro *et al.,* 1993). In the present study also, the combined effect of calcium and sucrose had a pronounced effect on callus proliferation and friability. Blanc *et al.,* (1999) has reported use of silver nitrate for *Hevea* callus proliferation. It was also observed that, 2-3 subculture of the proliferated calli in medium containing reduced level of silver nitrate (10 mg/l) helped in increasing the embryogenic potential of the callus. Vain *et al.,* (1989) has also reported that embryogenic callus initiation rate was improved in *Zea mays* when immature embryos were cultured on a modified Murashige & Skoog medium containing various concentrations of silver nitrate.

6.a.3. Embryogenic callus initiation and Somatic embryogenesis

Changes in culture atmosphere (Auboiron *et al.,* 1990) and water status of the medium and nature of explant (Etienne *et al.,* 1991; Van Winkle *et al.,* 2003) are influencing the embryogenic potential of the callus. In the present study also embryogenic callus could be initiated from the proliferated callus cultured for embryo induction with varied time and frequency depending on the type of callus. The influence of calcium in inducing callus friability in *Hevea* has been reported earlier also by Montoro *et al.,* (1993). Modified MS medium has been reported earlier to favor somatic embryogenesis in *Hevea* from anther and inflorescence explants (Jayasree *et al.,* 1999; Sushamakumari *et al.,* 2000). Montorro *et al.,* (1993; 1995) have reported the promotive effect of calcium on somatic embryogenesis. Etienne *et al.,* (1997) also reported that somatic embryogenesis was enhanced by using temporary immersion technique in medium containing higher concentration of calcium chloride. Similarly in the present study, the presence of calcium in the callus

induction and embryo induction medium had a promotive effect on embryogenesis from leaf derived callus. According to him calcium was found favorable for long term proliferation of friable embryogenic callus induced from internal integument of immature *Hevea* seed. Higher concentration of phytagel (5.0 g/l) was also found to favor formation of embryogenic callus. Similar observations had also been made by Li *et al.,* (1998) in loblolly pine where 4.0 g/l phytagel induced embryogenic extrusions, whereas Becwar *et al., (*1995) reported that reducing the gelling agent concentration significantly enhanced extrusion and proliferation. In red spruce, a phytagel concentration of 2.0-3.0 g/l could initiate embryogenic callus (Harry and Thorpe, 1991).

Sucrose has major influence on somatic embryogenesis of various species (Ammirato, 1983). In the present work also 60 g/l sucrose was found to favor embryogenesis. The findings of the present study is also in accordance with the findings of Sushamakumari *et al.*, (2000) who reported a higher level of sucrose favoring enhancement of embryo induction in presence of GA_3 in callus derived from immature inflorescence of *Hevea.* Similarly mineral and carbohydrate nutrition also influenced embryogenic calli formation, somatic embryogenesis and embryo maturation in *Hevea* (Etienne *et al.,* 1991; Cailloux *et al.,* 1996). Differential carbohydrate metabolism helps morphogenesis in embryogenic callus of *Hevea* (Blanc *et al.,* 1999 & 2002). According to them, regulation of endogenous hexose contents at a low level, through slow maltose hydrolysis, was a key element of the biochemical signal leading this callus towards somatic embryogenesis. GA_3 also regulated embryo induction and germination in *Hevea brasiliensis* (Jayasree *et al.,* 2001). The manner in which embryogenic potential of the cultured *Hevea* callus is affected leading to embryogenic calli formation followed by embryogenesis could be stabilized by modifying the culture conditions such as hormone balance and time of subculture Michaux-Ferriere and Carron, (1989). Embryogenic callus emergence was highly dependent on the source and maturity of the plant from where the leaves were collected.

Silver nitrate has been reported to have influence in inducing emrbyogenic potential of fresh callus in several crops (Giridhar *et al.*, 2003). In the present work, it was observed that callus proliferation in medium containing 20 mg/l silver nitrate and addition of 10 mg/l of the same in embryogenic callus induction medium favored embryogenesis. Culture medium compounds such as silver nitrate (Vain *et al.*, 1989) have also been reported to modify callus texture in *Zea mays*. It is reported that concentrations between 30–60 μM, $AgNO_3$ improved mbryo yield for the *Coffea canephora* Pierre genotypes with regard to somatic embryogenesis (Fuentes *et al.*, 2000). Silver ions in the form of nitrate, such as AgNO3, play a major role in influencing somatic embryogenesis, shoot formation and efficient root formation which are the prerequisites for successful genetic transformation (Bais *et al.*, 2000). In recent years, $AgNO_3$ has been employed in tissue culture studies for inhibiting ethylene action because of its water solubility and lack of phytotoxicity at effective concentrations. Addition of AgNO3 to the culture media greatly improved the regeneration of both dicot and monocot plant tissue cultures (Giridhar *et al.*, 2003). In carrot $AgNO_3$, at concentrations of 10–20 μM, caused a 2-fold increase in the number of somatic embryos without causing adverse effects on cell survival (Jean-Paul *et al.*, 1990).

There are several reports on GA_3 enhancing the rate of somatic embryo induction (Jayasree *et al.*, 2001; Lakshmi Sita *et al.*, 1979). Sushamakumari *et al.*, (2000) has also reported significant increase in embryo induction frequency in presence of GA_3. Etienne *et al.*, (1991) reports that water status of the medium and explant also influences the embryogenic potential. Stabilizing the water potential of the culture medium and callus helped in initiating somatic embryogenesis from internal integument of *Hevea* seeds. Becwar *et al.*, (1995) reported that the commonly used phytagel concentration (2.0 g l^{-1}) showed a higher frequency of callus proliferation during somatic embryogenesis in loblolly pine. Michaux –Ferriere *et al.*, (1989) reported that embryogenic potential in *Hevea* callus was affected by the hormone balance and the time of subculture. According to Zimmerman (1993)

endogenous auxin in the explant stimulated cell proliferation and initiated somatic embryogenesis. It was reported by Etienne *et al.* (1991) and Montorro *et al.,* (1992) that among the different media components, auxin /cytokinin content and the type of cytokinin had a dominant effect on both the frequency of embryogenesis and the quality of embryos. The effects of polyethylene glycol (PEG) and abscisic acid (ABA) were analysed earlier on *Hevea brasiliensis* somatic embryos development. The development of embryos with a desirable morphological appearance was observed in the presence of PEG in the solid culture media. The presence of osmoticum greatly reduced the phenomenon of secondary embryogenesis and improved the conversion of proembryonic masses (PEMs) into torpedo-shaped embryos, while the addition of exogenous ABA favoured only the formation of globular-stage embryos (Etienne *et al.*, 1993). This effect of ABA was PEG-dependent and the best development occurred in the presence of 140 g/l PEG and 10^{-5} M ABA. (Linossier *et al.,* 1997). In the present work also PEG at a concentration of 5.0 g/l and ABA at a concentration of (1.1µM) gave an embryo induction frequency of 65%. According to Carron *et al.* (1993) dessication, medium osmolarity and addition of ABA improve germinability of *Hevea* somatic embryos by setting up a maturation phase. Callus proliferation was considerably reduced with all the calli on the medium surface getting converted into pro embryos. Time of subculture was also found to be an important factor affecting somatic embryogenesis. Towards the end of the subculture period, callus growth became arrested and started browning. The callus in contact with the surface of the medium became pro-embryos, callus just above this was friable and proliferating and the callus present at the uppermost surface became whitish, spongy and died.

6.a.4. Embryo maturation and plant regeneration

During embryo maturation, the carbohydrate source of the medium influences further development of the embryos such as their enlargement and apical meristem induction. In the present study, it was observed that a higher sucrose (60 g/l) was favorable for embryo maturation. Higher sucrose has been reported to be good for embryo maturation in embryos developed from other explants of *Hevea.* Cailloux *et al.,* (1996) and Sushamakumari *et al.*, (2000) also reported that higher levels of

sucrose (234 mM) favored embryo maturation. The effect of different carbohydrates was tested on early embryogenesis of *Hevea* by Blanc *et al.,* (2000) and found that somatic embryo production was significantly higher with maltose. Earlier reports also show that reducing sucrose concentration and thereby reducing the osmotic potential encourages embryo germination (Carron *et al.,* 1995).

As in earlier reports with explants such as immature anther and immature infloresecence (Jayasree *et al.,* 2001; Suhamakumari, *et al.,* 2000), in the present study, with leaf explants also we have observed that cytokinins and GA_3 had a major role in embryo maturation and germination. In the earlier reports, cytokinins and GA_3 were found to have a major role in embryo maturation and germination (Jayasree *et al.,* 2001; Sushamakumari, *et. al,* 2000). Earlier reports showed that addition of Kin in the maturation medium and reducing the level of BA was found favorable for development of embryos (Jayasree *et al.,* 2005, Seneviratnae *et al.,* 1996). Organic supplements were found to have a pronounced effect on embryo maturation and germination. The positive effect of organic supplements on embryo germination has been reported earlier in several crops (Seneviratnae *et al.,* 1996). In somatic embryogenesis of *Santalum album* combination of casein hydrolysate and coconut water was found good for conversion of embryo to plantlet (Rugkhla and Jones, 1998). Embryo germination was obtained when mature apex induced embryos were transferred to culture tubes containing hormone free MS medium with 0.3% charcoal and solidified with 0.2% phytagel (Fig.2, C). Full plantlet development occurred within one month in this medium where the sucrose level was further reduced to 89 mM (Fig.2, D).

6.a.5. Secondary embryogenesis

A high frequency of secondary embryogenesis was induced from isolated early cotyledonary-stage somatic embryos of *Hevea brasiliensis* (Cailloux *et al.,* 1996.) Repetitive embryogenesis was also induced from primary somatic embryos derived from integumental tissue. Secondary embryogenesis has been developed in *H. brasiliensis* trees for improving the efficiency of somatic embryogenesis, which was

based on the use of budded-tree integuments as primary explants, mainly by reducing subculture number and duration (Lardet *et al.*, 2008). Though they reported a new process for obtaining embryogenic callus lines quickly based on secondary somatic embryogenesis, success rates were low and unpredictable. Different embryogenic lines were established and maintained by repetitive embryogenesis in multiplication medium containing BA and NAA. In *Hevea*, somatic embryos cultured on B-5 medium supplemented with NAA, KIN, IAA and 2,4-D enhanced repetitive embryogenesis (Asokan *et al.*, 2002). In the present work the case is different and secondary embryogenesis occurred from regenerating plantlet having both shoot and root.

