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FACTORS PROMOTING *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION EFFICIENCY IN *HEVEA BRASILIENSIS*

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In *Hevea brasiliensis,* since conventional methods of crop improvement are elaborate and time consuming, genetic transformation offers a viable approach to breeders for adding desirable traits in a relatively short period. Although, genetic transformation experiments in *Hevea brasiliensis* were initiated two decades ago, reports on the development of transgenic plants incorporated with genes coding for agronomically important traits is limited mainly due to the low efficiency of transformation and plant regeneration protocol in *Hevea brasiliensis*. Therefore the transformation and plant regeneration protocol needs further modifications for developing an efficient and reproducible transgenic plant regeneration system. In the present work, an attempt was made to identify the factors promoting *Agrobacterium* mediated genetic transformation frequency. The texture and friability of the target tissue (immature anther callus) was improved by pre-culturing it on medium containing varying concentrations of calcium nitrate (0-1200 mg/L). The transformation efficiency was significantly enhanced by using this soft friable callus as target tissue. The combined effect of callus pre-culture and acetosyringone concentration on transformation efficiency was also assessed by incorporating different concentrations of acetosyringone (0-200 mg/L) in the co-culture medium. The transformation frequency was increased from 6 to 14% by using the callus pre-cultured for fifteen days on medium containing 800 mg/L calcium nitrate and increasing the acetosyringone concentration to 80 mg/L in the co-culture medium. The transformation event was confirmed by PCR analysis with MnSOD and *npt*II gene specific primers.

Keywords: Acetosyringone, Callus pre-culture, Genetic transformation, Transformation frequency

INTRODUCTION

Increasing demand for natural rubber and the scarcity of land availability for rubber cultivation in the traditional rubber growing regions in India necessitate the expansion of rubber cultivation to the non-traditional areas, which are exposed to extreme climate such as cold, high temperature and high light intensities *etc*. Even though conventional breeding has achieved significant progress in improving the yield and secondary characters, genetic transformation has immense potential for crop improvement by transferring desirable genes within a short period.

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Among the different methods of gene transfer techniques available, *Agrobacterium tumefaciens* mediated genetic transformation is the most popular method used for foreign gene integration in *Hevea brasiliensis*. Transgenic plants integrated with manganese superoxide dismutase (MnSOD) gene were produced in *H. brasiliensis* clone RRII 105 using two month old anther callus with a transformation frequency of 6%. Since, a large number of transgenic plants from independent transformation events are required for the selection of plants with stable transgene expression, the transformation protocol reported earlier (Sobha *et al*., 2003; Jayashree *et al*., 2003) needs further modifications. In addition, there are several reports in other crops that the plant genetic transformation efficiency is highly influenced by the plant genotype, type of explant, texture of the target tissue, bacterial strain, addition of acetosyringone - a *vir*-gene inducing synthetic phenolic compound. Therefore, the type of explant and the textural characteristics of the target tissue should be taken into consideration before using it as target tissue for obtaining high frequency transformation. Hence, the present work was undertaken to increase the transformation efficiency in the *Hevea* clone, RRII 105, using anther callus by: 1) improving the texture of the target tissue by pre-culturing the callus on medium containing different concentrations of calcium nitrate and 2) amending the concentration of acetosyringone in the co-culture medium.

MATERIALS AND METHODS

Development of target tissue for *Agrobacterium* infection

The explant used for the present work was immature anther collected from 15-year-old *Hevea brasiliensis* trees (clone RRII 105) grown in the experimental fields of Rubber Research Institute of India. Callus was produced from the explant according to the protocol reported earlier (Jayasree *et al*.,

Table 1. **Composition of callus proliferation (CP) medium**

Ingredients	Concentration (mg/L)
Modified MS major salts	
NH_4NO_3	1000
KNO_3	1800
$CaCl_2$	333
$MgSO_4$	181
KH_2PO_4	170
$Ca(NO_3)_2.4H_2O$	0 - 1200
MS Minor salts	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2$	0.025
Fe Na EDTA	36.7
B5 vitamins	
Thiamine HCl.	10
Pyridoxine HCl.	1.00
Nicotinic acid	1.00
Myo-inositol	100
Casein hydrolyzate	400
Adenine	50
Sucrose	40
Coconut water	5%
Growth regulators	
2, 4-D	1.00
B A	0.50

1999). The target tissue for *Agrobacterium* infection was generated by subculturing the fresh anther callus on callus proliferation (CP) medium (Table 1). In order to determine the ideal concentration of calcium nitrate in producing soft friable callus, the CP medium was fortified with varying concentrations of calcium nitrate ranging from 0 to 1200 mg/L. The fresh anther callus was cultured over the proliferation medium for fifteen days and used for *Agrobacterium* infection.

