ROLE OF BACTERIAL STRAIN IN DETERMINING THE EFFICIENCY OF GENETIC TRANSFORMATION IN *HEVEA BRASILIENSIS*

R. Jayashree, K. Rekha, P. Venkatachalam*, S. Sushamakumari, S. Sobha, M. Vineetha, A.M Suni, P. Leda, A. Thulaseedharan and P.A. Nazeem**

Rubber Research Institute of India, Rubber Board, Kottayam-686009, Kerala, India
*Department of Biotechnology, Periyar University, Salem-636011, Tamil Nadu, India
**Department of Bioinformatics, Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University,Vellanikkara- 680656, Kerala, India

Received: 16 May 2013 Accepted: 04 November 2013

Jayashree, R., Rekha, K., Venkatachalam, P., Sushamakumari, S., Sobha, S., Vineetha, M., Suni, A.M., Leda, P., Thulaseedharan, A. and Nazeem, P.A. (2013). Role of bacterial strain in determining the efficiency of genetic transformation in *Hevea brasiliensis*. *Rubber Science*, **26**(2): 217-227.

Genetic transformation offers a viable approach in *Hevea brasiliensis* for crop improvement by adding valuable genes for specific characters in a relatively short period. 3-Hydroxy 3-methylglutaryl Coenzyme A reductase (HMGR) is considered as a key enzyme in the rubber biosynthetic pathway. With a view to increase the yield potential in *Hevea*, attempts were made to over-express this rate limiting enzyme by *Agrobacterium tumefaciens* mediated genetic transformation. Selection of an appropriate bacterial strain is one of the key parameters required for an efficient genetic transformation system. To identify a suitable bacterial strain giving maximum transformation efficiency with the *hmgr*1 gene in *Hevea*, three *Agrobacterium* strains (EHA 105, LBA4404 and pGV 1301) harbouring the transgene were experimented. The binary vector contained hygromycin phosphotransferase gene (*hpt*) as the plant selectable marker. The effect of different explants (primary callus, embryogenic callus and embryogenic suspension cultures) as well as co-cultivation temperature on transformation efficiency was evaluated. Highest transformation efficiency was observed with the strain EHA 105, irrespective of the target tissues tried for transformation. Low temperature incubation (20 ^{o}C) of the infected tissues during co-cultivation period improved the frequency of transformation. Among the different target tissues tried, embryogenic suspension cultures gave the maximum number of transgenic cell lines (32%). The presence of the transgene was confirmed in the transgenic cell lines by PCR using gene specific primers.

Keywords: *Agrobacterium tumefaciens*, Embryogenic suspension cultures, Genetic transformation

INTRODUCTION

The ultimate objective of breeding in natural rubber includes the development of superior clones with increased dry rubber yield. As *Hevea* clones cultivated in the south Asian countries originated from a small number of plant sources collected by Wickham, the genetic diversity is rather limited (Dijkman, 1951). Controlled hybridization allowed an increase in the latex yield of recommended clones to 2000 to 3000 kg/ha^{-1}yr^{-1} during 1980's (Omokhafe

Correspondence: Jayashree R. (Email: radha_jayasree@rediffmail.com)

and Ugwa, 1997). Latex yield in *Hevea* is a clonal character (Jacob *et al*., 1989), controlled by polygenes, and therefore the selection of parental clones with dominant genes for this trait is highly essential (Omokhafe and Nasiru, 2010). The increasing global demand for natural rubber has resulted in the development of *in vitro* techniques for crop improvement. Hence, biotechnological tools can be applied in parallel with the conventional breeding technologies for improving *Hevea* clones. Rubber breeding efficiency can be increased through biotechnological approaches, by transferring genes from any organism to the existing cultivar thereby accelerating the available gene pool for crop improvement. Tissue culture techniques combined with molecular biology strategies might help in improving the clones by incorporating specific traits by gene transfer. Genetic transformation attempts the transfer of selected genes in a single generation which is difficult through conventional breeding due to the heterozygous nature of the crop and the long juvenile phase which includes six to seven years before latex harvesting.

Natural rubber (*cis*-1, 4-polyisoprene) is a plant isoprenoid produced in the milky cytoplasm (latex) of specialized cells called laticifers. Laticifers are arranged as concentric sheaths in the phloem. Natural rubber is synthesized generally by the mevalonate pathway (MVA) with isopentenyl pyrophosophate (IPP) as the monomeric subunit. The renewed interest in the rubber biosynthesis and its pathway genes can be attributed to the diminishing acerage of rubber plantations coupled with the increasing demand of natural rubber. The enzyme 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the first step in isoprenoid biosynthesis, *i.e.,* the synthesis of mevalonate from HMG-CoA. This enzyme is considered to play a rate-limiting role in plant isoprenoid biosynthesis. In *Hevea*, HMGR comprise of a small gene family with three members namely *hmgr*1, *hmgr*2 and *hmgr*3 (Chye *et al*., 1991; 1992). It has been reported that *hmgr*1 is involved in rubber biosynthesis, *hmgr*2 with the defence responses induced by pathogens and wounding whereas *hmgr*3 is of a housekeeping nature (Chye *et al*., 1992). Studies in guayule showed a synchronized increase in the rubber formation during winter season with an increase in the HMGR activity of the plant (Ji *et al*., 1993). A positive correlation between hydroxymethyglutaryl coenzyme A synthase (HMGS) and *hmgr*1 mRNAs with the dry rubber content also proved the importance of this gene in the rubber biosynthetic pathway (Suwanmanee *et al*., 2007). Therefore, the over expression of this key rubber biosynthetic gene in transgenic *Hevea* plants through *Agrobacterium* mediated transformation can result in an enhanced latex yield.