6.b. Effect of source plant juvenility on somatic embryogenesis

Age of source plants, developmental stage of the leaf, size, preparation, and culture environment are important factors for the type of induced somatic embryogenesis indirect or direct and shortening of induction period (Dhavala *et al.*, 2009). According to them explant age and hormonal concentration play a pivotal role and constitute the primary step in the standardization of *in vitro* regeneration protocol. Similar observation was also made in the present study with the developmental stage of the leaf having a major influence in callus induction. In pistachio, regeneration of plantlets through somatic embryogenesis was obtained only from juvenile leaves cultured *in vitro*. In *Hevea* also leaves collected from juvenile sources showed more embryogenic capacity. In this case embryogenic callus formation was found to be dependent on both age of the explant and on the individual leaf cultured (Omay *et al.*, 2000). Cysteine HCl has been widely used by several groups mainly for developing regeneration systems from leaf explants in coffee and cocoa (Leroy *et al.*, 2000; Emilie *et al.*, 2007).

Callus induction in leaves collected from *in vitro* cultures was faster since they could be cultured without sterilization in *H. brasiliensis*. Rate of callus induction was also higher (80%), in leaves collected from *in vitro* plantlets developed through somatic embryogenesis and seedlings, since they were physiologically juvenile whereas in leaves collected from budded plants and mature trees which are

physiologically mature sources it was 60%. Texture of the callus was similar in cultures initiated from leaves of all sources when cultured in the same medium (Kala *et al.,* 2009).

The nature of the initial explant has been recognized for a long time as a determining factor for successful plant tissue culture (Bonga, 1982), particularly for somatic embryogenesis which is strongly influenced by the age of the donor plants (Bonga and von Aderkas, 1992). Somatic tissues of flowers of several species are reported to have a higher potential for vegetative reproduction than tissues from other plant parts, presumably because of their proximity to rejuvenating sexual cells (Bonga, 1982; Bonga and von Aderkas 1992). *Hevea brasiliensis* is one of a few arborescent species for which somatic embryos can be obtained from mature individuals via integument explants from seed (Carron *et al.,* 1995). According to them using primary explants obtained from the same seed integument tissues, collected at the same time and under the same conditions from donor plants of the same genotype, significant differences in capacity for somatic embryogenesis were observed in the same culture conditions based on the origins and age of these donor plants. The present study reports obvious difference in embryogenic competence of leaf explants collected from plants of different physiological maturity as shown by varied response in embryogenic tissue initiation and embryogenesis in *Hevea brasiliensis*. Simultaneous with the formation of embryogenic calli, embryo induction could also be observed in all regenerating clumps from which embryos became visible within 2 weeks. Once embryogenic callus was formed, rate of embryo induction from the proliferated embryogenic calli was similar irrespective of the explant source. The rate of embryo induction from the proliferated embryogenic callus was similar irrespective of the source of the leaf, whether *ex vitro* or *in vitro* derived (60-70 %).The physiological age of the source plant, from where the explants were collected, determines the embryogenic potential of the induced callus in leaf explants of *H. brasiliensis.* Interaction and balance of media constituents also determines the efficiency of a system along with the cultured tissue.

In the present study also initiation of embryogenic callus from leaf derived fresh callus was found to be strongly influenced by the physiological maturity of the source plant from where leaves were collected revealing its embryogenic capacity. Variation was observed in the rate and time taken for embryogenic callus initiation when proliferated calli obtained from different explant sources were cultured in the embryo induction medium standardized earlier (Kala *et al.,* 2006). The most juvenile tissue which were the leaves collected from *in vitro* plantlets derived through somatic embryogenesis gave the maximum response in a minimum time frame. Embryogenic callus initiation could be triggered by the combined effect of calcium, sucrose and phytagel concentration of the medium. It appears that a combination of water stress provided by phytagel and an osmotic stress caused by sucrose in conjunction with the presence of calcium imparts callus friability. This provided a favorable environment that triggered the emergence of embryogenic calli and simultaneous embryogenesis. Rate of embryogenesis was similar in all the explant sources. These observations confirm the fact that physiological age of the source plant from where explants were collected determines its embryogenic capacity. This was found to reduce with maturity of the source plant. Previous studies have shown that physiological aging negatively affects the micropropagation capacity of *H. brasiliensis* from microcuttings (Lardet *et al.* 1990; Perrin *et al.,* 1994). Interaction and balance of media constituents also determines the efficiency of a system along with the cultured tissue. Embryogenic callus emergence was highly dependent on the source and maturity of the plant from where the leaves were collected. Lardet *et al.,* (2009) also reported that the embryogenic capacity of integument explants was dependent on the physiological aging of the plants from where explants were taken, and initiation of embryogenic callus from primary somatic embryos derived from the different explant sources varied. The finding that these differences persisted during somatic embryogenesis further demonstrates the overriding influence of donor plant age, especially physiologic age and success rates of somatic embryogenesis (Bonga and von Aderkas, 1992, Dunstan *et al.,* 1995).

Liu and Pijut (2008) have also reported higher regeneration efficiency from juvenile source leaf explants of mature black cherry. Explants with a high ability for somatic embryogenesis generally originate from reproductive organs (e.g., anthers, inner integument of the seed and zygotic embryos), young seedlings (Dunstan *et al.,* 1995) and somatic-embryogenesis-derived plantlets. In *Picea abies* (L.) Karst., *in-vitro*-cultured somatic embryos showed higher ability for somatic embryogenesis than their similarly aged or older zygotic counterparts (Ruaud *et al.,* 1992). In *Larix decidua* Mill., somatic embryogenesis from cotyledons and needles has been successful only for tissues obtained from emblings or from immature and mature zygotic embryos (Lelu *et al.,* 1994). A higher organogenic responsiveness on a wider range of culture media for physiologically juvenile tissues compared to mature ones was observed in other species (Monteuuis, 1987; 2004), indicating that it may be a general phenomenon. This phase change phenomenon has been observed in many studies and is associated with a noticeable decline in the potential for micropropagation or somatic embryogenesis of most arborescent species (Bonga 1982; Hackett, 1985; Monteuuis, 1989; von Aderkas and Bonga, 2000).

6.c. *Agrobacterium* mediated genetic transformation

Transformation experiments were carried out using proliferated fresh leaf callus as the target tissue for *Agrobacterium* infection, following the earlier developed protocol (Jayashree *et al.,* 2003). Optimization of parameters for controlling bacterial overgrowth and improving the transformation efficiency were carried out. In the present study, addition of silver nitrate in the selection medium (20 mg/l) prevented the overgrowth of bacteria for about 3 weeks. Inclusion of silver nitrate in co-culture medium has been proven for its anti ethylene activity which is common with *in vitro* plant cultures. Inclusion of silver nitrate in co-culture medium has been proven for its anti ethylene activity which is common with *in vitro* plant cultures. The suppressed *Agrobacterium* growth on target explants could facilitate plant cell recovery and result in increased efficiency of transformation. The suppressed *Agrobacterium* growth on target explants could facilitate plant cell recovery and result in increased efficiency of transformation. Tissue damage by A. *tumefaciens* infection has been reported earlier

also and seems to be one of the major obstacles for *Agrobacterium* mediated transformation. Similar observation was also made by Orlikowska (1997) where presence of silver nitrate (100 mg/l) in the rose regeneration medium completely retarded bacterial overgrowth for about one month. In apple it has been reported that during *Agrobacterium* mediated transformation, ethylene production was increased which resulted in reduced efficiency of gene transfer mechanism (Seong *et al.,* 2005). Ozden *et al.,* (2004) have reported that involvement of ethylene production leads to tissue browning which can be reduced in the presence of silver nitrate. Addition of silver nitrate in the co-culture medium has shown to enhance stable gene transfer in maize (Armstrong *et al.,* 2001; Zhao *et al.,* 2001) and fuji apple (Zhang *et al.,* 2001). Orlikowska (1997) reported that addition of silver nitrate stimulated direct shoot regeneration in leaves taken from *in vitro* cultures and infected with *Agrobacterium.* It also inhibited bacterial growth after co cultivation with the explants. Opabode (2006) reported that silver nitrate is an anti necrotic compound which can reduce oxidative burst during the interaction between plant tissue and *Agrobacterium.* The use of silver nitrate in plant tissue culture have shown to have other important effects in plant tissue culture of cassava, such as improving somatic embryogenesis and micropropagation in many other species (Zhang *et al.,* 2001).

L-cysteine which is an amino acid with a thiol side chain is an important component of antioxidant glutathione. L-cysteine is an effective inhibitor for polyphenol oxidases, peroxidases and enzymatic browning. It is suggested that L-cysteine improves transformation efficiency by reducing plant defense response to pathogen attack, plant wounding and environmental stresses throughout the co-cultivation period. L-cysteine therefore reduced plant cell death, enzymatic browning of wounded sites and increased bacteral susceptibility which in turn improved transformation efficiency. In the present experiments also, presence of L-cysteine along with silver nitrate helped in reducing overgrowth and improving quality of callus in newly formed transgenic lines, along with improvement in transformation frequency. According to Olhoft *et al.,* (2003), inclusion of thiol compounds in the solid cocultivation medium enhanced T-DNA transfer into cotyledonary node cells

and genomic integration resulting in increased production of transgenic plants. Antioxidant effect of L-cysteine on *Agrobacterium* mediated transformation has been studied intensively in soyabean varieties (Olhoft *et al.*, 2001; Paz *et al.*, 2004 and Liu *et al.*, 2008). It has been repoted that addition of L-cysteine in the co-cultivation medium along with other thiol compounds and surfactants would increase the transformation efficiency significantly (Olhoft *et al.*, 2001; Liu *et al.*, 2008). Enriquez-Obregon *et al.*, (1999) also investigated the effects of these three compounds on the necrosis of shoot meristem explants prior to infection in rice transformation. The explants, which were incubated in a liquid medium containing 20 mg /1 ascorbic acid, 40 mg /1 cysteine, and 5 mg /1 silver nitrate for 6 h in the dark, had an average of 6% necrosis of explant area while it was 80% in explants without the antioxidant treatment. The antioxidant treatment increased rice transformation efficiency from 17 to 30%. Similarly, Olhoft *et al.*, (2001) increased *Agrobacterium* infection from 37 to 91% in the soybean cotyledonary node region by including 400 mg/l cysteine in cocultivation medium, subsequently, resulting in a two fold increase in transformation efficiency and simultaneously reducing browning/necrosis on the explant. Addition of 400 mg/1 cysteine in the co-culture medium increased both the frequency of transient β-glucuronidase (GUS) expression in target cells from 17 to 56% and the stable transformation frequency from 0.2 to 6.2% (Frame *et al.*, 2002). It was also found that the coculture of sugarcane leaf spindle sections with *A. tumefaciens* induced a rapid necrosis of the tissue (Enriquez-Obregon *et al.*, 1997, 1998). To minimize the necrosis, the leaf spindle explants were incubated in a liquid medium containing 15 mg/1 ascorbic acid, 40 mg/1 cysteine, and 2 mg/1 silver nitrate for 60 h in the dark prior to inoculation with *Agrobacterium* and then cocultured in a medium with the same antioxidants. By doing so, the percentage of the explant viability was increased from 10 to 90%. In addition, the percentage of GUS positive explants was increased from 0 to 100%. Zeng *et al.*, (2004) also reported that the inclusion of 400 mg/l cysteine in the cocultivation medium increased stable transformation from 0.2 to 5.9% in *Agrobacterium*-mediated transformation of soybean.

