**Title: Influence of juvenility on somatic embryogenesis and phase change related gene expression in Hevea brasiliensis**

# INTRODUCTION

*Hevea brasiliensis* (rubber tree) a native of Brazil, belonging to the family *Euphorbiacea*, is the most important source of natural rubber – a product of vital importance recovered from its latex. *H. brasiliensis* accounts for over 99% of the world's natural rubber because of its abundance in the latex, high quality and convenience of harvesting. Natural rubber is a biopolymer consisting of isoprene units $(C_5H_8)n$ linked together in a 1,4 *cis*-configuration. Rubber is harvested from the tree as latex, by controlled wounding of the bark in the main trunk of the tree, a process termed tapping. Since *H. brasiliensis* is cross pollinated, the seedlings are highly heterozygous. Genetic improvement of *H. brasiliensis* is very elaborate and time consuming as in many other perennial species. The major limitations are non-synchronous flowering, low fruit set, long gestation period, heterozygous nature and absence of fully reliable early selection parameters.

*In vitro* culture of *H. brasiliensis* was initiated in different laboratories with a view to develop protocols for micro propagation and genetic improvement through transgenic approaches. All the protocols developed for somatic embryogenesis and plant regeneration were from floral/fruit derived explants. Protocols for plant regeneration through somatic embryogenesis from different explants such as inner integument, immature anther and immature inflorescence have been reported in *H. brasiliensis.* Since flowering is seasonal and adverse environments during the season may hinder normal flowering, availability of viable explant is unpredictable. Development of alternate systems for plant regeneration through somatic embryogenesis using explants which are easily available throughout the year would be useful in *H. brasiliensis* crop improvement through micro propagation and genetic transformation. Leaf tissues have been reported to be an amenable explant for tissue culture. There are several reports in other crops, where leaf tissue has been mentioned as an ideal explant for micro propagation and a potential target tissue for *Agrobacterium* infection in genetic transformation experiments. However, so far there are no reports on development of a successful somatic embryogenesis and plant regeneration system from leaf explants of *H. brasiliensis.*

*H.brasiliensis* is predominantly cross pollinated and seeds are highly heterozygous. Hence the seedling derived materials which are most responsive to *in vitro* culture are not suitable for micro propagation. The current propagation method of bud-grafting on to unselected seedlings

maintains intraclonal heterogeneity for vigor and productivity. For the production of true to type individuals of selected genotypes, there is a need for the development of protocols for *in vitro* propagation of clonal materials. Leaf explants from clonal materials are available throughout the year from source plants of different maturity. It is necessary to compare the embryogenic capacity of the leaf explants collected from different sources to identify the one that is most amenable to *in vitro* culture and optimize the culture conditions suitable for *in vitro* plant regeneration.

Woody plants are reported to exhibit physiological phase changes. Responses that show marked differences between juvenile and adult stages include the ability to induce organogenesis/embryogenesis during *in vitro* culture, with juvenile tissues such as embryos and seedlings being the most responsive. Maturation is associated with changes in gene expression. *In vitro* regeneration is usually achieved by culturing tissues that are in a more juvenile, potentially regenerative state than most other tissues of the tree. *H. brasiliensis* tissues collected from mature trees are found to be highly recalcitrant to *in vitro* culture. In several other crops, comparison of cDNA of juvenile and mature trees indicated differences in gene expression. Differential gene expression in juvenile and mature tissues in turn influences *in vitro* culture response. So far no attempt has been made in *H.brasiliensis* to identify genes controlling juvenile/mature phase changes. Hence differential expression of these genes is to be studied and those having direct impact on *in vitro* culture response in terms of tissue recalcitrance are to be characterized.

The somatic embryogenesis protocols developed earlier for plant regeneration in *H.brasiliensis* from floral/fruit derived explants have been used for crop improvement through transgenic approaches. The somatic embryogenesis and plant regeneration prcotocol developed from leaf explants could also be utilized for *Agrobacterium* mediated genetic transformation. The feasibility of using the plant regeneration system from leaf explants for *Agrobacterium* mediated was explored so that the system developed could be used in future for crop improvement through transgenic approaches.