Agrobacterium mediated genetic transformation has remarkable advantages over direct transformation methods including preferential integration of defined T-DNA into transcriptionally active regions of the chromosome (Czernilofsky *et al.,* 1986; Koncz *et al*., 1989; Le *et al*., 2001; Olhoft *et al*., 2004). The transfer of T-DNA and its integration into the plant genome is influenced by many factors, one among them is the bacterial strain used in the transformation experiments (Montoro *et al*., 2000; Suzuki *et al*., 2001; Gelvin *et al*., 2003; Khanna *et al*., 2004; Wang *et al*., 2006). The type and texture of explant, antibiotic resistance gene in the binary vector and nature of the promoter which drives the transgene expression are other important determinants influencing the efficiency of transformation and further regeneration of

the transgenic tissues. The goal of this experiment was to identify a suitable *Agrobacterium* strain and optimum culture conditions giving successful genetic transformation in *Hevea brasiliensis* using different types of explants.

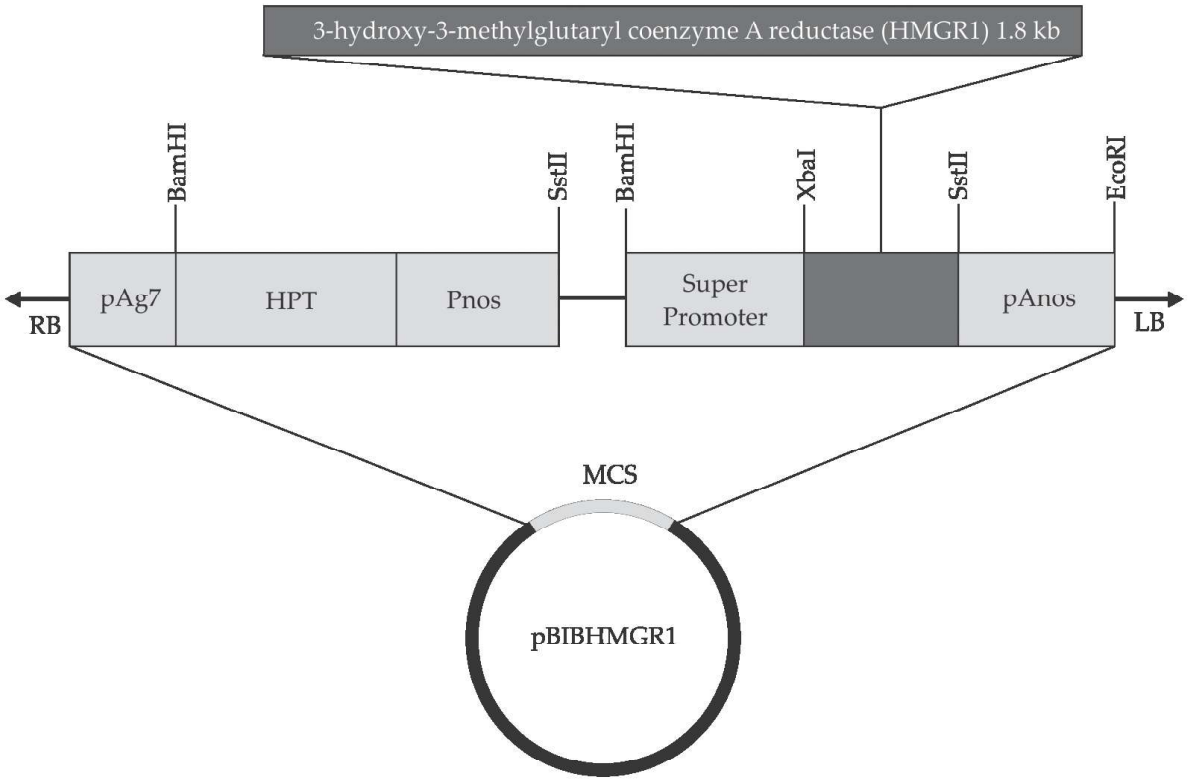
MATERIALS AND METHODS

Agrobacterium strain

Three different bacterial strains, namely LBA 4404, EHA 105 and p GV 1301, were used in the genetic transformation experiments. The *Agrobacterium tumefaciens* strain EHA 105, is a *vir*-helper, L-succinamopine type and a Km (S) derivative of EHA 101 (Rm^r) (Hood *et al*., 1993), whereas LBA 4404 is a *vir*-helper, octopine type which harbors the disarmed Ti plasmid pAL 4404, a T-DNA deletion derivative of pTiAch 5, (Ach5 pTiAch5) Sm/Sp (R) in the virulence plasmid (from Tn 904) (Ooms *et al*., 1982). The third one, pGV 1301 has a cured Ti plasmid, belonging to the nopaline type of strain, genotype C58. These three bacterial strains were successfully used in the genetic transformation of various crop plants. All the three bacterial strains harbouring the plasmid vector pBIB were maintained as glycerol stock (70%; v/v) at -80 ^{0}C for long-term storage.