Within a population of *Agrobacterium* transformed and non-transformed cells or tissues, lipoic acid promoted the differentiation, proliferation and regeneration of the transformed cells or tissues under appropriate selection pressure. It prevented the transformed cells or tissues, which were stressed by *Agrobacterium* and any unfavorable *in vitro* culture conditions, from browning and dying at an early stage of development and subsequently enhanced its transient GUS expression. In the present study, enhancement in transformation frequency by 5 - 10% was obtained with reduction in overgrowth when this was used along with silver nitrate and pluronic F68. In animals, free lipoic acid and dihydrolipoic acid are metabolic antioxidants that are able to scavenge reactive oxygen species to recycle other antioxidants such as vitamin C, glutathione, and vitamin B to increase the expression of genes involved in the regulation of normal growth and metabolism as well as redox regulation of gene transcription (Packer *et al.,* 1995, Packer and Tritschler 1996). Different concentrations of lipoic acid was applied in the co-culture medium of an *Agrobacterium*-mediated soybean transformation protocol (Dan *et al.,* 2004) and transgenic plants were generated. According to Dan *et al.,* (2009) within a population of *Agrobacterium* transformed and non-transformed cells or tissues, lipoic acid promoted the differentiation, proliferation, and regeneration of the transformed cells or tissues under appropriate selection pressure.

In this experiment, addition of the surfactant pluronic F68 (300 mg/l) in the infection medium containing silver nitrate and thiol compounds increased the transformation frequency further by 1-2%. Henrique *et al.,* (2004) reported that the addition of pluronic F68 (0.03%) to the inoculation medium dramatically increased transient GUS expression up to 100 fold in sorghum. Similar observations were also made here with pluronic F68 giving improvement in transformation frequency. Cheng *et al.,* (1997) has also reported that presence of pluronic F68 in the inoculation medium greatly enhanced T-DNA delivery in immature embryos of wheat. Surfactant added to the inoculation medium may play a role similar to vaccum infiltration, facilitating delivery of bacterial cells to closed ovules which are the primary target of *Agrobacterium* in plant transformation (Ye *et al.,* 1999, Bechold *et al.,* 2000). An

overall improvement in the transformation frequency by 10-15% was obtained by using the surfactant pluronic F68 along with silver nitrate and antioxidants in the modified infection, co-cultivation and selection medium.

6.d. Identification, Cloning and characterization of phase change related genes

The largest class of juvenile-induced genes was comprised of those involved in photosynthesis. It is also well established that photosynthetic rates and related physiological attributes differ between juvenile and reproductively mature individuals in nearly every woody species examined (Bond, 2000). Changes in chlorophyll content may be reflected in more general changes in the photosynthetic apparatus. This would suggest that genes encoding elements of the photosynthetic apparatus would be differentially expressed between juvenile and mature plants. Two steady-state Cab mRNA levels are relatively higher (40%) in newly expanding short shoot foliage from juvenile plants compared to mature plants in Eastern larch (*Larix laricina*) (Hutchison *et al.*,1990). In the present study also Chlorophyll a/b binding protein gene was found to be differentially expressed in juvenile and mature plants of *Hevea.* It was observed that chlorophyll a/b binding protein gene expression was more in juvenile source plants such as seedlings and *in vitro* somatic embryogenesis derived plants whereas in mature source plants such as bud grafted plants and mature trees it was comparatively less. In tree species, the general trend is towards lower instantaneous or integrated photosynthetic rates in reproductively mature individuals, but the inverse has also been reported in several species (Bond, 2000). In Eastern larch, juvenile and rejuvenated shoots also showed higher rates of photosynthesis and respiration, evidenced by faster O_2 evolution and consumption. This would suggest that genes encoding elements of the photosynthetic apparatus would be differentially expressed between juvenile and mature plants in. The photosynthetic rates were associated with more chlorophyll, especially chlorophyll *a*, in the juvenile and the rejuvenated shoots (Hutchison *et al.*, 1990)

Identification of genes differentially expressed in a phase-induced manner in maize leaf primordia by microarray analysis of juvenile, adult leaf and juvenile leaves

from culture-rejuvenated plants revealed that 221 and 28 genes that were up regulated in the juvenile and adult phases, respectively. Whereas photosynthetic genes have been long known to be induced by light, this observation suggests that in order to prime maize plants for energy production early in vegetative growth, the induction of photosynthetic genes relies on developmental cues (Strable *et al.*, 2008). The effect of maturation on the morphological and photosynthetic characteristics, as well as the expression of two genes involved in photosynthesis in the developing, current year foliage of Eastern larch. These data show that the maturation- related changes in morphological and physiological phenotypes are associated with changes in gene expression (Hutchison.*et al.*, 1990). Basheer- Salimia *et al.*, (2007) have identified some morphological, anatomical, and eco physiological markers with juvenility-maturity in olive tree. Their results with olive leaves could be used to assess the phase changes or the transition period in many of woody plants. They concluded that when the juvenile phase was compared to mature phase in olive trees, was physiologically characterized by higher photosynthetic rate and stomatal conductance and morphologically by smaller leaf size, smaller number of hairs and stomata, thicker leaves, and thicker palisade and spongy parenchymas. In contrary, mature phase has lower photosynthetic rate and stomatal conductance and decreased cell size in all leaf histological components. In all species of mediteranean oak, leaves on seedlings displayed a lower mass per unit area and lower nitrogen content than on mature trees. However, owing to their larger stomatal conductance, leaves on seedlings usually showed larger photosynthetic nitrogen-use efficiency and lower water-use efficiency than on mature trees. Stomatal conductance and CO_2 assimilation rate were lower in *Q. ilex* than in the two deciduous species at the mature stage (Sonia and Alfonso, 2003). *In vitro* shoot tips of *Sequoia sempervirens* including a juvenile, two adult, and two rejuvenated adult clones were examined for differences in basic physiological characteristics. Juvenile and rejuvenated shoots also showed higher rates of photosynthesis and respiration, evidenced by faster O_2 evolution and consumption. The photosynthetic rates were associated with more chlorophyll, especially chlorophyll *a*, in the juvenile and the rejuvenated shoots (Li *et al.*, 2003). The largest class of juvenile-induced genes was comprised of those involved in photosynthesis,

suggesting that maize plants are primed for energy production early in vegetative growth by the developmental induction of photosynthetic genes (Strable *et al.*, 2008).

A plant regeneration protocol from leaf explants of *Hevea brasiliensis* clone RRII 105 was developed. Optimization experiments of the medium involved in various steps of the pathway were done based on earlier reports of work carried out in related fields and results obtained were compared and discussed. It was observed that each plant species and even different tissues in a plant has its own unique set of nutritional requirement for *in vitro* culture. Regarding phase change related *in vitro response* and gene expression, in *Hevea* also, observations similar to those made by earlier workers in this area were found to be relevant and similarities found. Regarding genetic transformation, supplementing the medium with additives reported to be favourable for improving transformation in other crops, positive results were obtained and transformation frequency could be improved by 5-10%.

Summary & Conclusions

Chapter VI

SUMMARY AND CONCLUSIONS

Hevea brasiliensis belonging to the family *Euphorbiaceae* remains as the only cultivated species as a commercial source of natural rubber because of its abundance in the latex, high quality and convenience of harvesting. Since the tree is cross pollinated the seedlings are highly heterozygous and commercial propagation is by bud grafting. Genetic improvement of *H. brasiliensis* by conventional means is very elaborate and time consuming as in many other perennial species. *In vitro* culture of *H. brasiliensis* was initiated with a view to develop protocols for micropropagation and genetic improvement through transgenic approaches. Efficient protocols for plant regeneration through somatic embryogenesis from a range of explants such as inner integument, immature anthers and immature inflorescence have been reported in *H. brasiliensis.*

All the protocols developed earlier were for somatic embryogenesis in *H. brasiliensis*, floral/fruit derived explants. Since flowering in *H. brasiliensis* is seasonal and adverse environments during the season may hinder normal flowering, explant-availability and quality is unpredictable. Development of a plant regeneration system from *Hevea* leaves which is an easily available explant would be useful for crop improvement experiments through genetic transformation. The work also done to understand the effect of media constituents especially major salts, phytohormones, organic supplements and gelling agent on leaf derived somatic embryogenesis and plant regeneration in *Hevea.* Leaf cultures initiated with the medium mature leaves collected from glass house grown bud grafted plants could produce about 73% contamination free, viable cultures when surface sterilized with 0.15% (w/v) $HgCl_2$ for two minutes. Modified MS medium with addition of 1200 mg/l calcium nitrate, cysteine HCl (50 mg/l), casein hydrolysate (1.0 gm/l), B5 vitamins, sucrose (20g/l) and containing phyto hormones 2,4-D (5.4 μM), BA (4.4 μM) and NAA (1.08 μM) was identified as the most suitable medium for callus induction from leaf explants of bud grafted plants of *Hevea.* Embryo induction was obtained in modified MS medium ($Ca(NO_3)_2$. $4H_20$ -250

mg/l) containing B_5 vitamins, amino acids and phytohormones, BA (2.2 μM), GA_3 (2.9 μM), Kin (1.25 μM), ABA (0.75 μM) and NAA (0.54 μM). The medium also contained organic supplements such as coconut water (5 %), casein hydrolysate (300 mg/l), PEG (5.0 g/l), sucrose (60 g/l) and 0.2% activated charcoal. The medium was solidified with 5.0 g/l phytagel. Maturation and apex induction of embryos was obtained when cultures were dark incubated in WPM containing sucrose (60 g/l), amino acids such as glutamic acid (150 mg/l), arginine (40 mg/l) and glycine (10 mg/l), organic supplements and phytohormones BA (2.2 μM), (Kin 1.38 μM), IBA (0.49 μM) and GA_3 (5.3 μM). Plant regeneration was obtained in hormone free MS medium. Sixty percent of the plantlets transferred to sterile sand could withstand transplanting shock and were free of fungal attack after two weeks.

