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SOMATIC EMBRYOGENESIS FROM THE *IN VITRO* ROOT EXPLANTS OF TRANSGENIC *HEVEA BRASILIENSIS* AND VALIDATION OF STABLE GENE INTEGRATION

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Plant genetic transformation offers a potential tool for crop improvement in *Hevea brasiliensis* by the integration of agronomically useful genes with stable expression. The objectives of the present study were to develop a method for rapid regeneration of transgenic plants *via* somatic embryogenesis using root explants and validation of stable foreign gene integration. Actively growing root explants were collected from germinating *in vitro*-derived MnSOD transgenic somatic embryos (RRII 105), sliced into thin segments and placed on callus induction medium. Callus induction and proliferation were observed within four weeks of explant inoculation. Half-strength Murashige and Skoog (MS) basal medium containing B5 vitamins and growth regulators (1.4 µM each of 2,4-D and Kin, 1.0 µM each of NAA, BA and GA_3) showed the highest embryo induction frequency of 42 per cent. Maximum conversion of pro-embryos into heart, torpedo and cotyledonary stage was achieved by incorporating 40 g/L sucrose and 4 g/L phytagel in the embryo induction medium. The highest percentage of fully developed plantlets was obtained in half-strength modified MS basal medium containing 1.3 µM BA, 1.5 µM GA_3 and 2.5 µM IBA. Molecular confirmation of the presence of the transgene was performed by polymerase chain reaction with *npt*II and MnSOD gene-specific primers and Southern hybridization with *npt*II gene-specific probe. Regeneration of transgenic plants could be achieved within eight months using this protocol.

Keywords: Genetic transformation, *Hevea brasiliensis*, MnSOD, Somatic embryogenesis, Stable gene integration, Transgenic plant.

INTRODUCTION

Hevea brasiliensis (Para rubber), belonging to the family Euphorbiaceae, is the major source of commercial natural rubber, and accounts for 99 per cent of the world natural rubber (NR) production and 49 per cent of the elastomer sale market. Increasing demand for NR as well as pressure on availability of land and socio-economic constraints necessitate the expansion of rubber cultivation to the non-traditional/ marginal areas, which are exposed to severe environmental stresses such as extreme cold, elevated temperature and high light intensities, resulting in poor performance of the plants. Susceptibility of *H. brasiliensis* to

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various biotic and abiotic stress factors in the various agroclimatic conditions has been a major concern for crop production since the crop loss due to these factors is heavy. Attempts are being made to develop stable transgenic stress tolerant plants using molecular tools.

MnSOD transgenic *H. brasiliensis* plants were developed earlier under the control of FMV 34S promoter (Sobha *et al.*, 2003) and CaMV 35S promoter (Jayashree *et al.*, 2003) with the objective of enhancing oxidative stress tolerance, ultimately resulting in increased crop yield under environmental stress conditions. Confirmation of stable gene integration and expression in subsequent stages of vegetative propagation, including tissue culture, is also essential for the successful application of genetic engineering technology for crop improvement. A portion of the remaining calli generated during the development of transgenic plants in 2003 was maintained by serial subculturing. In order to ascertain the stability of the integrated foreign gene in the callus maintained through serial subculture, plant regeneration *via* somatic embryogenesis from these calli was attempted. Actively growing root tips of the germinating somatic embryos developed from the maintained callus offer a suitable explant for this purpose. This paper reports a method for plant regeneration through somatic embryogenesis from the actively growing root tips of the *in vitro*-derived germinating transgenic somatic embryos and validation of stable foreign gene integration in *H. brasiliensis*.

MATERIALS AND METHODS

Transgenic plants integrated with MnSOD gene under the control of FMV 34S promoter had been developed earlier through *Agrobacterium tumefaciens*-mediated genetic transformation of two-month-old anther-derived callus and foreign gene integration was confirmed by PCR and Southern hybridization analyses (Sobha, 2003). Calli generated during the transformation event were maintained by serial subculturing and these were used for generating somatic embryos for this study.