Agrobacterium tumefaciens strain and binary vector

The *Agrobacterium tumefaciens* strain used for transformation was EHA101. The plasmid vector contained β-glucuronidase (*uid*A) as the reporter gene, neomycin phosphotransferase (*npt*II) as the plant selectable marker gene and *Hevea brasiliensis* manganese superoxide dismutase (*HbMnSOD*) gene under the control of Figwort Mosaic Virus (FMV) 34S promoter (Fig. 1).

Preparation of the bacterial culture and foreign gene integration

The bacterial culture for *Agrobacterium* infection was prepared following the protocol after Dandekar *et al.* (1989). A single colony of *Agrobacterium* harbouring the binary vector was streaked on solid AELB (Luria-Bertani broth devoid of

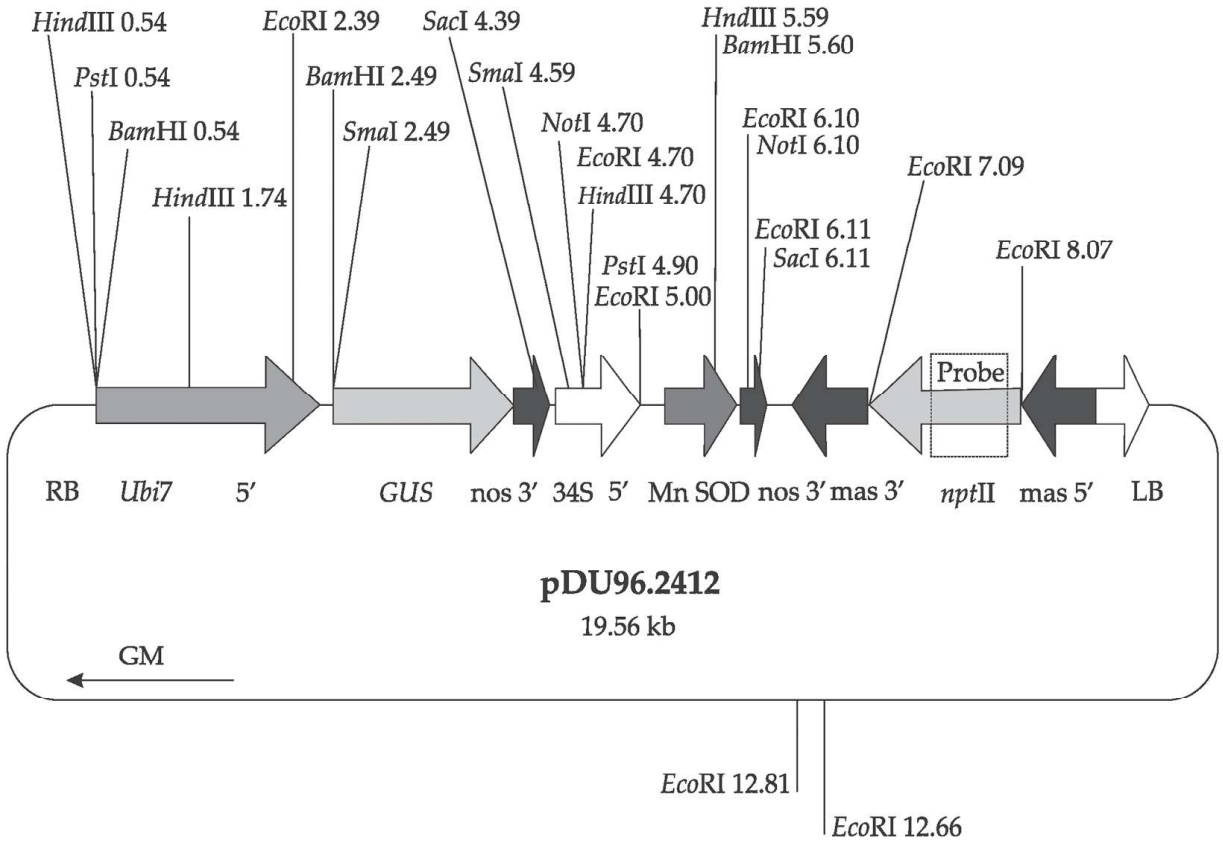


Fig. 1. Map of the binary vector used for the genetic transformation of *H. brasiliensis*.