Plasmid vector

The plasmid vector used was pBIB *hmgr*1 developed by Venkatachalam *et al*. (2009), where the full-length *hmgr*1 cDNA isolated from *Hevea* tree by a PCR based approach was used. The *hmgr*1 cDNA insert



RB - Right border; LB - Left border; HPT - Hygromycin; MCS - Multiple cloning site; nos - Nopaline synthase

Fig.1. The T-DNA map of pBIB *hmgr*1 binary vector

was placed between the super promoter and nos terminator elements of the binary plant transformation vector pBIB. The binary vector contained *hpt* gene as the selectable marker gene conferring tolerance to the antibiotic hygromycin. The resulting plasmid was designated as pBIB *hmgr*1 and the gene fusion details are shown in Fig. 1.

Preparation of *Agrobacterium* culture

The different *Agrobacterium* strains containing the plasmid vector was taken from the frozen glycerol stock and plated into LB (Luria-Bertani) medium containing antibiotic, 50 mg L^{-1} Kanamycin and 20 mg L^{-1} Rifampicin for bacterial selection. The culture plates were incubated at 28 °C for two days. The individual colonies formed in the culture plates were screened for the presence of the insert using colony PCR with gene specific primers. Colonies containing the inserts were grown in liquid LB medium containing antibiotics in an incubator shaker kept at 28 °C and 220 rpm. The bacterial cells were pelleted by centrifugation at 3000 Xg for 10 min and resuspended in the induction medium to get a density of 10^8 cells $mL^{-1.}$

Antibiotic sensitivity

The hygromycin phosphotransferase gene (*hpt*) conferring resistance to the antibiotic hygromycin was used for plant selection. A kill curve experiment was carried out using this selection antibiotic, by exposing the fresh anther derived callus to different levels of hygromycin (10 - 40 mg L^{-1}). The basal medium was autoclaved and cooled to 50 °C prior to the addition of hygromycin. Ten callus clumps were cultured per plate, replicated five times and the cultures were kept in dark. The response of the calli on exposure to the antibiotic was scored after one month of culture. The concentration of hygromycin at which 100 per cent of the control calli got dried up was chosen as the optimum one for selection.

Plant material

Explants used for genetic transformation include primary callus, embryogenic callus and embryogenic suspension cultures derived from immature anthers of *H. brasiliensis,* clone RRII 105.

Callus induction from anther tissue

The immature flowers of *H. brasiliensis*, clone RRII 105 was collected, surface sterilized with 0.15 per cent $HgCl_2$ for five to seven min. After thorough washing with sterile water, the anthers were dissected out and inoculated in the callus induction medium (Kumari Jayasree *et al*., 1999).

Embryogenic culture initiation

The basal medium used for embryogenic callus formation include modified MS fortified with growth regulators (0.1 mg L^{-1} 2,4-D; 0.2 mg L^{-1} BA; 0.5 mg L^{-1} GA3) and sucrose (60 g L^{-1}).

Initiation of cell suspensions from embryogenic cell aggregates

The embryogenic calli derived from the explants were transferred to 100 mL Erlenmeyer flask containing 25 mL ½ X MS basal medium supplemented with growth regulators along with sucrose (Jayashree *et al*., 2003). The suspension cultures were maintained at 25 °C under dark on an orbital shaker at 120 rpm. The suspension medium was replaced with fresh media at weekly intervals. One to five day old newly subcultured embryogenic cell suspensions were used for *Agrobacterium* infection.

Plant transformation protocol

The genetic transformation was performed according to the published protocol (Jayashree *et al*., 2003). The transformants were selected in the screening medium (SM), MS basal medium containing cefotaxime (500 mg L^{-1}) and hygromycin (the optimum concentration from the kill curve). The antibiotic cefotaxime (500 mg L^{-1}) was added to prevent bacterial overgrowth. The selection media contained a growth regulator combination of 2, 4-D (0.2 mg L^{-1}), Kin (0.5 mg L^{-1}) and BA (0.5 mg L^{-1}). The petri plates were sealed with parafilm and incubated in the dark at 25 °C. The infected calli were transferred to fresh selection media at monthly intervals until hygromycin resistant transgenic calli emerged from the cultures.

The frequency of transformation was assessed as,

$$\frac{\text{No. of transgenic cell lines emerged}}{\text{Total no. of callus clumps cultured}} \times 100$$

The resistant cell lines were sub cultured for proliferation and the transgene integration was confirmed by PCR analysis.