Callus induction and proliferation

Actively growing root tips of the germinating somatic embryos (Fig. 1A) were separated, cleaned thoroughly by dipping in sterile water and the excess water was removed using sterile tissue paper. The root tips were cut into thin segments and placed on modified MS medium for callus induction (Fig. 1B). The modification was made by using B5 vitamins and reducing the ammonium nitrate and potassium nitrate

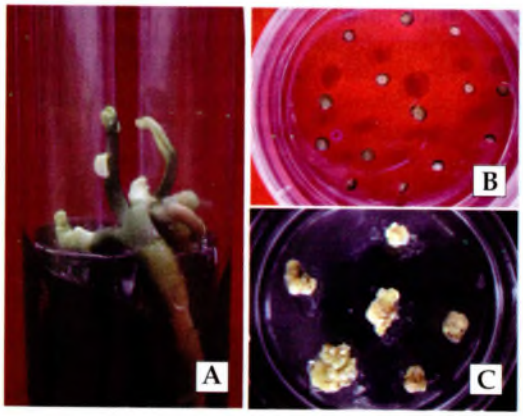

Fig. 1. Root explant, callus induction and proliferation
A- Actively growing root tip of the germinated transgenic somatic plant: the explant for callus induction
B- Freshly inoculated root segments in the callus induction medium
C- Callus induction and proliferation from the root explant

concentrations to 1000 and 1800 mg/L, respectively. The growth regulator concentrations tried for callus induction and proliferation were 0.0 - 10.0 μM 2, 4- D and 1.0 - 5.0 μM BA. Five replicate samples were taken and the experiment was repeated two times. The combined effect of 2, 4- D, BA and NAA was also assessed.

Embryo induction and maturation

The proliferated calli were subcultured on embryo induction medium. The basal medium used for embryo induction was half-strength modified MS containing amino acids proline, glutamine (200 mg/L each), arginine, aspartic acid and serine (20 mg/L each). Effect of growth regulators on embryo induction was assessed by incorporating different levels of NAA, BA and GA_3 ranging from 1.0 - 5.0 μM. The concentrations of 2, 4-D and Kin were kept constant at 1.4 μM each, based on earlier studies (Sobha, 2005). Effect of water stress on embryo maturation was evaluated by incorporating phytagel at different levels (2 – 8 g/L) and sucrose at 40 g/L in the embryo maturation medium.

Embryo germination and plant regeneration

For plant regeneration, individual mature embryos were cultured on germination medium. The effect of full-strength and half-strength Woody Plant Medium (Lloyd and McCown, 1981) and modified MS basal medium fortified with phytohormones on embryo germination and plant regeneration was studied. Effect of growth regulators on plant regeneration was assessed by adding different concentrations (0.5 - 3.0 μM) of GA_3 and IBA. The level of BA was fixed at 1.3 μM on the basis of an earlier report by Sobha (2005)

Culture conditions

Unless otherwise mentioned, the pH of each medium was adjusted to 5.7 with 1N KOH. Phytagel 0.2 per cent was added as gelling agent before autoclaving at 121 °C and 15 lb pressure for 15 min. Sucrose 2 per cent was used in the callus induction and plant regeneration media. Activated charcoal (0.2%) was also added in the embryo maturation and plant regeneration medium. For callus proliferation and embryo induction, cultures were maintained at 26 ± 2 °C in complete darkness, while for maturation and plant regeneration, the cultures were kept at 26 ± 2 °C under 16 h photoperiod (40 $\mu E/m^2/s$).

Molecular analysis of transgenic plants
Isolation of DNA

DNA was isolated from the leaves of two putative transgenic plants, developed from the root explants as explained above, and one control plant, following CTAB method (Doyle and Doyle, 1990). Plasmid DNA was isolated from the binary vector pDU96.2412 following the alkaline lysis method. The isolated DNA was used for PCR amplification and Southern hybridization.

