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BIOTECHNOLOGICAL APPROACHES FOR CROP IMPROVEMENT IN RUBBER

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1. INTRODUCTION

Hevea brasiliensis Muell.Arg., belonging to the family Euphorbiaceae, is the major source of natural rubber and the only commercially exploited species. It is believed that the present rubber trees, covering millions of hectares in South Asia, were derived probably from a few plants which survived from the Wickham's collection that originated from the Amazon river basin (Imle·1978). The plant is predominantly cross pollinated by insects. *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.

Natural rubber is synthesized in highly specialized cells called laticifers or latex vessels which are present in all parts of the tree except in the heart wood. These vessels originate

from the cells produced by the cambium and they are articulated and anastomosing (Hebant 1981). The milky cytoplasmic content (latex) of the latex vessels is collected by controlled wounding of the bark. The latex, upon coagulation and further processing, yields natural rubber. Chemically, natural rubber is cis-1,4-polyisoprene, having molecular weight 200,000 - 8000,000 and with visco-elastic properties. The flexibility of natural rubber to undergo vulcanization with sulphur under high temperatures is an important attribute. A major quantity of natural rubber produced is consumed by the automobile tyre industry. The higher strength, low heat build up and better resistance to wear and flex cracking, made natural rubber a suitable raw material for manufacture of the automobile tyre. It is a good insulator and can be easily manipulated. Being water-resistant, it finds use in the manufacture of water-proofing materials. More than 50,000 rubber based products such as hand gloves, toys, balloons, hoses, footwear etc are manufactured from rubber, and is also useful in soil stabilization, in vibration absorption, surfacing roads etc.

Guayule rubber (pronounced as: *why.you-lee*) (*Parthenium argentatum*) or Mexican rubber, which is a native of North Central Mexico and Southern Texas, provided about 10% of the worlds natural rubber in 1910 but no commercial cultivation was encouraged after 1929 in the US (Anonymous 1977). Rubber is produced in single, thin walled cells in all parts of the plant excepting leaves. The rubber particles are present as suspended in the cell sap. Rubber forms about 10% of the total dry weight with reports of as high as 26% in some strains after four years of growth. During active growth, the plant produces little rubber. The rubber containing cells in guayule are not inter connected and extraction is done by mechano-chemical means. Unlike *Hevea*, guayule can withstand a temperature range of -18° to +49 °C (George *et al.* 1980). This plant is now receiving considerable attention in USA where cultivation of *Hevea* is not possible. The advantages of guayule rubber are that it has low molecular weight and it is reported that there is no allergic response in humans with the use of finished products, especially useful in medical applications. Now, the USDA-ARS is putting efforts to develop it as an alternative source of commercial natural rubber (Wood 1999).

2. NEED FOR BIOTECHNOLOGICAL INTERVENTION

The global consumption of natural rubber is steadily increasing and the production has also increased so as to meet the demand. The major goals of crop improvement programme in *Hevea* include increased yield, tolerance to diseases, drought, physiological disorders like tapping panel dryness (TPD) etc. The long breeding cycle, highly heterozygous nature, the narrow genetic base etc. make conventional breeding rather difficult, elaborate and time consuming.

The elite clones of *H.brasiliensis* are commercially propagated by grafting buds from selected clones onto seedling rootstocks. This ensures genetic homogeneity of the scion in this highly heterozygous species. But rootstocks, 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 trials (Combe 1975; Seneviratne & Flegmann 1996). The micropropagation of elite clones with their own root system could reduce intra-

clonal variation due to stock-scion interaction. Many of the recently developed high yielding clones are susceptible to Tapping Panel Dryness, drought, and major diseases like abnormal leaf fall caused by *Phytophthora* sp., powdery mildew by *Oidium hevea*, leaf spot by *Corynespora cassicola* etc. There is an urgent need to introduce resistant genes into the high yielding clones through genetic manipulation. *Hevea* genetic transformation offers great potential to achieve tolerance to the above problem by introducing specific genes without disrupting their otherwise desirable genetic constitution.

DNA molecular marker based selection will be helpful to the plant breeder to identify and select the desired plants at an early stage and hasten the transfer of desirable genes into the cultivars and also to introduce novel genes from wild species (Mohan *et al.* 1997).

3. TISSUE CULTURE APPROACHES

Although elite clones of rubber plants can be propagated easily through bud grafting, the interaction of uniform scions with the unselected seed-derived stocks (stock-scion interaction) is a major problem. Development of protocols for the *in vitro* propagation of selected genotypes is highly desirable for mass producing true-to-type individuals. *Hevea* is highly recalcitrant to *in vitro* culture, and, only limited success has been achieved yet. Although, progress has been achieved in micropropagation using different explants, many problems still persist with *in vitro* explant viability, bud sprouting and shoot elongation (Seneviratne & Flegmann 1996). Additionally, *in vitro* rooting and survival of *in vitro*-produced plants remain constraints to more widespread application of micropropagation techniques to *Hevea.* Extensive work has been done over the past two decades; however, no large-scale commercial application of tissue culture techniques is available (Seneviratne *et al.* 1996a).

Most of the *in vitro* culture work in *Hevea* is on micropropagation and somatic embryogenesis and not much on haploid production, embryo culture, protoplast culture and genetic transformation. Plant regeneration through micropropagation, haplogenesis, organogenesis and somatic embryogenesis has been achieved. Studies on biochemical and nutrient requirements have also been performed using callus cultures. In addition to the regeneration techniques, a method for protoplast culture and genetic transformation has also been developed.

3.1. Micropropagation

The work on *in vitro* culture of *Hevea* is summarized in Table 1. Clonal propagation of *Hevea* by tissue culture methods stands to serve the rubber industry to a greater extent, if a reliable technique is developed for selected varieties, as, the conventional method of propagation by bud grafting led to undesirable stock-scion interaction. The yield of each individual tree in a plantation is becoming an important parameter in order to use the land efficiently, to increase the tapper productivity and also, to realize full capacity of the improved cultivars. The need to have many uniform individuals of a selected genotype led to a renewed interest for the development of *in vitro* culture techniques as a means of vegetative propagation. In the past, several reports have appeared on *Hevea*

Table 1. *Summary of Tissue Culture Studies on Hevea*

Technology	Explant/s	Results	References
Micropropagation			
	Shoot apices	PD, RD	Paranjothy & Ghandimathi (1975, 1976)
	Terminal buds from mature trees	PD, RD	Mascarenhas *et al.* (1982)
	Somatic embryos	PD	Carron & Enjalric (1982)
	Axillary buds from young trees	PD, RD	Enjalric & Carron (1982)
	Rubber rootstocks	PD	Carron & Enjalric (1983)
	Shoot tips from immature seedlings	PD	Gunatilleke & Samaranayake (1988)
	Shoot tips from clonal material	PD, RD	Asokan *et al.* (1988)
	Stem fragments	PD, RD	Chanasongkram & Jewtragoon (1991)
	Shoot tips, internodes	PD, RD	Sompong & Muangkaewngam (1992)
	Apices from mature shoots	PD, RD	Perrin *et al.* (1994)
	Axillary buds	PD, RD	Seneviratne *et al.* (1995a)
	Nodal explants from juvenile plants	PD, RD	Seneviratne & Flegmann (1996)
	Shoot tips	PD	Seneviratne (1996)
	Axillary buds from mature clones	PD, RD	Perrin *et al.* (1997)
	Axillary buds	PD	Seneviratne & Wijesekara (1996, 1997)
	Axillary buds	PD, RD	Mendanha *et al.* (1998)
	Axillary buds	PD	Lardet *et al.* (1998)
	Nodal explants	PD	Lardet *et al.* (1998)
Organogenesis			
	Seed embryos	PD	Muzik (1956)
	Mature embryonic axes	PD	Muzik (1956)
	Cotyledon, hypocotyl & epicotyl	RD	Paranjothy & Ghandimathi (1975, (1976)
	Mature embryonic axes	PD	Paranjothy & Ghandhimathi (1976)
	Decotyledonized embryos	PD	Toruan & Suryatmana (1977)
	Young stem	CD, RD	Bouychou (1953a, b); Chua (1966); Wilson & Street (1975)
	Stamens	CD,PD	Wilson *et al.* (1976); Audley & Wilson (1978)
	Dehusked seeds	CD	Paranjothy *et al.* (1979)
	Leaves	CD, ED	Carron & Enjalric (1982
	Leaves	CD, PD	Mendanha *et al.* (1998)

Contd.

