Review Article

Journal of Plantation Crops, 32 (Suppl.), December 2004 : 104-116

Biotechnology research in rubber: present status and future prospects

A. Thulaseedharan, P. Venkatachalam, R.G. Kala, I. Thanseem, P. Priya, A. Saleena and P. Kumari Jayasree

Rubber Research Institute of India, Kottayam-686 009, INDIA

Abstract

The recent developments in agricultural biotechnology offer great potential for the genetic improvement of *Hevea*. The goals of biotechnological research in *Hevea* 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, characterisation of laticiferous specific promoters, removal of latex allergens and development of transgenic plants for better agronomic qualities and production of recombinant proteins. Significant progress has been made over the last ten years. Efficient protocols for plant regeneration through somatic embryogenesis were developed using different explants. Field trials to evaluate the agronomic qualities of the somatic plants are going on. Methods were standardized for *Agrobacterium* mediated genetic transformation and transgenic rubber plants were developed with the objective of conferring tolerance to abiotic stresses as well as tapping panel dryness (TPD), and for the production of recombinant proteins. The stable integration and expression of the transgenes were confirmed. Several genes involved in the rubber biosynthetic pathway, disease tolerance, allergic responses etc. have been cloned and characterized. Tissue specific promoters have been isolated and attempts are going on to characterize more such promoters. Attempts to understand the molecular mechanism of TPD and tolerance to leaf fall diseases caused by *Phytophthora* and *Corynespora* are progressing. Different PCR techniques were successfully employed for the development of molecular markers, and use of these markers in developing linkage maps, clonal variability studies, characterization of pathotypes and cloning of agronomically important genes.

Key words: Biotechnology research, rubber, future prospects

Introduction

Natural rubber, cis-1,4 polyisoprene, produced in the milky cytoplasm (latex) of specialized cells called laticifers in certain plants is one of the most important biological macromolecules, which is used as an industrial raw material for the manufacture of a variety of products. Natural rubber has been found in the latex of over 2000 species of plants belonging to 311 genera of 79 families. However, *Hevea brasiliensis* has been the only commercial source of natural rubber mainly because of its abundance in latex, quality and convenience of harvesting. *H. brasiliensis* is now commercially grown in the tropical regions of Asia, Africa and South America, in countries like Indonesia, Thailand, Malaysia, India, China, Sri Lanka, Philippines, Vietnam, Nigeria, Cameroon, Ivory Coast, Liberia, Brazil, Mexico, etc. However, the major share comes from tropical Asia. The higher strength, low heat build up and better resistance to wear and flex cracking make natural rubber a suitable raw material for the manufacture of heavy duty automobile tyre industry. More than 50,000 rubber-based products are being manufactured now. The global rubber cultivation is in about 9.3 million hectares with an annual production of 8.0 million tonnes (*Rubber Statistical Bulletin*, 2004). In India, rubber is cultivated in 5.7 lakh hectars with an annual production of about 7.1 lakh tonnes (*Rubber Statistical News*, 2004). The global consumption of natural rubber is steadily increasing and the production has also to be increased so as to meet the demand.

The method of propagation of elite planting material for commercial cultivation is by grafting buds

from selected clones onto seedling stocks. This ensures genetic uniformity of the scion. But root stocks, which are derived from cross pollinated seeds, are heterozygous and hence lead to undesirable stock-scion interactions leading to intra-clonal variations in field performance (Combe 1975; Seniviratne & Flegmann 1996). The yield potential of each tree in a plantation is becoming an important parameter in order to improve the land use efficiency. The micropropagation of elite clones with their own root system could reduce intra-clonal variation due to stock-scion interaction.

Many of the elite *Hevea* clones are susceptible to one or a combination of undesirable traits, such as a physiological disorder called tapping panel dryness, drought, leaf fall diseases caused by *Phytophthora* and *Corynespora* etc. The crop loss due to these traits is very heavy. There is an urgent need to introduce resistant genes into the high yielding clones. The narrow genetic base of the cultivated clones, long juvenile, highly heterozygous nature of the seed propagated plants, poor seed set etc. are limitations of crop improvement programmes through conventional breeding. Genetic transformation offers a viable approach to overcome the above problems and for the introduction of specific agronomically important traits without disrupting their otherwise desirable genetic constitution.

The recently developed molecular technologies like RFLP, RAPD, AFLP etc. could be used efficiently for the development of molecular markers and genetic analysis in *Hevea* (Venkatachalam *et al.*, 2004; Birdu Roy *et al.*, 2004). DNA molecular marker based selection will help the plant breeder to identify and select the desired plants at an early stage and hasten the transfer of desirable genes into cultivars (Mohan *et al.*, 1997). The presence of ribosomes and polysomes in the latex of *Hevea* laticifers indicates active protein synthesis and metabolism in these cells (Tupy, 1988). By introducing suitable genes, the transgenic rubber tree could be used for the secretion of valuable proteins in its latex serum.

Tissue Culture Approaches

Most of the *in vitro* culture work in *Hevea* is directed towards micropropagation through shoot tip culture and somatic embryogenesis, and genetic transformation. Not much work has been done on development of haploid or triploid plants. Although, elite *Hevea* clones can be propagated easily by bud grafting, *in vitro* propagation is highly desirable since true-to-type plants could be produced without stock-scion interaction, as observed in bud grafting.

Micropropagation

There are several reports on *Hevea* micropropagation using different explants, mostly derived from seedlings (Thulaseedharan *et al.*, 2000). However, an efficient protocol for large-scale micropropagation of elite *Hevea* clones is yet to be evolved. Paranjothy and Ghandimathi (1976) had attempted shoot tip (2-3 cms long) culture, derived from aseptically grown seedlings. Although, these shoots rooted in liquid MS medium, they failed to grow on solid medium. Later Enjalric and Carron (1982), using shoots derived from 1-3 year old greenhouse grown seedlings as explants, developed rooted plantlets. Thereafter, plantlets with shoot and root could be successfully developed by different investigators (Gunatilleke and Samaranayake 1988; Carron *et al.*, 1989; Sompong & Muangkaewngam1992; Seneviratne & Flegmann 199[illegible]

The explants derived from elite clones of mature *Hevea* trees are highly recalcitrant. Only limited reports are available on successful micropropagation by using clonal material of *Hevea*. The major problem with clonal material from mature trees is the failure of producing adequate root system with taproot quality necessary for tree stability (Carron & Enjalric 1983). Rubber being a crop predominantly grown under tropical climates, the presence of bacteria and systemic fungal contamination in the field-grown mature plant derived explants is also a major limitation. Studies were therefore focused in this direction. Effective sterilization techniques for obtaining contamination free initial explants were developed (Enjalric *et al.*, 1987; Asokan *et al.*, 1988). Physiological stage of the explants was found to have a significant role in micropropagation and detailed stud[illegible] were carried out by many researchers. Rejuvenat explants by micrografting (Perrin *et al.*, 1994) and buds of nodal explants taken from dormant branches were found to exhibit better *in vitro* response (Seneviratne and Wijisekara 1997; Lardet *et al.*, 1998). Endogenous hormone levels were identified as markers for explant rejuvenation by Perrin et *al.*, (1997). Conditions for an efficient and reproducible system for *in vitro* micrografting 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).

Extensive studies were carried out at Rubber Research Institute of India with elite *Hevea* clones using shoot tip explants derived from mature trees (Sinha *et al.*, 1985; Sobhana *et al.*, 1986; Asokan *et al.*, 1988). Initially shoots have been regenerated from a few *Hevea* clones by Sinha *et al.*, (1985), who failed to obtain

rooting. Asokan *et al.*, (1988) cultured the shoot tips derived from clonal trees on a medium containing 1.5-3.0 mg/l IAA + 0.5-1.5 mg/l kinetin and reported shoot and root development. The rooted plantlets of four *Hevea* clones were successfully transplanted in the field and a field evaluation programme has been initiated (Thulaseedharan, 2002). In spite of these efforts over several years, no technique is available for large-scale micropropagation at a commercial scale.

Somatic embryogenesis

An efficient plant regeneration pathway through somatic embryogenesis is highly essential for crop improvement through transgenic approaches besides using this as a micropropagation system. Attempts to develop somatic embryogenesis as an *in vitro* propagation technique was started in the 1970s. Since then a number of studies have been reported. Paranjothy (1974) and Carron and Enjalric (1982) achieved differentiation of embryoids from the anther wall derived callus. Subsequently, shoot development was also achieved (Paranjothy and Ghandimathi 1975; Paranjothy and Rohani 1978). Later Wang *et al.*, (1980, 1984) and Wan *et al.*, (1982) successfully regenerated *Hevea* plants through somatic embryogenesis from anther walls. They also succeeded in transplanting plantlets into field. Carron (1981) used inner integument tissue of seeds for somatic embryogenesis and was successful in plantlet development. However, the frequency of somatic embryo induction was found to be very low and non-synchronous, its germination remained very difficult and thus *Hevea* embryogenic system needed further investigation (Carron *et al.*,1995; Linossier *et al.*, 1997).

