

Genetic transformation of *Hevea brasiliensis* Muell. Arg. using intact explants as target tissues for *Agrobacterium* infection

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Abstract

Genetic transformation is a promising tool for the improvement of the natural rubber producing tree, *Hevea brasiliensis*, through the incorporation of agronomically important genes. *Agrobacterium* mediated genetic transformation and regeneration of transgenic plants in *H. brasiliensis* was previously achieved using callus as the target tissue. The present study proves the feasibility of using intact explants directly as target tissue for *Agrobacterium* infection. Three target tissues, viz., leaf explants from glass house and pre-cultured in modified MS medium for one week, and leaf and root explants from *in vitro* developed somatic plants were used in the study. *A. tumefaciens* harbouring the binary vector carrying superoxide dismutase (MnSOD) gene for abiotic stress tolerance, GUS as reporter gene and *npt II* gene for antibiotic selection was used. Different explant pre-treatments such as air drying in laminar air flow hood, soaking in sterile water, sterile water containing acetosyringone (40 mg l⁻¹) and sterile water containing acetosyringone (40 mg l⁻¹) and picloram (2.0 mg l⁻¹) for 20 minutes were given prior to infection with *Agrobacterium*. Both precultured leaf and *in vitro* root explants soaked in sterile water containing acetosyringone (40 mg l⁻¹) and picloram (2.0 mg l⁻¹) responded well to bacterial infection with *in vitro* root explants giving maximum transformation efficiency (67 per cent). The optimum concentration of kanamycin required for selection of infected explants was 50 mg l⁻¹ and callus induction was obtained from the infected explants after three weeks. Newly formed callus from infected root explants were proliferated and the GUS histochemical assay was positive. The genomic DNA isolated from randomly selected putatively transgenic callus lines were subjected to PCR analysis with GUS, *npt II* and MnSOD gene specific primers. Gene amplification was obtained using GUS (650 bp), *npt II* (800 bp) and MnSOD (700 bp) gene specific primers.

Key words: *Agrobacterium*, GM, Intact Explant, MnSOD, *Hevea*, Transformation, Transgenic

Introduction

Hevea brasiliensis, a tropical tree crop belonging to the family *Euphorbiaceae* is the major commercial source of natural rubber. The tree being open pollinated, the seeds are heterozygous and clonal materials are propagated by bud grafting. Most of the *in vitro* culture works in *H. brasiliensis* are directed towards micropropagation through shoot tip culture, nodal cultures, somatic embryogenesis and genetic transformation. Somatic embryogenesis and plant regeneration systems have been developed in *Hevea* from a variety of explants

such as, inner integument (Carron et al., 1998), immature anther (Jayasree et al., 1999), inflorescence (Sushamakumari et al., 2000), leaf (Kala et al., 2005, 2006), and *in vitro* developed leaves and roots of somatic plants (Sushamakumari et al., 2006, 2010). The improvement of any crop through genetic engineering primarily involves introduction of exogenous genes in a heritable manner and secondarily, the expression of genes that confer the desired traits.

Extending rubber plantations to non-traditional areas is challenging due to the various abiotic

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stresses. In recent years, efforts on *H. brasiliensis* improvement have been focused on raising genetically altered plants with high yield potential, tolerant to abiotic stresses such as tapping panel dryness and capability to produce value added products such as recombinant proteins, according to the regional requirement of the crop. All earlier protocols for genetic transformation in *H. brasiliensis* used both fresh and embryogenic callus developed from different explants to produce transgenic cell lines. *Agrobacterium tumefaciens* mediated genetic transformation in *H. brasiliensis* was first attempted by Arokiaraj and Wan (1991) by infecting the calli derived from *in vitro* and *in vivo* seedling cultures. The first transgenic *Hevea* plant was produced by the incorporation of β -glucuronidase (GUS) and neomycin phosphotransferase (*nptII*) genes into callus cultures by particle gun method and complete plantlet regeneration (Arokiaraj et al., 1994). To date, the *Agrobacterium* mediated approach has been the most successful method for delivering foreign genes into *H. brasiliensis* and there are several reports of successful transgenic plant regeneration. Blanc et al. (2006) and Montoro et al. (2003) developed transgenic calli through infection of calli derived from inner integument tissues of immature fruits of *Hevea* clone PB260 with *Agrobacterium* strain EHA105 containing binary vector pCAMBIA2301 and led to high transient GUS activity in callus. Transgenic *Hevea* plants were also regenerated with the *MnSOD* gene under the control of CaMV 35S and FMV 34S (Figwort Mosaic Virus) promoters (Jayashree et al., 2003; Sobha et al., 2003, 2003a) and isopentenyl transferase (*ipt*) gene aiding cytokinin biosynthesis (Kala et al., 2003) using immature anther derived callus. Experiments were also carried out for recombinant protein production by incorporation of a TB antigen gene (Kala et al., 2006a).

