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REVIEW

TISSUE CULTURE OF RUBBER: PAST, PRESENT AND FUTURE PROSPECTS

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ABSTRACT

In rubber, the current propagation method of grafting on to unselected seedlings, maintains intraclonal heterogeneity for vigour and productivity and hence a great improvement may be expected by using micropropagation *in vitro.* Micropropagation with nodal and shoot tip explants derived from seedlings are possible as with mature clonal explants. The major problem in using clonal material from mature trees of *Hevea* is the failure to produce an adequate tap root system necessary for tree stability, and the poor response to culture conditions. The latter problem has been over come to a significant level by *in vitro* micrografting. Recently, there has been an increasing interest in the induction of somatic embryogenesis in *Hevea* using different explants, media compositions and conditions, especially for use in genetic transformation studies. Successful somatic embryo formation and plant regeneration have been reported by a few researchers in different countries using limited genotypes of *Hevea*. Attempts to induce somatic embryos from high yielding Sri Lankan clones have not been successful until the late 1990s. 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. There is no large scale commercial application of tissue culture techniques for mass propagation of clonal *Hevea* as yet, either by microcuttings or by somatic embryogenesis. However, there is sufficient progress at research level to suggest that tissue culture of *Hevea* can and should be further developed.

Key words: *Hevea brasiliensis*, micropropagation, microcuttings, somatic embryogenesis

INTRODUCTION

Hevea brasiliensis Muell Arg., the principal source of natural rubber, is an open pollinated crop belonging to the Family Euphorbiaceae. It is intensively cultivated in South East Asia. The main produces of natural rubber in the world are Thailand, Indonesia, Malaysia, India, Vietnam, China, Ivory Coast and Sri Lanka, in descending order of production (Rubber Statistical Bulletin, 2007). The higher strength, low heat build up, better resistance to wear and flex cracking make natural rubber a suitable raw material for the manufacture of heavy duty automobile tyres. Therefore, the global demand for natural rubber is steadily increasing and hence the production of rubber needs to be increased to meet the demand.

The genetic base of rubber is very narrow as it originated from about 10 mother trees grown from the 70,000 odd seeds collected by Wickham in 1876 on the banks of the Tapajo in Para, Brazil (Schultes, 1977). Rubber plantations were originally established with unselected seedlings, which resulted in considerable heterogeneity. From 1920 onwards, research was carried out along several lines to improve the quality of plant material.

The first difficulty in the use of cuttings of *Hevea* as planting material was the low rhizogenetic potential of the selected material and then the inadequate anchorage of the regenerated root system. It has been reported that the rhizogenetic capacity of *Hevea* is fugacious and soon gets exhausted during the development of the plant (Muzik and Cruzado, 1958). For a very long time, "selected" seeds were preferred to grafts because the resulting trees were more vigorous and displayed better resistance to diseases. However, between 1940-1950, almost all plantations had been established with grafted clones where the trees are comparatively homogeneous and also because of the difficulty in obtaining selected seeds in

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sufficient quantities for development programmes (Carron *et al.,* 1989).

As far as rubber yield in Sri Lanka is concerned, it has increased from 300 kg /ha/year when unselected seedlings were used during the early periods to over 2500 kg/year, using novel clones developed by the Rubber Research Institute of Sri Lanka, in the recent past. However, this trend must be continued in order to meet the world demand. Search for improved yields is all the more necessary since it is known that the full capacity of the species is far from what is being exploited. Generally mother trees produced by vegetative selection yield about 15-20 kg of dry rubber/tree/year, while the currently used grafted clones give about 4-6 kg/tree/year. This leaves room for potential improvement by 200%, which has been confirmed by theoretical studies on production factors (Sethuraj, 1981). This increase in productivity is directly linked to the improved quality of individual trees and to improved methods of harvesting the latex.

Hevea is still propagated by grafting, although the stocks produced from seeds maintain intraclonal heterogeneity and smaller production than the mother tree. Further, uniform growth and yield are not realized even under best management conditions (Combe, 1975). This shortfall in yield can be due to many reasons, amongst which soil heterogeneity may be one, but much of it could be due to the heterogeneous rootstock (Senanayake and Wijewantha, 1968). The yield potential of each tree in a plantation is becoming an important parameter in order to improve the land use efficiency. Thus, 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 the physiological disorder called tapping panel dryness, drought, leaf fall diseases caused by *Corynospora* and *Phytophthora*. The crop loss due to these traits is very heavy. Therefore, there is an urgent need to introduce resistant genes into 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 in 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 (Thulaseedharan *et al.,* 2004). Thus, a reproducible plant regeneration system for each genotype of *Hevea* through tissue culture is essential for crop improvement programmes.