sodium chloride) medium supplemented with 20 mg/L gentamycin and 50 mg/L kanamycin and grown overnight at 28 °C. After 24 hours, a single colony of the actively growing bacteria was suspended in 10 ml liquid AELB medium containing the above antibiotics in the same concentration and was allowed to grow over night at 28 °C in a rotary shaker at 220 rpm to get an optical density of 0.5 at 420 nm. This culture (1mL) was centrifuged at 5000 rpm for 10 min for separating the bacteria. Re-suspended the bacteria in 10 ml sterile anther callus induction medium containing 40 mg/L acetosyringone, 115 mg/L proline and 153 mg/L glycinebetaine to get a bacterial density of $1x10^8$ cells/ml**.** The bacterial cells were allowed to grow in this medium in a rotary shaker at 220 rpm for four hours at 28 °C. This bacterial culture was used for the infection of anther callus pre-cultured over medium containing calcium nitrate (0.0-1200 mg/L) for fifteen days. Approximately 2 g of the target tissue was taken in 30 mm sterile glass petri plates containing one ml of the *Agrobacterium* culture. The callus clumps were cut into small pieces and kept in the *Agrobacterium* inoculum for 10 min. The infected calli were blotted with sterile filter paper to remove the excess bacterial suspension and carefully transferred to solid co-culture medium. The basal media composition of the co-culture medium was same as that of the callus induction medium. Osmoprotectants like betaine hydrochloride (153/mg/L), proline (115/mg/L) and acetosyringone (20/mg/L) were added to the co-culture medium.

Combined effect of callus pre-culture and acetosyringone concentration on transformation efficiency

To assess the combined effect of callus pre-culture and acetosyringone concentration on transformation efficiency, *Agrobacterium* infection was carried out with the callus pre-cultured for fifteen days on medium containing 800 mg/L calcium nitrate and amending the co-culture medium by incorporating different concentrations of filter sterilized acetosyringone (0.0, 20, 40, 80, 100 and 200 mg/L). After *Agrobacterium* infection, co-cultivation was carried out for 72 hours in the dark at 26 ± 2 °C, air dried in the laminar flow hood and transferred to the selection medium.

The basal media composition of selection medium was the same as that of the callus induction medium except some specific modifications. Kanamycin 300 mg/L was added for the selection of transformed cell lines and cefotaxime 500 mg/L was supplemented to prevent overgrowth of the *Agrobacterium.* In the selection medium, three subcultures were made at three weeks interval for the elimination of escapes. After 40-50 days of culture in the selection medium, kanamycin resistant yellow friable callus emerged from the infected callus. The putatively transformed cell lines were selected after subjecting GUS histochemical staining (Jefferson, 1987).

Confirmation of gene integration by PCR

The GUS positive cell lines were selected and proliferated in medium containing 300 mg/L kanamycin. Untransformed callus maintained in the proliferation medium was taken as control. Five putatively transformed cell lines were randomly selected and DNA was isolated from these cell lines and also from negative control following CTAB method (Doyle and Doyle, 1990). Plasmid DNA (positive control) was isolated from the *E. coli* DH5 following the alkaline lysis method. The

PCR was performed in a 20 µL reaction mixture containing 40 ng template DNA, 250 nM each of forward and reverse primers, 0.5 U of *Taq* DNA polymerase, I X *Taq* buffer and 100 µM of DNTP's. The primers used for the amplification of *npt*II gene was, forward: 5'-GAGGCTATTCGGCTATGACT-3' and reverse 5'-AATCTCGTGATGGCAGGTTG-3' and MnSOD gene amplification with the primers 5'-ATGGCTCTGCGATCTCTA GTGACCC–3' (forward) and 5'CTAAG AAGAGCATTCTTTGGCAT-3' (reverse). The PCR conditions were: an initial denaturation at 93 °C for 2 minutes followed by 36 cycles of a denaturation at 92 °C for 1 minute, annealing at 55 °C for 1 minute and a 72 °C extension for 1 minute. A final extension step at 72 °C for 10 minutes was also given.