Genetic transformation was attempted with fresh and embryogenic callus derived from immature anther and ovule explants and a transformation frequency of 62 per cent was obtained with anther

derived embryogenic callus (Rekha et al., 2006). The protocol described by Horsch et al. (1985) for the transformation of tobacco leaf discs forms the basis of most modified procedures used with *A. tumefaciens*. Since then, there are several reports of the successful use of intact explants such as leaves and roots as target tissues for *Agrobacterium* infection in genetic transformation experiments in many other crop species (De Block et al., 1988). The leaf disc transformation and regeneration system was used for developing transgenic tomato plant expressing agronomically desirable traits (Murata et al., 1992). In mulberry, pre-culture of explants in regeneration medium for 5 days was found optimal for *Agrobacterium* infection in leaf, cotyledon and hypocotyls explants (Bhatnagar et al., 2004). In *Hevea*, explants from *in vitro* developed somatic plants showed more culture response than those from adult plants and it was assumed that they will respond similarly in genetic transformation (Kala et al., 2009). Hence leaves and root explants from the *in vitro* developed somatic plants were also used as target tissues in *Agrobacterium* mediated genetic transformation.

Superoxide dismutases (SODs) are metallo enzymes present in most aerobic and anaerobic organisms which catalyze the spontaneous dismutation of superoxide anions to hydrogen peroxide and molecular oxygen. Integration of this gene would impart abiotic stress tolerance by free radical scavenging (Bowler et al., 1992). The present work focuses on the feasibility of using leaves and root explants as target tissues for *Agrobacterium* infection for *MnSOD* gene integration and employing different pre-treatments for improving transformation efficiency. Gene insertion has been documented through GUS expression and molecular approaches.

Materials and Methods

Explants as target tissues

Three explants viz., pre-cultured leaves collected

from bud grafted plants grown *ex vitro*, leaf and root explants from *in vitro* derived somatic plants were used as targets for *Agrobacterium* infection. Leaf explants collected from newly formed flushes of six month old bud grafted plants of *H. brasiliensis* and maintained in glass house (clone RRII 105) were used for culture initiation. Selection of stage of leaf explant and culture initiation were carried out following the protocol developed by Kala et al., (2005). Surface sterilized explants were pre-cultured for one week in modified MS (Murashige and Skoog, 1962) medium and used for *Agrobacterium* infection. *In vitro* leaf and root explants were taken from plantlets regenerated through somatic embryogenesis from leaf explants collected from bud grafted plants grown in glass house. Callus induction, embryo induction and plant regeneration were obtained in earlier optimized medium (Kala et al., 2006).

Antibiotic sensitivity

Antibiotic sensitivity of the target explants was studied using different concentrations of the selection antibiotic, kanamycin (0-200 mg l⁻¹) in the earlier reported callus induction medium containing phytohormones 2,4-D (5.4 µM), BA (4.4 µM) and NAA (1.08 µM). The selection antibiotic was prepared as stock solution (10 mg l⁻¹), filter sterilized using 0.2 µm millipore filter and stored at -20°C. The neomycin phosphotransferase gene (*npt II*), conferring resistance to the antibiotic kanamycin was used for plant selection. Ten explants were cultured per plate and replicated three times. The cultures were kept in the dark. The response of the explants on exposure to the antibiotic was scored after one month of culture. The concentration of kanamycin at which majority of the explants perished was selected as the optimum concentration for selection of the transformants.

Pre-treatment of explants

Explants used as target tissues viz., leaves pre-cultured for one week and leaves and roots of plants

regenerated *in vitro* through somatic embryogenesis were transferred into small glass petriplates (30 mm). Different pre-treatments such as air drying the explants in laminar air flow hood (T1), soaking in sterile water (T2), sterile water containing acetosyringone (40 mg l⁻¹) (T3) and sterile water containing acetosyringone (40 mg l⁻¹) and picloram (2.0 mg l⁻¹) (T4) were given for 20 minutes prior to infection with *Agrobacterium* along with control. The pre-cultured and the leaves taken from *in vitro* somatic plants were cut into discs of 1x1 cm size and roots were cut into pieces of 1 cm length while in the preculture solution. About ten explants were used for each experiment.

Agrobacterium mediated genetic transformation

Transformation was carried out for integration of *MnSOD* gene into clone RRII 105. *Agrobacterium tumefaciens* strain EHA 101 harboring the construct PDU 96.2412 carrying *MnSOD* gene under the control of CaMV 35S promoter was used for genetic transformation. The binary vector contains β-glucuronidase (GUS) gene as the reporter gene and *nptII* gene for plant antibiotic selection.

Agrobacterium single colony was suspended in 10 ml sterile liquid AELB medium containing the antibiotics 50 mg l⁻¹ kanamycin and 20 mg l⁻¹ gentamycin. The cultures were incubated overnight at 28°C in a shaker. MS medium containing silver nitrate 10 mg l⁻¹, pluronic F68 (300 mg l⁻¹), α lipoic acid (50 mg l⁻¹), 100 µM aceto-syringone and 1.0 mM each of proline and betaine hydrochloride was used as infection medium. Bacteria were pelleted and resuspended in the infection medium so as to get a bacterial density of 5x10⁸ cells/ml. The pH of the medium was adjusted to 5.2 with 1 N KOH, filter sterilized and used for tissue infection.