The initiation of embryogenic callus from fresh callus derived from leaf explants was found to be influenced by the physiological maturity of the source plant from where the leaves were collected. The leaves collected from *in vitro* plantlets derived through somatic embryogenesis, the most juvenile tissue, gave the maximum positive response within the shortest time. Embryogenic callus initiation could be increased by the combined effect of calcium, sucrose and phytagel concentration of the culture medium. It was observed that a combination of water stress provided by phytagel and the osmotic stress caused by sucrose in conjunction with the presence of calcium imparts callus friability. This provided a favourable environment triggering the emergence of embryogenic calli and simultaneous embryogenesis. These observations confirm the fact that physiological age of the source plant from where explants were collected determines its embryogenic capacity. The embryogenic potential was found to reduce with maturity of the source plant, with leaves from mature trees having no embryogenic capacity. Between the remaining two sources, rate of embryogenesis from the proliferated embryogenic calli was similar irrespective of the explant source. The present study also reports obvious differences in embryogenic competence of leaf explants collected from source plants of different physiological maturity as shown by varied response in embryogenic tissue initiation in *Hevea brasiliensis*.

Efficient transformation systems using easily available explants are indispensible for genetic modification of important crop plants. The leaf is a favoured explant for most transformation experiments in other plants, since nutrients and *Agrobacterium* readily penetrate these tissues. Hence a leaf based regeneration would be very useful for the genetic improvement of *Hevea* through recombinant technology. The present study also reports the feasibility of using freshly proliferated leaf callus as target tissue for genetic transformation. The effect of factors that help to increase the transformation efficiency such as silver nitrate, surfactant pluronic-F68 and antioxidants, L- cysteine and α- lipoic acid in improving callus texture in the newly emerging transgenic cell lines and control overgrowth of bacteria were examined. It was observed that the infection, co-culture and selection media components, *Agrobacterium* strains and binary vectors significantly influence T-DNA delivery, integration and stable transformation. It was observed that addition of silver nitrate in the infection (10.0 mg/l), cocultivation (10.0 mg/l) and selection (10.0 mg/l) medium significantly suppressed bacterial overgrowth and improved the texture of callus in newly emerged lines. An overall improvement in the transformation frequency by 5 – 10 % was obtained by the inclusion of antioxidants L– cysteine (100 mg/l) or lipoic acid (50 mg/l) in the infection, cocultivation and selection medium and addition of the surfactant pluronic F68 (300 mg/l) in the infection medium. The transgenic callus obtained could be proliferated and somatic embryo induction obtained. Overall, it has become evident that each species and even tissues has its own unique set of requirements for effective transformation.

An attempt has been made for the isolation and characterization of genes differentially expressed in juvenile and mature tissues of *H. brasiliensis*. The chlorophyll a/b binding protein (Cab) gene was PCR amplified from the genomic DNA and cDNA of *H. brasiliensis* (clone RRII 105). The amplified sequence contains 525 bps. PCR amplified cDNA fragment was eluted, cloned in strataclone TA vector and sequenced. The sequence was compared with the earlier reported cDNA sequence of Cab gene from other plant species and BLAST analysis revealed sequence homology with Cab mRNA sequence (M60274) from *Ricinus communis* (91%) and the genomic sequence characterised was found to be intronless. This is the first report on isolation and characterization of chlorophyll

a/b binding protein gene partial cds from *H. brasiliensis*. Steady state Cab mRNA levels have been reported to be relatively higher in newly expanding short shoot foliage from juvenile plants compared to mature plants. Differential expression of the chlorophyll a/b binding protein gene was observed during the juvenile – mature phase change tissues derived from juvenile plants of *H. brasiliensis* showing more expression of the gene.

A plant regeneration system through somatic embryogenesis has been developed from leaf explants of *Hevea Brasiliensis*. The efficiency of the system was found to vary with juvenility of the source plant from where explants were collected with the leaves collected from in vitro developed plants being the most amenable. The system developed was also proved to be feasible for *Agrobacterium* mediated genetic transformation and subsequent plant regeneration. Isolation, cloning and sequence analysis of chlorophyll a/b binding protein gene which gave differential expression in tissues collected from juvenile and mature plants was done the expression of which may influence tissue recalcitrance during *in vitro* culture.

Leaf explants of *Hevea* were proved to be amenable for tissue culture and plant regeneration. The newly developed system for plant regeneration could be utilized in future for genetic transformation experiments in *Hevea* crop improvement. This opens up the scope of utilizing the leaf explant for developing a system for direct embryo induction in leaves without callus phase intervention by modifications in the already developed system through further optimization experiments. It would be a major breakthrough for genetic transformation and transgenic plant production.

References

REFERENCES

Adams CA, Rinne RW (1980) Moisture content as a controlling factor in seed development and germination. *Int Rev Cytol* **68**: 1-8

Alkhateeb AA (2006) Somatic embryogenesis in Date Palm (*Phoenix dactylifera* L.) cv. Sukary in response to Sucrose and Polyethylene Glycol. *Biotechnology (5)* **4**: 466-470

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410

Ammirato PV (1987) Organisational events during somatic embryogenesis. In: Green C.E., Somers D.A., Hackett W.O. & Biesboer D.D. (eds.) *Plant Tiss. Cell Cult.* (57-81). A R Liss, New York

Ammirato PY (1983) Embryogenesis. In *Handbook of Plant Cell Culture,* Vol. 1, eds Evans, D.A, Sharp, W.R., Ammirato, p.y. & Yamada, Y pp. 82-123. New York: Macmillan.

Annamma Y, Marattukalam JG, Premakumari D, Saraswathyamma CK, Licy J, Panikkar AON (1990) Promising rubber planting materials with special reference to Indian clones. *Proceedings of Planters Conference*. Kottayam, India.

Armstrong CL, Rout JR, (2001) A novel *Agrobacterium* – mediated plant transformation. *Method in plant publ. WOO1/09302 A2*

Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell,Tissue and Organ Culture* **69(3)**: 233-249

Arokiaraj P, Leelavathy R and Yeang HY (2009) The virulence PlasmidPTO47 from *Agrobacterium tumefaciens* A281 improves transformation efficiency of *Hevea brasiliensis. American journal of Biochemistry and biotechnology* **5(3)**: 137-141

Asokan MP, Kumari Jayasree P, Thomas V, Sushamakumari S, Kala RG, Jayasree R, Sethuraj MR, Thulaseedharan A (2002) Influence of 2,4-D and sucrose on repetitive embryogenesis in Rubber (*Hevea brasiliensis*). *J. Tree. Sci.* **21**:18-26

Asokan MP, Sobhana P, Sushmakumari S, Sethuraj MR (1988) Tissue culture propagation of rubber (*Hevea brasiliensis*) Wild ex Adr. De Juss. Muell. (Arg) clone GT1. *Indian. J. Nat. Rubb.* Res. **1**:10-12

Auboiron E, Carron MP, Michaux-Ferriere N (1990) Influence of atmospheric gases particularly ethylene on somatic embryogenesis of *Hevea brasiliensis. Plant cell Tiss. and Org. Cult* **21**: 31-37

Bais HP, Sudha G, Suresh B, Andravishankar GA (2000) $AgNO_3$ influences *in vitro* root formation in *Decalepis hamiltonii* Wight, Arn. *Current Science* **79**: 894-898

Basheer-Salimia R (2007) Juvenility, Maturity and Rejuvenation in Woody Plants. Hebran University Research Journal **3(1)**: 17-43

Bechold N, Jaudeu B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of T-DNA in plant transformation of *Arabidopsis thaliana. Genetics* **155**: 1875-1887

Becwar MR, Chesick EE, Handley LW, Rutter M.R (1995) Methods for regeneration of coniferous plants by somatic embryogenesis. US patent 5,413,930

Blanc G, Barakat A, Guyot R, Cooke R, Delseny M (2000) Extensive duplication and reshuffling in the *Arabidopsis* genome. *Plant Cell.* **12**: 1093- 1101

Blanc G, Lardet, L, Martin A, Jacob JL, Caron MP (2002) Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis* (Muell Arg.). *Journal of Experimental Botany* **53**: 1453-1462

Blanc G, Michaux-Ferriere N, Teisson C, Lardet L , Carron MP (1999) Effects of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis. Plant cell Tiss. and Org. Cult.***59**: 103-112

Block MD, Herrera, EL, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny. *EMBO J.*, **3**: 1681-1689

Bond BJ (2000) Age-related changes in photosynthesis of woody plants. *Trends Plant Sci.* **5**:349–353

Bonga JM (1982) Vegetative propagation in relation to juvenility, maturity, and rejuvenation. *In* Tissue Culture in Forestry. Eds. J.M.Bonga and D.J. Durzan. Martinus Nijhoff/Dr. W. Junk, The Hague, pp 387- 412

Bonga JM, von Aderkas P (1992) Rejuvenation of tissues from mature conifers and its implications for propagation *in vitro. In Clonal Forestry I: Genetics and Biotechnology*. Eds. M.R. Ahuja and W.J. Libby. Springer-Verlag, Berlin, pp 182–199

Bouychou J (1953) La culture *in vitro* des tissues d' *Hevea*. Proc. Rubb. Conf. Bogor. *Archives of Rubber Cultivation* **30**: 50-53

Cailloux F, Julien-Guerrier J, Linnossier L, and Coudret A (1996) Long-term somatic embryogenesis and maturation of somatic embryos in *Hevea brasiliensis*. *Plant Sci.* **120**:185-196

Carron MP, Enjalric F (1982) Studies on vegetative micropropagation of *Hevea brasiliensis* by somatic embryogenesis and *in vitro* microcutting. In: Fujiwara A (ed). *Plant Tiss. Cult.* (751-752) Maruzen, Tokyo

Carron MP, Enjarlic F (1983) Prospects for the micropropagation of *Hevea brasiliensis*. *Revue Generale des. Caoutch Plast.* **628**: 65-68

Carron MP, Enjarlic F, Deschamps A (1985) Progress of research into *in vitro* vegetative propagation of *Hevea brasilliensis Muell. Arg* IRCA, CR, Coll Exp Phys. Am *Hevea* IRRDB, IRCA, Monpellier. pp.427 - 435

Carron MP, Enjarlic F, Lardet L, Deschamps A (1989) Rubber (*Hevea brasilliensis* Muell.Arg) *In Bajaj YPS (Ed),Biotechnology in Agriculture and Forestry Trees II*, **5** Springer-Verlag,Berlin. 222-245

Carron MP, Etienne H, Lardet L, Campagha S, Perrin Leconte A, Chaine C (1995) Somatic embryogenesis in rubber (*Hevea brasiliensis* Mull. Arg.). *In* Somatic embryogenesis in woody plants (Eds.) S. M. Jain, P. Gupta, and R. Newton Kulwer Academic Publishers The Netherlands, pp 117-136

Carron MP, Lardet L, Dea BG (1998) *Hevea* micropropagation by somatic embryogenesis. *Plantations Research Development*. **5**: 187-92

Carron MP, Leroux ,Tison J, Dea BG, Caussanel V, Clairj KJ (2000) Compared root system architectures in seedlings and *in vitro* plantlets of *Hevea brasilliensis*, in the initial years of growth in the field. *Plant and soil* **223**:73-85