Improving transformation efficiency: Alteration in the incubation temperature

The *Agrobacterium* infected tissues were subjected to varying temperature regimes such as 4, 20 and 28 °C during co-cultivation. The incubation was continued for three days. After the period of incubation, they were transferred to the selection medium and dark incubated. Observations were recorded after fifty days of culture.

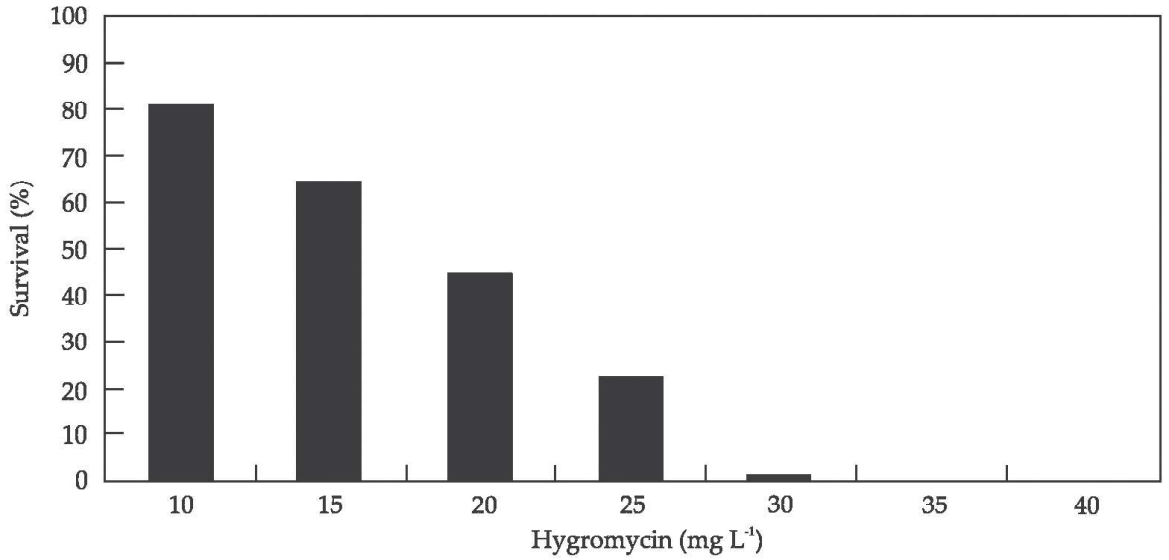
Molecular characterization of the transgenic callus

The genomic DNA was extracted from the transformed as well as untransformed control callus according to the CTAB method (Doyle and Doyle, 1990). PCR analysis was carried out using gene specific primers designed to amplify the *hmgr*1 gene fragment of approximately 640 bp. The forward and reverse primers for amplifying the *hmgr*-1 gene were 5′- CGCGTCGGCGACTAGAGCC-3′ and 5′- GCAAGTTGAGTTCCACCTC-3′, respectively. Plasmid DNA was used as a positive control whereas DNA from the untransformed calli served as the negative control. PCR reactions were carried out using 50 ng templates, 100 mM each of dATP, dGTP, dTTP and dCTP, 250 nM of each primer, 0.5 µl *Taq* DNA polymerase and 1.5 mM $MgCl_2$ in a final volume of 20 µl. The reaction mixture was incubated in a thermal cycler under the following reaction conditions. The initial denaturation step was carried out at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, with a final extension step of 72 °C for 7 min. The amplified PCR products were visualized on a 1.2 per cent agarose gel stained with ethidium bromide using 0.5 x TBE as the running buffer (Sambrook *et al*., 1989).

RESULTS AND DISCUSSION

Antibiotic sensitivity

The result of the kill curve experiment was summarized in Figure 2. As the concentration of the antibiotic increased, the percentage survival of the cells decreased. The callus was sub-cultured at an interval of 15 to 20 days, since the activity of the antibiotic is expected to perish after this period. The concentration at which proliferation of the callus was totally inhibited with the clumps getting dried up was selected as the optimum concentration for screening. The minimum concentration of hygromycin necessary for



The values given in the X-axis represent the concentration of hygromycin (10 – 40 mg L^{-1}) used in kill curve experiment and those given in the Y-axis represent the rate of survival of the callus on exposure to hygromycin.

Fig. 2. Sensitivity of *Hevea* callus at different levels of hygromycin

the selection was 30 mg L^{-1}, where the texture of the callus changed from brown to white and the calli dried up after one month. Effective selection of the transformants was possible at this concentration preventing any escapes. Parveez *et al*. (2007) used hygromycin for plant selection in castor and was found to be more efficient than kanamycin. Hygromycin has also been successfully used in monocots including rice, maize, sorghum *etc*. for selection as well as plant regeneration. In oil palm, it was noticed that a slightly higher concentration of hygromycin (50 mg L^{-1}) was essential for effective selection (Sujatha and Sailaja, 2005).