After four hours of growth in the infection medium, the bacterial culture was used to infect the pre-treated as well as control explants, for 15 min. The explants in the bacterial solution were slightly injured with a sterile scalpel blade to facilitate entry

of the bacteria. The infected explants were blotted dry and transferred onto filter papers kept above the solid co-cultivation medium. Modified induction, co-cultivation and selection medium containing surfactant pluronic F-68 (300 mg l⁻¹) in the infection medium and either of the antioxidants L- cysteine (100 mg l⁻¹) / α - lipoic acid (50 mg l⁻¹) in the infection, co-cultivation and selection medium (Kala et al., 2012b) were used in this experiment. Modified MS medium containing silver nitrate 10 mg l⁻¹ and lipoic acid (50 mg l⁻¹) was used for co-cultivation. The pH of the medium was adjusted to 5.6 with 1 N KOH before autoclaving. Phytohormones 2,4-D, BA and NAA and acetosyringone (100 μ m) and 1.0 mM each of proline and betaine hydrochloride were added after filter sterilization.

The tissues after co-cultivation were first transferred to the callus induction medium containing only selection antibiotic carbenicillin (400 mg l⁻¹) to control bacterial overgrowth. After two weeks, the tissues devoid of bacterial overgrowth were sub cultured in medium which contained both selection antibiotics carbenicillin (300 mg l⁻¹) and kanamycin sulphate (50 mg l⁻¹). About ten explants were cultured in petriplates of 90 x 15 mm size containing the selection medium. After the second subculture, carbenicillin was avoided from the selection medium used for subculture of the infected tissues without overgrowth. The newly emerged lines were further subcultured for proliferation after one month in selection medium which contained optimum kanamycin concentration (50 mg l⁻¹). The pH of the co-cultivation and selection medium was adjusted to 5.7 with 1N KOH before autoclaving at 121^o C for 15 min. Transformation efficiency was scored based on the number of lines that emerged from the infected tissues.

GUS histochemical assay

GUS activity assays were performed in the kanamycin resistant callus that emerged from the *Agrobacterium* infected explants following the method reported by Jefferson et al. (1987). Newly

emerged lines from pre-cultured leaf (SF1, SF2) and *in vitro* root (SR) were immersed in X-Gluc solution (2 mM X-Gluc, 100 mM NaH₂PO₄, 0.5 mM potassium ferricyanide and 50 mM ferro cyanide). The tissues were incubated at room temperature. Transient GUS expression (Tissue blue colouration) frequency was examined visually.

PCR amplification of MnsSOD gene from transformed callus

Randomly selected GUS positive callus obtained from *Agrobacterium* infected root and leaf explants were proliferated and used for DNA isolation (Doyle and Doyle 1990). Plasmid DNA was isolated from the binary vector pDU 96.2412 following the alkaline lysis method. PCR amplification of *nptII* gene was done using gene specific primers 5'GAGGCTATTCGGCTATGACT3' (F) and 5'AATCTCGTGATGGCAGGTTG3' (R). For the detection of GUS gene, specific primer 5'TAGAGATAACCTTCACCCGG3' (F) and 5'CGCGAACTGTGGAATTGA3' (R) were used. *MnSOD* gene specific primers 5'ATGGCTCTGCGATCTCT3' (F) and 5'CTAAGAAGAGCATTCTTTGGCAT3' (R) were used for detection of *MnSOD*. The PCR reactions were carried out in 20 μ l volumes containing 100 μ M dNTPs, 250 nM of each primer, 1.5 mM MgCl₂ and 0.7 U Taq DNA polymerase (Bangalore Genei, India) with 50 ng template DNA in a thermo cycler. The PCR conditions were: step I initial denaturation at 94^o C for 2min, step II - denaturation at 94^o C for 1min., annealing for 1min. at 55^o C and extension at 72^o C for 1 min. in (36 cycles) followed by step III final extension at 72^o C for 10 min. Amplified DNA was electrophoresed in 1.5% agarose gels, stained with ethidium bromide and the amplified DNA bands were visualised under UV transilluminator and documented.

Results and Discussion

Agrobacterium mediated genetic transformation was successfully carried out using intact plant parts such

as leaf and root as target tissues for bacterial infection. Explant pre-treatment helped in improving the efficiency of transformation. Infected tissues have been recovered without bacterial overgrowth and transgenic calli were obtained. Gene integration was confirmed by molecular analyses.