Amplification of *npt*II and *Hevea* MnSOD genes

For the detection of *npt*II gene, forward (5′-GAGGCTATTCGGCTATGACT-3′) and reverse (5′-AATCTCGTGATGGCAGGTTG-3′) primers corresponding to the *npt*II coding region of the plasmid vector were used. The transgene (*Hevea* MnSOD) was amplified with gene-specific primers 5′-ATGGCTCTGCGATCTCTAGTGACCC–3′ (forward) and 5′-CTAAGAAGAGCA TTCTTTGGCAT-3′ (reverse). The PCR reactions were carried out in 20 μl volumes

containing 100 µM dNTPs, 250 nM of each primer, 1.5 nM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Bangalore Genei, India) with 20 ng template DNA in a thermal cycler (Perkin Elmer-480). The PCR conditions were initial denaturation at 94 °C for 4 min followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The final elongation was at 72 °C for 7 min. Amplified DNA fragments were electrophoresed in 1.5 per cent agarose gels, stained with ethidium bromide and visualized under UV light.

Southern hybridization analysis

To confirm the integration of the *npt*II gene in the host genome, 15 µg of genomic DNA was digested with *Eco*RI. The fragments were separated in 1.0 per cent agarose gel and transferred on to nylon membranes (Hybond N^+, Amersham, U.K) by capillary blotting. PCR-amplified 804 bp internal fragment of *npt*II gene of the vector was used as the probe. The probe was radio-labelled with α-^{32}P dCTP (BARC, Mumbai, India) using multi-prime labelling kit (Amersham Pharmacia, UK). Hybridization was performed for 18 h at 65 °C. The membranes were then washed twice at room temperature in 2X SSC and 0.5 per cent SDS followed by two high stringent washes at 65 °C in 0.1X SSC and 0.1 per cent SDS for 30 min each. Final wash was in 0.2X SSC for 15 min at room temperature. The blots were sealed with saran wrap and exposed to X-ray film (X-Omat, Kodak) at -80 °C with intensifying screens.

RESULTS AND DISCUSSION

Callus induction and proliferation

Callus induction and proliferation were observed within four weeks of explant culture (Fig. 1C). Study on the effect of 2,4-D and BA on callus induction and proliferation revealed that in the absence of 2,4-D, swelling of the explant took place but there was no callus induction (Table 1). Callus induction was observed in medium fortified with 2,4-D and the callus induction efficiency increased with increasing concentrations of 2,4-D up to 4.2 µM. Lower levels of 2,4-D (2.4 - 4.2 µM) in combination with 1.4 - 2.0 µM BA, produced yellow friable calli. Although callus induction was observed at higher concentrations of 2, 4-D (above 5.6 µM), no embryo induction was observed on further subculturing of this callus on embryo induction medium.

Table 1. **Effect of 2, 4-D and BA on callus induction and proliferation**

2,4-D (µM)	BA (µM)	Callus induction and proliferation
0.00	1.00	–
1.20	1.20	+
2.40	1.40	++++
3.60	1.80	++++++
4.20	**2.00**	**++++++++**
4.80	2.40	++++++
5.60	2.80	+++++
6.80	3.20	++++
8.00	3.80	++
9.00	4.20	+
10.00	5.00	+

It is well documented that callus initiation in many plant species is facilitated by the addition of 2,4-D in the culture medium. In *H. brasiliensis*, several reports stated that callus induction from different explants such as anther (Wang *et al.*, 1980; Chen, 1984; Jayasree *et al.*, 1999; Asokan *et al.*, 2002) and leaf (Kala *et al.*, 2005) was favoured by the addition of 2,4-D in the culture medium. In carrot cells, Michalezuk