Cazaux E, d'Auzac (1994) Microcallus formation from *Hevea brasiliensis* protoplasts isolated from embryogenic callus. *Plant Cell Rep.* 13: 272–276

Chabaud M, Passiatore JE, Cannon F, Buchanan-Wallaston V (1988) Parameters affecting the frequency of kanamycin resistant alfalfa obtained by *Agrobacterium tumefaciens* mediated transformation. *Plant Cell Reports* **7(5)**: 12-16

Chen LP, Wang YJ, Xu CX (2006) *In vitro* propagation of *Lychens senno* Siebold et Zucc., a rare and ornamental value plant. *Scientia Horticulturae* **107**: 183-186

Cheng M, Fry JE, Pang S, Zhou H, Hironoka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol.*, **115**: 971-980

Chua SE (1966) Studies on tissue culture of *Hevea brasiliensis*: Role of osmotic concentration, carbohydrate and pH value in induction of callus growth in plumule tissue from *Hevea* seedling. *Journal of Rubber Research Institute of Malaya*. **19(5)**: 272-276

Citovsky V, Kozlovsky SV, Lacroix B, Zaltsman A, Dafni M, Vyas S, Tovkach A Tzfira, T (2007) Biological systems of the host cell involved in *Agrobacterium* infection. *Cell. Microbiol.* **9**, 9-20

Combe JC (1975) Demonstration of intraclonal variability in young graft trees. *Revue Generale des Caouth Plast.* **52**: 91-94

Corredoira E, Ballester A, Vieitez AM (2003) Proliferation, maturation and germination of *Castanea sativa* Mill. somatic embryos originated from leaf explants. *Annals of Botany* 92: 129-136

Dan Y, Charles L, Armstrong, Jimmy Dong, Xiaorong Feng, Joyce EF, Greg EK, Brian J, Martinell, Gail A, (2009) Lipoic acid—a unique plant transformation enhancer *In Vitro Cell.Dev.Biol.—Plant* **45**: 630–638

Dan Y, Munyikawa T, Rayford K, Rommens C (2004) Use of lipoic acid in plant culture media. US Patent Pub. No.: US 2004/0133938 A1

Das AB, Rout GR, Das P (1995) *In vitro* somatic embryogenesis from callus culture of the timber yielding tree *Hardwickia binata Roxb. Plant Cell Rep.* **15**: 147-149

Das G, Rout GR (2002) Direct plant regeneration from leaf explants of *Plumbago* species. *Plant Cell Tiss. Org. Cult.* **68**: 311-344

Day RB, OkadaM, Ito Y, Tsukada K, Zaghouani H, Shibuya N, Stacey G (2001) Binding site for chitin oligosaccharides in the soybean plasma membrane. *Plant Physiol.* **126**: 1162–1173

De Block M, D'Halluin K, Botterman J, Leemans J (1988) The use of phosphinothricin resistance as a selectable marker in tobacco protoplast transformation. *Curr. Plant Sci. Biotechnol. Agric. Dordrecht*, Kluwer Academic Publishers. p.389-390

De Bondt A, Eggermont K,Duart P, De Vil M,Goderis I, Vander layen Jand Brooekaert WF (1994) *Agrobacterium* mediated transformation of apple (*Malus* x *domestica* Borkh): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant cell Rep.* **13**: 587-593

Dhavala VN, Tejeswara Rao D, Yechuri VR, Prabavathi K (2009) Effect of explant age, hormones on somatic embryogenesis and production of multiple shoot from cotyledonary leaf explants of *Solanum trilobatum* L. *African Journal of Biotechnology*. **8 (4)**: 630-634

Dijkman MJ (1951) *Hevea*: *Thirty years of research in the Far East*. University of Miami Press, Florida, 5–7

Dodds JH, Roberts LW (1995) Experiments in Plant Tissue Culture, Cambridge University Press, Cambridge

Doyle JJ, Doyle JL (1990) A rapid total DNA preparation procedure for fresh plant tissue. *Focus* **12**: 13-15

Dunstan DI, Tautorius TE, Thorpe TA (1995) Somatic embryogenesis in woody plants. In *In Vitro* Embryogenesis in Plants. Ed. T.A. Thorpe. Kluwer Academic Publishers, Dordrecht, pp 471–538

Emilie M, Nicolas N, Soupi NMS, Sangarae A and Denis ON (2007) Implication of cysteine, glutathione and cysteine synthase in *Theobroma cacao L. Zygotic embryogenesis. Biotechnology* **6(1)**: 129-137

Enjalric F, Caron MP, Lardet L (1987) Contamination of primary cultures in tropical areas: the case of *Hevea brasiliensis*. International Symposium Cork, Ireland. *Acta Horticulture* **225**: 57-65

Enriquez-Obregon GA, Prieto-Samsonov, DL, Riva GA, de la Perez M, Selman-Housein G, Vazquez-Padron RI (1999) *Agrobacterium*-mediated japonica rice transformation: a procedure assisted by an antinecrotic treatment. *Plant Cell Tiss. Org. Cult.* **59(3)**: 159-168

Enriquez-Obregon GA, Vazquez-Padron RI, Prieto-Samsonov DL, de a Riva GA, Selman-Housein G (1998) Herbicide-resistant sugarcane (*Saccharum officinarum L.*) plants by *Agrobacterium* mediated transformation. *Planta.* **206**: 20-27

Enriquez-Obregon GA, Vazquez-Padron RI, Prieto-Samsonov DL, Perez M, Selman-Housein G (1997) Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using antioxidant compounds. *Biotecnol. Apl.* **14**: 169-174

Etienne H, Berger A, Carron MP (1991) Water status of callus from *Hevea brasiliensis* during induction of somatic embryogenesis. *Physiol. Plant.* **82**: 213-218

Etienne H, Lartaud M, Michaux-ferriere N, Carron MPB, Berthouly M, Teisson C (1997) Improvement of somatic embryogenesis in *Hevea brasiliensis* (Müll.

Arg.) using temporary immersion technique. *In vitro Cellular and Developmental Biology* - Plant, April 1997, vol. **33(2)**: 81-87

Etienne H, Michaux-ferriere N, Montoro P, Carron MP (1993a) Effects of dessication, medium osmolarity and abscisic acid on the maturation of *Hevea brasiliensis* somatic embryos. *J. Exp. Bot.* 44(10): 1613 - 1619

Etienne H, Sotta B, Montoro P, Miginiac E, Carron MP (1993b) Relations between exogenous growth regulators and endogenous indole-3-acetic acid and abscisic acid in the expression of somatic embryogenesis in *Hevea brasiliensis* (Mull. Arg). *Plant Science*. **88**: 91-96

Feher A (2006) Why somatic plant cells start to form embryos? In:Mujib, A.; Šamaj, J. (Ed.). **Somatic embryogenesis**. Berlin:Springer, (*Plant Cell Monographs*, **2**) 357p

Feher A (2008) The initiation phase of somatic embryogenesis: what we know and what we don't. *Acta Biologica Szegediensis* **52(1)**: 53-56

Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SEK, Li B, Nettleton DS, Pei D, Wang K (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiol.* **129**:13- 22

Fuentes SRL, Calheiros MBP, Manettifilho J, Vieira LGE (2000) The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora. Plant Cell, Tissue and Organ Culture* **60(1)**: 5-13

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension culture of soybean root cells. *Experimental Cell Research* **50**: 151-158

Gautheret RJ (1939) CR Acad Sci (Paris), **208**: 118-21

George L (1993) Somatic embryogenesis in peanut: influence of growth regulators and sugars. *Plant Cell, Tissue and Organ culture* **35**: 151-156

Giles KL, Morgan WM (1987) Industrial-scale plant micropropagation. *Trends in Biotechnology* **5(2)**: 35-39

Giridhar P, Indu EP, Vijaya Ramu D, Andravishankar GA (2003) Effect of silver nitrate on *in vitro* shoot growth of *Coffee. Tropical Science* **43(3)**: 144-146

Goldberg R, Liberman M, Mathieu C, Pierron M, Catesson AM (1987) Development of epidermal cell wall peroxidases along the Mung bean hypocotyls: Possible involvement in the cell wall stiffening process. *J. Experimental Botany* **38(193)**: 1378-1390

Greenwood MS (1995) Juvenility and maturation in conifers: current concepts. *Tree Physiol.* **15**: 433–438

Greenwood MS, Hopper CA, Hutchison KW (1989) Maturation in larch. I. Effect of age on shoot growth, foliar characteristics, and DNA methylation. *Plant Physiol.* **90**: 406 – 412

Gunatilleke ID, Samaranayake C (1988) Shoot tip as a method of micropropagation of *Hevea. Journal of Rubber Research Institute of Sri Lanka* **68**: 33-44

Guohua M, Qushang X (2002) Induction of somatic embryogenesis and adventitious shoots from immature leaves of cassava. *Plant Cell, Tiss. Org. Cult.* **70**: 281-288

Gupta PK, Timmis R, Mascarenhas AF (1991) Field performance of micropropagated forestry species. *In vitro Cell Dev. Bio.* **27**: 159

Haberlandt G (1902) (Translated by krikooriant Dand Berquam: Experiments on culture of isolated plant cells. *Bot.Rev.***35**: 68-88

Hackett PW, Murray JR (1996) Maturation or Phase change. In: Thompson Dwealander. (eds). Annual report of CA AIR3-CT94-2202. Molecular and morphological makers for juvenility, maturity, rejuvenation and somatic embryogenesis in woody plant species. *The Biotechnological approach CPG XII-E* pp7-26

Hackett WP (1985) Juvenility, maturation, and rejuvenation in woody plants. *Hortic. Rev.* **7**: 109–155

Hackett, W. P. and Murray, J. R. (1993) Maturation and rejuvenation in woody species. In *Micropropagation of Woody Plants* (ed. M. R. Ahuja), pp.93-105. Kluwer, Netherlands

Hadrami El I, Michaux-Ferriere N, Carron MP, d'Auzae J (1989) Polyamines, a possible limiting factor in somatic embryogenesis of *Hevea brasiliensis. Comptes Rendus de Academic des Sciences de Paris.* **308(III)**: 205-211

Haffner VF, Enjalric L, Lardet MP, Carron (1991) Maturation of woody plants: a review of metabolic and genomic aspects. Ann. Sci. For. **48**: 615–630

Hamama L, Baaziz M, Letouze R (2001) Somatic embryogenesis and plant regenerarion from leaf tissue of jojoba. *Plant, Tiss. Org Cult.* **65**: 109-113

Hammatt N (1993) Micropropagation of fastigiated bird cherry *(Prunus padus L.)* and adventitious shoot formation from leaves. *J. Hort. Sci.* **68**: 975-981