TISSUE CULTURE APPROACHES

Most of the *in vitro* culture work in *Hevea* is directed towards micropropagation through shoot tip culture, nodal cultures, somatic embryogenesis and genetic transformation. 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 yield increment per tapping will be a great attribute to a tree crop like *Hevea* with an economic life span of about 30 years.

The first known work on *in vitro* culture of *Hevea* was carried out by Bouychou (1953) of the Institut Francais Caoutchouc, with the aim of using calli to obtain convenient material for the study of the laticeferous system. This line of research was taken up again by Chua (1966) of the Rubber Research Institute of Malaysia and later by Wilson (Wilson and Street, 1975), with the backing of the Malaysian Rubber Producers' Research Association.

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 developed. Paranjothy and Gandhimathi (1976) have attempted shoot tip (2-3 cm long) culture, derived from aseptically grown seedlings, for the first time. 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 shoots and roots could be successfully developed by different investigators (Gunatilleke and Samaranayake, 1988; Carron *et al.,* 1989; Sompong and Muangkaewngam 1992; Seneviratne and Flegmann, 1996).

The explants derived from elite clones of mature *Hevea* trees are highly recalcitrant. Only limited reports are available on successful micropropagation using clonal material of

Hevea. The major problem with clonal material from mature trees is the failure to produce an adequate root system with tap root quality necessary for tree stability (Carron & Enjarlic, 1983). Moreover, explants of mature origin posed problems both of establishment in culture and of reluctance to proliferate *in vitro* (Seneviratne, 1991). Rubber being a crop predominantly grown under tropical climates, the presence of bacterial and systemic fungal contamination in the field grown mature plants derived from explants is also a major limitation. Studies were therefore focused in this direction and effective sterilization techniques for obtaining contamination free initial explants were developed (Enjarlic *et al.,* 1987; Asokan *et al.,*1988; Seneviratne, 1991). The physiological stage of the explants was found to play a significant role in micropropagation and detailed studies were carried out by Carron *et al.* (1985) and Seneviratne (1991). However, they reported that the multiplication rate was low and the plants lacked the advantages of the embryogenic route, *i.e*. both shoot and tap root development.

Micrografting have shown very promising results for enhancement of shoot development from microcuttings of mature materials of *Hevea* although the plants produced by this method lacked the advantages of the embryogenic route, *i.e*. shoot and root (with a tap root) development simultaneously. Rejuvenated 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 Wijesekara, 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 plant material difficult to root plant were standardized by Kala *et al.* (2002). Studies on micrografting of selected high yielding Sri Lankan clones of *Hevea* are being carried out at the Rubber Research Institute of Sri Lanka for optimization of conditions needed. Encouraging results have been obtained by Edusuriya (2004) and Jayatillake (2007).

Studies were carried out at the 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 were 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 and reported shoot and root development. The rooted plantlets of four *Hevea* clones were successfully transplanted in the field and a field evaluation programme was initiated (Thulaseedharan, 2002). In spite of these efforts over several years, no reliable technique is currently available for large scale micropropagation of *Hevea* on a commercial scale.

Somatic embryogenesis

An efficient plant regeneration pathway through somatic embryogenesis is essential for crop improvement through transgenic approaches besides using this as a micropropagation system. Although micropropagation of clonal *Hevea* using axillary shoot proliferation has been achieved to a progressive level (Senevirathne, 1991), there are a number of drawbacks in this system. A single plant could be produced from a single nodal explant and the plants that were produced were without a tap root which is undesirable in clonal tree propagation. Further, it cannot be utilized effectively in genetic transformation studies due to formation of chimeras which lead to formation of different genotypes within the tissue (Personal communication, Genetics and Plant Breeding Department, RRISL).

Attempts to develop somatic embryogenesis as an *in vitro* propagation technique was started in the early 1970s by a Chinese team at the Rubber Cultivation Research Institute (Baodao) and a Malaysian team at the Rubber Research Institute of Malaysia, simultaneously. The Institute de Recherche sur le Caoutchouc (France) began work in this field in 1979 (Carron, 1980). The Rubber Research Institute of Sri Lanka initiated callus culture in 1972 (Anon., 1972) and expanded work on somatic embryogenesis in 1984 (Anon., 1984).