Table 1. *Contd.*

Technology	Explant/s	Results	References
Haplogenesis			
	Anther	CD	Satchuthananthavale & Irugalbandra (1972)
	Anther	CD	Satchuthananthavale (1973)
	Anther	CD, ED	Paranjothy (1974)
	Anther	CD, CD	Paranjothy & Ghandimathy (1975, (1976); Ghandimathi & Paranjothy (1975)
	Anther	CD, ED, PD	Paranjothy & Rohani (1978)
	Anther	CD, ED, PD	Chen *et al.* (1978, 1979, 1981,1982); Chen (1984)
	Ovules	CD, ED, PD	Guo *et al.* (1982)
	Anther	CD, ED	Cen *et al.* (1981)
	Anther	CD, ED, PD	Shijie *et al.* (1990)
	Anther	CD, ED	Das *et al.* (1994)
	Anther	CD	Seneviratne *et al.* (1996a, b or c)
	Ovules	CD, ED	Yang *et al.* (1997)
Somatic Embryogenesis			
	Anther	CD, ED, PD	Parnajothy (1974)
	Anther	CD, ED	Paranjothy & Ghandimathi (1975)
	Anther	CD, ED, PD	Paranjothy & Rohani (1978)
	Anther	CD, ED	Wang *et al.* (1980)
	Stem	CD, ED, PD	Carron (1980, 1981)
	Anther	CD, ED, PD	Wan *et al.* (1982)
	Integuement tissue	CD, ED	Carron & Enjalric (1982)
	Integument tissue	CD, ED	Carron *et al.* (1985); Carron & Enjalric (1984)
	Integument tissue	CD, ED	El Hadrami *et al.* (1989a,b)
	Integument tissue	CD, ED	El Hadrami *et al.* (1991)
	Integument tissue	CD, ED	El Hadrami & d'Auzac (1992)
	Integument tissue	CD, ED	Etienne *et al.* (1991, 1993a, b)
	Integument tissue	CD, ED	Montoro *et al.* (1993)
	Integument tissue	CD, ED	Veisseire *et al.* (1994a, b)
	Stamens	CD, ED, PD	Wang & Chen (1995)
	Anther	CD, ED	Seneviratne *et al.* (1996a, b)
	Integument tissue	CD, ED, PD	Etienne *et al.* (1997a, b)
	Anther & stamen	CD, ED, PD	Wang *et al.* (1998)
	Immature anthers	CD, ED, PD	Kumari Jayasree *et al.* (1999)
Protoplast Culture			
	Immature leaves	PI, PF, FD	Cailloux & Lleras (1979)

Contd.

Table 1. *Contd.*

Technology	Explant/s	Results	References
	Stem pith cell suspensions	PI, FD	Rohani & Paranjothy (1980)
	Stem tissues	PI, FD	Wilson & Power (1989)
	Anther callus & cell suspension	PI, PF	Haris *et al.* (1993)
	Embryonic callus	PI, PD, MCF	Cazaux & d'Auzac (1994)
	Stem tissues	PI, FD	Cazaux & d'Auzac (1995)
Genetic Transformation			
	In vitro & *in vivo* seedlings	TF	Arokiaraj & Wan (1991)
	Anther derived callus	GT, TC, TE	Arokiaraj *et al.* (1994)
	Anther derived callus	GT, TC, TE, TP	Arokiayaj *et al.* (1996; 1998)

CD-callus development; PD-plantlet development; RD-root development; ED-embryo development; PI-protoplast isolation; PF-protoplast fusion; FD-failed for division; MCF-Microcallus formation; TF-Tumour formation; GT-genetic transformation; TC-transformed callus; TE-transformed embryos; TP-transgenic plant.

micropropagation with varying degrees of success, using different explants, mostly derived from seedlings (Seneviratne *et al.* 1993, Seneviratne 1996).

Paranjothy and Ghandimathi (1975, 1976) tried the culture of shoot apices (2-3 cm long) derived from 2 to 3 week old aseptically-grown seedlings. They obtained rooted plantlets within four weeks of culture in liquid MS medium. However, these shoots failed to grow on solid medium. Mascarenhas *et al.* (1982) reported the development of up to three shoots per explant when terminal buds from 10-20 year old trees were cultured on solid MS medium supplemented with 0.5 ppm kinetin (Kin) 2 ppm 6-benzyladenine(BA), 200 ppm casein hydrolysate, 0.1 ppm calcium pantothenate and 0.1 ppm biotin. These shoots were separated individually and rooted on White's medium containing 10 ppm each of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPA) and α-naphthalane acetic acid (NAA). The maximum rooting obtained was 60%.

Enjalric & Carron (1982) reported that stem nodes from young greenhouse plants (1 to 3 year old) sprouted in basal medium containing 0.5% activated charcoal and 6% sucrose without any growth hormones. These explants were pretreated with 5 ppm IBA and 10 ppm BA for 2 h before being cultured. They obtained rooting of sprouted shoots when dipped in 5 ppm each of IBA and NAA solution for five days and then cultured on nutrient medium without any growth regulators. A major problem with *Hevea* explants is the high rate of systemic contamination derived from field grown plants (Enjalric & Carron 1982; Seneviratne *et al.* 1995a). Enjalric & Carron (1982) developed plants via micropropagation from eight clones using seedling derived explants. These authors, as well as Carron *et al.* (1998), cultured somatic embryos as explants and developed shoots via micropropagation. Carron & Enjalric (1983) reported that the main problem with the propagation of elite rubber clone stock material from cuttings has been the failure to produce an adequate root system with taproot quality necessary for tree stability. However,

shoot and root development was successfully obtained from seedlings by Carron *et al.* (1989). Shoots have been regenerated from axillary bud explants of 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 for the first time. Rooted plantlets were successfully transplanted in the field.

Gunatilleke & Samaranayake (1988) cultured shoot tips from aseptically grown rubber seedlings on MS liquid medium with half-strength salts containing 0.5 mg/l BA + 0.05 mg/l IBA or on MS solid medium with full-strength salts with or without 0.5 mg/l BA + 0.05 mg/l IBA. BA was better for axillary shoot proliferation than other cytokinins tested (kinetin and 2ip). Stem segments from seedlings (3-8 months old) were cultured on basal medium containing activated charcoal, sucrose (6%), IBA at 5 ppm and BA at 10 ppm. Bud growth and new leaves developed within one month of culture. These shoots were then rooted on basal medium without growth regulators. Both adventitious as well as pseudo-tap roots were reported (Chanasongkram & Jewtragoon 1991).

Sompong & Muangkaewngam (1992) cultured various explants derived from *in vitro* seedlings on MS medium with different levels of NAA and BA or with BA alone at various levels. They obtained multiple shoots from 100 % of nodal as well as shoot tip explants with BA alone. Rooting of shoots was achieved on MS medium with 5 mg/l IBA. Successful micropropagation has been carried out with apices from mature shoots of two *Hevea* genotypes micrografted onto three week-old seedlings grown *in vitro* (Perrin *et al.* 1994). This indicates that physiological juvenility of explant is perhaps highly important for successful micropropagation. Seneviratne *et al.* (1995b) described that the root system of plants produced from axillary buds did not produce a tap root, unlike plants propagated by other methods like bud grafting or embryo culture. However, tree growth in the field was similar for plants produced by all three methods. Shoot explants from five rubber clones were cultured on MS medium. Shoot tips sterilized with sodium hypochlorite promoted phenolic browning. Use of mercuric chloride instead of sodium hypochlorite reduced browning. Pretreatment of explants with antioxidants increased phenolic browning, however, incorporation of these antioxidants into solid MS medium decreased browning (Seneviratne & Wijesekara 1996). Seneviratne & Flegmann (1996) obtained multiple axillary shoot production from nodal explants of juvenile origin on a medium containing thidizuron at 0.02 ppm in combination with NAA 0.2 ppm within four weeks. Axillary buds showed satisfactory elongation on transfer onto a growth regulator free medium. Elongated axillary shoots produced root in the presence of IBA at 2 ppm.