Explants as target tissues

The leaves from *ex vitro* sources when cultured in the modified MS medium swelled after one week and these explants were found to yield good response for *Agrobacterium* infection. Previously, in *Malus*, *Populus* and *Prunus*, it was reported that the pre-culture of the explants prior to co-cultivation shall enhance susceptibility of cells to bacterial infection and T-DNA transfer (Schaart et al., 1995; Tsai et al., 1994; Miguel and Olivera, 1999). Plants were regenerated from the embryos obtained from embryogenic callus derived from leaf cultures initiated and maintained from earlier experiments. Leaf and root explants taken from these plants were used successfully for *Agrobacterium* infection.

Antibiotic sensitivity

It was observed with regard to antibiotic sensitivity when tested with *in vitro* root explants that any concentration above 25 mg l⁻¹ was lethal to the tissues and though tissue viability was observed at 50 mg l⁻¹ after two week culture, gradual death occurred (Table.1). It was observed that antibiotic sensitivity varied with the explants (Table 2.). Using the other two explants also, after three weeks culture, tissue death was gradually observed in cultures containing kanamycin concentration above 25 mg l⁻¹. With *in vitro* developed leaf explants, the tissues could not tolerate a concentration above 25 mg l⁻¹ for two weeks, while at 50 mg l⁻¹ kanamycin, the pre-cultured leaf explants remained viable for three weeks. Even at the same antibiotic concentration, the time required for tissue death varied with the explant. Tissue death was gradual at this antibiotic concentration with all explants losing their viability after one month. Hence

Table 1. Antibiotic sensitivity of *in vitro* root explants after two weeks culture

Kanamycin conc. (mg l ⁻¹)	Tissue viability (mean)
0	90 (74.89)
25	46.67 (43.08)
50	13.33 (21.14)
75	—
100	—
150	—
200	—

CD (P<0.05) =9.99. Results are from 10 replications. Experiment repeated thrice
The data was analyzed using Arc sine transformation and the transformed values are given in parenthesis

Table 2. Response of explants in selection medium with different Kanamycin concentration after two weeks culture

Kanamycin (mg l ⁻¹)	<i>In vitro</i> root	<i>In vitro</i> leaf	Precultured leaf
25	TV	TV	TV
50	TV	TD	TV
75	TD	TD	TD
100	TD	TD	TD
200	TD	TD	TD

TD- Tissue died, TV- Tissue viable

50 mg l⁻¹ kanamycin was used for selection of transformed tissues. Similar to this observation, Mazumdar et al., (2010) also used 40 mg l⁻¹ kanamycin, along with 150 mg l⁻¹ cefotaxime, to screen the transformed regenerants, while transferring the *LIF* gene into the *J. curcas* genome using leaf discs as explants.

Agrobacterium mediated genetic transformation

After *Agrobacterium* infection and co-cultivation for three days, the target explants were recovered without bacterial overgrowth. The infected explants were blotted dry in the laminar flow hood and transferred to selection medium containing the antibiotic carbencillin to prevent bacterial overgrowth. Infected explants in control cultures gradually died. After two weeks these were transferred to selection medium containing both

carbenicillin and kanamycin and callus induced within four weeks of incubation. Though callus could be induced in all three target explants, proliferation of the callus obtained from *in vitro* leaf explants was low. Proliferation of fresh callus obtained from precultured leaf and *in vitro* root explants could be achieved after one month in selection medium and the calli had a friable texture with pale yellow appearance (Fig.1.A and B). Kala et al.(2012a) has reported that inclusion of silver nitrate (10.0 mg l⁻¹) in the infection and co-cultivation media and 20.0 mg l⁻¹ in the selection medium, controlled bacterial overgrowth, reduced tissue damage and improved the callus texture in newly emerged putatively transgenic cell lines. Similar results were obtained from this study also. Ten per cent increase in transformation frequency was obtained earlier by the inclusion of the surfactant pluronic F-68 (300 mg l⁻¹) in the infection medium and addition of antioxidants L- cysteine (100 mg l⁻¹) /α- lipoic acid (50 mg l⁻¹) in the infection, co-cultivation and selection media (Kala et al., 2012b).

Different pre-treatments have been tried in order to increase the transformation efficiency of *Agrobacterium* in leaf and root explants of *Hevea brasiliensis*. It may be noted from Table 3 that transformation efficiency could be improved by explant pre-treatment. Among the four pre-treatments compared, the *in vitro* root and pre cultured leaves soaked for 20 min. in acetosyringone (40 mg l⁻¹) and picloram (2.0 mg l⁻¹) showed the highest transformation efficiency, improved callus texture and good transgenic callus proliferation (Table 3). The explants treated with acetosyringone alone also gave good response regarding transformation efficiency but further callus proliferation was less. Among the three explants tried, response was best in the *in vitro* developed roots (67 per cent) followed by pre-cultured leaves. Callus initiation was found to be much faster in these tissues compared to the tissues which received other pre-treatments. Explants from *in vitro* developed somatic plants usually show more culture response

Table 3 Transformation efficiency of explants with different pre-treatments

Pre-treatment	Transformation efficiency (mean)		
	Pre-cultured leaf	<i>In vitro</i> leaf	<i>In vitro</i> root
0	13.33±3.33	03.33±3.33	13.33±3.33
T1	13.33±3.33	13.33±3.33	26.67±3.33
T2	16.67±3.33	23.33±3.33	16.67±3.33
T3	33.33±3.33	16.67±5.77	53.33±6.67
T4	43.33±3.33	26.67±5.77	66.67±3.33

Mean ± SE Results are from 10 replications. Experiment repeated thrice

than those from adult plants due to their physiological juvenility. The same has been already proven in *Hevea* by Kala et al., (2009) and it was observed that they responded similarly in genetic transformation using *in vitro* root explants also, giving maximum response.