The first anther derived callus which could be sub cultured was produced at the Rubber Research Institute of Sri Lanka in 1972 (Satchuthananthavale and Irugalbandara, 1972). Later, this line of work was followed by the Chinese and Malaysian teams. Paranjothy (1974) achieved differentiation of embryoids from anther wall derived callus for the first time. Subsequently, shoot development was also achieved (Paranjothy and Ghandhimathi, 1975; Paranjothy and Rohani, 1978).

Initiation and growth of *Hevea* cell suspension cultures have also been studied.

Callus obtained from stem segment cultures on semi-solid medium failed to yield a good cell suspension when transferred to agitated, modified MS liquid medium (Wilson and Street, 1975). Studies have also been made to determine the effect of photoperiod on growth and differentiation of anther wall callus (RRIM, 1981). Chen *et al.* (1979) reported the production of the first batch of anther derived plants after the production of the first three pollen plants in 1977 (Anon, 1977).

Wang *et al.* (1980) reported for the first time, the establishment in the field, of *in vitro* cultured *Hevea brasiliensis* plants of anther-wall derived callus. However, according to Wan Abdul Rahaman *et al.* (1981), clonal differences had been observed in the frequency of embryogenesis of anther-wall derived callus and therefore the reported technique had not been applicable to all clones of *Hevea brasiliensis*. Carron (1981) used inner integument tissue of seeds for somatic embryogenesis and was successful in plantlet development. Carron and Enjarlic (1982) also achieved differentiation of embryoids from the anther wall derived callus.

During 1979-1989, Shiji *et al.* (1990) produced 1700 plants out of 52,896 embryoids by inoculating 31,584 anthers in tubes. They successfully transplanted and established 539 plants of 13 clones. However, great variation among the clones for their induction frequency was reported. Some clones such as Haiken 2 had given high induction rates for both embryod and plantlet formation, while on the same media, other clones had not responded at all.

Extensive experiments were carried out by many researchers to enhance the frequency of somatic embryo induction and plant regeneration in *Hevea*. Studies were conducted to optimize culture conditions, nutritional and hormonal requirements during somatic embryogenesis. EI-Hadrami *et al.* (1989) compared the effect of polyamines on two embryogenic clones with two non-embryogenic clones and revealed quantitative differences in the polyamine (PA) contents. Also in their evolution during culture; more embryogenic calli were, overall richest, in polyamine. Hormonal balance (Michaux-Ferrier 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 abscisic acid on embryo induction (Veisseier *et al.*, 1994a, b; Linossier *et al.*, 1997) and carbohydrate types (Blanc *et al.*, 2002) have also been evaluated.

Wang and Chen (1995) made attempts to regenerate plantlets through somatic embryogenesis from stamen cultures. They optimized the temperature requirements for callus induction, somatic embryogenesis and plant regeneration at 26^0 C, 24-25^0 C and 26-27^0 C respectively and regeneration frequencies up to 40.5% were 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 a commercial scale. In most of the above studies, inner integument tissue was used as the explant. It is reported that the calli obtained from the integuments of immature seeds frequently displayed browning (necrosis) leading to tissue degeneration and 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). For the past ten years, research is being carried out at the Rubber Research Institute of India to develop a plant regeneration system through somatic embryogenesis for the Indian clones of *Hevea*. In order to identify a suitable explant source, a variety of explants such as leaf, tender shoots, integumental tissues of immature fruit, immature anther, immature inflorescence etc., have been tried.

Immature anthers (before microsporogenesis) (Kumari Jayasree *et al.*, 1999) and immature inflorescence (Sushamakumari *et al.*, 2000) have been identified as the suitable explant sources and protocols have been developed for high frequency somatic embryo induction and plant regeneration for RRII-105, the most popular Indian clone. Kumari Jayasree *et al.* (1999) reported the standardized protocol for the induction of friable embryogenic callus, somatic embryogenesis 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*. (2000) developed a technique for somatic embryogenesis and plant regeneration using immature inflorescence as explants. They also studied the role of sucrose and abscisic acid on embryo induction. A higher sucrose level was found to be essential for effective embryo induction as well as maturation. However, lower levels were 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*. (1999) have attempted induction of multiple shoots on germinating somatic embryos thereby enhancing the efficiency of plantlet formation. They could induce on average 3.45 micro-shoots per explant by manipulation of the levels of BA and thidiazuron in the medium, as well as by wounding of the shoot primordial of the somatic embryos.