Perrin *et al.* (1997) reported induction of endogenous cytokinin levels during *in vitro* shoot organogenesis and rhizogenesis in rubber. Slow-growth of axillary buds is one of the key reasons for the slow progress in micropropagation of clonal *Hevea*. Axillary buds, induced in the presence of cytokinins, elongated satisfactorily in the presence of gibberellic acid (GA3) (2-4 mg/l) (Seneviratne & Wijesekara 1997). They explained that the growth

phase of explants had an effect on *in vitro* axillary bud elongation. Nodes harvested from young shoots during active growth showed the least elongation, while explant removed during the stationary phase of growth showed the highest elongation rates. The difference may be attributed to endogenous levels of growth regulators. Mendanha *et al.* (1998) described shoot development from axillary buds cultured on MS medium supplemented with 1.0 mg/l each of kin and 2,4-dichlorophenaxy aeeric acid (2,4-d), 2% sucrose and 4% agar. Shoots developed from axillary buds rooted vigorously when cultured on MS medium with NAA, IAA and IBA. Nodes, when used for micropropagation, exhibited better growth (shoot elongation) (Lardet *et al.* 1998).

At the Rubber Research Institute of India (RRII), Kottayam, the present authors have developed the following method for micropropagation using shoot tips as explants (unpublished). Shoot tips taken from 2 to 4 week old seedlings and mature trees were surface sterilized in commercial bleach (5%) in the presence of Tween-80 for 5 min followed by 0.1% mercuric chloride solution for 5 min. They were rinsed several times with sterile distilled water to remove excessive sterilants, which is toxic to the explants. Mature leaves are removed from the sterilized shoots except tip portion containing 2 to 4 young leaves. The shoot tips are grown on MS medium containing 3-4% sucrose with 1.0 mg/l of each BA and kin and 2.0 mg/l of each IAA, IBA and NAA (Fig.1a). New leaves are grown and elongation is occurred. These elongated shoots are rooted on the same medium or on subculture into fresh medium within 6-8 weeks of culture (Fig.1b). Well-developed plants are transferred to soil in polybags after hardening (Fig. 1c).

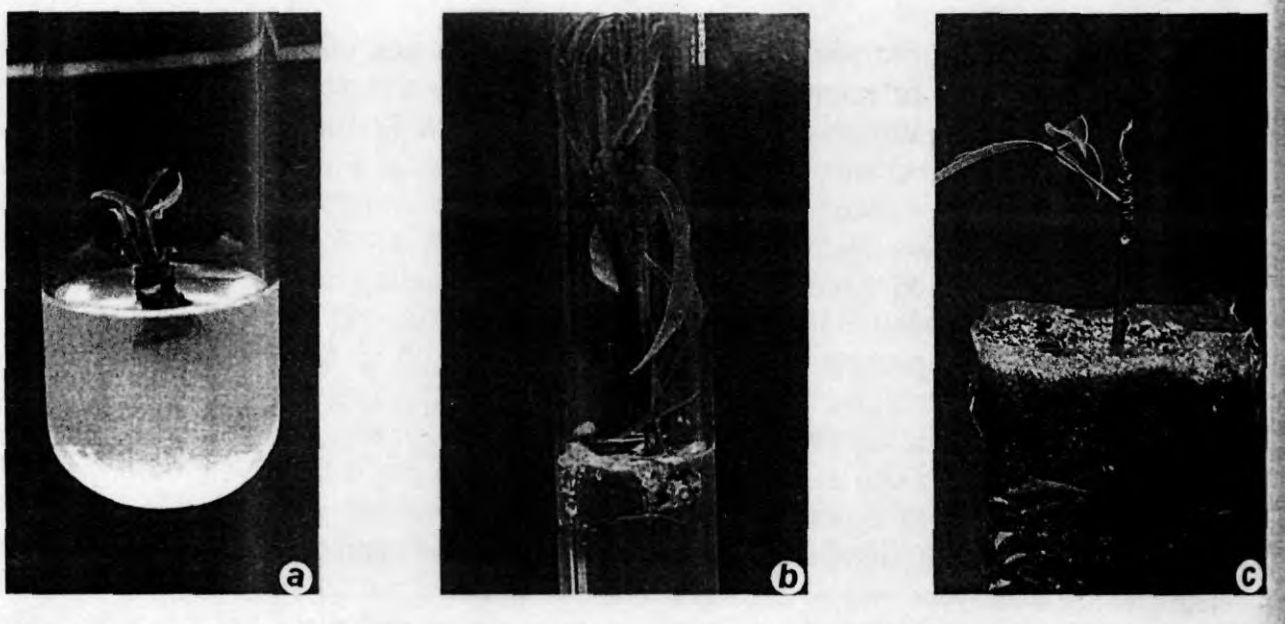

Fig. 1. Micropropagation and Plantlet Regeneration in *Hevea brasiliensis:* (*a*) A shoot tip growing on medium (*b*) Development of roots from elongated shoot tip on rooting medium (*c*) A shoot tip derived plantlet established in polybag containing soil

3.2. Organogenesis

Attempts to induce shoot organogenesis from somatic tissues of *Hevea* species have not been promising and only vaguely reported (Seneviratne *et al.* 1996b). Successful regeneration from *Hevea* has been limited to a few clones. Additional work to improve regeneration frequency as well as to induce regeneration from somatic tissues of other commercial popular *Hevea* genotypes is still needed. Various workers have investigated propagation of *Hevea* via tissue culture in the past.

The first tissue culture work on *Hevea* was carried out in 1953 by Bouychou (1953a, b) with the aim of using callus to obtain convenient material for the study of the laticiferous system. Muzik (1956) found that mature embryos developed into plants rapidly on basal medium, but immature embryos failed to develop further. Chua (1966) found cell proliferation from epicotyl and green stems of young seedlings on MS medium with high sucrose concentration (100 g/l). Subsequently, Wilson and Street (1975) reported the initiation of cell suspension by subculturing this callus in liquid medium. Wilson *et al.* (1976) used such cell suspensions for cytological and chromosome analysis. Later, green shoots were obtained from these cell suspensions on medium containing 2-chloroethylphosphonic acid (2-CEPA) (Audley *et al.* 1975; Audley & Wilson 1978). Attempts to regenerate plants from mature embryonic axes were a failure unless a portion of the cotyledon was attached (Muzik 1956; Paranjothy & Ghandimathi 1976). Toruan and Suryatmana (1977) developed seedlings from decotyledonized embryos in MS medium and subsequently established in soil. Dehusked seeds from pods or freshly fallen seeds collected from the ground germinated and developed into full plantlets when cultured on nutrient media containing 3% sucrose (Paranjothy *et al.* 1979).

Explants derived from young *Hevea* seedlings developed profuse callusing on media containing IAA, NAA or naphthoxy aeetic acid (NOA) and subsequently rooting was observed (Chua 1966; Wilson & Street 1975; Paranjothy & Ghandimathi 1975, 1976). The callus from hypocotyl explants was usually hard and compact and some samples released latex when excised for subculture. Cotyledon explants also produced roots, even in media without growth regulators (Paranjothy & Ghandimathi 1975, 1976). Rooting got suppressed in such explants with addition of 2,4-D or chlorophenoxyacetic acid (p-CPA) in the medium. However, this callus failed to regenerate. Paranjothy and Ghandimathi (1975, 1976) could induce callus from segments of epicotyl or hypocotyl and pieces of cotyledons on MS medium with low levels of hormones or in the absence of hormones. Carron and Enjalric (1982) demonstrated callus induction from leaf explants on basal medium supplemented with auxins (2,4-D and IAA at 0.3 to 2 ppm and 1 to 5 ppm respectively) and cytokinins (kinetin and BA at 1 to 5 ppm, each) but, no organogenesis was observed. Recently, Mendanha *et al.* (1998) demonstrated abundant callus initiation from leaf explants, but no regeneration was observed when the calli from different media were transferred to MS medium without growth regulators. On this basal medium, callus cultures became necrotic and dried later.