Kanamycin (50 mg l⁻¹) was used as antibiotic for selection of transformants since the binary vector carries the *nptII* which confers resistance to kanamycin promoting the growth of transformants alone. Carbenicillin (400 mg l⁻¹) was also included in the medium which prevent the overgrowth of *Agrobacterium* along with the putatively transformed explants. In strawberry, leaf disc explants showed kanamycin resistance after 16 weeks in a medium containing 25 mg l⁻¹ of this antibiotic. The transgenic nature of several shoots was also confirmed by the GUS assay and PCR analysis (Barcel ò et al., 1998).

Acetosyringone (AS) is produced during wounding of plant cells and induces transcription of the virulence genes of *Agrobacterium tumefaciens* by interacting with a transmembrane receptor protein (Satchel et al., 1985). Transformation efficiency could be enhanced from 6 to14 per cent in *H. brasiliensis* by pre-culture of callus used as target tissue, in medium containing calcium nitrate and increased levels of acetosyringone (Sobha et al., 2010). James et al. (1993) reported that acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*

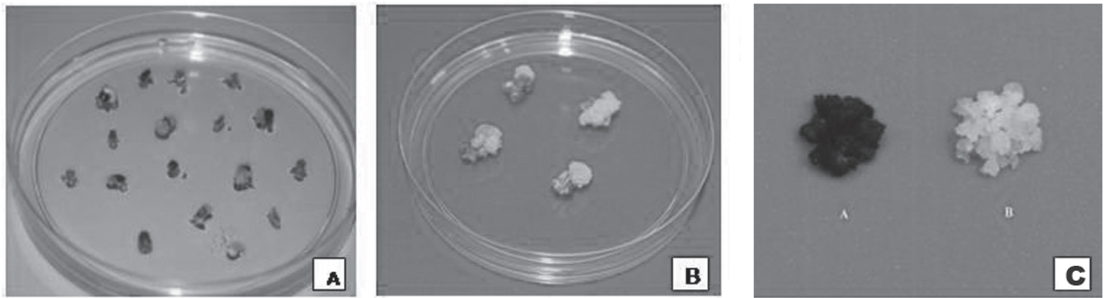


Figure 1 A and B Transgenic callus proliferation in in vitro root and precultured leaf explants. Figure 1 C. GUS expression in transgenic and non transgenic callus

mediated transformation of apple. Further, the transformation enhancement capability of acetosyringone is already reported by other researchers also. Pre-induction of *Agrobacterium* with 400 μ M acetosyringone prior to co-cultivation facilitated rice transformation (Aldemita and Hodges, 1996). Similarly, Khanna and Raina (1999) stated that 400-500 μ M acetosyringone enhanced the transformation of rice. According to Rao and Rao (2007), phenolics like acetosyringone may not be essential for induction of *vir* genes and development of transgenic *indica* rice is feasible under acetosyringone free conditions. The role of picloram has been mainly understood as an inducer of somatic embryogenesis (Mendoza and Kaeppler, 2002; Castillo et al., 1998; Kordestani and Karami, 2008)

PCR amplification of *MnSOD* gene from transformed callus

The proliferated putatively transformed callus from leaf and root explants when incubated with X-gluc, was oxidized and dimerised into an intense blue coloured product which showed the presence of GUS gene incorporated in the callus (Fig. 1.C). Good quality genomic DNA was isolated from the GUS positive callus cell lines of pre-cultured leaf explants and *in vitro* roots (Fig. 2.A). DNA isolated from pre-cultured leaf and *in vitro* root explants' callus were subjected to PCR amplification and compared with untransformed callus as negative control. The plasmid vector pDU 96.2412 was used as positive control. The presence of *nptII* gene was confirmed by the amplification of 800 bp fragment in the transformed cell lines derived from leaf and

