Chapter VI

SUMMARY AND CONCLUSION

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

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

The present study aimed at improving the responsiveness of the tissue to various factors, have been proven successful in improving the embryogenic process. Before inoculation on solid medium, explants pretreated in liquid medium reduced the duration of callus induction. Immature anthers pretreated in liquid medium for 10 d followed by 25 d culturing on solid callus induction medium was found to be the most suitable. Results revealed that short duration pretreatments in liquid increased the production of embryogenic callus, but, prolonged
pretreatment adversely affected the embryogenic calli formation. After primary callus induction, when maintained in 2, 4-D containing callus induction medium, influenced embryogenic capacity of callus. Results indicated that the optimal time of callus transfer for embryogenesis is 50th d (35 d + 15 d) of explant inoculation. On the other hand, by increasing the time of callus maintenance in 2, 4-D containing medium, embryogenic calli formation was decreased. The effect of polyamine showed that spermine does not enhance embryogenesis. However, spermidine was found to slightly improve the embryogenesis when used at a concentration of 0.5 mg/l. In brief, polyamines had no significant stimulatory effect on embryogenesis. Among various amino acids, inclusion of alanine and arginine showed no effect on embryo enhancement. With asparagine, all tested concentrations allowed embryo differentiation similar to that of control. By the addition of glutamine, embryo induction was increased and maximum response on embryo induction was obtained at higher concentration of glutamine (200mg/l). Similarly, casein hydrolysate also improved embryogenesis and maximum number of embryos was produced when medium was enriched with 400 mg/l casein hydrolysate.

Efforts were also directed towards the optimization and enhancement of embryo germination and plant regeneration. On hormone free medium, mature embryos were converted into plantlets and 27% of embryos were germinated into full plantlets. However, by the inclusion of cytokinins combined with GA3, the somatic embryo germination and plantlet development was significantly enhanced. By the inclusion of BA and ZEA, maximum response was obtained at 0.5 and 2.0 mg/ respectively. Of the four cytokinins tested, the response of TDZ at 0.25 mg/l concentration was found to be more effective for embryo germination as well as plant regeneration followed by BA and ZEA. Response with KIN was found to be low compared with other cytokinins.

For long term maintenance of embryogenic cultures, a subculture interval of 50 d was found to be ideal. When embryogenic callus aggregates, derived from primary somatic embryogenesis, were used as target tissue, the embryogenic potential could be maintained up to 3 years by serial subculture of 50 d interval. When proline was included in embryo induction medium, embryo induction frequency was increased with maximum being at 100 mg/l. Similarly, AC was found to be essential for long term maintenance of cultures. Embryogenesis was reached maximum at 0.1% AC. Embryogenic lines could also be maintained up to 1 year by inducing secondary embryos from primary embryos. Among different developmental stages of embryos, secondary embryos were higher in immature cotyledon stage embryos.
Explants incubated both under dark and light conditions induced callus, however, further proliferation of calli was found to be affected by light. The studies indicated that *Hevea* needed continuous darkness till the acquisition of embryogenic competence or embryo induction. Further embryo development and maturation was favoured by light. Similarly light is a critical factor for plant regeneration though few plantlets could be regenerated under dark.

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

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

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

During different developmental stages of somatic embryogenesis, different protein profiles were observed. More protein bands were observed in embryogenic calli as well as in embryos. Compared to embryogenic calli, embryos showed a relative abundance of both low
and high molecular weight proteins. Embryogenic calli and non embryogenic calli significantly varied in their protein profiles. No clear protein bands were observed in non embryogenic calli. The protein electrophoretic analysis of somatic embryos at different stages of development revealed that the overall pattern of proteins was similar in all 3 embryos, however, varied in their relative expression. When embryos proceeded from globular stage to next stages of development, proteins were expressed in a much lower intensity.

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

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

For a recalcitrant crop like rubber tree, transformation techniques provide an attractive tool for crop improvement; however, the key to transformation appears to be the development of in vitro methods. Therefore, besides transformation, a more important goal is the development of a suitable high frequency plant regeneration system via somatic embryogenesis. The ability of rubber trees to respond well in tissue culture, particularly plant
regeneration via somatic embryogenesis has allowed the application of various powerful biotechnology tools for genetic improvement. In future, there is a necessity to concentrate efforts for the development of more transgenic rubber plants with new genes encoding resistance to biotic and abiotic stresses. Engineering of *Hevea* with rubber biosynthesis genes may contribute revolutions in rubber industry. Similarly, improvement of wood quality and volume by the generation of timber clones is other promising future objectives. Besides their usefulness as potential valuable breeding material, somatic embryogenesis opens the perspective of exploitation of *Hevea* transgenic plants for the production of recombinant proteins. Further prospects will focus on the production of plants via somatic embryogenesis utilizing bioreactor and synthetic seed technology. Nevertheless special attention has to be paid to the use of automation which could enhance the use of long term somatic embryogenesis for micro-propagation by reducing the cost and time. Therefore, the protocol developed in the present study would serve as a useful tool for desirable gene integration aiming at the genetic improvement of *Hevea* in the future.