3.3. Haplogenesis

Isolated anther/microspore culture is an important tool for production of homozygous doubled haploid plants. Anther culture may reduce the time needed to reach homozygosity by spontaneous or induced doubling of the haploid chromosome number. It allows for an increase in selection efficiency due to better discrimination between genotypes within any generation and efficient retention of desirable genes in later generations. Microspore embryogenesis is also an elegant system for gene mapping, genetic transformation as well as for selection for dominant and recessive traits. Although, anther culture is much simpler in handling, microspore culture has several advantages (a) formation of calli and embryos that often form on somatic tissues of anther could be avoided (b) there is direct access to microspores, which speeds up the optimization of culture conditions. The production of doubled haploids 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. The major limitation of successful incorporation of the anther culture technique to *Hevea* breeding programmes was the difficulty in obtaining doubled haploid populations large enough for all kinds of genetic recombination. However, *in vitro* approaches to induce haploids in *Hevea* have had only limited success in comparison with other plant species (Seneviratne *et al.* 1996c).

As reported by Shijie *et al.* (1990), pollen plants from about 13 clones have successfully been transplanted and established in the field. With only a few papers dealing with *in vitro* *Hevea* regeneration, it can be said that the regeneration ability of *Hevea* is highly genotype dependent, and, a specific protocol must be developed for each clone or cultivar (Shijie *et al.* 1990). Induction of embryogenesis and limited plant formation has been reported from anther cultures of *Hevea*. Satchuthananthavale and Irugalbandra (1972) cultured *Hevea* anthers for the first time, with the aim of obtaining haploid plants. Satchuthananthavale (1973), Ghandimathi and Paranjothy (1975) as well as Paranjothy and Ghandimathi (1976) obtained callus formation from anther tissues and pollen grains after 4-5 weeks in culture and the callus continued to maintain good growth on the same medium through six subcultures over a period of six months. Chen *et al.* (1978, 1979), however, first reported the production of pollen plantlets in *Hevea*. Since then, reports on haplogenesis in *Hevea* by several investigators have been published in China and pollen-derived plantlets have been established in soil (Chen 1984). The frequency of viable pollen plantlets has been enhanced to 3% (Chen *et al.* 1981, 1982). Guo *et al.* (1982) reported that two plantlets were recovered from unpollinated ovules of *Hevea*. The ploidy level of these plantlets was not reported. Das *et al.* (1994) found callus induction from anthers of several clones. Anthers of all clones produced calli on a medium with 6% sucrose. Cold treatment of anthers at 8-10°C for 24h had a positive effect on callus development. Frequency of embryogenesis was enhanced in the presence of BA and isopentenyladenine (2ip). Recently, callus induction and embryoid formation has been achieved from unfertilized ovule of *Hevea* (Yang & Fu 1997).

A protocol used for anther culture is described below (Chen 1984). Anthers and flower

buds used for inoculation should have well-developed microspores with good viability and, therefore, male-sterile clones should be avoided. Collection of anthers must be carried out when most of the pollen grains are at the uninucleate stage. A majority of the flower buds at the uninuclear stage are 3-3.5 mm in length with greenish-yellow colour. However, these characteristics may vary with the clone, season etc., hence, microscopic observation is essential before each clone is used for culture. The stage of flower bud used to harvest anthers seems to be important in producing haploids (Shijie *et al.* 1990). The inside of the flower bud is usually in aseptic condition, only surface sterilization is needed with either 5% sodium hypochlorite or 0.1% mercuric chloride solution for 5 and 3 minutes, respectively, followed by rinsing several times with sterilized distilled water. The induction of pollen plants is carried out in three steps. The first step involves inoculation of anthers on primary medium to induce callus formation, in which, the microspores grow and develop into multicellular masses, haploid embryos or pollen calli. The walls of the anther proliferate into a mass of callus during the first 3-4 weeks of culture. This somatic callus then disintegrates while callus and embryo development occurs vigorously from microspores (Carron *et al.* 1989). Proliferation of both haploid and somatic cells in cultured anthers is common and an important strategy for obtaining haploid plantlets, and eventually, in suppression of somatic callus proliferation. This is achieved by incorporation of coconut water and high sucrose level in the medium. A higher concentration of total nitrogen is required for induction of pollen embryos. A high concentration of KH_2PO_4 is also important for induction of high frequency of callus as well as embryo formation. Under favourable conditions, there are two different processes that exist simultaneously: the formation of somatic calli and the initiation of microspore development into small embryos or pollen calli. It is very difficult to distinguish development of microspores from somatic calli. Therefore, much attention should be paid to the development of microspores.

The second step in anther culture is transfer of the anther-derived callus to a differentiation medium after 50 days in culture. The pollen calli differentiate into small embryoids that subsequently develop into embryos visible to the naked eye. The time of transfer of the callus to differentiation medium is critical in order to promote further development and differentiation of haploid embryos (Chen *et al.* 1979, Cen *et al.* 1981). Three growth regulators i.e., kinetin, NAA and GA_3 are required for differentiation of embryos. Gibberellic acid (GA_3) promotes embryo growth as well as development. In the last step, well-developed microspore embryos are transferred to plant regeneration medium for getting fully developed plantlets. Conversion of embryos into plantlets requires mainly a progressive increase in the amount of GA_3.

3.4. Somatic Embryogenesis

Successful plant regeneration via somatic embryogenesis in *Hevea* can provide a rapid *in vitro* method for mass propagation of this plant, as well as this opens up new avenues for crop improvement. However, like in many other woody species, this phenomenon is still infrequent because of low germination percentage and plant conversion (Cailloux *et al.* 1996; Linossier *et al.* 1997). Although somatic embryogenesis has the

potential as a very efficient method for *Hevea* propagation, the mechanisms involved in embryogenic callus induction and differentiation are still poorly understood (Etienne *et al.* 1993a). For many *Hevea* clones induction and expression of somatic embryogenesis can be induced either with the same hormonal treatment or with different hormonal sequences (El Hadrami *et al.* 1991). In most cases, auxin is necessary for the induction, but cultures fail to produce embryo upon continuous exposure to auxin. Thus, a major limitation of the *Hevea* embryogenic system observed by the earlier workers has been the low and non-synchronous production of somatic embryos. In fact, the germination of embryos remained very difficult and the evaluation of their quality need further investigation (Etienne *et al.* 1993b; Carron *et al.* 1995; Linossier *et al.* 1997). Recently, there has been an increasing interest in the development of plantlets through somatic embryogenesis especially for use in genetic transformation (Kumari Jayasree *et al.* 1999).

Although, there are a few reports on regeneration of shoots and/or plantlets from somatic explants/tissues of *Hevea*, regeneration of plantlets via somatic embryogenesis from somatic tissues of different genotypes remains difficult with a low frequency of occurrence. Reliable somatic embryo formation is limited to only a few genotypes of *Hevea* (Kumari Jayasree *et al.* 1999). Paranjothy (1974) achieved differentiation of embryoids from the anther wall derived callus. The embryoids that develop from the anther wall derived callus are usually nodular or spindle shaped with clear evidence of bipolarity. These embryos generally turned green with a pair of cotyledons on incubation under light. Subsequently, shoot development was also achieved (Paranjothy & Ghandimathi 1975; Paranjothy & Rohani 1978). Later, Wang *et al.* (1980, 1984) and Wan *et al.* (1982) successfully regenerated *Hevea* plants through somatic embryogenesis from anther walls. They succeeded in transplanting several plantlets into soil. Carron and Enjalric (1982) cultured anther wall for callus induction and subsequent embryoid development. Carron (1980, 1981) used inner integument tissue of seed for somatic embryogenesis and subsequent plantlet development. The whole process of somatic embryogenesis obtained using inner integument callus involved four successive phases i.e., caullogenesis, embryoid differentiation, multiplication of embryos and development of embryos into plants (Carron 1984; Carron *et al.* 1985).