## OBJECTIVES

The present study was undertaken with the following objectives.

1. Development of a protocol for the induction of somatic embryogenesis and plant regeneration from leaf explants of *H. brasiliensis.*
2. Identification of suitable source and stage of explant with better *in vitro* response.
3. Comparison of the embryogenic competence of callus derived from leaf explants collected from source plants of different physiological maturity.

4. Identification and characterization of genes related to juvenile-mature phase change in *H. brasiliensis* with respect to tissue recalcitrance.
5. Study the feasibility of using the developed plant regeneration system for crop improvement through transgenic approaches.

## BRIEF OUTLINE OF THE MATERIALS AND METHODS

### I. Somatic Embryogenesis and Plant Regeneration from Leaf Explants

Leaf explants collected from newly formed flushes of six month old bud - grafted plants of *H. brasiliensis* (clone RRII 105) maintained in glass house were used for developing the somatic embryogenesis and plant regeneration system. Surface sterilization of the explants were tried with both $HgCl_2$ (0.1- 0.2 %) and NaOCl (1-5%). Leaf sections with and without liquid preculture were tried for callus induction. Explants were cultured with their adaxial surface in contact with the callus induction medium. Two different basal media MS (Murashige and Skoog) and WPM (Woody Plant Medium) as such and with modifications and containing B5 vitamins, myo-inositol, L-cysteine HCl and sucrose were tried. The phytohormones used were different concentrations of auxins 2,4-D and NAA (0-10 µM) in combination with cytokinins BA and KIN (0-10 µM). The effect of different concentrations of calcium nitrate ($Ca(NO_3)_2 \cdot 4H_20$) (0-2.0 g/l) and sucrose (10-50 g/l) on the rate and quality of callus induction was studied. L-cysteine HCl at different concentrations (0-200 mg/l) was also added to the medium to find its effect on tissue browning and callus induction. Callus formed were excised from the explant surface after 40 days and sub cultured for proliferation in fresh / modified callus induction medium. The effect of reduced concentrations of calcium nitrate and 2,4-D along with different levels of sucrose (20-60 g/l) and silver nitrate (0-50 mg/l) were tried to find their effect on callus proliferation and callus texture improvement.

Proliferated friable callus were tried for embryogenesis. Both embryogenic callus initiation and embryo induction were attempted in modified MS and WP basal medium containing $Ca(NO_3)_2 \cdot 4H_20$- (0-2.0 g/l), B5 vitamins, amino acids, organic supplements and sucrose. Different combinations and concentrations of phytohormones such as BA, $GA_3$ and Kin at concentration ranging from 0-10 µM and NAA (0-5 µM) were supplemented in the medium to find their optimal concentration for embryogenic callus initiation. Silver nitrate (0-50 mg/l) was also added to the medium to find its effect on embryogenic callus formation. The effect of different amino acids such as glutamine (0-1.0 g/l), proline (0-500 mg/l), arginine (0-200 mg/l), L- cysteine (0-200 mg/l) and glycine (0-20 mg/l) were also studied. Proliferation of the embryogenic callus and further embryo induction was tried by subculture of the callus in fresh medium at every 40-50 days interval. The same basal medium that initiated embryogenic callus and supplemented with different combinations and concentration of phytohormones such as BA (0- 8.8 µM), $GA_3$ (0- 5.8 µM), Kin (0- 4.6 µM)

and NAA (0- 2.7 µM) were used. Basal medium contained B5 vitamins, amino acids, organic supplements and sucrose. Experiments were carried out to increase the rate of embryo induction from the proliferated embryogenic calli. The concentration of calcium nitrate, phytohormones and phytagel were varied during further subcultures according to the texture of the callus to be sub cultured. During subculture for further embryo induction from the proliferated callus, polyethylene glycol (MW 8000) at different concentrations (0 - 10.0 g/l) was tried along with ABA (0-5 µM). The effect of charcoal and gelling agents such as bacto agar (15.0-25.0 g/l), phytagel (4.0-10.0 g/l) and agar-agar (10.0-20.0 g/l) (Sigma) at various concentrations on the rate and quality of embryo induction were also tried.