Extensive experiments were carried out by many researchers to enhance the frequency of somatic embryo induction and plant regeneration. Studies were conducted to optimize cultural conditions, nutritional and hormonal requirements during somatic embryogenesis. The parameters include, the effect of polyamines (El-Hadrami *et al.*, 1989), hormone balance (Michaux-Ferriere and Carron 1989), water status of the medium and explant (Etienne *et al.*, 1991a), mineral and carbohydrate nutrition (Etienne *et al.*, 1991b), interaction of growth regulators, sucrose and calcium on callus friability (Montoro *et al.*, 1993, 1995), role of sucrose and ABA on embryo induction (Veisseier *et al.*, 1994a,b; Cailloux *et al.*, 1996; Linossier *et al.*,1997) and carbohydrate types (Blanc *et al.*, 2002). Wang and Chen (1995) made attempts to regenerate plantlets through somatic embryogenesis from stamen cultures. They optimized the temperature requirement for callus induction, somatic embryogenesis and plant regeneration as 26^0C, 24-25^0C and 26-27^0C respectively and regeneration frequencies up to 40.5 % was obtained (Wang *et al.*,1998). In spite of all the above studies, the plant regeneration frequency remains very low and the technology could not reach into a commercial scale. In most of the above studies inner integument tissue is used as the explant. It is reported that the calli obtained from the integuments of immature seeds frequently display browning (necrosis) leading to tissue degeneration and a loss of embryogenic competence (Housti *et al.*, 1991; Veisseier *et al.*, 1994b).

Recently, there has been a renewed interest in *Hevea* for the development of techniques for plant regeneration through somatic embryogenesis especially for use in genetic transformation (Kumari Jayasree *et al.*, 1999). Extensive research work has been carried out at the Rubber Research Institute of India for the past ten years to develop an efficient plant regeneration system through somatic embryogenesis for the Indian clones of *Hevea*. In order to identify the suitable explant source, a variety of explants such as leaf, tender shoots, integumental tissues of immature fruit, immature anther, immature inflorescence etc. were tried. Attempts were also made to standardize the optimum growth regulator concentration and the balance between different growth regulators, the nutritional requirements and the physical factors such as light and temperature for maximum callus proliferation, embryo induction and subsequent regeneration of normal and healthy plantlets. As a result initially immature anthers (before microsporogenesis) (Kumari Jayasree *et al.*, 1999) and immature inflorescence (Sushamakumari *et al.*, 2000a) were identified as the suitable explant source and protocols were developed for high frequency somatic embryo induction and plant regeneration for RRII-105, the most popular Indian clone. Kumari Jayasree *et al.*, (1999), for the first time, reported the standardized protocol for the induction of friable embryogenic callus, somatic embryogeneis and further plant regeneration from the immature anthers. 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. Cytological analysis revealed that all the plantlets tested were diploid. Sushamakumari *et al.*, (2000a) perfected 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 essential for effective embryo induction as well as maturation. However, lower level was found to be beneficial for plant regeneration. Further, efforts have been made to enhance the embryo induction and plant regeneration frequency by the manipulation of the nutrients and hormonal combinations. Sushamakumari *et al.*, (1999a) have attempted induction of multiple shoots on germinating somatic embryos thereby enhancing the efficiency of plantlet formation. They could induce an average 3.45 micro-shoots per explant by manipulation of the levels of BA and thidiazuron in the medium as well as wounding of the shoot primordia of the somatic embryos.

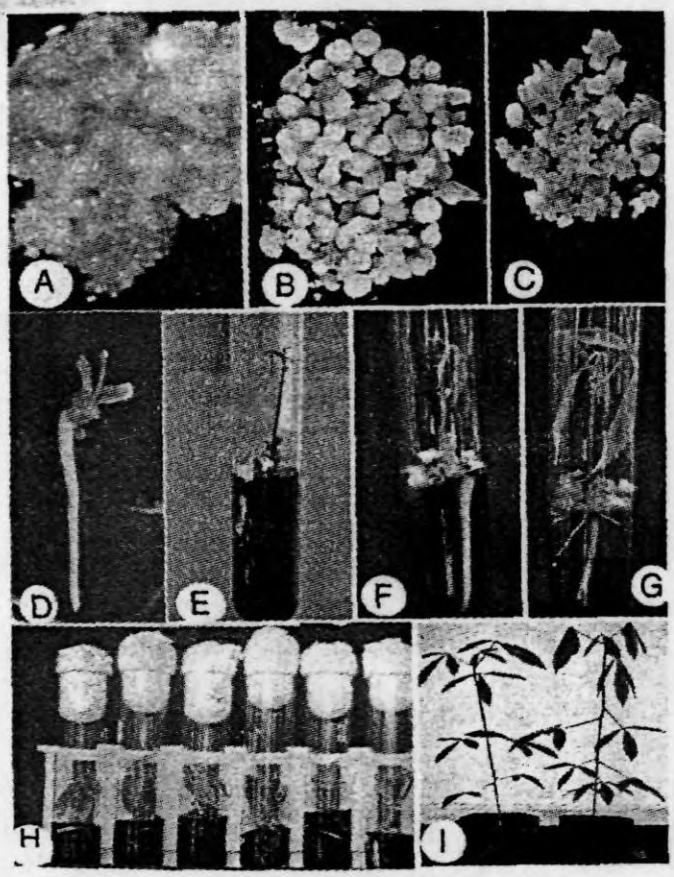

Fig. 1. Pathway for plant regeneration through somatic embryogenesis

Culture conditions and other nutritional requirements for improving the efficiency of somatic embryo induction and germination were investigated by Kumari Jayasree *et al.*, (2001a). Immature anthers precultured in liquid medium for 10 days followed by 25 days culture in solid medium was found to promote callus induction. The embryo induction efficiency was promoted by supplementing 200 mg/l glutamine and 400 mg/l casein hydrolysate in embryo induction medium. Dark incubation favoured callus induction and proliferation as well as induction of embryogenesis, whereas plantlet regeneration was found to be light dependent. 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). A detailed investigation was done on the response of various cytokinins such as BA, ZEA, KIN and thidiazuron (TDZ) on germination of somatic embryos derived from immature anther explants. TDZ was proved to be superior to BA and ZEA while KIN showed the least response. Maximum germination (80%) and plantlet development (82%) was obtained when the medium was supplemented with TDZ (Kumari Jayasree *et al.*, 2001 b). An isozyme study revealed a clear difference between embryogenic and nonembryogenic callus as well as for different stages o embryogenesis, and markers could be developed (Asokan *et al.*, 2001)). Repetitive embryogenesis 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). Methods were also standardized for the maintenance of embryogenic cultures for over three years for retaining the embryo induction and plant regeneration potential (Kumari Jayasree and Thulaseedharan, 2004). Recently attempts were also made for the induction of callus and somatic embryogenesis using leaf and ovule as explants. Good callus induction and high frequency somatic embryogenesis were obtained. A few plantlets were also obtained and attempts are going on to obtain high frequency plant regeneration (Kala *et al;* Jayashree *et al.* personal communication).

Several *in vitro* plants have been raised through somatic embryogenesis from immature anther as well as immature inflorescence of the *Hevea* clone RRII-105. Morphologically all these plants were uniform. The preliminary molecular analysis carried out with these plants also revealed genetic uniformity. These plantlets could be hardened, established in soil and large-scale field trials have been initiated to characterize their agronomic qualities (Thulaseedharan, 2002). Carron *et al.*, (2000) have also studied the field performance of the *in vitro* plants and found clearly better growth for the *in vitro* plants compared with the seedling control, and the annual increment was consistently higher giving an increasing gap between two treatments. The *in vitro* trees

also had a higher degree of trunk base conicity resulting in 34% more trunk biomass in the tappable zone.