Wang & Chen (1995) regenerated plantlets through somatic embryogenesis from stamen cultures. They optimized the temperature requirement for callus induction, somatic embryogenesis and plant regeneration as 26°C, 24-25°C and 26-27°C respectively (Wang *et al.* 1998). Regeneration frequencies as high as 40.5% were obtained with the optimized condition. Veisseire *et al.* (1994a, b) induced embryogenic calli from integument tissue of immature seeds on medium containing 234 mM sucrose, 9µM BA and 9µM 2,4-D. Abscisic acid (ABA) stimulated embryo development in liquid medium. In the presence of cytokinins, embryos turned green within one month (Veisseire *et al.* 1994a, b). Sucrose and ABA treatments enhanced the embryo germination and plantlet development, (Etienne *et al.* 1993b). ElHadrami *et al.* (1989a, b) as well as El Hadrami & d'Auzac (1992) found that callus induction from inner integument tissue of *Hevea* seeds was not affected by addition

of polyamine biosynthesis inhibitors, but reduced somatic embryo formation. Montoro *et al.* (1993, 1995) tested various growth regulators, sucrose and calcium on callus friability and somatic embryogenesis. 2,4-D, kin and higher level of sucrose and calcium content induced the formation of friable calli as well as embryonic cell suspensions (Montoro *et al.* 1992). Etienne *et al.* (1993) found that the low endogenous ABA levels was measured in embryogenic callus whereas non-embryogenic calli accumulated high levels of ABA.

Etienne *et al.* (1991) studied the importance of water and plant growth regulator status of culture medium on somatic embryogenesis in *Hevea*. Higher relative water content (93-95%) and water potential of callus with lower concentration of 2,4-D, BA and ABA in the medium favoured embryogenic potential of the callus. El Hadrami *et al.* (1991) obtained embryogenic callus from inner integuments of *Hevea* seeds using lower levels of 2,4-D and BAP in the medium. Cailloux *et al.* (1996) observed a high frequency secondary embryogenesis from isolated early cotyledonary-stage somatic embryos of *Hevea*. The addition of 234 mM sucrose and 10^{-5} M ABA to the culture medium enhanced the maturation of somatic embryos, however, the conversion to plantlets remains a major problem. Linossier *et al.* (1997) found that PEG was necessary to obtain more of torpedo-shaped embryos, and the number of embryos increased by addition of ABA in the medium. Etienne *et al.* (1997a) reported that the regeneration potential of embryogenic callus was stimulated by high $CaCl_2$ concentration. Induction of somatic embryogenesis was enhanced by temporary immersion of embryos in liquid medium (Etienne *et al.* 1997b). These observations confirm the idea that the interaction and balance of all the plant growth regulators, osmoticum and nutrients are important in the control of embryogenesis and plantlet formation in *Hevea*. Most of the aforementioned investigators used inner integument tissues of immature fruit as the explant source for embryogenesis. However, somatic embryogenesis in rubber remains difficult because calli obtained from the integuments of immature seed frequently display browning (necrosis) leading to tissue degeneration and a loss of embryogenic competence (Housti *et al.* 1991; Veisseire *et al.* 1994a). Recently, a protocol has been developed to induce high frequency somatic embryogenesis using immature anther as explant (Kumari Jayasree *et al.* 1999). This procedure greatly increased the percentage of calli showing somatic embryos as well as improved plantlet conversion rate.

In the authors' laboratory, the following protocol was developed for induction of somatic embryogenesis and plant regeneration from immature anthers *of Hevea* (Kumari Jayasree *et al.* 1999). Floral buds, about 0.5-1.0 mm long, were used for callus initiation on modified MS medium with B_5 vitamins and 5% sucrose. Microscopic observations were carried out and the correct stage with only diploid tissues i.e. before microsporogenesis were identified. Best callus induction was obtained with 2.0 mg/l 2,4-D and 0.5 mg/l kn. Embryo induction and development was achieved on a medium containing 0.7 mg/l kin and 0.2 mg/l NAA with 7% sucrose and 0.2% activated charcoal (Fig.2a, b). Conversion of somatic embryos into full plantlets was achieved on a hormone free medium (Fig. 2c). A cytological study was carried out and the regenerated plants studied were diploids. Well-developed plantlets were transferred to polybags (Fig. 2d) and subsequently established in the field for further

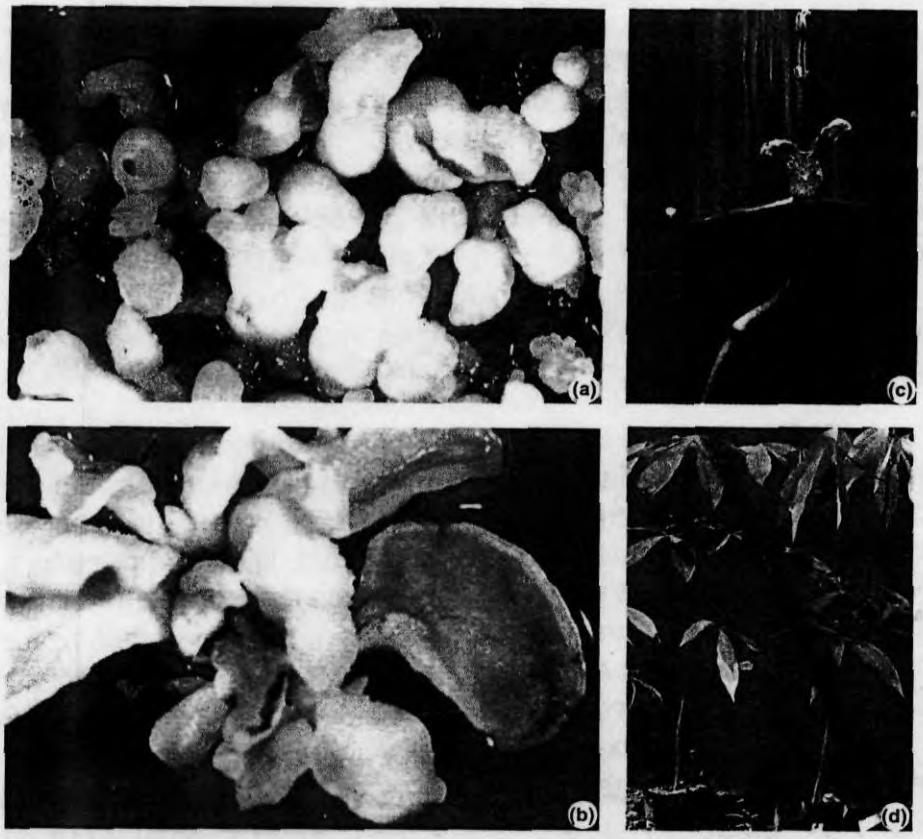

Fig. 2. Somatic Embryogenesis and Plantlet Regeneration in *Hevea brasiliensis:* (*a*) Globular and heart-shaped somatic embryos (*b*) Maturation of embryos (Arrow indicates the matured dicotyledonary embryo with shoot plumule) (*c*) Germination of embryos (*d*) Somatic embryo derived plantlets established in polybags containing soil

evaluation of the performance of somatic embryogenesis derived plants over the conventionally propagated ones. The preliminary RAPD profiles generated with 10 primers showed uniformity (Unpublished data).

3.5. Protoplast Culture

Protoplast culture technology applied to *Hevea* has the advantage that genetic material can be freely exchanged. This means that both closely related and distant genotypes can be hybridized, there by avoiding the problems of conventional plant breeding. Single gene transfer into protoplasts can also potentially be used for the incorporation of genes coding for key enzymes for desired qualities. Improvements in *Hevea*, such as in latex yield, disease resistance etc., have been generally achieved through conventional breeding. New possibilities for improving *Hevea* may be provided by protoplast technologies involving somatic hybridization, somaclonal variation and genetic transformation. There are only limited published reports on *Hevea* protoplast isolation and culture.