Maturation and apex induction of the somatic embryos were tried in both MS and WP basal medium containing additives such as vitamins; amino acids, organic supplements and sucrose. Phytohormones such as BA (0 - 8.8 µM), $GA_3$ (0 - 8.7 µM), Kin (0 - 9.2 µM) and IBA (0.49 µM) at different concentrations and combinations were supplemented in the medium. The effect of organic supplements such as CW (5 - 10%), malt extract (0 - 200 mg/l) and casein hydrolysate (0-500 mg/l) and different levels of sucrose (30 - 80 g/l) on embryo maturation was also studied. The embryos were cultured individually and kept in both light and dark for maturation.

Plant regeneration was tried with the apical meristem induced mature embryos. Both full and half MS and WP major salts containing MS minor, MS vitamins, myo- inositol (100 mg/l), organic supplements such as coconut water (5 %), malt extract (100 mg/l) and sucrose (30 g/l) were tried. Phytohormones used were BA (1.32 µM, 2.2 µM, 3.52 µM) and GA (2.9, 4.4µM) along with IBA (0.49 µM). 10% of the regenerating plantlets showed secondary embryogenesis from the hypocotyls region and these were also experimented for plant regeneration.

Plantlets after complete development were transferred to small cups (5 cm diameter x 8 cm height) containing sterile sand, kept in the growth chamber (temp. - $27^0$C, RH-85) and watered daily. After 10 days, the plantlets that survived the transplantation shock and showing continued growth were transferred to small poly bags (30 x15 cm) containing potting mixture (mixture of sand + soil + siolrite) and maintained in the growth chamber. The plants were watered on alternate days and supplemented with ¼ x Hoagland solution at two weeks interval. After emergence of 2-3 whorls of leaves, the plants were transferred to big poly bags containing sand and soil, kept in the glass house and watered on alternate days. The plants were transferred to the shade house after one month and watered once in three days.

**II. Identification of suitable source and ideal growth stage of explants**

Leaves were collected from different explant sources such as *in vitro* plantlets regenerated through somatic embryogenesis, newly formed flushes of six month old bud- grafted plants grown in poly bags in the glass house and 15 year old mature trees in the field to identify the ideal source

of explants. Leaves of three growth stages such as immature, medium mature and mature were used for the experiments, with explants collected from different sources, to identify the ideal stage for culture initiation. To initiate aseptic and viable cultures the concentration of sterilant and time of sterilization had to be changed with stage of the leaf. Callus induction was tried in the optimized medium. After twenty days, cultures were observed for contamination. Sterile and viable cultures were scored based on the response of cultures in terms of the rate, time of callus induction and quality of induced callus.

### III. Effect of Source Plant Juvenility on Somatic Embryogenesis

Embryogenic callus initiation medium developed earlier was used for culture of proliferated callus for embryo induction and rate and time taken for embryogenic callus initiation were observed. Further, the medium was modified to trigger formation of embryogenic callus. The callus obtained from source plant showing maximum culture response was used for the experiments. Osmotic stress was provided by increasing sucrose concentration and physical restriction of water availability was given by varying the level of phytagel. To find the combined effect of calcium and sucrose on embryogenic callus initiation, different concentrations of $Ca(NO_3)_2 \cdot 4H_20$ (0-2 g/l) was tried along with different levels of sucrose (20-100 g/l) for varying osmolarity, while the concentration of phytagel was kept at 0.3%. The medium combination from which embryogenic callus emerged in shortest time was used to test the effect of water stress by using different concentrations of phytagel (2.0-8.0 g/l). The optimized medium was further used for embryogenic callus initiation in proliferated fresh leaf callus derived from leaf explants collected from different source plants. Embryogenic callus obtained were then proliferated and experimented for somatic embryogenesis and plant regeneration in the earlier standardized medium.