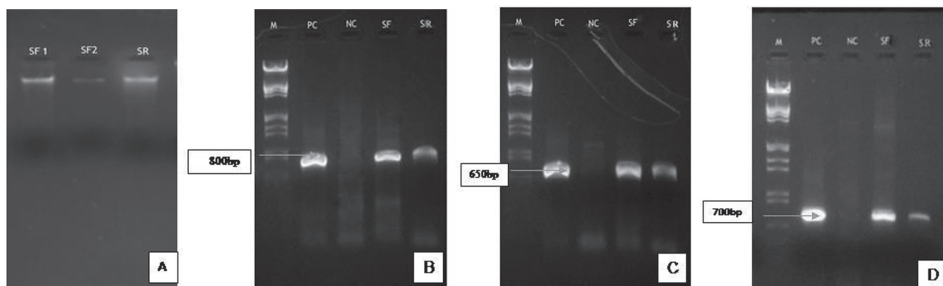


Figure 2 A. DNA isolated from transgenic callus SF1 and SF2 genomic DNA from precultured leaf explant callus, SR-genomic callus from *in vitro* root callus. Figure 2 B. PCR amplification with *nptII* specific primer. M-Marker, PC positive control(plasmid DNA) NC- negative control (Lamda DNA), SF- amplified DNA of pre cultured leaf, SR-amplified DNA of *in vitro* root Figure 2 C and D PCR amplification with GUS and SOD gene specific primer.M-Marker, PC positive control(plasmid DNA) NC- negative control (Lamda DNA), SF- transgenic callus of pre cultured leaf explant, SR- amplified DNA of *in vitro* root

root explants and in positive control. No amplification was detected in the untransformed callus (Fig. 2.B). The presence of GUS gene was also confirmed by the amplification of 650 bp fragment which was not detected in the untransformed callus (Fig. 2.C). When PCR was performed with gene specific primer for *MnSOD*, a 700 bp fragment was amplified in both the transformed cell lines and in the positive control plasmid. This 700 bp fragment was absent in the untransformed callus (Fig.2.D).

Agrobacterium tumefaciens mediated *MnSOD* gene insertion in intact explants was successfully achieved. Enhancement of transformation efficiency by explant pre-treatment was also observed. Among the three explants tried, response was higher in the *in vitro* developed roots followed by pre-cultured leaves. Highest response was obtained with the *in vitro* developed roots. Callus initiation was found to be much faster in these when compared to other tissues. Pre-treatment of explants by soaking in water containing acetosyringone (40 mg l⁻¹) and picloram (2.0 mg l⁻¹) for 20 minutes prior to bacterial infection gave the maximum efficiency. Along with the transformation efficiency studies, kanamycin sensitivity of the explants was also studied, which showed that after three weeks culture, tissue death occurred in cultures containing kanamycin above 50 mg l⁻¹. When the proliferated callus was subjected to GUS histochemical assay, cell lines were found to be GUS positive. Further PCR analysis also confirmed the presence of transgene in the callus. Development of genetic transformation protocols with improved efficiency would be useful for incorporation of desired genes for crop improvement. This is the first report on successful use of intact explants for bacterial infection in *Agrobacterium* mediated genetic transformation of *Hevea*. Optimization of the developed protocols using suitable target tissues more amenable for *Agrobacterium* infection and providing suitable conditions aiding effective transformation would speed up genetic modification in *Hevea*.

References

- Aldemita, R.R., and Hodges, T. 1996. *Agrobacterium tumefaciens* mediated transformation of japonica and indica rice varieties. *Planta*, 199: 612-617.
- Arokiaraj, P. and Wan, A.R. 1991. *Agrobacterium* mediated transformation of *Hevea* cells derived from *in vitro* and *in vivo* seedling cultures. *Nat. Rub. Res.*, 6: 55-61.
- Arokiaraj, P., Jones, H., Cheong, K.F., Coomber, S. and Charlwood, B.V. 1994. Gene insertion into *Hevea brasiliensis*. *Pl. Cell Rep.*, 13: 425 – 431.
- Barcelò, M., El-Mansouri, I., Mercado, J.A., Quesada, M.A. and Alfaro, F.P., 1998. Regeneration and transformation via *Agrobacterium tumefaciens* of the strawberry cultivar Chandler. *Pl. Cell Tiss. Org. Cul.*, 54: 29–36.
- Bhatnagar, S., Kaptur, A. and Khurana, P. 2004. Evaluation of parameters for high efficiency gene transfer via *Agrobacterium tumefaciens* and production of transformants in Indian mulberry, *Morus indica* cv. K2. *Pl. Biotech.*, 21: 1-8.
- Blanc, G., Baptiste, C., Oliver, G., Martin, F. and Montorro, P. 2006. Efficient *Agrobacterium tumefaciens* – mediated transformation of friable calli and regeneration of *Hevea brasiliensis* Mull Arg. plants. *Pl. Cell Rep.*, 24: 724–733.
- Bowler, C., Montagu, M.V. and Inze, D. 1992. Superoxide Dismutase and Stress Tolerance. *Ann. Rev. Pl. Physiol. Pl. Mol. Biol.* 43: 83-116.
- Carron, M.P., Lardet, L., Dea, B.G. 1998. *Hevea* micropropagation by somatic embryogenesis. *Plantations Res. Dev.*, 5: 187-92.
- Castillo, A., Egena, B., Sanz, J. and Cistue, L. 1998. Somatic embryogenesis and plant regeneration from barley cultivars grown in Spain. *Pl. Cell Rep.*, 17: 902–906.
- De Block, M., D'Halluin, K., Botterman, J., Lemans, J. 1988. The use of phosphinothricin resistance as a selectable marker in tobacco protoplast transformation. In: *Progress in Plant Protoplast Research*, Eds., Springer, The Netherlands, pp 389-390.
- Doyle, J.J., and Doyle, J.L. 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus*, 12: 13-15.
- Horsch, R., Fry, J.E., Hoffman, N.C., Eichholtz D., Rogers, S. and Fraley, R.T. 1985. A simple general method for transferring genes into plants. *Science*,