Cailloux and Lleras (1979) used young leaves and stem for protoplast isolation. After isolation, they cultured protoplasts in liquid medium containing PEG for fusion and obtained

a protoplast 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 that pith from young shoots and suspension cultures of anther derived callus were the most promising source. Fine suspension cultures were necessary for isolation of viable protoplasts. They obtained 90% of viable protoplasts (by fluorescence tests). The isolated protoplasts were washed and cultured in MS liquid medium supplemented with 2% glucose, 6.5% mannitol, 0.1% casein hydrolysate, 0.025% ammonium nitrate and 0.025% $CaCl_2.2H_2O$ at pH 6.8 where cell wall regeneration was noticed within seven days, but cell divisions were not observed.

Haris *et al.* (1993) isolated protoplasts from cell suspensions derived from anther callus using a mixture of hemicellulase, cellulase, and macerase. Protoplast purification was achieved using a floating method with a concentration gradient of 20% sucrose and 12% mannitol. Preliminary experiments on protoplast fusion and culture were also conducted. Wilson and Power (1989) isolated protoplasts from stem tissues of *Hevea*. They included several antibiotics in the plasmolysis and enzyme solution for eliminating contamination from protoplast culture. These antibiotics promoted rubber protoplast survival also. Cell wall regeneration and divisions were observed occasionally but rapid protoplast degeneration was noticed. Cazaux and d' Auzac (1994) obtained microcalli for the first time from protoplasts of friable embryogenic callus. Protoplasts, derived from embryonic callus, regenerated cell walls and underwent division when embedded in alginate bead and cultured on a modified MS medium containing 9µM 2,4-D, 0.6 µM glucose, 0.93 µM kinetin in the presence of tobacco nurse cells. In 1995, Cazaux and d'Auzac reported that protoplasts from stems of *Hevea* are recalcitrant to division. They explained that *Hevea* stem protoplasts are associated with high polyamine content which inhibits the protoplast division by enhancing the ethylene production in the culture. Sushamakumari *et al.* (1998) developed an efficient pathway for callus induction from protoplasts. After evaluating different explant sources they obtained considerably high yield of good, viable protoplasts from 2-month-old embryogenic cell suspensions which originated from immature inflorescence derived callus. Cell division and microcalli with a plating efficiency of 20% were obtained when plated over *Lolium* nurse culture.

4. GENETIC TRANSFORMATION

Genetic improvement of tree species by classical breeding methods is a rather slow and difficult process. Recently, developed gene transfer technologies could provide a direct route for the introduction of a specific genetic change within a short period of time. One of the prerequisites for successful plant transformation is the availability of a regeneration protocol that is compatible with the gene transfer method of the target species. Exploitation of the natural gene transfer system, *Agrobacterium* is the most widely used strategy for plant transformation offering an additional tool for plant breeding. 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. Therefore, the incorporation of new characters such as disease resistance, drought tolerance etc., into *H. brasiliensis* by genetic

engineering has the potential to increase the productivity of this economically important species. To date, there have been very limited reports on genetic transformation.

Arokiaraj and Wan (1991) have done co-cultivation of *in vitro* and *in vivo* propagated plantlets with *Agrobacterium tumefaciens* strain 541 and cultured on MS medium without growth regulators. Cocultivated explants developed tumours and produced octopine indicating that transformation had taken place. Subsequently, Arokiaraj *et al.* (1994, 1996) developed a transformation system for both the direct introduction of foreign DNA using microprojectile bombardment method as well as *Agrobacterium*-mediated method. Anther derived calli were transformed with vectors GV2260 (p35GUSINT) and LBA4404 (pAL4404/ pMON9793) harbouring thes ß-glucuronidase (GUS), neomycin phosphotransferase (NPTII) and chloramphenicol acetyl transferase (CAT) genes. Genetic transformation was confirmed by histochemical staining, fluorometric assay for GUS activity, ELISA for detecting expression of the NPT gene and direct enzyme assay for detection of CAT gene expression. The presence of foreign gene in the transformed callus, embryoids and transgenic plants was further confirmed by the polymerase chain reaction (PCR) analysis. Recently Arokiaraj *et al.* (1998) genetically transformed anther derived calli of *Hevea* using *Agrobacterium* GV2260 (p35SGUSINT) harbouring the GUS and NPTII genes. GUS protein was expressed in the leaves of transformed plants grown on kanamycin medium. The presence of GUS gene in the transgenic plants was confirmed by southern blot analysis. They observed GUS expression in the latex, phloem and laticifers.

5. GERMPLASM CONSERVATION

The urgent need for plant germplasm conservation is becoming increasingly important; since, plant production is threatened globally by several pests, diseases, and field germplasm collections have been set up to support different breeding programmes. Conventionally, plant germplasm is conserved through seeds, tubers, roots, bulbs, corms, rhizomes, buds, cuttings etc. In *Hevea*, both *in situ* conservation of genotypes in their original habitats and *ex situ* conservation in special nurseries or fields are feasible, of which the later is widely adopted. The storage of *Hevea* seeds to prolong seed viability is important because seeds are used for the production of seedling root stocks and as planting material. However, the germplasm of a number of plantation crops and trees cannot be preserved as their seeds are 'recalcitrant' and the embryos degenerate early. Moreover, germplasm of vegetatively propagated crops cannot be stored on a long-term basis, but it must be grown and multiplied periodically in nurseries or fields. Thus, it is exposed to unpredictable weather and/or diseases and instances are known when entire genetic stocks have been lost. So, unconventional methods are being developed for the storage, maintenance, conservation and international exchange of germplasm and to establish germplasm banks for rare plant materials. In *Hevea*, the germplasm is presently maintained as field genebank as active collection for regeneration, multiplication, distribution and characterization. For this a core collection or a condensed assembly of germplasm should be identified and established for efficient conservation and finally a working population need to be identified to suit short term needs of individual breeders and breeding programmes (Varghese 1992). There are, however, several problems with these field

gene banks. Conservation of genetic resources of *Hevea* is indeed elaborate, expensive and difficult and requires much space and infrastructure facility.

In view of these problems, *in vitro* approaches are to be established. Like other crop plants, one approach is to store germplasm *in vitro* at 25°C. However, this requires periodic transfer to fresh media, involving not only manpower and high costs, but also the hazards of contamination and sometimes the loss of the entire material. Various constraints are encountered in the establishment and maintenance of embryogenic cultures, including difficulties in producing embryogenic material, progressive decrease of embryogenic potential over time which can occur over extended culture periods and management of a large number of cultures. The *in vitro* maintenance of germplasm, is also constrained by the occurrence of somaclonal variation.

Cryopreservation is based on the reduction and subsequent arrest of metabolic functions of biological materials, while maintaining viability. At the low temperature of liquid nitrogen all the metabolic activities of cells are at a standstill and they can be preserved in such a state for extended periods. Hence, cryo-preservation is considered to be the only valid alternative for long term preservation of *Hevea* germplasm, because at ultra-low temperatures, physical and chemical reactions are arrested and time-related changes are eliminated (Engelmann *et al.* 1997). It has been well known that plant materials such as seeds, pollen, tubers, *in vitro* cultured cells, embryos etc., which resist intensive dehydration, retain their viability after direct immersion in liquid nitrogen from room temperature. In *Hevea*, a very simple cryo-preservation method with potential application to a wide range of cultivars representing the different genomic combinations is desired. Although cryo-preservation protocols have been established well for many crops, only limited work has been done in *Hevea*. A method for cryo-preservation of embryogenic cell suspensions was developed by Veisseire *et al.* (1993). Their study focussed mainly on the effect of pregrowth and preculture conditions on survival. Hamzah and Chan (1996) preserved pollen in liquid nitrogen for one and a half months, retaining viability and fertility. Recently, two cryo-preservation protocols, one using a classical freezing process and the other a simplified freezing process, were developed for embryogenic calli of a commercial clone of *Hevea* (Engelmann *et al.* 1997). They have used both 1M sucrose and 10% DMSO as cryoprotectants for *Hevea* embryogenic calli and frozen in a programmable freezer at the rate of 0.2°Cmin^{-1} down to -40°C or in a simple device consisting of an isopropanol bath enclosed in a polystyrene box, placed in a -80°C deep freezer, thus achieving an average cooling rate of 0.2°C min^{-1} down to -40°C. High survival, and rapid regrowth, as well as production of somatic embryos and plant regeneration were obtained with calli cryo-preserved using both freezing protocols. Hamzah *et al.* (1999) reported that two cross combinations were carried out using pollen cryo-preserved for three and seven months respectively and yielded viable seeds and seedlings. More work is to be done in *Hevea* to establish protocol for cryo-preservation of germplasm.