### IV. Characterization of phase change related genes and its expression

The genes known to have control of phase change in other crops and aiding adventitious rooting were identified from literature and primers were designed. The genes selected were Glossy 15 gene, Dihydro flavonol reductase, AAA-ATPase, Chlorophyll a/b binding protein gene (cab gene), QRCPE, Zinc finger print protein gene, NAC1 gene, Rol c genes and SQUINT genes. PCR amplification was attempted with genomic DNA and cDNA from leaves of the *H.brasiliensis* (clone RRII 105) after optimizing the conditions using specific gene primers. Amplified fragments were cloned, sequenced and analyzed using bioinformatics' tools. Chlorophyll a/b binding protein gene (cab gene) was amplified through PCR using genomic DNA as template. Total RNA was isolated from leaves of *in vitro* plantlets regenerated through somatic embryogenesis, seedlings, newly formed flushes of six month old bud-grafted plants grown in poly bags in the glass house and 15 year old mature trees in the field. Cab gene expression was studied following northern analysis of

leaf samples collected from different source plants. Gene expression was also studied following RT-PCR using cDNA prepared from different samples.

### V. *Agrobacterium* mediated genetic transformation using leaf callus

Although a protocol for *Agrobacterium* mediated genetic transformation is available in *H.brasiliensis*, efficiency of the system was found to vary with each target tissue and new infection. The newly developed plant regeneration system from leaves was experimented for transgenic plant production. The protocol developed earlier with other explants was used for transformation experiments. *Agrobacterium* infection was done using proliferated fresh leaf callus and embryogenic callus as the target tissue. *Agrobacterium s*trains EHA 101 containing isopentenyl transferase gene and LBA 4404 containing a TB antigen gene were used for tissue infection. Experiments were carried out to control overgrowth of bacteria and improve callus texture in transformed cell lines by modifying the infection, cocultivation and selection medium with factors reported to enhance transformation efficiency in other crops. The effect of silver nitrate in controlling bacterial over growth and improving the texture of callus, thiol compounds such as L-cysteine, α-lipoic acid and dithiothreitol which are antioxidants and surfactant pluronic F68, in improving the transformation efficiency were studied.

## RESULTS

### I. Somatic Embryogenesis and Plant Regeneration from Leaf Explants

Leaf explants, collected from newly formed flushes of glass house grown six month old bud-grafted plants of *H.brasiliensis* (clone RRII 105) were used for initiating contamination free and viable cultures. The optimal stage of leaf explants for callus induction was identified as the intermediate between the immature and mature stage, when the leaf blade attains a light green colour with a shiny appearance. **S**terile and viable cultures initiated with explants of the optimal stage could be recovered with minimum culture contamination (<10%) during summer months when surface sterilized with 0.15% (w/v) $HgCl_2$ for two minutes. Liquid preculture of the explants helped in reducing the time taken for callus induction but culture contamination was more. Dissecting the sterilized explants in hormone solution also helped in increasing rate of callus induction. Callus induction could be obtained within 4 weeks in modified MS medium with addition of calcium nitrate (1200 mg/l), casein hydrolysate (1.0 g/l), B5 vitamins, sucrose (20 g/l) and phytohormones 2,4-D (5.4 µM), BA (4.4 µM) and NAA (1.08 µM ). L- cysteine hydrochloride at a concentration of 50 mg/l prevented phenolic exudation and favored callus induction. Callus proliferation was obtained by subculture of the callus in fresh callus induction medium containing reduced calcium nitrate (800 mg/l) and 2,4-D (1.8 µM) along with increased sucrose (40 g/l). With 2-3 subcultures in proliferation medium by gradually increasing the cytokinin/auxin ratio during

subculture, the texture of the callus was improved. Silver nitrate (20 mg/l) helped further improvement in the callus texture and favored embryogenic callus initiation. Proliferated callus was light yellow and friable.