- 227: 1229-1231.
- James, D.J., Uratsu, S., Cheng, J, Negri, P., Visserie, P. and Dandekar, A.M., 1993. Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium* mediated transformation of apple. *Pl. Cell Rep.*, 12: 559-563.
- Jayasree, P.K., Asokan, M.P., Sobha, S., Sankari Ammal, L., Rekha, K., Kala, R.G., Jayasree, R., Thulaseedharan, A. 1999. Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell.Arg). *Curr.Sci.*, 76:1242-1245.
- Jayashree, R., Rekha, K., Venkatachalam, P., Uratsu, S.L., Dandekar, A.M., Jayasree, P. K., Kala, R.G., Priya, P., Sushamakumari, S., Sobha, S., Asokan, M.P., Sethuraj M.R., and Thulaseedharan, A. 2003. Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. *Pl. Cell Rep.*, 22: 201-209.
- Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W., 1987. Gus fusions: β -glucuronidase as a sensitive and versatile gene marker in higher plants. *EMBO Journal*, 6: 3901-3907.
- Kala, R.G., Kumari Jayasree, P., Sobha, S., Sushamakumari, S., Jayasree, R., Rekha, K., Venkatachalam, P and Thulaseedharan, A. (2003). Introduction of the gene coding for isopentenyl transferase into *Hevea brasiliensis*: Effect on plant regeneration. In: Abstracts - 10th Congress of FAOBMB, December 7-11, Bangalore, India. pp 120.
- Kala, R.G., Jayasree, P.K., Sushamakumari, S., Jayashree, R., Rekha, K., Sobha, S. and Thulaseedharan, A. 2005. *In vitro* regeneration of *Hevea brasiliensis* from leaf explants. In: Abstracts - ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies, Trichur, Jan. 10-12, India, pp 105-106.
- Kala, R.G., Jayasree, P.K., Sobha, S., Rajani, J. and Thulaseedharan, A. 2006. Plant regeneration from leaf derived somatic embryos of *Hevea brasiliensis*. In: Abstracts International Symposium on Frontiers in Genetics and Biotechnology – Retrospect and Prospect, Jan 8-10, Osmania University, Hyderabad, India, pp 161.
- Kala R.G., Anu K. S., Manesh K., Saleena A., Jayasree P. K., Narayanan P. R., Thomas G. and Thulaseedharan A. 2006a. *Agrobacterium* Mediated Genetic Transformation in *Hevea brasiliensis* for Recombinant Protein Production. *J.Plant.Crops* , 34(3): 582-586.
- Kala, R.G., Gimisha, G.C., Jayasree, P.K., Sushamakumari, S., Sobha, S., Jayasree, R., Rekha, K., and Thulaseedharan, A. 2009. Somatic embryogenesis in leaf cultures of *Hevea brasiliensis*: effect of source plant. *Nat. Rub. Res.*, 22: 117-126.
- Kala, R.G. Abraham, V. Sobha, S. Jayasree, P.K., Suni, A.M. and Thulaseedharan, A. 2012a. *Agrobacterium* mediated genetic transformation and somatic embryogenesis from leaf callus of *Hevea brasiliensis*: effect of silver nitrate. In: *Prospects in Bioscience: Addressing the Issues*, Eds. Sabu A. and Augustine A., Springer, India, pp.303-315.
- Kala, R.G., Anju, V., Sobha, S., Jayasree, P.K., Suni, A.M. and Thulaseedharan, A. 2012b. Genetic transformation in *Hevea brasiliensis*: Effect of *Agrobacterium* strain, surfactant and antioxidants. In: *Proc. National Seminar “GGES”*, Periyar University, Salem 27th - 28th Feb. 2012 , pp. 54.
- Khanna, H.K. and Raina, S.K., 1999. *Agrobacterium* mediated transformation of indica rice cultivars using binary and super binary vectors. *Aus. J. Pl. Phy.*, 26: 311-324.
- Kordestani, G.K. and Karami, O., 2008. Picloram-induced somatic embryogenesis in leaves of strawberry (*Fragaria ananassa* L.). *Acta Biologica Cracoviensia Series Botanica*, 50: 69–72.
- Kumari Jayasree, P., Asokan, M.P., Sobha, S., Sankari Ammal, L., Rekha, K., Kala, R.G., Jayasree, R. and Thulaseedharan, A. 1999. Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* (Muell. Arg). *Curr. Sci.* 76: 1242-1245.
- Mazumdar, P., Basu, A., Paul, A., Mahanta, C. and Sahoo, L., 2010. Age and orientation of the cotyledonary leaf explants determine the efficiency of *de novo* plant regeneration and *Agrobacterium tumefaciens* mediated transformation in *Jatropha curcas* L. *South African J. Bot.*, 76: 337–344.
- Mendoza, M.G. and Kaeppler, H.F., 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). *In Vitro Cell. Dev. Biol. Plant*, 38: 39–45.
- Miguel, C. and Oliveira, M.M., 1999. Transgenic almond (*Prunus dulcis* Mill.) plants obtained by