et al. (1992) reported that 2,4-D promoted callus induction. They also reported that both exogenous and endogenous auxins were closely involved in the process of somatic embryogenesis and the competence of the cells to initiate embryogenesis was improved by exposure to 2,4-D. For somatic embryogenesis in rice also, an auxin treatment was essential to induce embryogenic competence (Heyser *et al.*, 1983; Abey and Futsuhara, 1986; Rueb *et al.*, 1994). Padmanabhan *et al.* (2001) reported that sweet potato exhibited a greater response to 2,4-D for callus induction and gave rise to embryos having high regeneration potential. In *Populus* also, 2,4-D was supplemented in the medium to initiate embryogenic callus (Park and Son, 1988). In citrus, 2,4- D has been used to induce embryogenic calli from nucellar and ovular explants of *Citrus limon* and *C. volekameriana* (Saad, 1975). It is evident from all these reports that among the various types of auxins, 2, 4-D is the most popularly used auxin for callus initiation.

Naphthalene acetic acid was also used alone or in combination with 2,4-D for callus initiation and embryo induction. In the present study, callus induction and proliferation were improved by the addition of NAA along with 2,4-D and BA. Addition of low concentrations of NAA along with 2,4-D and BA in the callus induction medium improved embryogenesis due to the synergistic effect of these two growth regulators. The optimum concentration identified for efficient callus induction and proliferation was 4.2 µM 2,4-D, 1.0 µM NAA and 2.0 µM BA. A callus induction frequency of 80 per cent was obtained with this hormone combination. This study revealed that combination of two auxins was superior to a single auxin in promoting callus induction and proliferation. Similar effects were reported in the somatic embryogenesis of blue pearl where a high frequency callus formation was obtained from leaf explants in media containing 2,4-D and NAA (Suzuki and Nakano, 2001).

Embryo induction and maturation

Proliferated calli, on further subculture in the embryo induction medium, formed embryogenic calli within two months (Fig. 2A). Effect of BA, NAA and GA_3 on embryo induction revealed that addition of 1.0 µM each of BA, NAA and GA_3 in the presence of 1.4 µM each 2,4-D and Kin, favoured the conversion of embryonic mass to globular/torpedo stage embryos (Fig. 2B). It was also observed that incorporation of 1.0 µM BA improved embryo induction, but at higher concentrations the embryo induction frequency was reduced due to callus proliferation (Table 2). Contradictory reports are available regarding the beneficial effect of cytokinin on somatic embryo induction. Embryogenesis in some *Citrus* species was inhibited by the exogenous application of cytokinins such as BA, Kin and Zea or auxins like IAA, NAA in the culture medium but in some species supplementation of basal medium with cytokinins such as BA, Kin and Zea resulted in embryoid formation (Kochba *et al.*, 1972; Chaturvedi and Mitra, 1975; Bhansali and Arya, 1978; Hidaka and Kajiura, 1988).

Incorporation of GA_3 in the embryo induction medium favoured the conversion of embryonic clusters to globular embryos. It was also noticed that the efficiency of embryo induction was more dependent on the combination as well as concentration of auxin, cytokinin and GA_3. The optimum concentration of growth regulators identified for efficient embryo induction was 1.0 µM each of NAA, BA and GA_3, in combination with 1.4 µM each of 2, 4-D and Kin.

Table 2. **Effect of BA, NAA and GA_3 in presence of 1.4 μM each of 2,4-D and Kin on embryo induction**

BA and NAA (μM)	GA_3 (μM)	Embryo induction (%)
0.5	0.50	13.00
	1.00	15.66
	1.50	16.00
	2.00	11.33
	2.50	8.00
	3.00	7.00
1.0	0.50	31.00
	1.00	42.00
	1.50	36.00
	2.00	30.00
	2.50	24.00
	3.00	12.00
1.5	0.50	27.00
	1.00	38.00
	1.50	31.00
	2.00	28.00
	2.50	20.00
	3.00	13.00
2.0	0.50	18.00
	1.00	34.00
	1.50	28.00
	2.00	22.00
	2.50	18.00
	3.00	11.00
2.5	0.50	16.00
	1.00	30.00
	1.50	24.00
	2.00	17.00
	2.50	13.00
	3.00	6.66
3.0	0.50	8.00
	1.00	22.00
	1.50	11.00
	2.00	8.00
	2.50	7.00
	3.00	6.00
CD (P = 0.05)		3.06