6. MOLECULAR APPROACHES

The past three decades witnessed tremendous advancements in the understanding of the molecular mechanism of plant growth and development. However, most of these studies were carried out in annuals, and very few in the tree species. The advanced state of cell wall development, their long generation time, presence of high amount of phenolics, terpenoids, tannin etc. which can react with proteins and nucleic acids have made the molecular studies in *Hevea* rather difficult. In spite of all such problems, attempts have been made at different laboratories to study laticifer specific gene expression, rubber biosynthesis, disease resistance etc. Although extensive studies have been carried out on the physiological and biochemical understanding of rubber biosynthesis (d'Auzac 1989), not much has been done at the molecular level. Efforts are on for the characterisation of germplasm using molecular techniques for breeding purpose.

6.1. Gene Expression in Laticiferous Cells

Natural rubber, Cis-1,4 polyisoprene, is produced as a colloidal fluid called latex in the laticifers of *Hevea*. The economic importance of natural rubber has prompted active investigations on the biochemical and molecular aspects of rubber biosynthesis and the laticifer specific gene expression. A unique feature of the laticifers is the absence of cytoplasmic connections or plasmodesmata between these cells and their neighbouring cells (Hebant 1981). Therefore, the exuded latex should represent only the cytoplasmic contents of the laticifers uncontaminated by those of other cells. Since, latex can be readily obtained in large quantities, it provides an opportunity to investigate the biochemical properties of a single specialised cell type (Kush *et al.* 1990).

The presence of ribosomes and polysomes in the latex of *Hevea* laticifers indicates active protein synthesis and metabolism in these cells (Tupy 1988). Development of protocols for the isolation of translatable mRNA from the latex have made the studies on gene expression easier in the laticifer system. The expression levels of various genes present in laticifers have been shown to be markedly different from those of leaves. This has been evidenced by the protein profiles and northern blot analysis of leaf and latex. Northern blot analysis demonstrated that laticifer RNA is 20 to 100 fold enriched in transcripts encoding the enzymes involved in rubber biosynthesis. Plant defense gene encoding chitinase, pathogenesis related proteins like phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, cinnamyl alcohol dehydrogenase and 5-enolpyruvylshikimate 3- phosphate synthase were showed a 10 to 50 fold higher expression in laticifers than in leaves. Cellulase and polygalacturonase were highly expressed in laticifers. However, photosynthetic genes encoding ribulosebisphosphate carboxylase small subunit and chlorophyll a/b binding protein were not expressed at a detectable level in laticifers (Kush *et al.* 1990). Chye *et al.* (1995) isolated a cDNA clone encoding ß-1,3 glucanase in the laticifer cells using a heterologous probe derived from *Nicotiana*. This gene was preferentially expressed in the laticifer system than in leaves.

Wound sealing during latex tapping and protection from fungal and other pathogen

attack is very important in *Hevea,* as the latex is collected by successive tapping of the tree. It is believed that the plugging of the wound during latex collection is mediated through some substance released from certain membrane bound particles called lutoids (d'Auzac 1989). A single chain chitin binding protein called 'hevein' is a major protein present in the lutoids (Archer *et al.* 1969). It is suggested that 'hevein' plays a major role in the protection of wound sites from fungal attack (Archer *et al.* 1969; Parijs 1991). Using polymerase chain reaction with mixed hevein oligonucleotide primers, a cDNA clone encoding hevein was isolated from *Hevea* latex cDNA library (Broeakaert *et al.* 1990). They demonstrated that wounding as well as application of the plant hormones (abscisic acid and ethylene) stimulated the accumulation of hevein transcripts in leaves, stems and latex.

Through the efforts of several laboratories, it is established that cis-1,4- polyisoprene is synthesised on particles suspended in the cytosol of laticifers (Hepper & Audley 1969). The biochemical pathway leading to rubber biosynthesis begins with the synthesis of isopentenyl pyrophosphate, the isoprene unit which then undergoes polymerisation to from polyisoprene. The chain length of the rubber particles is determined by the interaction of rubber elongation factor (REF), a 14.6 kd protein with the prenyltransferase on the surface of preformed rubber particles (Dennis & Light 1989).

Using synthetic oligonucleotide primers for farnesyl diphosphate (FDP) synthase protein, Adiwilaga and Kush (1996) isolated a full-length cDNA clone, which encodes a 47 Kda protein having strong homology to FDP from many other species. This gene was expressed in laticiferous cells as well as epidermal region of *Hevea* suggesting a dual role for the protein in the biosynthesis of rubber and other isoprenoids. The regeneration of latex due to tapping increases the expression level.

The gene coding for the important enzymes involved in the rubber biosynthetic pathway namely 3-hydroxy-3-methyl glutaryl co-enzyme A reductase (HMGR), rubber elongation on factor (REF) and farnesyl diphosphate synthase (FDP synthase) have already been documented. Chye *et al.* (1991) isolated cDNA clone for HMGR using a heterologous probe and its expression was studied. Three members of a gene family *i.e.*, hmg1, hmg2 and hmg3 encode the transcript for HMGR. The HMG1 expression is laticifer specific and is induced by ethylene, whereas HMG3, which does not have a clear cell type specificity remain unchanged on ethylene treatment (Chye *et al.* 1991, 1992). The HMG1 genomic fragment under the control of CaMV 35S promoter was introduced into tobacco through *Agrobacterium* mediated transformation and as a result, overproduction of sterol was observed (Schaller *et al.* 1995).

Attanayaka *et al.* (1991) screened a *Hevea* latex cDNA library with REF specific antibody and cloned a cDNA insert of 681 base pairs in length for rubber elongation factor. The clone encodes the entire coding region of the mature REF. This cDNA coding for REF protein was cloned into the plant transformation vector with CaMV 35S promoter and transformed to the tobacco plants. The REF gene expression in transgenic plants was confirmed by southern blot analysis (Attanayaka *et al.* 1998).

6.2. DNA Markers

In a tree crop like *Hevea* with a long breeding cycle and gestation period, identification of parents with desired characters and generation of new clones through conventional breeding is an arduous task. With the use of the recently developed molecular techniques like, RFLP, RAPD, AFLP, STS etc. it would now be possible to identify potential parents at the seedling stage itself for a controlled breeding programme. A major limitation in rubber is the long breeding cycle and it needs a long time to construct segregating population or near iso-genic lines (NILS) which are traditionally used to obtain useful DNA markers. DNA markers for desired characters may be obtained in properly selected population with contrasting characters, (Shoucai *et al.* 1994; Thulaseedharan *et al.* 1997).