RESULTS AND DISCUSSION

Effect of callus pre-culture on transformation frequency

In the callus induction medium, swelling of the explant was noticed 2-3 weeks after anther inoculation, followed by callus formation within 40-50 days. The friability of the callus was improved by subculturing the fresh callus on medium containing calcium nitrate. From the optimization experiments, it was found that soft friable callus was obtained at two concentrations of calcium nitrate *viz*., 600 and 800 mg/L (Fig. 2 a & b). However, the callus was hard and compact, when the concentration was below 600 mg/L; where as above 1000 mg/L the callus became white and watery (Table 2). In *Hevea* somatic embryogenesis, the beneficial effect of high concentration of calcium on inducing friable callus was reported by different authors for different explants *viz*. inner integument of fruit, clone PB 260 (Montoro *et al*., 1993); leaf

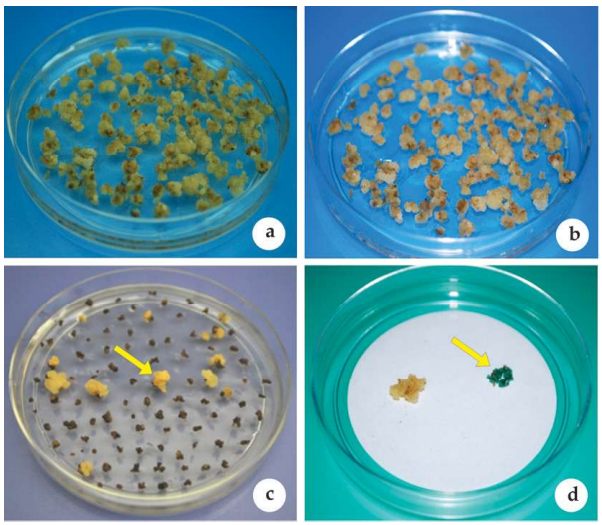


Fig. 2. a-b: Proliferation of callus in medium containing Ca $(NO_3)_2$. $4H_2O$
a - 600 mg/L
b - 800 mg/L
c - Emergence of transformed cell lines (yellow color)
d - Histochemical staining for GUS expression in the callus (blue colour)

Table 2. **Effect of calcium nitrate on transformation frequency**

Concentration of Ca $(NO_3)_2$. $4H_2O$ (mg/L)	Nature of callus	Transformation frequency (%)
0	Hard & compact	4 (2.20)
200	Compact	4 (2.08)
400	Soft & compact	6 (2.63)
600	Soft, friable	8 (2.99)
800	Translucent, soft & friable	12 (3.60)
1000	White and translucent	2 (1.66)
1200	White and watery	0 (1.00)
		CD (5%) = 0.92

Values are average of 50 replicate samples and the experiment was repeated three times

Data was analyzed by square root transformation (√x+1) and values are given in parenthesis

explants of clone RRII 105 (Kala *et al*., 2005) and immature inflorescence of clone RRII 414 (Sushamakumari *et al*., 2008). According to the authors, the friable callus could be induced from different explants by amending the calcium levels in the callus induction or proliferation medium and the optimum concentration was depended on the genotype and type of explant used. In the present work also a fifteen day pre-culture of the fresh anther callus on medium containing calcium nitrate improved the callus friability. Spontaneous occurrence of callus friability during somatic embryogenesis due to genetic factors was also reported in white oats (Bregitzer *et al*., 1989).

This study demonstrates that *Agrobacterium* mediated genetic transformation frequency was enhanced by pre-culturing the anther callus for fifteen days on medium containing calcium nitrate. The transformation frequency increased with increasing concentration of calcium nitrate in the proliferation medium and the highest transformation frequency (12%) was obtained with the callus pre-cultured on medium fortified with 800 mg/L calcium nitrate, whereas with the compact callus the transformation frequency was 4 per cent. Further, it was noted that the concentration of calcium nitrate was crucial in converting the callus soft and friable thereby improving its competence for foreign gene integration. The callus cultured over medium containing 1200 mg/L calcium nitrate became white and watery. The watery callus when subjected to *Agrobacterium* infection after mechanical wounding, no transformation events occurred. It was observed that the watery callus, when subjected to mechanical wounding became a paste rather than clear wounded callus usually obtained with soft friable callus. It was also noticed that the wounded watery callus after infection could not be made dry by blotting with sterile filter paper. Moreover, in the selection medium, overgrowth of the *Agrobacterium* was observed over the watery callus. All these factors might have contributed to lower transformation efficiency with the watery callus.