Harris R, Wright M, Byrne M, Varnum J, Brightwell B, Schubert K (1988) Callus formation and plant regeneration from protoplast derived suspension cultures of wheat (*Triticum aestivum* L) *Plant Cell Reports* 7: 337-340

Harry IS, Thorpe TA (1991) Somatic Embryogenesis and plant regeneration from mature zygote embryos of red spruce. *Botanical Gazette*, **152**: 446-452

Hatanaka T, Arakawa T, Yasuda T, Uchida N, Yamaguchi T (1991) Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora. Plant Cell Rep.* **10**: 179-182

Henrique CS, Carvalho, Usha B, Gunaratna ZN, Anderson J, Halina H, Kononwiz, Thomas KH, John DA (2004) *Agrobacterium* mediated transformation of sorghum: factors that affect transformation efficiency. *Gactus and Mol. biology* **27(2)**: 259-269

Hoagland DR, Arnon, DI (1950) The water culture method for growing plants without soil. *Calif. Agri. Exp. Stn. Circ.*, 347: 39

Huang CL, Hsieh MT, Hsieh WC, Sagare AP, Tsay HS (2000) *In vitro* propagation of *Limonium wrightii* (Hance) Ktze. (Plumbaginaceae), an ethnomedicinal plant, from shoot-tip, leaf- and inflorescence-node explants. *In Vitro Cellular and Developmental Biology- Plant.* **36**: 220-224

Hulme JS, Higgins ES, Shields R (1992) An efficient genotype independent method for regeneration of potato plants from leaf tissue. *Plant Cell Tiss. Org. Cult.* **31**: 161-167

Hutchison KW, Sherman CD, Weber J, Smith SS, Singer PB, Greenwood MS (1990) Maturation in larch II: Effects of age on photosynthesis and gene expression in developing foliage. *Plant Physiol.* **94**:1308–1315

Ignacimuthu S, Arockiasamy S, Antonysamy M, Ravichandran P (1999) Plant regeneration through somatic embryogenesis from mature leaf explants of *Eryngium foetidum*, a condiment. *Plant Cell, Tiss. Org. Cult.* **56**: 131-137

James DJ, Passey AJ, Malhotra SB (1984) Organogenesis in callus derived from stem and leaf tissues of apple and cherry root stocks. *Plant Cell.Tiss. Org. Cult.* **3**: 333-341

Jansen MAK, Booij H, Schel JHN, de Vries SC (1990) Calcium increases the yield of somatic embryos in carrot embryogenic suspension cultures. *Plant Cell Rep* **9**: 221–223

Jayashree R, Rekha K, Sushamakumari S, Thulaseedharan A (2005) Establishment of callus cultures from isolated microspores of *Hevea brasiliensis. National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops*, Trissur, pp 176-178

Jayasree PK, Asokan MP, Sobha S, Sankari Ammal L, Rekha K, Kala RG, Jayasree R, Thulaseedharan A (1999) Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell Arg.) *Curr. Sci.* **76**: 1242-1245

Jayasree R, Rekha K, Uratsu SL, Venketachalam P, Jayasree PK, Kala RG, Priya P, Sushamakumari S, Sobha S, Asokan MP, Sethuraj MR, Thulaseedharan A, Dandekar M (2003) *Agrobacterium* mediated genetic transformation and regeneration of transgenic plants *Hevea brasiliensis* Muell Arg, via somatic embryogenesis. *Plant Cell Rep.* **22**: 201-209

Jayasree T, Pavan U, Ramesh M, Rao AV, Reddy KJM, Sadanandam A (2001) Somatic embryogenesis from leaf cultures of potato. *Plant Cell, Tiss. Org. Cult.* **64**: 13-17

Jean-Paul Roustan, Alain Latche, Jean Fallot (1990) Control of carrot somatic embryogenesis by $AgNO_3$, an inhibitor of ethylene action: Effect on arginine decarboxylase activity. *Plant Science* **67(1)**: 89-95

Jefferson RA, Karanagh TA, Beven MW (1987) GUS fusion: b- Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6(13)**: 3901-3907

Kala RG, Asokan MP, Kumari Jayasree P, Sobha S, Jayasree R, Rekha K Thulaseedharan A (2002) *In vitro* micrografting in *Hevea brasiliensis:* optimisation of conditions. *Indian Journal of Natural Rubber Research.*Vol.**15** (2)

Kala RG, Gimisha GC, Kumari Jayasree P, Sushamakumari S, Sobha S, Jayasree R, Rekha K, Thulaseedharan A (2009) Somatic embryogenesis in leaf cultures of *Hevea brasiliensis:* effect of source plant. *Natural Rubber Research* **22(1&2):** 117

Kala RG, Kumari Jayasree P, Sobha S, Rajani Joseph, Thulaseedharan A (2006) Plant regeneration from leaf derived embryos of *Hevea brasiliensis.* International Symposium on Frontiers in Genetics and Biotechnology-Retrospect and Prospect, Jan. 8-10, Osmania University, Hyderabad.

Kala RG, Kumari Jayasree P, Sushamakumari S, Jayashree R, Rekha K, Sobha S, Thulaseedharan A (2005) *In vitro* regeneration of *Hevea brasiliensis* from leaf explants. *ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies*, Trichur, India, pp 105-106

Kala RG, Leda Pavithran, Thulaseedharan A (2004) Effect of fungicides and antibiotics to control microbial contamination in *Hevea* cultures. *Plant Cell Biotechnology and Molecular Biology* **5**: 51-58

Kintzios S, Drossopoulos JB, Lymperopoulos C (2001) Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from leaves of chilli pepper. *Plant Cell Tiss. Org.Cult.* **67**:55-62

Kumar V, Parvatam G, Ravishankar GA (2009) $AgNO_3$ - a potential regulator of ethylene activity and plant growth modulator. *Electronic Journal of Biotechnology* **12(2)**: 0717-3458

Kumari Jayasree P, Thulaseedharan A (2001) Gibberllic acid regulated embryo induction and germination in *Hevea brasiliensis* (Muell. Arg.). *Indian Journal of Natural Rubber Research* **14**: 106-111

Kumari Jayasree P, Thulaseedharan A (2004) Initiation and maintenance of long term somatic embryogenesis in *Hevea brasiliensis. IRRDB, Biotechnology Workshop*, Kuala Lumpur, Malaysia, **56**

Kumari Jayasree P, Vinoth Thomas, Saraswathyamma CK, Thulaseedharan A (2001) Optimization of parameters affecting somatic embryogenesis in *Hevea brasiliensis. Indian Journal of Natural Rubber Research* **14**: 20-29

Kush A, Goyvaerts E, Chye ML,Chua NH (1990) Laticifer-specifc gene expression in *Hevea brasiliensis* (rubber tree), *Proceedings of the National Academy of Sciences of USA.* **87**: 1787–1790

Lakshmi Sita G, Rhagava Ram NV, Vaidyanathan CS (1979) Differentiation of embryoids and plantlets from shoot callus of sandalwood. *Plant Sci. Lett.* **15**: 265-270

Landi L, Mezzetti B (2006) TDZ, auxin and genotype effects on leaf organogenesis in Fragaria. *Plant Cell Rep.* **25**: 281–288

Lardet L, Aguilar, ME, Michaux- Fermere N, Berthouly M (1998) Effect of strictly plant related factors on the response of *Hevea brasiliensis* and *Theobroma cacao* nodal explants cultured *in vitro. In Vitro Celular Development Biology (Plant)* **34**: 34-40

Lardet L, Dessailly F, Carron MP, Monteuuis O (2009) Influence of aging and cloning methods on the capacity for somatic embryogenesis of a mature *Hevea brasiliensis* genotype. *Tree Physiology* **29**: 291-298

Lardet L, Dessailly F, Ferrière N, Carron MP, Montoro P (2008) First evidence of secondary somatic embryogenesis in *Hevea brasiliensis* (Müll. Arg.) as an alternative process for long-term somatic embryogenesis. *In Vitro Cell. Devel. Biol. Plant* 2008. (In press)

Lardet L, Enjalric F, Carron MP (1990) Culture *d'apex d'Hevea brasiliensis (Müll. Arg.)*. Influence du stade morphogénétique et de l'âge des explants. *C. R. Acad. Sci. Paris* 1990; **310**(Série III):195-202

Lardet L, Martin F, Dessailly F, Carron MP, Montoro P (2007) Effect of exogenous calcium on post-thaw growth recovery and subsequent plant regeneration of cryopreserved embryogenic calli of *Hevea brasiliensis* (Mull. Arg.). *Plant Cell Reports* **26**: 559-569

Lavee S, Avidan N, Haskal A, Ogrodovich A (1996) Juvenility period reduction in the olive seedlings - a tool for enhancement of breeding. *Olivae* **60**: 33-41

Lelu MA, Klimaszewska K, Charest P (1994) Somatic embryogenesis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of Larix. *Can. J. For. Res.* **24**: 100–106

Leroy T, Henry AM, Royer M, Altosaar I, Frutos R, Duris D, Philippe R (2000) Genetically modified coffee plants expressing the *Bacillus thuringiensis cry* 1Ac gene for resistance to leaf miner. *Plant Cell Reports* **19(4)**: 382-385

Li XY, Huang FH, Gbur Jr EE (1998) Effect of basal medium growth regulators and phytagel concentration on initiation of embryogenic cultures from immature zygotic embryos of loblolly pine (*Pinus taeda l.*). *Plant Cell Reports* **17**: 298-301

Li-Chun Huang, Jui-Hsi Weng, Chiu-Hui Wang, Ching-I Kuo, and Yuh-Jang Shieh (2003) Photosynthetic potentials of *in vitro*-grown juvenile, adult, and rejuvenated *Sequoia sempervirens* (*D. Don*) *Endl. Shoots. Bot. Bull. Acad. Sin* **44**: 31-35

Linossier L, Veisseire P, Cailloux F and Coudret A (1997) Effect of abscisic acid and high concentrations of PEG on *Hevea brasiliensis* somatic embryos development. *Plant Science* **124**: 183-191

Litz RE, Gray DJ (1995) Somatic embryogenesis for agricultural improvement. *World Journal of Microbiology and Biotechnology.* **11**: 416- 425

Liu SJ, Wei ZM, Huang JQ (2008) The effect of co-cultivation and selection parameters on *agrobacterium* mediated transformation of Chinese soybean varieties. *Plant Cell Rep.* **27**: 489-498

Liu X, Pijut PM (2008) Plant regeneration from *in vitro* leaves of mature black cherry (*Prunus serotina*). *Plant Cell Tissue and Organ Culture* **94**: 113-123

Lloyd G, Mc Cown G (1980) Commercially feasible micropropagation of Mountain Laurel, *Kalmia latifolia*, by use of shoot tip culture. Comb. Proc. Intern. Plant Prop. Soc. **30**:421-427

Martino-Catt SJ, Sachs ES (2008) Editor's choice series: the next generation of biotech crops. *Plant Physiol* **147**: 3–5

Mathias RJ, Boyd LA (1986) Cefotaxime stimulates callusgrowth, embryogenesis and regeneration in hexaploid breadwheat (*Triticum aestivum* L. EM. THELL). *Plant Sci.* **46**: 217-223

Mendanha ABL, Torres RAA, de Freire (1998) Micropropagation of rubber trees (*Hevea brasiliensis* Muell. Arg). *Gene. Mol. Biol.* **21**: 395-398

Michaux-Ferriere N, Carron MP (1989) Histology of early somatic embryogenesis in *Hevea brasiliensis*: the importance of the timing of subculturing. *Plant Cell Tissue and Organ Culture* **19**: 243-256

Monteuuis O (2004) *In vitro* rooting of juvenile and mature *Acacia mangium* microcuttings with reference to leaf morphology as a phase change marker. *Trees* **18**: 77–82

Monteuuis O (1987) *In Vitro* meristem culture of juvenile and mature *Sequoiadendron gigantum* during rest and growth phases. *Tree Physiol* **3**: 265-272

Monteuuis O (1989) Maturation concept and possible rejuvenation of arborescent species. Limits and promises of shoot apical meristems to ensure successful cloning. *In:* Gibson G.L., Griffin A.R., Matheson A.C. *editors.* Breeding Tropical Trees: Population Structure and Genetic Improvement Strategies in Clonal and Seedling Forestry; *Proc. Conference IUFRO*; *Pattaya, Thailand.. p.* 106-118.