Genetic transformation

Genetic improvement of tree species by conventional methods is a rather slow and difficult process. The recent developments in recombinant DNA and *in vitro* plant regeneration techniques could provide a direct route for the introduction of specific genes controlling agronomic traits into crop plants. The transfer of selected genes in a single generation by genetic transformation is especially interesting for the species *H. brasiliensis*, since its improvement is limited by long breeding cycles and high levels of heterozygosity. The major objective of *Hevea* genetic transformation in different laboratories are (a) improvement of agronomic traits of elite *Hevea* clones and (b) production of pharmaceuticals and other valuable recombinant proteins in the transgenic rubber tree. In the improvement of agronomic traits, the underlying direction is towards development of transgenic rubber tree with increased rubber biosynthesis and timber volume, resistance to diseases, various abiotic stresses etc. (Thulaseedharan, 2002; Yeang 2004). Arokiaraj *et al.*, (1994, 1996) developed transgenic plants with the marker genes coding for b-glucuronidase and neomycin phosphotransferase (NPT-II) using microprojectile bombardment as well as *Agrobacterium* methods. They observed GUS expression in the leaves and latex of transgenic plants (Arokiaraj *et al.*, 1998). Transgenic plants integrated with the cDNA coding for human serum albumin and for a single chain variable fragment antibody (ScFv4715) against the coat protein of an oral dental bacterium, *Streptoccocus sanguis* under the control of CaMV 35S promoter have been developed, to explore the possibility of producing the recombinant proteins in the latex. Analysis of the recombinant proteins at different growth stages of the transgenic plants indicated an increasing trend in the concentration of the recombinant proteins in the latex as the plants aged (Arokiaraj *et al.*, 2002). A main problem faced by the earlier workers for the rapid progress in *Hevea* genetic transformation work was the lack of an efficient system for the development of transgenic plants, particularly a plant regeneration system for the most desired genotypes containing the transgene (Arokiaraj *et al.*, 2004).

After developing efficient plant regeneration protocols through somatic embryogenesis, Rubber Research Institute of India has started active research in the development of transgenic plants integrated with genes for desired agronomic traits. Very efficient *Agrobacterium* mediated genetic transformation system as well as methods for high frequency transgenic plant regeneration have been developed (Jayashree *et al.*, 2000, 2003; Sobha *et al.*, 2003a). Initial focus was to develop transgenic plants tolerant to abiotic stresses like elevated temperature and light, drought and tapping panel dryness. With the above objectives four genes, namely the genes coding for sorbitol-6-phosphate dehydrogenase, isopentenyl transferase, superoixde dismutase and antisense sequence for ACC synthase were identified, and genetic transformation experiments carried out. The callus transformed with the foreign genes were selected over the antibiotic Kanamycin and somatic embryogenesis technique was followed for further transgenic plant regeneration (Thulaseedharan, 2002; Kala *et al.*, 2003). Transgenic plantlets integrated with the superoxide dismutase gene under the control of different promoters such as CaMV 35S promoter and FMV 34S were developed separately from different cell lines obtained through independent transformation events. The stable integration and copy number of the transgene were confirmed through molecular analysis (Jayashree *et al.*, 2000, 2003; Sobha *et al.*, 2003a). The preliminary biochemical studies conducted with superoxide dismutase transformed callus cultures revealed over expression of superoxide dismutase and related enzymes such as catalase and peroxidase (Sobha *et al.*, 2003b).

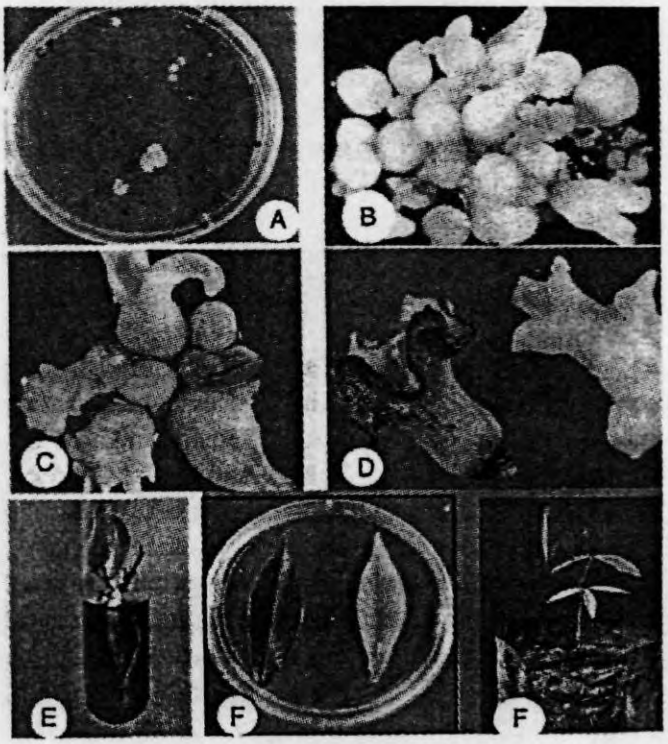

Fig.2. Pathway for the development of transgenic *Hevea* plants

Development of haploids and triploids

Production of homozygous doubled haploids via anther/microspore culture would offer new possibilities for genetic studies and breeding, especially in perennial tree species which are characterized by a long reproductive cycle, with many years of juvenile phase and a high degree of heterozygosity. However, *in vitro* approaches to induce haploids in *Hevea* have had only limited success in comparison with other plant species (Seneviratne *et al.*, 1996). Satchuthananthavale and Irugalbandra, (1972) cultured *Hevea* anthers for the first time, with the aim of obtaining haploid plants. Later, Satchuthananthavale, (1973), Gandimathi and Paranjothy, (1975) induced callus from anther tissues. Chen *et al.*, (1979), however, first reported the production of pollen plantlets in *Hevea*. Since then, reports on haplogenesis in *Hevea* by several investigators have been published from China and anther derived plantlets have been established in field (Chen, 1984). Guo *et al.*, (1982) produced two plantlets from unpollinated ovules of *Hevea*. The ploidy level of these plantlets was not reported. Shijie *et al.*, (1990) developed pollen plants through anther culture from 13 clones, transplanted and established in the field. Das *et al.*, (1994) induced callus from anthers of several clones on medium with 6% sucrose. Although, the above investigators named the plantlets they developed as pollen plantlets, most of them used only mature anther as explants. It is reported that, the pollen derived calli would selectively proliferate, suppressing the anther wall derived callus, 3-4 weeks after culture in a medium containing high sucrose levels (Carron *et al.*, 1989). Haploid calli from pollen culture could be obtained for the *Hevea* clone RRII 105 by the recent efforts made at RRII (Jayashree *et al*, personal communication). Similarly methods were also standardized for callus induction, somatic embryogenesis and triploid plant regeneration from the endosperm cultures of *Hevea* (Rekha *et al.* personal communication).

Protoplast Culture

Protoplast culture technology applied to *Hevea* has the advantage that both closely related and distant genotypes can be hybridized, thereby avoiding the barriers of conventional breeding. Only limited reports are available on *Hevea* protoplast isolation and culture. Cailloux and Lleras, (1979) isolated protoplasts from young leaves and stem. They cultured the protoplasts in liquid medium containing PEG for fusion and obtained a fusion-index of 10-15%. However, no division was observed. Rohani and Paranjothy, (1980) attempted isolation of protoplasts from a variety of *Hevea* tissues and found both young shoots and suspension cultures of anther-derived calli as the most promising sources. Cazaux and d'Auzac, (1994) obtained microcalli for the first time from protoplasts of friable embryogenic callus. Extensive studies have been carried out by Sushamakumari *et al.*, (1999b; 2000) at RRII to identify the suitable explant source for the isolation of viable protoplasts and to optimize the culture conditions for plant regeneration. Embryogenic cell suspension was found to be the ideal explant source (Sushamakumari *et al.*, 1999b). Good microcolony formation was observed with lolium cell suspension culture as a nurse culture and god plant regeneration was obtained (Sushamakumari *et al.*, 2000b).

In vitro fertilisation and plant recovery

In *Hevea,* the major constraints in conventional breeding programme are low fruit set, seasonal flowering and lack of synchrony in flowering among clones. The brief and periodic nature of flowering impedes the progress of pollination programmes. The inaccessibility of flowers makes the programme labour intensive and limits the number of crosses that could be performed in a season. In this context, extensive work has been carried out and the conditions for effective *in vitro* pollination and *in vitro* fertilization were standardized. The system could be further utilized for interspecific and intergeneric hybridization, genetic transformation and induction of haploid plants (Rekha *et al.*, 2002).

Molecular studies

Although the past three decades witnessed tremendous advancement towards understanding the molecular mechanism of plant growth and developmen most of these studies were carried out in annuals and studies in the tree species are very limited (Thulaseedharan *et al.*, 2000). In *Hevea* extensive studies have been carried out on the physiological and biochemical understanding of rubber biosynthesis (d'Auzac, 1989), but not much has been done at the molecular level. Recently attempts have been initiated at different laboratories in the characterization of rubber biosynthesis genes, latex allergens, to understand the laticifer specific gene expression and molecular mechanism of disease tolerance. Another area in which research is currently focused is in the characterization of laticifer specific promoters and on the possible use of *Hevea* laticifer system for producing recombinant proteins of therapeutic and nutritional value (Arokiaraj *et al.*, 2004).

Laticiferous specific gene expression and characterization of rubber biosynthesis genes.