Transformation efficiency: influence of the bacterial strain and the target tissue

The results show the importance of the bacterial strain in determining the transformation efficiency in *Hevea*. The type and texture of the explant is another factor contributing towards efficient transformation. The succinamopine type of strain, EHA 105, gave the highest transformation efficiency with each kind of explant used. The variations in the efficiency

Table 1. **Influence of *Agrobacterium* strain and explant type on transformation efficiency (%) in *H. brasiliensis***

Explant	Bacterial strain		
	EHA 105	pGV 1301	LBA4404
Primary callus	9.0(2.99)	6.80 (2.56)	—
Embryogenic callus	15.2(3.88)	8.0 (2.79)	—
Embryogenic suspension cultures	27 (5.19)	16 (3.99)	—
CD (P = 0.05)	0.32	0.42	

Twenty-five callus clumps were used per treatment and each treatment contained four replications. The values given in parenthesis are the transformed values

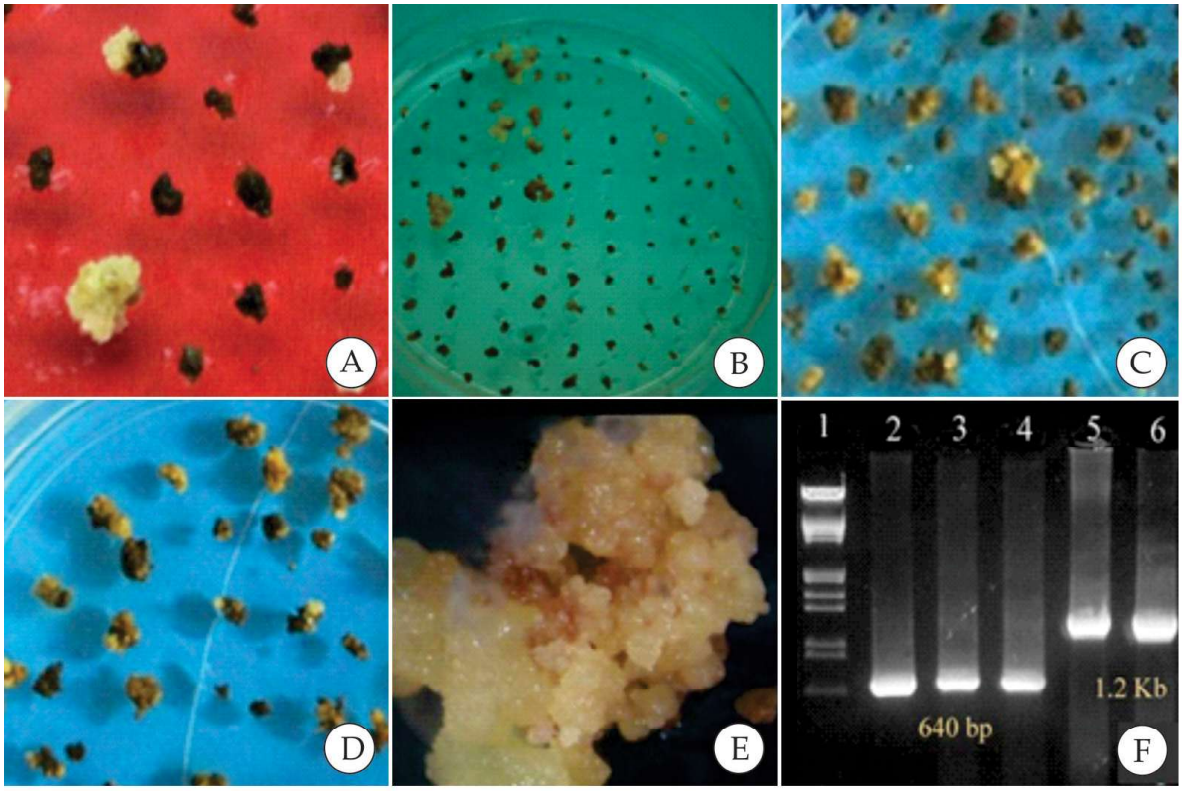


Fig. 3. Emergence of transgenic cell lines from different explant types and their PCR analysis
A Transgenic cell lines from the primary callus
B Transgenic cell lines from embryogenic callus
C,D,E Emergence of cell lines from embryogenic suspension cultures
F PCR analysis of the transgenic cell lines, lane (1) Molecular weight marker (2) Positive control (3 & 4) Transgenic cell lines and (5 & 6) Non transgenic tissues

of transformation between the three tested *Agrobacterium* strains are shown in Table.1. The observations showed that the strain, EHA 105 gave a frequency of 9 per cent using primary callus (Fig. 3.A), 15 per cent with the embryogenic calli (Fig. 3.B) and 27 per cent using the three-day old embryogenic suspension cultures (Fig. 3.C, D, and E). Irrespective of the type of the tissues used for transformation, highest efficiency was obtained with the strain EHA 105 which was the best one.