Conflicting reports are available regarding the influence of GA_3 on somatic embryo induction. Addition of autoclaved or filter-sterilized GA_3 to the embryo induction medium or embryo development medium increased the number of explants producing embryos in fennel and it was also noticed that the embryogenic frequency of petiole explants of several fennel genotypes, previously considered as poorly responding, was highly enhanced by the addition of GA_3 to the culture medium (Hunault and Maatar, 1995). Effect of GA_3 on somatic embryogenesis of *H. brasiliensis* from different explants was studied in detail by different groups (Sushamakumari *et al.*, 2000; Jayasree and Thulaseedharan, 2001), who reported that GA_3 has a crucial role in embryo induction, maturation and plant regeneration. In *Santalum album* L. also, GA_3 enhanced somatic embryogenesis (Sita *et al.*, 1979; Sita, 1986). Higher concentration of GA_3 in combination with IAA in the initiation phase enhanced embryoid formation in spinach (Xiao and Branchard, 1993). However, embryo induction was inhibited by the addition of GA_3 in carrot cells (Tisserat and Murashige, 1977), ovule culture of *Citrus sinensis* (Kochba *et al.*, 1978) and soybean (Phillips and Collins, 1981).

Embryo maturation was initiated by the conversion of globular stage embryos to heart, torpedo and finally to cotyledonary stage embryos (Fig. 2C). In the maturation medium, along with transition process from globular to cotyledonary stage, many secondary embryos were also developed from the primary embryos. Supplementation of 4 g/L phytagel and 40 g/L sucrose in the embryo maturation medium promoted embryo maturation. Phytagel (synthetic agar), 2 g/L, is normally used as a gelling agent for the preparation of tissue culture

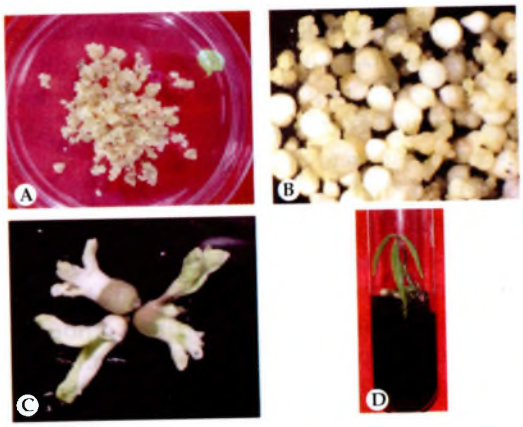

Fig. 2. Different stages of somatic embryogenesis
A - Induction of embryogenic callus
B - Globular stage embryos
C - Mature somatic embryos
D - Fully developed plantlet

medium. The use of high concentrations of phytagel (4-8 g/L) as gelling agent makes the medium hard as well as dry and thereby reduces the availability of water to the cultures. In the present study, culturing somatic embryos on medium containing 4 g/L phytagel favoured embryo maturation. The beneficial effect of water stress was the desiccation, which might have promoted cell morphogenesis. Above 6 g/L, there was reduction in fresh weight of the embryos and they were highly dehydrated. This dehydration led to cell necrosis and loss of embryogenic potential.

The importance of water relations in controlling embryo maturation was proposed by Fischer *et al.* (1987) and has been supported by evidence from both zygotic and somatic embryo culture experiments (Xu *et al.*, 1990) and *in situ* studies (Saab and Obendorf, 1989). In rapeseed embryo development, attempts to stimulate the *in vivo* environment through modification of the composition of maturation medium showed increased storage compound levels and desiccation tolerance (Finkelstein and Crouch, 1986; Xu *et al.*, 1990). Desiccation is a natural event observed during the late phase of embryogenesis. A severe decrease in moisture content experienced by fully developed seed embryos is a key event required for the termination of the developmental processes in preparation for germination (Kermode, 1995). Induction of water stress is also required in the *in vitro* culture for increasing the conversion frequency of the embryos (Attree *et al.*, 1991).