Embryogenic callus initiation and proliferation, with simultaneous embryo induction, was obtained in modified MS medium ($Ca(NO_3)_2 \cdot 4H_20$ - 800 mg/l; $KH_2PO_4$ – 270 mg/l) containing $B_5$ vitamins, silver nitrate (10 mg/l), sucrose (60 g/l) and phyto hormones, BA (2.2 µM), $GA_3$ (2.9 µM), Kin (1.25 µM) and NAA (1.08 µM). The medium also contained glutamine (500 mg/l), proline (200 mg/l), L-cysteine HCl (100 mg/l), serine (20 mg/l), arginine (40 mg/l) and organic supplements such as (5 %) coconut water, casein hydrolysate (300 mg/l) and solidified with 0.3% phytagel.

Embryo induction was obtained from the proliferated embryogenic calli when the cultures were dark incubated in modified MS basal medium ($Ca(NO_3)_2 \cdot 4H_20$ – 250 mg/l and $KH_2PO_4$ – 270 mg/l) containing B5 vitamins, amino acids, organic supplements such as coconut water (5%), malt extract (50 mg/l), casein hydrolysate (300 mg/l) and sucrose (60 gm/l). Phytohormones used were BA (2.2 µM), $GA_3$ (2.9 µM), Kin (1.25 µM), and NAA (0.54 µM). Polyethylene glycol (MW 8000) at 5.0 g/l along with ABA (0.75 µM) helped in improving embryo induction. It was observed that bacto agar was not suitable for both embryogenic callus formation and embryo induction while phytagel (5.0 g/l) and agar- agar (18.0 g/l) were good. The embryos formed in medium solidified with agar- agar were small compared to embryos obtained in medium solidified with phytagel. Though embryogenic callus formation and embryo induction was observed in medium with and without charcoal, presence of charcoal was found more favorable.

Maturation and apex induction of embryos was obtained when cultures were dark incubated in WPM containing sucrose (60 g/l), amino acids such as glutamic acid (150 mg/l), arginine (40 mg/l) and glycine (10 mg/l), organic supplements and phytohormones BA (2.2 µM), (Kin 1.38 µM), IBA (0.49 µM) and $GA_3$ (5.3 µM). Organic supplements in the medium such as coconut water, casein hydrolysate and malt extract favored embryo maturation. Apex induced embryos at the cotyledonary stage were transferred to light for plant regeneration. Hormone free MS medium containing MS vitamins, sucrose (30 g/l), organic supplements such as coconut water (5 %), malt extract (100 mg/l) and casein hydrolysate (400 mg/l) was found good for plant regeneration.

Fifty to sixty percent survival of the plants was observed in the growth chamber during the initial transfer. The plants that showed continued growth were transferred to potting mixture after 10-15 days and watered once in three days. During the second transplant 30% of the plants continued to survive. After two months, plants with 2-3 whorls of leaves were transferred to big poly bags containing sand and soil, kept in the glass house and watered on alternate days. The plants were transferred to the shade house after one month and watered once in three days.

The rate of embryo maturation in the secondary embryos was similar in all media tried, showing the presence of enough endogenous reserves for their development. Apex induced embryos were big and seemed to be more healthy but only 10% showed continued growth during plant regeneration. Regenerating plantlets were healthy and they showed good growth.

## II. Identification of suitable source and ideal growth stage of explants

Cultures were recovered with minimum culture contamination (> 10%) during summer months when explants were surface sterilized with 0.15% (w/v) $HgCl_2$ for two minutes. The optimal stage of the explants for initiating viable cultures was identified as the intermediate stage between the immature and mature phase for explants collected from glass house grown bud grafted plants and field grown mature trees. In the case of explants collected from *in vitro* cultures, viable cultures could be initiated from both immature and medium mature leaves since no surface sterilization was needed.