There are several reports in other crop plants that *Agrobacterium* mediated transformation efficiency is highly dependent on the type of explant, the texture and developmental stage of the target tissue *etc*. (Stachel *et al*., 1985; James *et al., 199*3; Dillen *et al*., 1997). In *H. brasilensis* also, the transformation frequency was influenced by the type and texture of the callus. Pre-culturing of the callus on medium containing 800/mg/L calcium nitrate made the callus soft, friable and translucent and the transformation frequency was enhanced. One probable reason for obtaining higher transformation frequency during *Agrobacterium* infection was that the translucent friable calli were easily separated by enhancing the callus cell surface contact with the bacterial culture when compared with the hard compact callus. Further, as the callus became soft and friable, the permeability of the cells to T-DNA transfer was facilitated and enhanced the transformation frequency. It was also noticed that as the calcium nitrate content was high (1200/mg/L), the callus became white and watery with little competence for foreign gene transfer and the transformation frequency was low. The role of calcium on promoting transformation frequency is also dependent on *Agrobacterium* strain as well as the plant genotype. In the *Hevea* clone PB 260, Montoro *et al*. (2003) reported that the transformation frequency was decreased when *Agrobacterium* infection was carried

out in the callus maintained long-term in the medium containing high calcium chloride (9 and 18 mM). They have carried out transformation experiments with different *Agrobacterium* strains using the callus pre-cultured in medium containing with and without calcium chloride. It was found that out of five *Agrobacterium* strains tested, the effect of pre-culture remained specific to the EHA105 and pC2301 strains which were most effective in transforming the PB 260 clone. Hence they suggested that the role of calcium chloride on decreasing the transformation efficiency was limited to specific *Agrobacterium* strains and *Hevea* clones. In *Quercus suber L.* (cork oak), Alvarez and Ordas (2007), tested the effect of pre-culture of the target tissue (embryogenic callus) on transformation frequency and reported that transformation efficiency was greatly improved by callus pre-culture. Saini and Jaiwal, (2007) also observed that pre-culture and wounding of cotyledonary node explants and manipulations in the inoculation and co-cultivation conditions played a significant role in influencing the tissue competence for foreign DNA delivery and transformation frequency.

Combined effect of callus pre-culture and acetosyringone concentration on transformation efficiency

Since acetosyringone is produced during wounding of plant cells and induce transcription of the virulence genes of *Agrobacterium tumefaciens* by interacting with the trans membrane receptor protein, the concentration of acetosyringone had a crucial role in determining the transformation efficiency (Stachel *et al*., 1985). The combined effect of pre-culture of the callus on medium containing calcium nitrate (800 mg/L) and addition of acetosyringone (0-200 mg/L) in the co-culture medium, on transformation efficiency was also assessed (Table 3). Significant differences among treatments were observed. No transformation event was noticed in the absence of acetosyringone or at high concentration (200 mg/L) in the co-culture medium. Transformation frequency of 6, 10 and 14 per cent were obtained with the acetosyringone concentrations 20, 40 and 80 mg/L respectively.

There are similar results in the genetic transformation of apple (James *et al*., 1993). They suggested that acetosyringone and osmoprotectants like betaine or proline synergistically enhanced *Agrobacterium* mediated genetic transformation. In rice transformation, contradictory reports are available regarding the effect of acetosyringone on transformation efficiency. Aldemita and Hodges (1996) reported in rice that pre-induction of *Agrobacterium* with 80 mg/L acetosyringone prior to co-cultivation facilitated rice transformation and when acetosyringone was omitted, no stable transformed plants could be regenerated. In

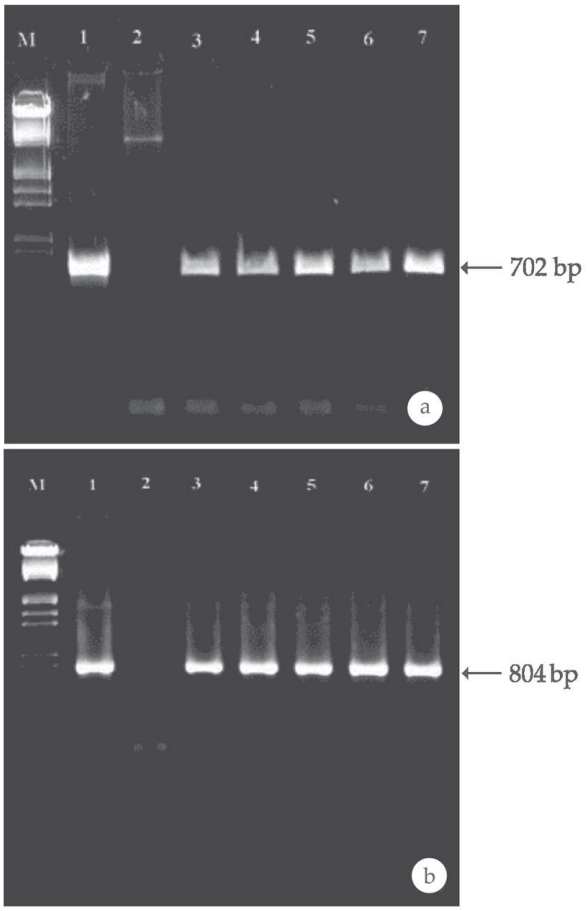
Table 3. **Effect of acetosyringone on transformation frequency**