Since latex could be readily obtained in large quantities, it provides an opportunity to investigate the biochemical properties of a single specialized cell type (Kush *et al.*, 1990). Development of simple but efficient protocols for the isolation of translatable mRNA from the latex has made gene expression studies easier in the laticifer system (Venkatachalam *et al.*, 1999). The expression levels of various genes present in the laticifer system have been shown to be markedly different from those of leaves. Laticifer RNA is 10 to 100 fold enriched in transcripts encoding the enzymes involved in rubber biosynthesis. The PR proteins also showed a 10-50 fold higher expression in laticifers than in leaves (Kush *et al.*, 1990; Chye *et al.*, 1995). The cloning and analysis of genes/cDNAs for enzymes involved in the rubber biosynthesis is more recent compared to the long history of biochemical understanding. One of the first genes to be cloned in this pathway from *Hevea* is 3-hydroxy-3-methyl glutaryl co-enzyme A reductase (HMGR). Three forms of HMGR namely hmgr 1, hmgr 2 and hmgr 3 were identified, where the transcripts level differed in their tissue specificity (Chye *et al.*, 1992). The cloning of the cDNA encoding the complete protein of rubber elongation factor involved in the polymerization of isoprene units was also done earlier (Goyvaerts *et al.*, 1991; Attanayaka *et al.*, 1991). The other recently characterized genes in this pathway are, farnasyl diphosphate (FDP) synthase (Adiwilaga and Kush, 1996), small rubber particle protein (SRPP) (Oh *et al.*, 1999), isopentenyl disphosphate (IDP) isomerase (Oh *et al.*, 2000), 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co A) synthase (Suwanmanee *et al.*, 2002; 2004) and cis-prenyl transferase (Asawatreratnaakul *et al.*, 2003). Chow *et al.*, (2003) isolated unique cDNAs encoding the rubber biosynthesis stimulator protein (RBSP) which was previously identified as the eukaryotic translation initiation factor eIF5A, a protein having significant role in the regulation of latex biosynthesis. A cDNA clone encoding geranylgeranyl diphosphate synthase was characterized, and its role in rubber biosynthesis examined (Takaya *et al.*, 2003). Another important protein characterized from the latex at the biochemical and molecular level is hevein a single chain chitin binding protein which plays a crucial role in wound sealing after tapping, protection of wounded sites from fungal attack and also is a major latex allergen (Broeakaert *et al.*, 1990; Parijs, 1991). *Hevea* latex contains hundreds of proteins and several of them cause allergenic reactions in sensitized persons, particularly health care workers who routinely wear latex gloves. Thirteen proteins were currently recognized as latex allergens out of which 7 are considered major latex allergens (Yeang, 2004). Latex cDNA libraries have been constructed by Han *et al.*, (2000) to investigate the genes expressed in the latex. Analysis of more than 20,000 cDNA-AFLP based transcription derived fragments (TDF) and 1176 ESTs revealed that, seven gene families, accounted for more than 51% of the latex transcriptome. Among them, two of the most abundant ESTs were the genes encoding the rubber particle proteins, REF and SRPP, comprising 29% of the total ESTs (Ko *et al.*, 2003). The partial promoter sequences of certain genes which are overexpressed in the laticiferous cells such as hevein (Pujade-Renand *et al.*, 2003), β-1,3-glucanase (Thanseem, 2004) and REF (Priya *et al.*, personal communication) were isolated. After characterization, these promoters could be used for the over expression of recombinant proteins in the latex vessels.

Molecular markers and gene mapping

Conventional genetic analysis in *Hevea* is difficult because of its perennial nature, long breeding and selection cycles and difficulties in raising F2 progenies. Genetic linkage maps were developed for many annual crops in the late 1980's and information from such maps were used for breeding purpose (Helent-Jaris *et al.*, 1985; Bernatzky and Tanksley, 1986). However, these molecular techniques were used in *Hevea* only very recently. With the advent of PCR technology in the nineties, there has been a remarkable progress in the development of potential molecular markers, based on RFLP, RAPD, AFLP, micro-satellites etc. for the characterization of plant genome. Available reports on *Hevea* describes the successful application of RFLP or RAPD markers for the assessment of genetic variability in cultivated clones and wild accessions (Besse *et al.*, 1994), clonal identification and evaluation of genetic diversity in popular clones using RAPDs (Varghese *et al.*, 1997; Venkatachalam *et al.*, 2002), estimation of phylogenetic relationships from mitochondrial DNA RFLPs (Luo *et al.*, 1995) and identification of markers for mildew resistance genes (Shoucai *et al.*, 1994). DNA markers for tapping panel dryness (TPD) tolerance (Thulaseedharan *et al.*, 1997) and dwarf genome specific marker (Venkatachalam *et al.*, 2004) were also developed through RAPD. Lespinasse *et al.*, (2000) constructed a genetic linkage map of *Hevea* using different molecular markers. Low *et al.*, (1996) detected microsatellites for the first time in *Hevea* through the analysis of *Hevea* sequences available in the database. Microsatellites were also used for the diversity analysis of wild germplasm

and cultivated clones of *Hevea* (Lekawipat *et al.*, 2003). Several microsatellites having different types of repeat motifs comprising dinucleotides, trinucleotides, tetranucleotides etc. were characterized from *Hevea* (Bindu Roy *et al.*, 2004). A combination of four microsatellite markers were successfully used to discriminate *Hevea* clones and clone specific allelic profiles were generated by Saha *et al.*, (2004). The recent sequence analysis of the repeat region at the 3'UTR of the HMGR containing clusters of AG repeats revealed the existence of two alleles based on the repeat length polymorphism. More studies in this direction would significantly contribute to the understanding of the regulation of gene expression in laticiferous cells (Saha *et al.*, 2004).

Biotic and abiotic stresses

Although, crop loss due to tapping panel dryness, a serious physiological disorder at the tapping panel and other diseases are very heavy, attempts to control these problems through molecular intervention started only very recently. DNA markers conferring tolerance to TPD were developed through RAPD (Thulaseedharan *et al.*, 1997). A decreased level of trans-zeatin riboside and SOD with increased free radicals were reported in the bark of TPD affected plants (Krishnakumar *et al.*, 1997; Das *et al.*, 1998). Therefore, a high level of SOD in the bark and latex could counteract the action of free radicals effectively, providing protection against TPD. Transgenic *Hevea* plants integrated with SOD gene were developed to explore the above possibility (Sobha *et al.*, 2003b; Jayashree *et al.*, 2003). A preliminary RAPD analysis conducted recently shows that, the genetic distance between the stock and scion of TPD susceptible plants are more than that of tolerant ones (Sobhana *et al.*, 2004). Chen *et al.*, (2003) identified a Myb transcription factor gene from the bark of *Hevea* trees and studied its association with TPD syndrome. Myb gene is expressed in leaves, bark and latex of rubber trees but its expression is significantly decreased in the bark of TPD trees. In order to characterize more genes linked to the TPD incidence, two subtracted cDNA libraries were constructed at RRII. From the subtracted cDNA library of healthy trees, 3 novel sequences and from TPD specific cDNA library 25 novel sequences were identified. The stress related cDNAs were highly abundant in the cDNA library of TPD affected trees. The preliminary northern analysis revealed a lower expression for a Myb transcription factor gene and a translationally controlled tumour protein (TCTP) (Venkatachalam *et al.*, personal communication).

Severity of various diseases in different rubber growing regions is highly dependent on the virulence of the pathotypes. Molecular marker technologies were used to characterize various isolates. Genetic variability studies of several fungal isolates using RAPD and rDNA analysis were carried out and putative markers for virulence identified for *Corynespora cassiicola,* (Saha *et al.*, 2000; Atan and Hamid, 2003) and *Colletotrichum acutatum* (Saha *et al.*, 2002). Characterization of various *Phytophthora* isolates infecting rubber using rDNA RFLP was also attempted (Philip *et al.*, 2004)

Shoucai *et al.*, (1994) characterized DNA markers conferring tolerance to powdery mildew through RAPD. A single chain chitin binding protein, 'hevein' with antifungal activity have been characterized (Parijs *et al.*, 1990) and the cDNA been cloned from latex (Broeakaert *et al.*, 1990). Hevamine, an enzyme with chitinase/ lysozyme activity, which is important for plant defense against pathogenic fungi and bacteria, was purified fro lutoid bodies (Jekel *et al.*, 1991). The cDNA sequence of hevamine was isolated (Bokma *et al.*, 2001) and the active sites of the enzyme were determined (Bokma *et al.*, 2002). The expression of the â-1,3 glucanase, a PR protein involved in the control of many pathogenic fungi has been studied (Kush *et al.*, 1990; Philip *et al.*, 2002, Thanseem and Thulaseedharan, 2004). The cDNA clone for â-1,3-glucanase has been isolated from latex cDNA library and the northern hybridization showed constitutive expression in the laticifers (Chye and Cheng, 1995). A cDNA coding for a pathogen induced isoform of â-1,3-glucanase was characterized from *Hevea* clone RRII 105 and its role in tolerance to abnormal leaf fall disease was studied in detail (Thanseem, 2004). It was found that this anti-fungal gene was differentially expressed at the time of pathogen infection in different clones of rubber. Although, the induction of this gei has occurred in both tolerant and susceptible clones, the timing and magnitude of induction were different. In tolerant clones very high induction has occurred. The high, rapid and prolonged induction of this protein may be a factor that determines the tolerance/susceptibility to the disease (Thanseem *et al.*, 2004). This is more important in the case of abnormal leaf fall, as the causative organism belongs to the class of Oomycetes which lack chitin in their cell walls.