Montoro P, Etienne H, Carron MP (1995) Effect of calcium on callus friability and somatic embryogenesis in *Hevea brasiliensis* Muell.Arg. Relations with callus mineral nutrition, nitrogen metabolism and water parameters. *Journal of Experimental Botany* **46**: 255-261

Montoro P, Etienne H, Michaux- Ferriere N , Carron MP (1993) Callus friability and somatic embryogenesis in *Hevea brasiliensis. Plant Cell Tiss. Org. Cult.* **33**: 331-338

Montoro P, Teinseree N, Rattana W, Kongsawadworakul P, Michaux-Ferriere N (2000) Effect of exogenous calcium on *Agrobacterium tumefaciens*-mediated gene transfer in *Hevea brasiliensis* (rubber tree) friable calli. *Plant Cell Reports* **19**: 851-855

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant.* **15**: 473-497

Murray JR, Concepcion Sanchez M, Smith AG, WP Hackett (1994*a*) Differential competence for adventitious root formation in histologically similar cell types. *In* Biology of Adventitious Root Formation. Eds. T.D. Davis and B.E. Haissig. Plenum Press, New York, pp 99-110

Murray JR, AG Smith, Hackett WP (1994*b*) Differential dihydroflavanol reductase transcription and anthocyanin pigmentation in the juvenile and mature phases of ivy (*Hedera helix* L.). *Planta* **194**: 102-109

Murray JR, Hackett WP (1991) Dihydroflavanol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature *Hedera helix* L. *Plant Physiol.* **97**: 343-351

Nakagawa, H, Saijyo T, Yamauchi N, Shigyo M, Kako S, Ito A (2001) Effects of sugars and abscisic acid on somatic embryogenesis from melon (*Cucumis melo* L.) expanded cotyledon. *Sci. Hort.* **90**:85–92

Nayanakantha NMC, Seneviratne P (2007) Tissue Culture of Rubber: past, present and future prospects *Cey. J. Sci. Bio. Sci.* **36 (2)**: 116-125

Nester EW, Gordon MP, Amasino RM, Yanofsky MF (1984) Crown gall: a molecular and physiological analysis. *Annual Review of Plant Physiology* **35**: 387-413

Niinemets Ü (2002) Stomatal conductance alone does not explain the decline in foliar photosynthetic rates with increasing tree age and size in *Picea abies* and *Pinus sylvestris*. *Tree Physiology* **22**: 515-535

Olhoft PM, Flagel LE, Donovan CM, Somers DA (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta* **216**: 723-735

Olhoft PM, Lin K, Galbraith J, Nielson NC, Somers DA (2001) The role of thiol compounds in increasing *Agrobacterium* mediated transformation of soybean cotyledonary node cells. *Plant Cell Rep.* **20**: 731-737

Omay A, Jeffrey CE (2000) Somatic embryogenesis in Pistachio (*Pistachia vera*) Somatic Embryogenesis in Woody Plants, Volume 6 Edts. By S. Mohan Jain, Pramod K.Gupta, RonaLD J Newton, Kluwer Academic Publishers

Opabode JT (2006) *Agrobacterium* mediated transformation of plants: emerging factors that influence efficiency. *Biotechnology and Molecular Biology Review* **1(1)**: 12-20

Orlikowska T (1997) Silver nitrate inhibits bacterial growth in plant tissue cultures. *Agri cell Reports* **29(4)**: 25

Overvoorde PJ, Grimes HD (1994) The role of calcium and calmodulin in carrot somatic embryogenesis. *Plant Cell Physiol* **35**: 135–144

Ozden-Tokatli Y, Ozudogru, EA, and Akrin A (2004) *In vitro* regeneration of Pistachio through organogenesis: effect of silver nitrate, polyvinylpyrolidone and citric acid. *Proceedings of '2004 World Congress on In Vitro Biology'* **40**: 46-50

Packer L, Tritschler H (1996) Alpha-lipoic acid: The metabolic antioxidant free Radical. *Biol. Med.* **20**: 625–626

Packer L, Witt E, Tritschler H (1995) Alpha-lipoic acid as a biological antioxidant. Free Radical Biol *Med* **19**: 227–250

Pacques MJ, Bercetches, Harvengt L (1997) Somatic embryogenesis: a way to recover clones from needles of selected Norway spruce. *Proc. Joint Meeting of IUFRO Working Parties 2.04.07 and 2.04.06*, Québec City, 4 p.

Paranjothy K (1974) Induced root and embryoid differentiation in *Hevea brasiliensis* in culture. *3rd International Congress of PlantTissue and Cell Culture. Univ. Leicester Abstract* No. 67

Paranjothy K, Ghandimathi H (1975) Morphogenesis in callus cultures of *Hevea brasiliensis* Muell. Arg. *Proceedings of Plant Tissue Culture Symposium, Kuala Lumpur, Malaysia.* 19-25

Paranjothy K, Gandimathi H (1976) Tissue and Organ Culture of *Hevea. Proceedings of International. Rubber Conference, Kuala Lumpur, Malaysia.* 59-84

Paranjothy K, Rohani O (1978) Embryoid and plantlet development from cell culture of *Hevea. 4th International Congress of Plant. Tissue and Cell Culture, University of Calgary. Abstract* No. **134**

Paz MM, Shou H, Guo Z, Zhang Z, Banerjee AK, Wang K (2004) Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant. *Euphytica* **136**: 167–179

Pedroso MC, Pais MS (1993) Direct embryo formation in leaves of *Camellia japonica L. Plant Cell Rep.* **12**: 639-643

Perrin Y, Lardet, L, Enjalric F, Carron MP (1994) Rejuvenation of mature clones of *Hevea brasiliensis* (Muell. Arg) by *in vitro* micrografting. *Canadian Journal of Plant Science*, Kluwer Academic Publisher, The Netherlands. **74**: 623-630

Perrin Y, Doumas P, Lardet L, Carron M P (1997) Endogenous cytokinins as a biochemical marker of rubber tree (*Hevea brasiliensis* Mull.Arg.) clone rejuvenation. *Plant Cell, Tissue and Organ Culture* **47**: 239-245

Prevost I, LePage-Degivry MTh (1985) Inverse correlation between ABA content and germinability throughout the maturation and the *in vitro* culture of the embryo of *Phaseolus vulgaris*. *J Expt Bot* **36**: 1457-1464

Pullman GS, Namjoshi K, Zhang Y (2003) Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): improving culture initiation with abscisic acid and silver nitrate *Plant Cell Rep (2003)* **22**: 85–95

Rao PS, Vijayakumar KR (1992) Climatic requirements.In: Sethuraj, M.R. and Mathew, N.M. (eds.) *Natural Rubber: Biology, Cultivation and Technology.* Elsevier, Amsterdam, 200–219

Rohani O, Paranjothi K (1980) Isolation of *Hevea* protoplasts. *Journal of Rubber Research Institute of Malaysia* **28**: 61-66

Romano A, Barros S, Martins-Loucao MA (2002) Micropropagation of the Mediterranean tree *Ceratonia Siliqua. Plant Cell Tiss. Org. Cult.***68**: 35-41

Ruaud JN, J Bercetche, Paques M (1992) First evidence of somatic embryogenesis from needles of 1-year-old *Picea abies* plants. *Plant Cell Rep.* **11**: 563–566

Rugkhla A, Jones MGK (1998) Somatic embryogenesis and plantlet formation in *Santalum album* and S. *spicatum. J. of Exptl. Bot.* **49**: 563-571

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sambrook J, Russell DW (2001) Molecular cloning. A laboratory manual, vol 1, 3rd edn. Cold Spring Harbor Laboratory press, New York, pp 6.33–6.44

San Jose MC, Vieitez AM (1993) Regeneration of *Camellia* plantlets from leaf explant cultures by embryogenesis and caulogenesis. *Sci Hort.* **54**: 303-315

Seneviratne P (1991) Micropropagation of juvenile and mature *Hevea brasiliensis.* PhD.Thesis, University of Bath, UK. Pp 48-167

Seneviratne P, Flegmann AW (1996) The effect of thidiazuron on axillary shoot proliferation of *Hevea brasiliensis in vitro. J. Rubb. Res. Inst. Sri Lanka.* **77**: 1-14

Seneviratne P, Gammanaliyanage, SS Wijesekera GAS (1998) The effect of the origin of explant on the *in vitro* growth of axillary buds of *Hevea brasiliensis. Tropical Agricultural Research and Extension* **1(2)**: 98-102

Seneviratne P, Wijesekara GAS (1997) Effect of episodic growth pattern on *in vitro* growth of *Hevea brasiliensis. Journal of Plantation Crops.* **25**: 52-56

Seneviratne P, Withange SP, Nugawela A,Wijesekera GAS Oysa GM de (1996) Somatic embryogenesis for *Hevea.* The technique for anther culture. *J. Rubb. Res. Inst. Sri Lanka.* **78**:79-99

Seong ES, Song KJ, Jegal S, Yu CY, Chung IM (2005) Silver nitrate and amino ethoxyvinylglycine affect *Agrobacterium* mediated apple transformation. *Plant growth Regulation* **45**:75-82