Leaves of optimal growth stage, collected from different sources, could induce callus with varied time and frequency in the standardized callus induction medium. Rate of callus induction was higher (80%) in leaves collected from *in vitro* somatic embryogenesis derived plantlets since they were physiologically juvenile and at the same time needed no sterilization. In leaves collected from bud-grafted plants and mature trees which are physiologically mature sources, callus induction rate was lower (50%). Texture of the callus was similar in cultures initiated from leaves of all sources when cultured in the same medium. Silver nitrate (20 mg/l) in the proliferation medium helped in improving the texture of the callus. The proliferated callus was friable yellow.

## III. Effect of Source Plant on Somatic Embryogenesis

Embryogenic callus formation and simultaneous embryo induction was obtained from the proliferated leaf callus in modified MS medium ($Ca(NO_3)_2 \cdot 4H_20$- 800 mg/l; $KH_2PO_4$-270 mg/l) containing B5 vitamins, silver nitrate (10 mg/l) and phytohormones, BA (2.2 µM), $GA_3$ (2.9 µM), Kin (1.25 µM), ABA (0.75 µM) and NAA (0.54 µM). Amino acids present in the medium were glutamine (500 mg/l), proline (200 mg/l), L-cysteine HCl (100 mg/l), serine (20 mg/l) and arginine (40 mg/l). The medium also contained organic supplements such as coconut water (5 %) and casein hydrolysate (300 mg/l), sucrose (60 g/l), 0.2% activated charcoal and solidified with 3.0 g/l phytagel. In the callus developed from *in vitro* derived somatic plants, embryogenic callus initiation was observed within three months with a higher frequency (55 %). In callus obtained from leaves of bud-grafted plants, the rate was 20 % and time taken was about six months. Embryogenic calli originated from more than one region of each callus clump as a small yellow lump and proliferated to form a mass of friable golden yellow callus. The callus derived from mature tree leaves showed no embryogenic potential.

Modification of the standardized medium for faster embryogenic callus initiation showed that $Ca(NO_3)_2 \cdot 4H_20$ (500 mg/l) and sucrose (80 g/l) could trigger induction of embryogenic callus from the proliferated friable callus obtained from somatic embryogenesis derived *in vitro* plants at a phytagel concentration of 0.3 %. Further, in this combination ($Ca(NO_3)_2 \cdot 4H_20$ – 500 mg/l and sucrose 80 g/l) when different levels of phytagel were tried, 0.5% phytagel was found to be optimum for embryogenic tissue initiation.

Proliferation of the embryogenic calli was obtained on subculture to fresh medium. Embryo induction from these was obtained in MS medium modified with $Ca(NO_3)_2 \cdot 4H_20$ (250 mg/l ), $KH_2PO_4$ (270 mg/l), B5 vitamins, amino acids, organic supplements, sucrose (60 g/l) and phytohormones BA (2.2 µM), $GA_3$ (2.9 µM), Kin (1.25 µM) and NAA (0.54 µM). The embryo induction medium contained the amino acids, glutamine (300 mg/l), proline (100 mg/l), L-cysteine HCl (50 mg/l), serine (20 mg/l) and arginine (40 mg/l) along with organic supplements, coconut water (5%), casein hydrolysate (400 mg/l) and malt extract (50 mg/l). Presence of polyethylene glycol (5.0 g/l) along with ABA (0.75 µM) favored embryo induction during subculture of the proliferated callus. Embryo induction (60%) was obtained from the proliferated embryogenic calli. Rate of embryo induction, embryo maturation and plant regeneration was similar in the proliferated embryogenic calli derived from all explant sources.