Using the *Agrobacterium* strain pGV 1301, the frequency was lower with all the type of explants used. Genetic transformation using primary callus resulted in a frequency of 7 per cent which was slightly improved to 8 per cent using embryogenic callus. The low frequency of transformation with the primary callus may be due to the lack of friability and low phenol content. When the three-day-old embryogenic suspensions were used for infection, the frequency could be raised to 16 per cent. Depending on the friability of the initial explant, the frequency of transformation also varied, the most friable one giving highest number of transgenic cell lines. Suspension cultures were attempted earlier by many researchers on less responsive genotypes due to their beneficial effect. Similar observations were noticed in

rice where a higher frequency of transformation (60-times higher than the control) was obtained using three-day-old suspension cultures (Ozawa and Takaiwa, 2010). It was reported a higher frequency of transformation using suspension cultures in tobacco was reported (An 1985) and this protocol was further elaborated and used in *Arabidopsis* where stably transformed cultures were recovered (Forreiter *et al*., 1997). In sweet potato cultivars, a successful transformation and plant regeneration system was developed from suspension cultures (Xing *et al*., 2008). Recently in recalcitrant citrus cultivars, a good transformation system was established using the embryogenic cell suspensions obtained from unfertilized ovules (Dutt and Grosser, 2010).

The strain, LBA4404 which was the least virulent one responded poorly towards transformation. Transgenic cell development was absent from the cultures infected with this strain. According to Jones *et al*. (2005), the strain LBA 4404 was considered to be weak when it carries the native binary vector**.** The efficiency of a strain or the host range of a particular strain can be attributed to the interactions of the Ti plasmid with certain bacterial chromosomal background (Gelvin, 2003). Generally, *Agrobacterium* strains differ in their ability to infect and transfer their T-DNA (Suzuki *et al*., 2001; Khanna *et al*., 2004). In cauliflower, a high level GUS expression was observed with the strain GV2260, while co-cultivation with LBA4404 gave a very low expression of the integrated gene (Chakrabarty *et al*., 2002). The transformation experiments performed in *Vitis vinifera* also confirmed the superiority of the virulent strain EHA 105 over the three others tried (Torregrosa *et al*., 2002). Thus in recalcitrant crops, the frequency of transformation are determined by the virulence of the *Agrobacterium* strain or by the use of super-binary vectors (de la Riva *et al*., 1998). The transgenic cell lines obtained from different cultures were separated and proliferated.

After identifying the best strain and the suitable explant giving higher rate of transformation with *hmgr*1 gene in *Hevea*, the effect of incubation temperature on transformation frequency was also monitored. Among the different temperatures tried, incubation at 20 °C was most appropriate for the emergence of transgenic cell lines (Table 2). The infected callus remained yellow after two sub-cultures and putatively transgenic cell lines were obtained after 50 days of culture (32%). Incubation at 4 °C resulted in bacterial overgrowth free cultures but the infected calli turned white in the co-culture medium. New cell emergence was also absent from these cultures. Co-cultivation of the transgenic callus at 28 °C resulted in a frequency of 27 per cent, which was the control treatment. Considering these parameters, it can be stated that genetic transformation using the *hmgr*1 gene was more effective at 20 °C, leading to production of new transformed cell lines. In an earlier study transgenic cell emergence in *Hevea,* was noticed at an efficiency of 4 per cent, using the infected primary callus of anther, when the co-cultivation was carried

Table 2. **Effect of co-cultivation temperature on transformation efficiency**

Treatment	Transformation efficiency (%)
28 °C	26.8 (5.23)
20 °C	32 (5.74)
4 °C	— (1.0)
CD (P = 0.05)	0.33

Each treatment contained twenty five callus clumps replicated five times. Analysis was carried out using square root transformation and the transformed values were given in parenthesis

out at 28 °C (Jayashree *et al*., 2003). A different gene construct (*HbSOD)* was used for transformation in this study and low temperature incubation was also not attempted. In maize, a higher transformation frequency was reported when the co-culturing was performed at 20 °C (Frame *et al*., 2002). Low temperature incubation (19 °C) also improved the transformation efficiency in cotton (Jin *et al*., 2005) which was in agreement with our results. A possible explanation for the favourable effect of lower incubation temperature on transformation frequency may be the temperature lability of the pilus. According to Gelvin (2003), although *vir* gene induction is maximal at approximately 25 to 27 °C, the pilus of some *Agrobacterium* strains are most stable at lower incubation temperatures (approximately 18-20 °C). Therefore, co-cultivating *Agrobacterium* cells with the plant tissues at lower incubation temperatures could augment the efficiency of transformation.

DNA was extracted from the transformed as well as non-transgenic cell lines. Primers were designed to amplify a DNA fragment of approximately 640 bp with the *hmgr*1 gene specific forward and reverse primers. Positive amplification was observed in the transgenic lines whereas the corresponding band was absent in the non-transformed ones. The native *hmgr*1 gene (1.2 kb) was amplified in the non-transgenic tissues using the gene specific primers. The presence of the integrated *hmgr*1 transgene was confirmed in all the transgenic cell lines and the positive control (plasmid DNA), where a DNA fragment of 640 bp was amplified (Fig. 3.F).