Conclusion and Future Prospects

The recent advances in cell culture and recombinant DNA techniques have tremendous application for crop improvement in *Hevea*. Development of protocols for micropropagation will greatly facilitate production of true-to-type elite planting materials eliminating stock-scion

interaction leading to intraclonal variations in field performance. The recently developed protocols for high frequency somatic embryogenesis and plant regeneration have opened up new avenues for mass propagation besides using as a tool for genetic transformation. After evaluation of the agronomic qualities of the plants regenerated through somatic embryogenesis, over years, if found superior to the bud grafted ones, commercial planting could be effected. In *Hevea*, increased growth and vigour have already been reported for plants regenerated through tissue culture (Carron *et al.*, 1995; 2000). Even a very small increment in yield per tapping will be of great attribute to a tree crop like *Hevea* with an economic life span of about 30 years.

Breeding for high latex yield with increased timber volume is one of the priority areas of rubber research now. Many of the genes involved in the rubber biosynthesis pathway have been cloned and characterised. Efforts are already going on to achieve increased latex and timber yield through transgenic approaches. In future, efforts are also to be directed for integrating drought and disease resistance genes including TPD tolerance to high yielding clones. The molecular marker assisted selection in *Hevea* is still at the preliminary stage. Further efforts are to be made in this direction. The development of haploid and triploid plants, *in vitro* fertilization techniques, the advances in DNA marker assisted selection and genetic transformation approaches would complement the conventional breeding efforts by increasing the diversity of genes for incorporation and also shortens the time required for the development of new varieties. The laticiferous cells contain all the essential cytoplasmic components required for the biosynthesis of proteins and secondary products. At present only the rubber particles are exploited from latex. There is enormous potential to produce novel recombinant proteins, carbohydrates, vaccines and other valuable compounds in the laticiferous system by genetic manipulation of *Hevea* plants with appropriate genes. The over expression could be very well achieved by modifying the genes with laticferous specific promoters. Attempts have already been initiated in this direction.

Acknowledgement

The authors are grateful to Dr. N.M. Mathew, Director, RRII, for encouragements and facilities.

Reference

Adiwilaga, K. and Kush, A. 1996. Cloning and characterization of cDNA encoding farnesyl diphosphate synthase from rubber tree (*Hevea brasiliensis*). *Plant Mol. Biol.* **30**: 935-946

Arokiaraj, P., Jones, H., Cheong, K.F., Coomber, S. and Charlwood, B.V. 1994. Gene insertion into *Hevea brasiliensis*. *Plant Cell Rep.* **13**: 425-431.

Arokiaraj, P., Jones, H., Jaafar, H., Coomber, S. and Charlwood, B. V. 1996. *Agrobacterium*-mediated transformation of *Hevea* anther calli and their regeneration into plantlets. *J. Natl. Rubb. Res.* **11**:77-87.

Arokiaraj, P., Razak, S.B.A., Jaafer, H., Arif, S.A.M. and Yeang, H.Y. 2004. Transgenic rubber trees: where are we now? Report on the *IRRDB Biotechnology workshop*, Sungai Buloh, Malaysia. pp. 60-62.

Arokiaraj, P., Yeang, H. Y., Cheong, K.F., Hamzah, S., Jones, H., Coomber, S. and Charlwood, B. V. 1998. CaMV 35S promoter directs â-glucuronidase expression in the laticiferous system of transgenic *Hevea brasiliensis* (Rubber tree), *Plant Cell Rep.* **17**: 621-625.

Arokiaraj, P., Jaffar, H., Arif S.A.M., Bahri, S., Badarudin, B.E., and Yeang, H.Y. 2002. Prospects and recent developments in *Hevea* genetic transformation at Malaysian Rubber Board. *Rubber Planters Conference, India*. pp. 141-145.

Asawatreratanakul, K., Zhang, Y.W., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S., Rattanapittayaporn, A., and Koyama, T. 2003. Molecular Cloning, Expression and Characterisation of cDNA encoding cis-prenyl transferases from *Hevea brasiliensis*. *Eur. J. Biochem.* **270**: 4671-4680.

Asokan, M. P., Sobhana, P., Sushamakumari, S. and Sethuraj, M.R. 1988.Tissue culture propagation of rubber (*Hevea brasiliensis* Willd ex Adrg. De Juss. Muell. Arg) clone GT1. *Ind. J. Natl. Rubb. Res.* **1**:10-12.

Asokan, M..P, Narasimhan, K., Kumari Jayasree, P., Thulaseedharan, A., Sushama Kumari, S., Kala, R.G. and Jayashree, R. 2001. Isozyme markers for distingushing embryogenic callus from non embryogenic during somatic embryogenesis in *Hevea brasiliensis* Muell. Arg. *J. Tree Sci.* **20**:78-85

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

Atan, S. and Hamid, N.H. 2003. Differentiating races of *Corynespora cassiicola* using RAPD and internal transcribed spacer markers. *J. Nat. Rubb. Res.* **6**: 58-64.

Attanayaka, D. P. S. T. G., Kekwick, R.G.O. and Franklin, F. C. H. 1991. Molecular cloning and nucleotide sequencing of the rubber elongation factor gene from *Hevea brasiliensis*. *Plant Mol. Biol.* **16**: 1079-82.

Bernatzky, R. and Tanksley, S. D. 1986. Towards a saturated linkage map in tomato based on isoenzyme and random cDNA sequences. *Genetics* **112**: 889.

Besse, P., Seguin, M., Sebrun, P. and Lanand, C. 1993. Ribosomal DNA variations in wild and cultivated rubber tree (*Hevea brasiliensis*). *Genome* **36**:1049-1057.

Besse, P., Seguin, M., Lebrun, P., Chevallier, M. H., Nicolas, D. and Lanand, C. 1994. Genetic diversity among wild and cultivated populations of *Hevea brasiliensis* assessed by Nuclear RFLP analysis. *Theor. Appl. Genet.* **88**:199-207.

Bindu Roy, C., Nazeer, M.A. and Saha, T. 2004. Identification of simple sequence repeats in rubber (*Hevea brasiliensis*). *Curr. Sci.* **87:** 807-811.

Blanc, G., Lardet, L., Martin, A., Jacob, J. L. and Carron, M.P. 2002. Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis*. (Mull. Arg.). *J. Exp. Bot.* 53:1453-1462.

Bokma, E., Spiering, M., Chow, K.S., Mulder, P., Subroto, T. and Beitema, J.J. 2001. Determination of cDNA and genomic DNA sequences of hevamine, a chitinase from the rubber tree *Hevea brasiliensis*. *Plant Physiol. Biochem.* **39**: 367 – 376.

Bokma, E., Rozeboom, H.J., Sibbald, M., Dijkstra, B.W. and Beitema, J.J. 2002. Expression and characterisation of active site mutants of hevamine, a chitinase from the rubber tree *Hevea brasiliensis*. *Eur. J. Biochem.* **269**: 893 – 901.

Broeakaert, W, F., Lee, H., Kush, A., Chua, N. H. and Raikhel, N. 1990. Wound induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). *Proc. Natl. Acad. Sci. USA* **87**:7633-7637.

Cailloux, M. and Lleras, E. 1979. Fusao de protoplastos de *Hevea brasiliensis e Hevea pauciflore*. Estabelecimento de tecnica. Acta *Amazonica*. **9:** 9-13.

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

Carron, M.P., Etienne, H., Lardet, L., Campagha, S., Perrin, Y., Leconte, A and Chaine C. 1995. Somatic embryogenesis in rubber (*Hevea brasiliensis* Muell. Arg.) In: Jain SM., Gupta, P and Newton, R (Eds.), *Somatic embryogenesis in woody plants*, Vol.2, pp 117-36, Kluwer Academic publishers, The Netherelands.

Carron, M.P., Leroux, Y., Tison, J., Dea, B.G., Caussanel, V. and Clairj Keli J. 2000. Compared root system architectures in seedlings and *in vitro* plantlets of *Hevea brasiliensis*, in the initial years of growth in the field. *Plant and Soil* **223:** 73-85.