Genetic linkage maps were developed for many annual crop plants in the late 1980's and information from such maps were used for breeding purpose (Helent Jaris *et al.* 1985; Bernatzky & Tanksley 1986). However, these molecular techniques were used in *Hevea* only very recently for identification of DNA markers and characterisation of germplasm (Low & Gale 1991; Low *et al.* 1996; Varghese *et al.* 1997). Low & Gale (1991) used random probes from *Hevea* genomic library and heterologous probes to study the DNA polymorphism, employing RFLP technique. Restriction fragment length polymorphisms were detected between *Hevea* spp. and clones. Besse *et al.* (1994) surveyed 92 wild accessions from the three states of Brazil (Acre, Rondonia and Mato-grosso) and 72 cultivated Wickham clones through RFLP analysis using a random probe generated from *Hevea.* Despite their narrow genetic base and high level of inbreeding, cultivated Wickham clones show a high level of polymorphism, however, variation was less compared with wild accessions, and they are very close to the Mato-grosso clones. The genetic variability of the wild population of *H.brasiliensis* is clearly organised on the basis of the geographical location of the areas sampled. It is also observed that there is a clear-cut division between the three populations originating from Acre, Rondonia and Mato-Grosso. A preliminary RFLP analysis carried out by Low *et al.* (1996) with limited clones could not observe much polymorphism between commonly cultivated clones. However, they could establish polymorphism between different *Hevea* spp. and distinguish between clones sharing common parents.

In addition to genomic DNA variations, ribosomal DNA (rDNA) variation was also used to characterise the *Hevea* germplasm. A survey of ribosomal DNA variation was done by Besse *et al.* (1993) to assess the genetic variability among wild and cultivated *Hevea* clones using a heterologous probe pTa71 (Gerlach & Bedbrook 1979) derived from wheat and a high level of rDNA variation was observed among Wickham clones. A 4.5 kb mt DNA fragment showing a high RFLP polymorphism between various *Hevea* genotypes were identified by Luo & Boutry (1995). Primers were designed for a 1.4 kb fragment and they could amplify a segment of about 0.9 kb from 23 representatives. Complete DNA sequences from four genotypes showed between 6.7% and 20.2% nucleotide diversity, suggesting the presence of a hyper variable or hot spot region. The phylogenetic relationship inferred from this sequence comparison was in general agreement with the results obtained from the mtDNA RFLP analysis data (Luo & Boutry 1995). A reduction in the number of

rDNA genes was also observed during cell culture. This was observed when, pTa71, a wheat rDNA probe was hybridized with genomic DNA of *in vitro* plants (Low *et al.* 1996).

PCR based RAPD, AFLP, DAF and microsatellite techniques were also used in *Hevea* for identification of DNA markers and phylogenetic analysis. Polymorphism was observed between several selected cultivars and *in vitro* plants (Low *et al.* 1996). In our laboratory, RAPD experiments were conducted with somatic embryogenesis derived plants from two cultivated *Hevea* clones using 10 random primers and no polymorphism was detected (unpublished data). Varghese *et al.* (1997) developed RAPD profile of 24 clones with 42 random primers and used the data for the estimation of genetic distance. The genetic distance of tested clones facilitated identification of genetically divergent clusters. Among the different clones tested 'RRIC 100' displayed the highest mean genetic distance. Use of these clones as parent in hybridisation programmes has resulted in highly heterotic hybrids (Licy *et al.* 1996). The RAPD profiles using 200 random primers with plants raised through seeds showed distinct plant to plant variations so as to consider each plant as a separate genotypes (Thulaseedharan *et al.* 1997). Like many other crop plants, in *Hevea* also RAPD technique was exploited for identification of DNA markers conferring disease tolerance. DNA markers for Powdery mildew caused by *Oidium* (Shoucai *et al.* 1994; Shoucai *et al.* 1999a) were developed from the selected tolerant as well as susceptible genotypes through RAPD technique.

Microsatellites [$(GA)_9$, $(TA)_{16}$ and $(TTA)_8$] were developed from the sequence of the gene coding for the enzyme hydroxymethylglutarylcoenzymeA reductase (HMGR). The microsatellite $(GA)_9$ was found to be suitable for detecting polymorphism in *H. brasiliensis* clones (Low *et al.* 1996). The recently developed more sensitive and reliable AFLP technique (combining the RFLP and PCR techniques) was also employed for studying genetic diversity and clonal identification in *Hevea* (Anding *et al.* 1999).

6.3. Molecular Studies on Tapping-Panel Dryness (TPD)

Tapping Panel Dryness (TPD), often associated with high yielding clones with extensive tapping of latex, is a serious physiological disorder in *Hevea*. This syndrome is characterised by the browning of bark followed by cessation of latex flow causing considerable yield loss. There is an urgent need to study the molecular mechanism of wound healing, stability of bark tissues during latex tapping and control of tapping panel dryness in the bark of the *H. brasiliensis*. A simple and reliable method for the isolation of RNA from bark tissues which is a pre-requisite for successful use of the molecular biology techniques to study the functioning of bark during latex tapping has been achieved recently (Venkatachalam *et al.* 1999).

It is reported that the trans-zeatin riboside (t ZR) levels in the bark samples of TPD tolerant plants are higher compared to susceptible plants (Krishnakumar *et al.* 1997). It has been widely documented that TPD is always associated with an accumulation of free radicals. Therefore, a high level of superoxide dismutase (SOD) in the bark as well as latex can counteract the action of free radicals effectively thus providing protection against

TPD. Similarly, endogenous level of ethylene may also lead to the TPD syndrome. After generating RAPD profiles of selected TPD-tolerant as well as susceptible plants, two DNA markers, conferring tolerance to TPD, were detected and one of the markers has been cloned and sequenced recently (Thulaseedharan *et al.* 1997). Shoucai *et al.* (1999b) subjected the total RNAs from latex and bark of healthy and TPD affected trees for differential display reverse trancriptase polymerase chain reaction (DDRT-PCR). One marked common band was detected in the latex and bark of all healthy plants studied. But the band was very weak or not detected at all in all the tested clones. Work is going on in different laboratories to find out an early solution for TPD.

7. CONCLUSION

Biotechnology could play a major role in the crop improvement of *Hevea*. However, *in vitro* culture techniques are still at a rudimentary stage. Additional work will be necessary to integrate successfully biotechnological approaches with conventional methods of *Hevea* improvement. Development of protocols for micropropagation will greatly facilitate production of true-to-type elite planting material eliminating stock scion interaction. The greatest technical limitation to micropropagation is the low degree of culture response observed with mature tree explants. Recent advances in somatic embryogenesis and protoplast cultures of *Hevea* have opened up new avenues for mass propagation and genetic transformation of this important tree species. For large-scale plant production to become a reality, higher conversion frequencies will have to be attained, which is one of the goals of current research. Protoplasts of *Hevea* have been isolated and grown for short periods of time but have not as yet been amenable for regeneration. In *Hevea,* an increased growth and vigour has already been reported for plants propagated through tissue culture (Carron *et al.* 1995). Even a very small increment in yield per tapping will be of great attribute to a tree crop like *Hevea* with a life span of 30 -35 years. To achieve the crop improvement through molecular intervention, much attention is required in the genetic transformation of *Hevea.* Regeneration of transformed cells are confronted with innumerable technical constraints. Efficient protocols should be developed for the incorporation of foreign genes to *Hevea* and further regeneration to plantlets. Because of the narrow genetic base and the continuing habitat destruction in areas of wild *Hevea* species, germplasm collection and storage are also receiving much attention in recent years. Techniques for the preservation of the short-lived *Hevea* embryos through growth limitation *in vitro,* or for the cryopreservation of embryos or other tissues and their subsequent recovery and growth in culture will have significant application.

The molecular marker assisted selection (MAS) in *Hevea* is still at the preliminary stage. The advances in DNA marker technology combined with marker assisted selection and genetic transformation would complement plant breeding efforts by increasing the diversity of genes for incorporation and also shorten the time required for the production of new varieties. Breeding for high latex yield with increased timber volume is another aspect to be given priority. Future efforts should be directed for integrating drought and disease resistance genes including TPD tolerance to high yielding clones. The laticiferous cells contain all the essential cytoplasmic components required for the biosynthesis of

protein and many other secondary products. At present only the rubber particles are exploited from latex. 60% of latex is a cytoplamic fluid (serum) which is now going as a waste. There is a great potential to produce novel foreign proteins, vaccines and other useful compounds in the laticiferous system by genetic manipulation of *Hevea* plants with appropriate genes. These products can be easily purified from the serum phase of the latex after harvesting by a simple tapping method.

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