- Agrobacterium*-mediated transformation of leaf explants. Plant Cell Rep., 18: 187-193.
- Montoro, P., Rattana, W., Pujade-Renaud, V., Michaux-Ferriere, N., Monkol-sook, Y., Kanthapura, R. and Adunsadthapong, S. 2003. Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. Pl. Cell Rep., 21: 1095-1102.
- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.
- Murata, N., Nishizawa, I.O., Higashi, H., Tasaka, Y. and Nishida, I., 1992. Genetically engineered alteration in the chilling sensitivity of plants. Nature, 356: 710-713.
- Rao, M.V.R and Rao, G.J.N., 2007. *Agrobacterium* mediated transformation of *indica rice* under acetosyringone-free conditions. Pl. Biotech., 24: 507-511.
- Rekha, K., Jayashree, R., Jayasree, P.K., Venkatachalam, P., Jinu, C. and Thulaseedharan, A., 2006. An efficient protocol for *Agrobacterium* mediated genetic transformation in rubber tree (*Hevea brasiliensis*). Pl. Cell Biotech. Mol. Biol., 7 : 155-158.
- Satchel, S. E., Messens, E., Montague, M.V. and Zambryski, P. 1985. Identification of signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature. 318: 624-629.
- Schaart, J.G., Pute, K. J., Kolova, L., Pogrebnyak, N., 1995. Some methodological aspects of apple transformation. Euphytica, 85: 131-134.
- Sobha, S., Sushamakumari, S., Thanseem, I., Jayasree, P., Rekha, K., Jayashree, R., Kala, R.G., Asokan, M.P., Dandekar, A.M. and Thulaseedharan, A. 2003. Genetic transformation of *Hevea brasiliensis* with the gene coding for superoxide dismutase with FMV 34S promoter. Curr. Sci., 85: 1767 – 1773.
- Sobha, S., Sushama Kumari, S., Thanseem, I., Rekha, K., Jayashree, R., Kala, R.G., Kumari Jayasree, P., Asokan, M. P., Sethuraj, M. R., Dandekar, A. M. and Thulaseedharan, A. 2003a. Abiotic stress induced over-expression of superoxide dismutase enzyme in transgenic *Hevea brasiliensis*. Ind. J. Nat. Rub. Res., 16: 45-52.
- Sobha, S., Reshma, R.S., Sushama kumari, S., Rekha, K., Jayashree, R., Kala, R.G., Jayasree, P., Supriya, R., and Thulaseedharan, A. 2010. *Agrobacterium* mediated genetic transformation of *Hevea brasiliensis*: Effect of acetosyringone and pre-culture of the target tissue on transformation efficiency. In: Abstracts 19th Symposium on Plantation Crops: Climate change and Plantation Crops, Rubber Research Institute of India, Kottayam. 7-10 December, pp 75-76.
- Sushamakumari, S., Sobha, S., Rekha, K., Jayasree, R. and Asokan, M.P. 2000. Influence of growth regulators and sucrose on somatic embryogenesis from immature inflorescence of *Hevea brasiliensis* (Muell. Arg.). Ind. J. Nat. Rub. Res., 13: 19-29.
- Sushamakumari, S., Sobha, S., Rekha, K., Jayashree, R. and Thulaseedharan, A., 2006. Plant regeneration via somatic embryogenesis from root explants in *Hevea brasiliensis*. In: Abstracts International Symposium on Frontiers in Genetics and Biotechnology- Retrospect and Prospect, Jan 8-10, Osmania University, Hyderabad, India, pp- 162.
- Sushamakumari, S., Kala, R.G., Sobha, S., Rekha, K., Jayashree, R. and Thulaseedharan, A., 2010. Regeneration and acclimatization of somatic plants from *in vitro* derived leaf explants of *Hevea brasiliensis*. In: Abstracts 19th Symposium on Plantation Crops: Climate change and Plantation Crops, Rubber Research Institute of India, Kottayam. 7-10, December, pp 74.
- Tsai, J., Podila, G.K. and Chiang, V.L. 1994. *Agrobacterium*-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants. Plant Cell Rep., 94-97.