Carron, M.P and Enjalric, F. 1982. Studies on vegetative propagation of *Hevea brasiliensis* by somatic embryogenesis and *in vitro* microcutting. In: Fujiwara A (Ed), Plant Tissue Culture pp. 751-752, Maruzen, Tokyo.

Carron, M.P. 1981. Germination *in vitro* d'embryons immatures d'hevea. *Caoutch. Plast.* 612: 93.

Carron, M.P., Enjalric, F and Deschamps, A. 1985. Progress of research into *in vitro* vegetative propagation of *Hevea brasiliensis* Muell. Arg. IRCA. CR. Coll Exp Phys. AM *Hevea* IRRDB. IRCA, Monpellier pp. 427-435.

Carron, M.P., Enjarlic, F., Lardet, L and Deschamps, A. 1989. Rubber (*Hevea brasiliensis* Muell. Arg). In: Bajaj Y.P.S. (Ed), *Biotechnology in Agriculture and Forestry. Trees II.* Vol **5**, Springer –Verlag, Berlin pp. 222-245.

Carron,M.P. and Enjalric, F. 1983. Prospects for the micropropagation of *Hevea brasiliensis*. *Revue Generale des. Caoutch Plast.* **628:** 65-8.

Cazaux, E. and d'Auzac, J. 1994. Microcallus formation from *Hevea brasiliensis* protoplasts isolated from embryonic callus. *Plant Cell Rep.* **13**: 272-276.

Chen, C., Chen, F., Chien, C., Wang, C., Chang, S., Hsu, H., Ou, S., Ho, Y. and Lu, T. 1979. A process of obtaining pollen plants of *Hevea brasiliensis* Muell. Arg. *Sci. Sin.* **22**: 81-90.

Chen, Z. 1984. Rubber (*Hevea*). In: Sharp, R., Amiratto, D.A. and Yamada, Y (Eds), *Handbook of Plant Cell Culture*. Vol.2 Crop Species. MacMillan Publishers pp. 546-571.

Chen, S., Peng, S., Huang, G., Wu, K., Fu, X. and Chen, Z. 2003. Association of decreased expression of a Myb transcription factor with the TPD (tapping panel dryness) syndrome in *Hevea brasiliensis*. *Plant Mol. Biol.* **51**: 51-58.

Chow, K.S., Lim, S.M.and Han, K.H., 2003. Characterisation of a family of eIF. 5A cDNAs from. *Hevea brasiliensis*. *J. Rubb. Res.* **6**:107-120.

Chye, M. L., Tan, C. T., and Chua, N. H. 1992. Three genes encode 3-hydroxy 3-methylglutaryl coenzyme A reductase in *Hevea brasiliensis*: hmg 1 and hmg3 are differentially expressed. *Plant Mol. Biol.* **16**: 473-484.

Chye, M. L. and Cheung, K. Y. 1995. â-1,3-glucanase is highly expressed in laticifers of *Hevea brasiliensis*. *Plant Mol. Biol.* **29**: 397-402.

Combe, J.C. 1975. Demonstration of intraclonal variability in young graft trees. *Revue Generale des Caouth Plast.* **52:** 91-94.

d'Auzac, J. 1989. Factors involved in the stopping of flow after tapping in d'Auzac J. Jacob J L & Chrestin H (Eds.), *Physiology of rubber tree latex*, CRC Press. Florida, USA pp. 257-285.

Das, G., Raj, S., Pothen, J., Sethuraj, M.R., Sinha, T.P. and Sen-Mandi, S. 1998. Status of free radical and its scavenging system with stimulation in *Hevea brasiliensis*. *Plant Physiol. Biochem.* **25**: 47-50.

Das, K., Sinha, R.R., Potty, S.N., and Sethuraj, M.R. 1994. Embryogenesis from anther derived callus of *Heva brasiliensis*Muell. Arg. *Ind. J. Hill Farming*.7: 90-95.

El Hadrami, I., Michaux-Ferriere, N., Carron, M. P. and d'Auzac, J. 1989. Polyamines, a possible limiting factor in somatic embryogenesis of *Hevea brasiliensis*. *Comptes Rendus de l' Academic des Sciences de Paris 308, Serie* III: 205-211

Enjalric, F and Carron, M.P. 1982. Microbouturage *in vitro* de jeunes plants d' *Hevea brasiliensis*. *CR Acad. Sci. Paris* **295:** 259-264

Enjalric, F., Carron, M.P. and Lardet, L. 1987. *Acta Hortic.* **225:** 5 65

Etienne, H., Berger, A. and Carron, M.P. 1991a. Water status of callus from *Hevea brasiliensis* during induction of somatic embryogenesis. *Physiologia Plantarum* **82**: 213-218.

Etienne, H., Montoro, P. and Carron, M.P. 1991b. Incidence des parameters hydreques sur le development des cals d'*Hevea brasiliensis* en culture *in vitro*. *Annales des Sciences Forestieres* **48:** 235-265.

Gandimathi, H. and Paranjothy, K. 1975. Anther culture-attempts at induction of haploidy in *Hevea* and other plants.*Proc. Natl.Plant Tissue Cult. Symp.* Kuala Lumpur, Malaysia pp. 32-5.

Goyvaerts,E., Dennis, M., Light, D. and Chua, N 1991. Cloning and Sequencing of the cDNA Encoding the Rubber Elongation Factor of *Hevea brasiliensis*. *Plant Physiology* **97:** 317-321.

Gunatilleke, I. D. and Samaranayaka, C. 1988. Shoot tip culture as a method of micropropagation of *Hevea*. *J. Rubb. Res. Inst. Sri Lanka* **68**: 33-44.

Guo, G., Jia, X. and Chen, L. 1982. Induction of plantlet from ovules *in vitro* of *Hevea brasiliensis*. *Hereditas* **4**: 27-28.

Han. K.H.. Shin. D.H.. Yang. J.. Kim. I.J.. OH. S.K.. and Chow K.S. 2000. Genes expressed in the latex of *Hevea brasiliensis* . *Tree Physiol.* **20**: 503-510.

Helent Jaris. T., King. G., Slocum. M., Sidenstrang. C. and Wegman. S. 1985. Restriction fragment length polymorphisms as probes for plant diversity and their development as tools for applied plant breeding *Plant Mol. Biol.* **5**: 109-121.

Housti. F., Coupe. M. and d'Auzac. J. 1991. Facteurs enzymatiques du brunissement in vitro et capacite embryogene des cals d' *Hevea brasiliensis*. *C.R. Acad Sci. Paris* (III). **313:** 461-466

Jekel. P.A.. Hartmann. B.H. and Beintema. J.J. 1991. The primary structure of hevamine. an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex. *Eur. J. Biochem.* **200**: 123 – 130.

Jayashree. R.. Rekha. K.. Sobha. S.. Sushama Kumari. S., Kala. R.G., Kumari Jayasree. P., Thanseem. I.. Asokan. M.P., Sethuraj. M.R. and Thulaseedharan, A. 2000. *Agrobacterium* mediated genetic transformation in *Hevea brasiliensis*. *Intl. Symposium on Plantation Crops*. PLACROSYM XIV. Hyderabad, India.

Jayashree. R., Rekha. K., Venkatachalam. P., Uratsu. S.L., Dandekar. A.M.. Kumari Jayasree. P., Kala. R.G., Priya. P., Sushama Kumari. S.. Sobha. S., Asokan. M.P., Sethuraj. M.R. and Thulaseedharan. A. 2003. Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis Muell. Arg*) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. *Plant Cell Rep.* **22:** 201-209.

Kala. R.G., Asokan. M.P., Kumari Jayasree. P., Sobha. S.. Jayashree.R., Rekha. K. and Thulaseedharan. A. 2002. *In vitro* micrografting in *Hevea brasiliensis*: optimization of conditions. *Indian. J. Nalt. Rubb. Res.* **15:** 165-171.

Kala. R.G., Kumari Jayasree. P., Sobha. S., Sushamakumari. S., Jayasree. R., Rekha. K., Venkatachalam. P. and Thulaseedharan. A. 2003. Introduction of the gene coding for isopentenyl transferase into *Hevea brasiliensis:* Effect on plant regeneration. 10th international congress of federation of Asian and Oceanian Biochemists and Molecular Biologists. Dec 7-11, Bangalore, India.

Ko. J-H.. Chow, K-S. and Han, K-H. 2003. Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of *Hevea brasiliensis* (para rubber tree). *Plant Mol. Biol.* **53**: 479-492.

Krishnakumar. R., Sasidhar. V. R. and Sethuraj. M. R. 1997. Influence of TPD on cytokinin level in *Hevea* bark. *Indian J. Nat. Rubb. Res.* **10**: 107-109.

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

Kumari Jayasree, P. and Thulaseedharan, A. 2001. Gibberellic acid regulated embryo induction and germination in *Hevea brasiliensis*. *Indian J. Nat. Rubb. Res.* **14:** 106-111.