CONCLUSION

The importance of the bacterial strain in determining the transformation efficiency with the *hmgr* 1 gene in *Hevea brasiliensis* was evaluated. Three *Agrobacterium* strains were tested in the present study where the superiority of the strain EHA 105 was highlighted. The efficiency of this strain remain unchanged with every type of explant used. The embryogenic suspension culture proved to be the most responsive target tissue for genetic transformation. Low temperature incubation (20 °C) during co cultivation improved the frequency of transformation. The transgene integration was confirmed in the cell lines by PCR analysis using gene specific primers. The PCR confirmed transgenic cell lines were proliferated and subsequently cultured for embryogenesis. The method optimized in the present study could be utilized for *Hevea* genetic transformation more efficiently with desirable trait coding genes.

REFERENCES

An, G. (1985). High efficiency transformation of cultured tobacco cells. *Plant Physiology*, **79**: 568-570.

Chakrabarty, R., Viswakarma, N., Bhat, S.R., Kirti P.B., Singh, B.D. and Chopra,V.L. (2002). *Agrobacterium*-mediated transformation of cauliflower: Optimization of protocol and development of Bt-transgenic cauliflower. *Journal of Biosciences*, **27**(5): 495-502.

Chye, M.L., Kush, A., Tan, C.T. and Chua, N.H. (1991). Characterization of cDNA and genomic clones encoding 3-hydroxy-3-methylglutaryl Coenzyme A reductase from *Hevea brasiliensis*. *Plant Molecular Biology*, **16**: 567-577.

Chye, M.L., Tan, C.T. and Chua, M.N. (1992). Three genes encode 3-hydroxy-3-methylglutaryl Coenzyme A reductase in *Hevea brasiliensis*: *hmg*1 and *hmg*3 are differentially expressed. *Plant Molecular Biology*, **19**: 473-484.

Czernilofsky, A.P., Hain, R., Baker, B. and Wirtz, U. (1986). Studies of the structure and functional

organization of foreign DNA integrated into the genome of *Nicotiana tabacum DNA,* **5**: 473-478.

De la Riva, G.A., Gonzalez-Cabrera, J. Vazquez-Padron, R. and Ayra-Pardo, C. (1998). *Agrobacterium tumefaciens*: A natural tool for plant transformation. *Electron. Journal of Biotechnology,* **1**: 1–16.

Dijkman, M.J. (1951). *Hevea*: *Thirty years of research in the Far East*. University of Miami Press, Florida, 5–7.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of DNA from small amount of plant tissues. *Focus,* **12**: 13-15.

Dutt, M. and Grosser, J.W. (2010). An embryogenic suspension cell culture system for *Agrobacterium*-mediated transformation of Citrus. *Plant Cell Reports*, **29**: 1251-1260.

Forreiter, C., Kirschner, M. and Nover, L. (1997). Stable transformation of an arabidopsis cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity in *vivo*. *The Plant Cell,* **9**: 2171-2181.

Frame, B.R., Shou, H., Chikwamba, R.K., Zhang, Z., Xiang, C., Fonger, T.M., Pegg, S.E.K., Li, B., Nettleton, D.S., Pei, D. and Wang, K. (2002). *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. *Plant Physiology,* **129**: 113-122.

Gelvin, S.B. (2003). *Agrobacterium*- mediated plant transformation: the biology behind the "gene jockeying" tool. *Microbiology and Molecular Biology Reviews,* 16-37.

Hood, E.E., Gelvin, S.B., Melchers, L.S. and Hoekema, A. (1993). New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research,* **2**: 208–218.

Jacob, J.L., Pre´voˆ t. J.C. and Roussel, D. (1989). *Hevea brasiliensis* yield-limiting factors, latex physiological parameters, latex diagnosis and clonal typology. In: *Physiology of rubber tree latex*. (Eds) d'Auzac J, Jacob J.L., Chrestin H. Boca Raton, FL: CRC Press Inc. pp. 345–382.

Jayashree, R., Rekha, K., Venkatachalam, P., Uratsu, S.L., Dandekar, A.M., Kumari Jayasree, P., 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. *Plant Cell Reports,* **22**: 201-209.

Jones, H. D., Doherty, A. and Wu, H. (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated genetic transformation of wheat. *Plant Methods,* **1**: 5.

Ji, W., Benedict, C.R. and Foster, M.A. (1993). Seasonal variations in rubber biosynthesis, 3-Hydroxy-3- Methylglutaryl-Coenzyme A reductase, and rubber transferase activities in *Parthenium argentatum* in the Chihuahuan desert. *Plant Physiology*, 1**03**: 535-542.

Jin, S., Zhang, X., Liang, S., Nie, Y., Guo, X. and Huang, C. (2005). Factors affecting transformation efficiency of embryogenic callus of Upland cotton (*Gossypium hirsutum*) with *Agrobacterium tumefaciens. Plant Cell Tissue and Organ Culture,* **81**(2): 229-237.

Khanna, H., Becker, D., Kleidon, J. and Dale, J. (2004). Centrifugation assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. 'Cavendish' AAA and 'Lady finger' AAB). *Molecular Breeding,* **14:** 239-252.