Acetosyringone concentration (mg/L)	Transformation frequency (%)
0.0	0 (1,00)
20.0	6 (2.63)
40.0	10 (3.31)
80.0	14 (3.85)
100.0	4 (2.23)
200.0	0 (1.00)
	CD (5%) = 0.54

Values are average of 50 replicated samples and the experiment was repeated three times

Data was analyzed by square root transformation ("x+1) and values are given in parenthesis

Indica rice, omission of acetosyringone in both pre-induction and co-cultivation medium induced higher levels of transient GUS expression than that was obtained with the addition of acetosyringone to either of the stages (Hiei *et al*., 1977; Rao and Rao, 2007). Khanna and Raina, (1999) also stated that addition of acetosyringone in the co-cultivation medium enhanced rice transformation frequency. The beneficial effect of acetosyringone on transformation efficiency was reported in several crops such as blackgram (Saini and Jaiwal 2007), barley (Shrawat *et al*., 2007; Kumlehn *et al*., 2006), onion (Zheng *et al*., 2001) and in plantain (Pineda *et al*., 2002). In tree crops like conifers, Ellis (1989) reported that the efficiency of *Agrobacterium* mediated genetic transformation was dependent on physiological state and developmental stage of tissue inoculated and the presence of acetosyringone in the co-cultivation medium. In black poplar clones Confalonieri *et al*. (1995) observed significant difference in transformation frequency when acetosyringone was included in the co-cultivation medium. All these reports suggested that acetosyringone had a crucial role in promoting the T-DNA transfer.



M : Lambda marker, wide range (0.5 - 21 kb)
1 : +ve control (plasmid)
2 : -ve control (non - transgenic)
3-7 : transgenic callus a) MnSOD, b) npt II

Fig. 3. Detection of transgenes by PCR analysis
a - with MnSOD gene specific primer (Lanes: M-Lambda marker, wide range (0.5-21 kb); 1- positive control (plasmid); 2- untransformed control callus; 3-7: transgenic callus
b - with nptII gene-specific primers (Lanes: M-Lambda marker, wide range (0.5-21 kb); 1- positive control (plasmid); 2- untransformed control callus; 3-7: transgenic callus

Confirmation of gene integration

Antibiotic resistant yellow friable callus emergence was observed from the *Agrobacterium* infected callus, after three sub cultures at three weeks interval in the selection medium (Fig. 2c). These cell lines were selected after GUS histochemical staining. The callus in which gene integration had occurred showed dark blue color through GUS histochemical staining (Fig. 2 d). The presence of transgene was further confirmed by PCR analysis with gene specific primers. The presence of *npt*II gene was confirmed by the amplification of 804 bp in all the cell lines tested and in positive control and no amplification was observed in the non-transgenic (negative control). When PCR was performed with MnSOD gene specific primers, a 702 bp fragment was amplified in

all the transgenic callus and in the positive control (plasmid). This fragment corresponds to the cDNA sequence coding for a 702 bp MnSOD transgene integrated in *Hevea* callus. This 702 bp fragment was absent in the genome of untransformed callus (Fig. 3a & b). All the transformed cell lines were further proliferated and cultured for embryo induction.

CONCLUSIONS

In conclusion this study reports that *Agrobacterium tumefaciens* mediated genetic transformation frequency in *Hevea brasiliensis* (clone RRII 105) with the gene coding for MnSOD was significantly improved by pre-culturing the fresh anther callus for fifteen days on proliferation medium containing calcium nitrate, and increasing the acetosyringone concentration in the co-culture medium. Transformation frequency of 14 per cent was obtained by culturing the target tissue on medium containing 800 mg/L calcium nitrate and incorporating 80 mg/L acetosyringone in the co-culture medium. Foreign gene integration was confirmed in the GUS positive cell lines through PCR analysis with MnSOD and *npt*II gene specific primers.

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