Kumari Jayasree, P., Thomas,V., Saraswathyamma, C.K. and Thulaseedharan, A. 2001a. Optimisation of parameters affecting somatic embryogenesis in *Hevea brasiliensis*. *Indian J. Nat. Rubb. Res.* 14: 20-29.

Kumari Jayasree, P. Sunitha, S.S. and Thulaseedharan, A. 2001b. Effect of cytokinins on in vitro germination of Hevea *somatic embryos. Symposium on* " Natural Rubber Research", *CIRAD, France.*

Kumari Jayasree, P. and Thulaseedharan, A. 2004. Initiation and maintenance of long term somatic embryogenesis in *Hevea brasiliensis*. *IRRDB Biotechnology Workshop*, 9-11 Feb 2004, Kuala Lumpur, Malaysia. P.56.

Kush, A., Goyvaerts, E., Chye. M. L. and Chua, N. H. 1990. Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree). *Proc. Natl. Acad. Sci. USA* **87**:1787-1790.

Lardet. L.. Aguilar, M. E., Michaux. Fermere, N. and 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 Cell. Dev. Biol. (Plant)* **34:** 34-40.

Lespinasse. D., Rodier-Goud. M., Grivet, L., Leconte, A., Legnate. H. and Seguin. M. 2000. A saturated genetic linkage map of rubber tree (*Hevea* spp.) based on RFLP, AFLP, microsatellite, and isozyme markers. *Theor. Appl. Genet.* **100:** 127-138.

Lekawipat. N., Teerawatanasuk. K., Rodier-Goud. M. Seguin. M., Vanmavichit. A., Toojinda. T. and Tragoonrung. S. 2003. Genetic diversity analysis of wild germplasm and cultivated clones of *Hevea brasiliensis* Muell. Arg. by using microsatellite markers. *J. Rubber Res.* **6:** 36-47.

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 Sci.* **124:**183-191.

Low. F. C.. Atan S., Jaffar. H. and Tan. H. 1996. Recent advances in the development of molecular markers for *Hevea* studies. *J. Natl. Rubb. Res.* **11**:32-44.

Luo. H. and Boutry, M. 1995. Phylogenetic relationships within *Hevea brasiliensis* as deduced from a polymorphic mitochondrial DNA region. *Theor. Appl. Genet.* **91**:876-884.

Michaux-Ferriere, N. and Carron, M.P. 1989. Histology of early somatic embryogenesis in *Hevea brasiliensis*: the importance of the timing of subculturing. *Pl. Cell Tiss. Org. Cult.* **19:** 243-256.

Mohan, M., Nair, S., Bhagawat. A., Krishna, T.G., Yano, M.., Bhatia, C.R. and Sasaki, T. 1997. Genome mapping , molecular markers and marker assisted selection in crop plants. Mol. Breed. **3:** 87-103.

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

Montoro, P., Etienne, H. and Carron, M.P. 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. *J. Exp. Bot.* **46**: 255-261.

Oh, S.K., Kang, H., Shin, D.H., Yang, J., Chow, K., Yeang, H.Y., Wagner, B., Breiteneder, H. and Han. K. 1999. Isolation, characterization, and functional analysis of a novel cDNA clone encoding a small rubber particle protein from *Hevea brasiliensis*. *J. Biol. Chem.* **274**: 17132-17138.

Oh, S.K., Kang, H.S., Kim. I.J., Shin, D.H., Yang, J.M., and Han , K.H., 2000. Molecular Cloning and characterization of a cDNA encloding Isopentenyldiphosphate isomerase gene from *Hevea brasiliensis*. *J. Plant Physiol.* **157**: 549-557.

Paranjothy, K. 1974. Induced root and embryoid differentiation in *Hevea brasiliensis* in culture. *3rd Intl. Cong. ,Plant Tiss. Cell Cult.* Univ. Leicester Abst. No.67.

Paranjothy, K. and Gandimathi, H. 1976. Tissue and organ culture of *Hevea Proc. Intl. Rubb. Conf.* Kuala Lumpur *II*.pp. 59-84.

Paranjothy, K. and Ghandimathi, H. 1975. Morphogenesis in callus cultures of *Hevea brasiliensis* Muell. Arg. *Proc. Natl. Plant Tissue Cult. Sym.* KualaLumpur pp. 19-25.

Paranjothy, K. and Rohani, O. 1978. Embryoid and plantlet development from cell culture of *Hevea. 4th Int. Cong. Plant Tiss. Cell Cult.* Univ. Calgary Abstr. No.134.

Parijs, J. V., Broekaert, W. F., Goldstein, I. J. and Peumans, W. J. 1991. Hevein: an antifungal protein from rubber tree (*Hevea brasiliensis*) latex. *Planta* 183: 258-264.

Perrin, Y., Doumas, P., Lardet, L. and Carron, M. P. 1997. Endogenous cytokinins as biochemical markers of rubber tree (*Hevea brasiliensis*) clone rejuvenation. *Plant Cell Tiss. Org. Cult.* **47**: 239-245.

Perrin, Y., Lardet, L., Enjalric, F. and Carron, M. P. 1994. Rejuvenation of mature clones of *Hevea brasiliensis* (Muell. Arg) by *in vitro* micrografting. *Can J. Plant Sci.* **74**:623-630.

Philip, S., Joseph, A., Zachariah, C.A., George, G., Prem E., and Jacob, C.K. 2004. Molecular characterization of *Phytophthora* isolates infecting traditional rubber growing regions through rDNA RFLP. In: *National Symposium on Crop Surveillance: Disease Forecasting and Management*, New Delhi, Feb. 19 –21, 2004.

Pujade-Renaud, V., Arokiaraj, P., Jones, H., Sanier, C., Camvillau L., Tharreau, D., Narangajavana, J. and Chrestin, H. 2003. Functional Analysis of Promoter sequences from *Hevea brasiliensis* hevein genes, in rice and rubber tree. *International Congress of Plant Molecular Biology*, Barcelona.

Rohani, O. and Paranjothy, K. 1980. Isolation of *Hevea* protoplasts *J. Rubb. Res. Inst. Malaysia* **28**: 61-66.

Rekha, K., Jayasree, R., Kumari Jayasree, P., Kala, R.G., Sobha, S., Sushamakumari, S. and Thulaseedharan, A. 2002. *In vitro* micrografting in *Hevea brasiliensis*: A preliminary investigation PLACROSYM XV, Dec. 10-13, 200, Mysore, India.

Rubber Statistical Bulletin 2004. Rubber Study Group, Wembley, UK. **59** p.2

Rubber Statistical News 2004, Rubber Board, Kottayam, India. **63**.

Saha, T., Kumar, A., Sreena, S., Joseph, A., Jacob, C.K., Kothandaraman, R. and Nazeer, M.A. 2000. Genetic variability of *Corynespora cassiicola* infecting *Hevea brasiliensis* isolated from the traditional rubber growing areas in India. *Ind. J. Nat. Rubb. Res.* **13**: 1-10.

Saha, T., Kumar, A., Ravindran, M., Jacob, C.K., Bindu Roy, C. and Nazeer, M.A. 2002. Identification of *Colletotrichum acutatum* from rubber using random amplified polymorphic DNAs and ribosomal DNA polymorphisms. *Mycol. Res.* **106**: 215-221.

Saha, T., Bindu Roy C. and Nazeer, M.A. 2004. Microsatellite variatbility and its use in the characterization of cultivated clones of *Hevea brasiliensis*. *Plant Breeding* (in press)

Satchuthananthavale, .R 1973. *Hevea* tissue culture Q.*J. Rubb. Res. Inst. Ceylon* **50:** 91-97.

Satchuthananthavale, R. and Irugalbandra, Z. E. 1972. Propagation of callus for *Hevea* anthers. Q*J. Rubb. Res. Inst. Ceylon*. **49**: 65-68.

Seneviratne, P and Flegmann, A.W. 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. and Wijesekara, G. A. S. 1997. Effect of episodic growth pattern on *in vitro* growth of *Hevea brasiliensis J. Plantation Crops* **25**: 52-56.

Seneviratne, P. and Wijesekara, G. A. S. 1996. The problem of phenolic exudates in *in vitro* cultures of mature *Hevea brasiliensis*. *J. Plantation Crops* **24**:54-62.

Seneviratne, P., Withangem S. P., Nugawela, A., Wijesekera, G. A. S. and Zoysa, G. M. 1996. Somatic embryogenesis for *Hevea*. The technique for anther culture. *J. Rubb. Res. Instt. Sri Lanka* **78**: 69-78.

Shijie, Z., Zhenghua, C. and Xueng, X. 1990. A summary report on anther culture for haploid plants of *Hevea brasiliensis*. *In: Pro IRRDB Symp. Breed. Hevea brasiliensis*, pp. 69-78, Kunming, China.