Koncz, C., Martini, N., Mayerhofer, R., Koncz-kalman, Z., Korber, H., Redei, G.P. and Schell, J. (1989). High frequency T-DNA- mediated gene tagging in plants. *Proceedings of the National Academy of Science of the United States of America*, **86**(21): 8467-8471.

Kumari Jayasree, P., Asokan, M.P., Sobha, S., Sankariammal, L., Rekha, K., Kala, R.G., Jayasree, R. and Thulaseedharan, A. (1999). Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis*. *Current Science*, **76**(9): 1242-1245.

Le, V.Q., Belles-Isles, J., Dusabenyagusani, M. and Tremblay, F.M. (2001). An important procedure for production of white pruce (*Picea glauca*) transgenic plants using *Agrobacterium tumefaciens*. *Journal of Experimental Botany,* **52**: 289-300.

Montoro, P., Tcinscree, N., Rattana, W., Kongsawadworakul, P. and Michaux-Ferriere, N. (2000). Effect of exogenous calcium on *Agrobacterium tumefaciens* mediated gene transfer in *Hevea brasiliensis* (rubber tree) friable calli. *Plant Cell Reports,* **19**: 851-855.

Olhoft, P.M., Flaye, L.E. and Somers, D.A. (2004). T-DNA locus structure in a large population of soybean plant transform using the *Agrobacterium*-mediated cotyledonary node methods. *Plant Biotechnology Journal,* **2**: 289-300.

Omokhafe, K.O. and Ugwa, I.K. (1997). Breeding and selection for high latex yield in *Hevea brasiliensis. Thirty sixth Annual Conference of Science Association of Nigeria*, 7–11 April 1997. Delta State University, Abraka.

Omokhafe, K.O. and Nasiru, I. (2010). Polygene inheritance of latex yield in *Hevea brasiliensis* Muell.Arg. *Plant Genetic Resources Newsletter,* **140:** 48-50.

Ooms, G., Bakker, A., Molendijk, L., Wullems, G. J., Gordon, M. P., Nester, E. W. and Schilperoort, R.A. (1982). T-DNA organization in homogeneous and heterogeneous octopine-type crown gall tissues of *Nicotiana tabacum*. *Cell,* **30**: 589–597.

Ozawa, K. and Takaiwa, F. (2010). Highly efficient *Agrobacterium*-mediated transformation of suspension-cultured cell clusters of rice (*Oryza sativa* L.). *Plant Science,* **179**(4): 333-337.

Parveez, G. K. A., Majid, N.A., Zainal, A. and Rasid. O. (2007). Determination of minimal inhibitory concentration of selection agent for selecting transformed immature embryos of oil palm. Asia Pacific. *Journal of Molecular biology and Biotechnology*, **15**(3): 133-146.

Sambrook J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork.

Sujatha, M. and Sailaja, M. (2005). Stable genetic transformation of castor (*Ricinus communis* L.) *via., Agrobacterium tumefaciens*-mediated gene transfer using embryo axes from mature seeds. *Plant Cell Reports*, **23**: 803-810.

Suwanmanee, P., Sirinupong, N., Nunthanuwat, W., Chatree, T., Chocknukul, S., Seedungnu, N,Y. and Suwachitanont, W. (2007). Expression of HMG-CoA synthase (*hmgs*) and HMG-CoA reductase-1 (*hmgr*-1) reveal coordinated regulation of rubber biosynthesis in *Hevea brasiliensis* (B.H.K.) Mull. Arg. *TERPNET* . April 30 – May 4, 2007 Strasbourg, France.

Suzuki, K., Iwata, K. and Yoshida, K. (2001). Genome analysis of *Agrobacterium tumefaciens*: construction of physical maps for linear and circular chromosomal DNAs, determination of copy number ratio and mapping of chromosomal virulence genes. *DNA Research,* **8**: 141–152.

Torregrosa, L., Iocco, P. and Thomas, M. R. (2002). Influence of *Agrobacterium* strain, culture medium, and cultivar on the transformation efficiency of *Vitis vinifera* L. *American Journal of Ecology and Viticulture,* **53**: 183-190

Venkatachalam, P., Priya, P., Jayashree, R., Rekha, K. and Thulaseedharan, A. (2009). Molecular cloning and characterization of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (*hmgr*1) gene from rubber tree (*Hevea brasiliensis* Muell. Arg.): A key gene involved in isoprenoid biosynthesis. *Physiology Molecular Biology Plants*, **15**(2): 133-143.

Wang, T., Atkinson, R. and Janssen, B. (2006).The choice of *Agrobacterium* strain for transformation of kiwifruit ISHS. *Acta Horticulturae*. **753**: VI International Symposium on Kiwifruit.

Xing, Y., Ji1, Q., Yang, Q., Luo1, Y., Li, Q. and Wang.X. (2008). Studies on *Agrobacterium*-mediated genetic transformation of embryogenic suspension cultures of sweet potato. *African Journal of Biotechnology,* **7**(5): 534-540.