### 1V. Characterization of phase change related genes and its expression

A PCR amplified product of approximately 0.5 kb was obtained from genomic DNA of the clone RRII 105 using the primer pair designed for Chlorophyll a/b binding protein (cab protein) gene. RT-PCR with the same primers for chlorophyll a/b binding protein gene using first strand cDNA synthesized from total RNA of leaves of different source plants of clone RRII 105 such as somatic embryogenesis derived *in vitro* plants, bud-grafted plants, mature trees, and seedlings also yielded a product of approximately 0.5kb in size. The fragment was similar in size with the genomic DNA and further cloning and sequencing confirmed that the PCR amplified portion is exactly similar to the PCR amplified genomic DNA indicating the absence of introns in the amplified region. The sequence has been registered in NCBI with the accession no: HM803119.1. The sequence comparison using CLUSTAL W of the isolated sequence with the reported cDNA sequence of cab gene (NCBI accession No: M60274) showed 91% sequence homology in the 525 base pair region from *Ricinus communis* and from other species like *Manihot esculenta* (88%). Further full length amplification (0.8 kB) of the genomic DNA from the translation initiation codon to termination codon was also obtained using a different set of primers.

To study differential expression of Chlorophyll a/b binding protein gene in the juvenile and mature phase, cDNA was PCR amplified with gene specific primers designed for Chlorophyll a/b binding protein gene. Cab gene showed difference in gene expression pattern when RT PCR was

carried out with the tissues of juvenile/mature plants. Seedlings and *in vitro* derived somatic plants showed more expression of the gene. Northern analysis using RNA isolated from leaf samples collected from young seedlings, bud- grafted plants grown in glass house, *in vitro* developed somatic plants and mature trees of *Hevea* clone (RRII 105) showed differential expression of the gene in tissues from juvenile and mature plants. The cab protein gene was found to be expressed more in tissues collected from juvenile explant sources.

### V. *Agrobacterium* mediated genetic transformation using leaf callus

Proliferated fresh leaf callus which was friable was identified as a suitable target tissue for A*grobacterium* infection. A transformation frequency of 9% was obtained when proliferated fresh leaf callus was infected with *Agrobacterium* strain EHA101 containing isopentenyl transferase gene and a frequency of 12% with *Agrobacterium* strain LBA 4404 containing TB antigen gene using the earlier developed protocol with other explants. Bacterial overgrowth and failure of the transgenic callus lines to proliferate and induce embryos were the major obstacles in transgenic plant production. It was observed that addition of 10.0 mg/l silver nitrate in the infection and co cultivation medium and 20.0 mg/l in the selection medium significantly suppressed bacterial overgrowth and improved the texture of callus in newly emerged putatively transgenic cell lines. Inclusion of dithiothreitol (5 mg/l) in the infection and co-cultivation medium, L– cysteine (100 mg/l) in the infection, cocultivation and selection medium, α-lipoic acid (50 mg/l) in the cocultivation and (50 mg/l) in the selection medium and addition of the surfactant pluronic F68 (300 mg/l) in the infection medium helped in improving the transformation frequency by 10-15%. The transgenic callus obtained was proliferated, somatic embryogenesis and plant regeneration was also obtained.

## SUMMARY

A protocol was developed for callus induction, somatic embryogenesis and plant regeneration from leaf explants of *Hevea*. The leaf source, ideal growth stage of leaf, culture conditions and media requirements in each step of the somatic embryogenesis and plant regeneration pathway were optimized. Embryogenic competence of the explant in terms of embryogenic callus initiation and further embryo induction was highly dependent on the source plant and was found to be reduced with maturity of the source plant. Chlorophyll a/b binding protein gene could be amplified from genomic and cDNA. Amplified product was cloned and sequenced. The sequence showed 91 % sequence homology in the 525 base pair region of Cab protein gene from *Ricinus communis*. Difference was observed in gene expression pattern in juvenile and mature tissues through RT PCR and northern analysis. The protocol developed somatic embryogenesis and plant regeneration from leaf explants in *H.brasiliensis* was also proved to be useful for crop improvement through transgenic approaches.