Shoucai, C., Hansuaang S, Dongqiong H, Sheng L and Xucquin 1994 Identification of mildew resistance gene from *Hevea* tree by RAPD technique. *Chinese J. Trop. Crops* **15:** 21-26.

Sinha, R.R., Sobhana, P. and Sethuraj, M.R. 1985. Axillary buds of some high yielding clones of *Hevea* in cultue. *First IRRDB Hevea tissue culture workshop*. Kuala Lumpur, Malaysia p. 16.

Sobha, S.,Sushamakumari, S., Thanseem, I., Rekha, K., Jayashree, R., Kala, R.G., Kumari Jayasree, P., Asokan, M.P., Sethuraj M.R., Dandekar, A.M. and Thulaseedharan, A. 2003a. Abiotic stress induced over-expression of superoxide dismutase enzyme in transgenic *Hevea brasiliensis*. *Indian J. Nat. Rubb. Res.* **16**: 45-52.

Sobha, S., Sushamakumari, S., Thanseem, I., Kumari Jayasree, P., Rekha, K., Jayasree, R., Kala, R.G, Asokan, M.P., Sethuraj, M.R., Dandekar, A.M. and Thulaseedharan, A. 2003b. Genetic transformation of *Hevea brasiliensis* with the gene coding for superoxide dismutase with FMV 34S promoter. *Curr. Sci.* **5:** 176 1773.

Sobhana, P., Sinha, R.R. and Sethuraj, M.R 1986. Micropropagation of *Hevea brasiliensis*: retrospects and prospects. In: *International Congress of Plant Tissue and Cell Culture*, Minnesota, USA

Sobhana, P., Thomas M . and Jacob J. 2004. Analysis of the genetic distance between the root stock and scion of healthy and TPD affected trees of *Hevea brasiliensis*. *Indian J. Nat. Rubb. Res.* (In press).

Sompong, T. C. and Muangkaewngam, A. 1992. Tissue culture of rubber I. *In vitro* micropropagation of rubber. Songklanakarin *J. Sci. Technol.* **14**:123-132.

Sushamakumari, S., Rekha, K., Thomas, V., Sobha, S. and Jayasree, R. 1999a. Multiple shoot formation from somatic embryos of *Hevea brasiliensis* (Muell.) Arg. *Indian J. Nat. Rubb. Res.* **12:** 23-28.

Sushamakumari, S., Sobha, S., Jayasree, R. and Asokan, M.P. 1999b. Evaluation of parameters affecting the isolation and culture of protoplasts of *Hevea brasiliensis* (Muell.) Arg. *Curr. Sci* **77:** 1580-1581.

Sushamakumari, S., Sobha, S., Rekha, K., Jayasree, R. and Asokan, M.P. 2000a. Influence of growth regulators and sucrose on somatic embryogenesis from immature inflorescence of *Hevea brasiliensis* (Muell.) Arg. *Indian J. Nat. Rubb. Res.* **13**: 19-29

Sushamakumari, S., Asokan, M.P., Anthony P., Lowe, K.C., Davey, M.R., and Power J.B., 2000b. Plant regeneration from embryogenic suspension derived protoplast of rubber (*Hevea brasiliensis*). *Plant Cell Tissue Org. Cult.* **61**: 81-85.

Suwanmanee, P., Suvachittanont, W. and Fincher, G.B. 2002. Molecular Cloning and sequencing of a cDNA encoding 3 hydroxy-3 methylglutaryl coenzyme A synthase from *Hevea brasiliensis* Muell. Arg. *Science Asia.* **28:** 29-36.

Suwanmanee, P., Sirinupong, N., and Suvachittanont, W. 2004. Regulation of the expression of 3 hydroxy-3-methyl glutaryl-coA synthase gene in *Hevea brasiliensis*. *Plant Sci.* **166**: 531-537.

Takaya, A., Zhang, Y.W., Asawatreratanakul, K., Wititsuwannakul, D., Wititsuwannakul, R., Takahashi, S. and Koyama, T. 2003. Cloning, expression and characterisation of a functional cDNA clone encoding geranylgeranyl diphosphate synthase of *Hevea brasiliensis*. *Biochim Biophys Acta* **1625**: 241 – 220.

Thanseem, I.,Venkatachalam, P. and Thulaseedharan, A. 2003. Sequence characterisation of β-1,3-glucanase gene from Hevea brasiliensis through genomic and cDNA cloning. Ind. J. Nat. Rubb. Res. **16**: 106-114.

Thanseem, I., Joseph, A. and Thulaseedharan, A. 2004. Differential expression of β-1,3- glucanase in *Hevea* clones during pathogenesis. *IRRDB Biotechnology Workshop*, 9-11 Feb 2004, Kuala Lumpur, Malaysia. P.57.

Thanseem, I. 2004. Ph D thesis. Isolation and molecular characterisation of β-1,3-glucanse gene and its role in combating abnormal leaf fall disease in rubber tree (*Hevea brasiliensis* Muell. Arg). Mahatma Gandhi University, Kottayam, Kerala, India.

Thulaseedharan, A., Soni, K. B., Asokan, M. P., Chattoo, B.B. and Sethuraj, M. R. 1997. Identification, cloning and sequencing of a DNA marker linked to tapping panel dryness in *Hevea brasiliensis*. *66 Annual Meet. Society of Biological Chemists* (India) pp. 166.

Thulaseedharan, A. 2002. Biotechnological approaches for crop improvement in natural rubber at RRII – Present status. In : Proceedings of the *Rubber Planters' Conference*, India. 2002. pp. 135-140.

Thulaseedharan, A., Kumari Jayasree, P. and Venkatachalam, P 2000. Biotechnological approaches for crop improvement in rubber. In: *Biotechnology in Horticultural and Plantation Crops*. Chadha KL, Ravindran PN and Sahijram L (Eds) Malhotra Publishingg House (Calcutta) pp: 323-351

Tupy, M. 1988. Ribosomal and polyadenylated RNA content of Rubber tree latex, association with sucrose level and latex pH. *Plant Sci.* **55:** 137-144.

Varghese, Y. A., Knaak, C., Sethuraj, M. R. and Ecke, W. 1997. Evaluation of random amplified polymorphic DNA (RAPD) markers in *Hevea brasiliensis*. *Pl. Breed.* **116** :47-52.

Veisseire, P., Cailloux, F. and Coudret, A. 1994a. Effect of conditioned media on the somatic embryogenesis of *Hevea brasiliensis*. *Pl. Physiol. Biochem.* **32**:571-76.

Veisseire,. P, Linossier, L. and Coudret, A. 1994b. Effect of abscisic acid and cytokinins on the development of somatic embryos in *Hevea brasiliensis*. *Plant. Cell. Tiss. Org. Cult.* **39**:219-23.

Venkatachalam, P., Thanseem, I. and Thulaseedharan, A. 1999. A rapid and effective method for isolation of RNA from bark tissues of *Hevea brasiliensis*. *Curt Sci.* **77:** 101-103.

Venkatachalam, P., Thomas, S., Priya, P., Thanseem, I., Gireesh, T., Saraswathyamma, C.K., and Thulaseedharan, A. 2002. Identification of DNA polymorphism among clones of *Hevea brasiliensis* Muell. Arg. Using RAPD analysis. *Ind. J. Nat. Rubber Res.* **15**: 172-181.

Venkatachalam, P., Priya, P., Saraswathy Amma, C.K. and Thulaseedharan, A. 2004. Identification, cloning and sequence analysis of a dwarf genome specific RAPD marker in rubber tree (*Hevea brasiliensis*) *Plant Cell Rep.* **23**:327-332

Wan, A. R., Ghandimathi, H., Rohani, O. and Paranjothy, K. 1982. Recent developments in tissue culture of *Hevea*. In: Rao A 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. and Lu, W. 1980. Induction of rubber plantlets from anther of *Hevea brasiliensis* Muell. Arg. *In vitro. Chin. J. Trop. Crops* **1**:25-26.

Wang, Z., Wu, H., Zeng, X., Chen, C. and Li, Q. 1984. Embryogeny and origin of anther plantlet of *Hevea brasiliensis*. *Chin. J. Trop. Crops* **5**:9-13.

Wang, Z. Y and Chen, X.T. 1995. Effect of temperature on stamen culture and somatic plant regeneration in rubber, *Acta Agronomica Sinica* **21**:723-726.

Wang, Z.Y., Wu, H.D, and Chen, X.T. 1998. Effects of altered temperatures on plant regeneration frequencies in stamen culture of rubber trees. *J. Trop. Subtrop. Bot.* **6**:166-168.

Yeang, H.Y., 2004. Country Report (Malaysia). IRRDB Biotechnology Workshop, Kuala Lumpur, Malaysia. pp: 40-43.