Culture conditions and other nutritional requirements for improving the efficiency of somatic embryo induction and germination were investigated by Kumari Jayasree *et al.* (2001). A study on the gibberellic acid requirement on embryo induction and maturation revealed that incorporation of GA3 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 GA3 levels (Kumari Jayasee and Thulaseedharan, 2001). An isozyme study revealed a clear difference between embryogenic and non-embrogenic calli, as well as between different stages of embryogenesis where markers could be developed (Asokan *et al*., 2001). Repetitive embrogenesis was also induced from primary somatic embryos derived from integumental tissue. Somatic embryos cultured on B-5 medium supplemented with 0.5 mg/l NAA, 2.0 mg/l KIN, 0.5 mg/l IAA and 4.0 mg/l 2,4-D enhanced repetitive embryogenesis and 5% sucrose was found to be optimum (Asokan *et al.*, 2001). Embryogenic cultures had been maintained for over three years for retaining the embryo induction and plant regeneration potential (Kumari Jayasree and Thulaseedharan, 2004).

Several *in vitro* plants have been raised through somatic embryogenesis from immature anther as well as immature inflorescence of the *Hevea* clone RRII-105 (Thulaseedharan, 2002) and they confirmed that those plants were morphologically as well as genetically uniform. Carron *et al.* (2000) have also studied the field performance of the *in vitro* plants regenerated *via* somatic embryogenesis and found clearly better growth of the *in vitro* plants compared with the seedling control, and the annual increment was consistently higher giving an increasing gap between the two treatments.

Several factors such as the developmental stage and type of the explant, quantity of growth regulators and other growth enhancing substances, basal media composition, light intensity, etc. appear to play a crucial role in the induction and maintenance of somatic embryogenesis in many plants including *Hevea*. At present, reliable somatic embryo formation is limited to only a few genotypes of *Hevea, i.e.*, RRII-105 (Sobha *et al.*, 2003a), SCATC 93/114, PB 260, PB 235, PR 107, RRIM 600 and GT 1 (Montoro *et al.*, 1993), Haiken, 2, Haiken 1 and SCATC 88-13 (Shiji *et al.*, 1990). Somatic embryogenesis in *Hevea* is highly fugaceous (genotypic dependent and strictly specific to genotype - medium interaction), non-synchronous and its germination remained very difficult and there is a very low rate of conversion of the embryos into plantlets (Carron *et al.*, 1995; Linossier *et al.*, 1997) which necessitates to optimize the culture conditions for each genotype of *Hevea.*

The results made on somatic embryogenesis and plant production through anthers and nucellus tissues at the Rubber Research Institute of Sri Lanka in the past were not successful (Seneviratne *et al.*, personal communication). Although good callus induction was possible with above explant types, somatic embryogenesis has not been possible. Studies on somatic embryogenesis in *Hevea* was in its infancy in other Rubber Research Institutes of the world when major research was carried out in Sri Lanka, until the year 2000. Even published protocols were non reproducible for Sri Lankan clones. This has led to the temporally suspension of further studies on this aspect.

CONCLUSIONS

Development of protocols for micropropagation will greatly facilitate production of true-to type elite planting material eliminating stock-scion interaction leading to intraclonal variation in field performance. Currently two types of *in vitro* propagation are being studied *viz*. microcuttings and somatic

embryogenesis, in Rubber Research Laboratories world wide. However, no reliable tissue culture method for propagation of *Hevea* is available on a commercial scale, though steady progress is being made.

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 characterized. Recently developed protocols for somatic embryogenesis and plant regeneration have opened up new avenues and tools for genetic transformation rather than for mass propagation in *Hevea* (Jayashree *et al.*, 2000, 2003; Sobha *et al.*, 2003 a, b). In the improvement of agronomic traits, the underlying direction is towards development of transgenic rubber trees with increased rubber biosynthesis and timber volume, resistance to diseases, various abiotic stresses, etc. (Thulaseedharan *et al.*, 2004, Yeang, 2004).

At present, attempts are being made by the Rubber Research Institute of Sri Lanka to re-initiate strategically, somatic embryogenesis, with novel literature. Also, to evaluate the effect of different parameters that significantly influence somatic embryogenesis in high yielding clones of *Hevea* grown in Sri Lanka. Further, to utilize this system in crop improvement programmes since we are now in the 'transgenic era'.

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