Sharma KK, Bhatnagar-Mathur P, Thorpe TA (2005) Genetic transformation technology: status and problems. *In Vitro Cell. Dev.Biol.* Plant. **41**: 102-112

Shiji Z, Zherghua C, Xueng X (1990) A summary report on anther culture for haploid plants of *Hevea brasiliensis. Plant Cell Environment.* **4**: 81-83. In: *Proceedings ofIRRDB symposium on Breeding of Heveabrasiliensis, Kuming, China, 5-6 October.* 69-78

Sinha RR, Sobhana P, Sethuraj MR (1985) Axillary buds of some high yielding clones of Hevea in culture. First IRRDB Hevea tissue culture workshop. Kuala Lumpur, Malaysia. Pp. 16

Smith EF, Towsend CO (1907) A plant tumor of bacterial origin. *Science* **25**: 671-673

Sobha S, Reshma RS, Sushama Kumari S, Rekha K, Jayashree R, Kala RG, Jayasree P, Supriya R, Thulaseedharan A (2010) *Agrobacterium* mediated genetic transformation of *Hevea brasiliensis:* Effect of acetosyringone and pre-culture of the target tissue on transformation efficiency. *Placrosym* .Dec.7-10

Sobha S, Sushama Kumari S, Thanseem I, Rekha K, Jayashree R, Kala RG, Kumari Jayasree P, Asokan MP, Sethuraj MR, Dandekar AM, Thulaseedharan A (2003) Genetic transformation of *Hevea brasiliensis* with the gene coding for superoxidedismutase with FMV 34S promoter. *Current Science* **5**: 1767-1773

Sobha S, Sushamakumari S, Venkatachalem P, Kala RG, Rekha K, Jayashree R, Leda P, Thulaseedharan A (2009) Somatic embryogenesis from the *in vitro* root explants of transgenic *Hevea brasiliensis* and validation of stable gene integration. *Indian Journal of Natural Rubber Research* **22(1&2)**: 43

Sobhana P, Sinha RR, Sethuraj MR (1986) Micropropagation of *Hevea brasiliensis*: retrospect and prospects. In: International Congress of Plant Tissue and Cell Culture, Minnesota, U.S.A

Sompong TC, Muangkaewngam A (1992) Tissue culture of rubber *In vitro* micropropagation of rubber. Songklanakarin *Journal of Science and Technology* **14**: 123-132

Sonia, Alfonso (2003) Mature trees versus seedlings: Differences in leaf traits and gas exchange patterns in three co-occurring Mediterranean oaks *Ann. For. Sci.* **60** 455–460

Steele MJ, Coutts MP, Yeoman MM (1989) Developmental changes in Sitka spruce as indices of physiological age. I. Changes in needle morphology. *New Phytol.* **113**: 367-375

Steward FC, Mapes MO and Mears K (1958) Growth and organised development of cultured cells.II.Organisation in cultures grown from freely suspended cells *Amer.J.Bot.* **45**: 705-708

Strable J, Borsuk L, Nettleton D, Patrick SS, Irish EE (2008) Microarray analysis of vegetative phase change in maize *The Plant Journal* **56**: 1045–1057

Subramaniam S, Midrarullah SR, Rathinam X (2009) Preliminary Factors Influencing Transient Expression of *Gusa* in *Dendrobium* Savin White Protocorm-like Bodies (PLBs) using *Agrobacterium*-mediated Transformation System *World Applied Sciences Journal* 7 (10): 1295-1307

Su YJ, Chen JT and Chang WC (2002) Efficient and repetitive production of leaf-derived somatic embryos of *Oncidium. Biologia Plantarum* **50(1)**: 107-110

Sushmakumari S, Rekha K, Thomas V, Sobha S, Jayasree R (1999) Multiple Shoot formation from somatic embryos of *Hevea brasilliensis* (Muell.)Arg. *Indian.J. Nat.Rubb.Res.***12**: 23-28

Sushamakumari S, Sobha S, Rehka K, Jayasree R, Asokan MP (2000) Influence of growth regulators and sucrose on somatic embryogenesis and plant regeneration from immature inflorescence of *Hevea brasiliensis. Indian Journal Natural Rubber Research* **13**: 19-29

Tao Huang, Shaolin P, Gaofeng D, Lanying Z, Gengguang L (2002) Plant regeneration from leaf derived callus in *Citrus grandis*: Effects of auxins in callus induction medium. *Plant Cell,Tiss.Org.Cult.* **69**: 141-146

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence

weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **11(22)**: 4673-80

Thulaseedharan A (2002) Biotechnological approaches for crop improvement in natural rubber at RRII—present status. *Proceedings of the Rubber Planters' Conference*. Kottayam, India, April 28–30, pp. 135–140

Thulaseedharan A, Kumari Jayasree P, Venkatachalam P (2000) Biotechnological approaches for crop improvement in rubber. In, *Biotechnology in Horticultural and plantation Crops.* Chadha K.L. Ravindran, P. N. and Sahijram L. (*Eds.*) Malhotra Publishing House (Calcutta) Pp: 323-351

Thulaseedharan A, Venkatachalam P, Kala RG, Thanseem I, Priya P, Saleena A, Kumari Jayasree P (2004) Biotechnology research in rubber: present status and future prospects. *Journal of plantation crops*, **32 (suppl.)**: 104-116

Vaeck M, Reynaerts A, Hofte H, Jansens S, De Beuckeleer M, Dean C, Zabeau M, Van Montague M AND Leemans J (1987) Transgenic plants protected from insect attack. *Nature* **328**: 33-37

Vain P, Yean H, Flament P (1989) Enhancement of production and regeneration of embryogenic type 11 callus in Zea mays L. by $AgNO_3$ *Plant Cell, Tissue and Organ Culture* **18**: 143-151

Van Attikum H, Hooykaas PJJ (2003) Genetic requirements for the targeted integration of *Agrobacterium* T-DNA in *Saccharomyces cerevisiae. Nucleic Acids Res* **31:** 826–832

Van Winkle SC, Jhonson S, Pullman GS (2003) The impact of Gelrite and activated carbon on the elemental composition of two conifer embryogenic tissue initiation media. *Plant Cell Reports,* **21**: 1175-1182

Varghese YA, Mydin KK (2000) Genetic improvement. In: George, P.J. and Jacob, C.K. (eds.) *Natural Rubber. Agromanagement and Crop Processing.* Rubber Research Institute of India, Kottayam, 36–46

Varghese YA, John A, Premakumary D, Panikkar AON, Sethuraj MR (1992) Early evaluation in *Hevea*: Growth and yield at the juvenile phase. *Indian Journal of Natural Rubber Research* **6 (1, 2)** 19–23

Veisseire P, Cailloux F, Coudret A (1994a) Effect of conditioned media on the somatic embryogenesis of *Hevea brasiliensis. Plant Physiology and Biochemistry* **32**: 571–576

Veisseire P, Linossier L, Coudret A (1994b) Effect of Abscisic acid and cytokinins on development of somatic embryos in *Hevea brasiliensis. Plant Cell Tissue and Organ Culture* **39**: 219-223

Veluthambi K, Aditya KG, Sharma A (2003) The current status of plant transformation technologies. Curr. Sci., **84(3)**: 368–37

von Aderkas P, Bonga JM (2000) Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree Physiol.* **20**: 921–928

Wan Abdul Rahaman, Ghandimathi WY, Rohani H, Paranjothy K (1981) Recent developments in tissue culture of *Hevea.* In: RaoA.N (Ed.) *Tissue Culture of economically important plants.* Costed, Singapore, Pp 152-158

Wang Z, Zeng X, Chen C, Wu H, Li Q, Fan G, Lu W (1980) Induction of rubber plantlets from anther of *Hevea brasiliensis* Muell. Arg. *In vitro Chinese Journal of Tropical Crops* **1**: 25-26

Watson GA (1989) Climate and soil. In: Webster, C.C. and Baulkwill, W.J. (eds.) *Rubber.* Longman Scientific and Technical, Essex, 125–164

Wendt ALS, Silveira V, Steiner N, Vidor M, Guerra MP (2002) Somatic Embryogenesis in Parana Pine (*Araucaria angustifolia* (Bert.) O. Kuntze) *Braz. arch. biol. Technol.* **45(1)**: 97 - 106

Wernicke W, Bretell R (1980) Somatic embryogenesis from soyabean bicolour leaves *Nature* **287**:138-139

Wernicke W, Potrykus I, Thomas E (1982) Morphogenesis from cultured leaf tissue of *Sorghum bicolor*—The morphogenetic pathways. *Protoplasma.* 111(1), 53-62

Wilson ZA, Power JB (1989) Elimination of systemic contamination in explants and protoplast cultures of rubber (*Hevea brasiliensis)* Muell. Arg. *Plant Cell Reports* 7: 622-625

Wilson HM, Street HE (1975) The growth, anatomy and morphogenetic potential of callus and cell suspension cultures of *Hevea brasiliensis.* Annals of Botany **39**: 671-682

Wilson HM, Street HE (1974) The growth, anatomy and morphogenetic potential of callus and cell suspension cultures of *Hevea brasiliensis. Physiol. Plant. 36*: 399-402

Woo HH, Hackett WP Das A (1994) Differential expression of a chlorophyll a/b binding protein gene and a proline rich protein gene in juvenile and mature phase English ivy (*Hedera helix*). *Physiol. Plant.* **92**: 69–78

Ye G-N, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) Arabidopsis ovule is the target for *Agrobacterium* in planta vaccum infilteration transformation. *Plant J.* **19**: 249-257

Yusuf A, Tyagi RK, Malik SK (2001) Somatic embryogenesis and plantlet regeneration from leaf segments of *Piper colubrinum*. *Plant Cell, Tissue and Organ Culture* **65(3)**: 255 - 258

Zeng P, Vadnais DA, Zhang Z, Polacco JC (2004) Refined glufosinate selection in *Agrobacterium*-mediated transformation of soybean Glycine max (L.) Merrill. *Plant Cell Rep.* **22(7)**: 478-482

Zhang PS, Phansiri J, Puonti-Kaerlas (2001) Improvement of Cassava shoot organogenesis by the use of silver nitrate *in vitro*. *Plant Cell, Tiss. and Org. Cult.* **67**:47-54

Zhang Q, Chen J, Henny RJ (2005) Direct somatic embryogenesis and plant regeneration from leaf, petiole and stem explants of Golden pothos. *Plant Cell Rep.* **23**: 587-595

Zhao ZY, Gu W, Cai T, Tagliani L, Hondered D, Bond D, Schroeder S, Ruder M, Pierce D (2001) High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. *Molecular breeding* **8**: 323-333

Zimmerman JL (1993) Somatic embryogenesis: A model for early development in higher plants. *Plant Cell* **5**: 1411-1423

Zupan JR, Zambryski PC (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiology* **107**: 1041 – 1047