Embryo germination and plant regeneration

The mature embryos germinated and developed into plantlets after five to six weeks when individual embryos were cultured on modified half- strength MS medium fortified with growth regulators (Fig. 2D). Incorporation of GA_3 in combination with BA and IBA in the germination medium favoured bipolar differentiation and improved embryo germination. An embryo germination frequency of 20 per cent was achieved with the hormonal combination GA_3 (1.5 µM) and IBA (2.5 µM), in the presence of 1.3 µM BA (Table 3).

Even though the exact mechanism of the beneficial effect of GA_3 on embryo germination is not clear, ultrastructural studies carried out by Choi *et al.* (1999) showed that somatic embryos developed *in vitro* could be dormant after maturing requiring dormancy breaking treatment. The beneficial effect of GA_3 on somatic embryo germination has been reported in grapes (Mullins and Srinivasan, 1976) and in *Panax ginseng* (Chang and Hussing, 1980; Choi *et al.*, 1999). *Citrus sinensis* embryos derived from ovules were germinated by supplementing

Table 3. **Effect of GA_3 and IBA in presence of 1.3 μM BA on embryo germination**

IBA (μM)	GA_3 (μM)	Embryo induction (%)
0.5	0.50	5.00
	1.00	9.33
	1.50	15.00
	2.00	12.00
	2.50	9.00
	3.00	6.33
1	0.50	6.66
	1.00	11.00
	1.50	17.00
	2.00	14.00
	2.50	10.66
	3.00	7.00
1.5	0.50	7.00
	1.00	9.00
	1.50	17.66
	2.00	15.00
	2.50	12.00
	3.00	8.33
2	0.50	8.00
	1.00	15.66
	1.50	18.00
	2.00	15.00
	2.50	12.00
	3.00	8.66
2.5	0.50	5.00
	1.00	16.00
	1.50	20.00
	2.00	17.33
	2.50	9.00
	3.00	3.66
3	0.50	2.00
	1.00	5.00
	1.50	11.00
	2.00	6.00
	2.50	5.00
	3.00	3.00
CD (P = 0.05)		2.11

GA_3 (1.0 μM) in the basal medium and the presence of GA_3 in the germination medium stimulated rooting as well as stem elongation (Button and Bonman, 1971). In the growth and development of tomato embryos, it was observed that low concentrations of GA_3 appeared to stimulate growth following germination but IAA was not as effective. Combinations of Kin and GA_3 or Kin and IAA were the most beneficial for these embryos (Neal and Topoleski, 1985). GA_3 when used at concentrations from 10^{-8} to 10^{-6} M, promoted the growth of caraway somatic embryos, while some embryos grown in media with GA_3 exhibited extensive root growth (Ammirato, 1997). It is well documented in *Hevea* that GA_3 has a crucial role in somatic embryo germination and full plantlet development (Chen *et al.*, 1984; Carron *et al.*, 1995; Sushamakumari *et al.*, 2000). The total duration from callus induction to regeneration of plant was eight months in this study.

Molecular confirmation of stable gene integration

DNA isolated from two putative transgenic plants developed from root explants and one non-transformed plant as negative control was used in PCR analysis using MnSOD and *npt*II gene-specific primers. DNA from the plasmid vector was used as the positive control. When PCR was performed with MnSOD gene-specific primers, a 702 bp fragment was amplified in all transgenic plants and in the positive control plasmid (Fig. 3A). This fragment corresponds to the cDNA sequences coding for MnSOD gene that has been integrated into the plant genome. This 702 bp fragment was absent in the non-transformed plant. An extended PCR was carried out for the amplification of the genomic *Hevea* MnSOD gene in both the control (non-transformed) and transformed

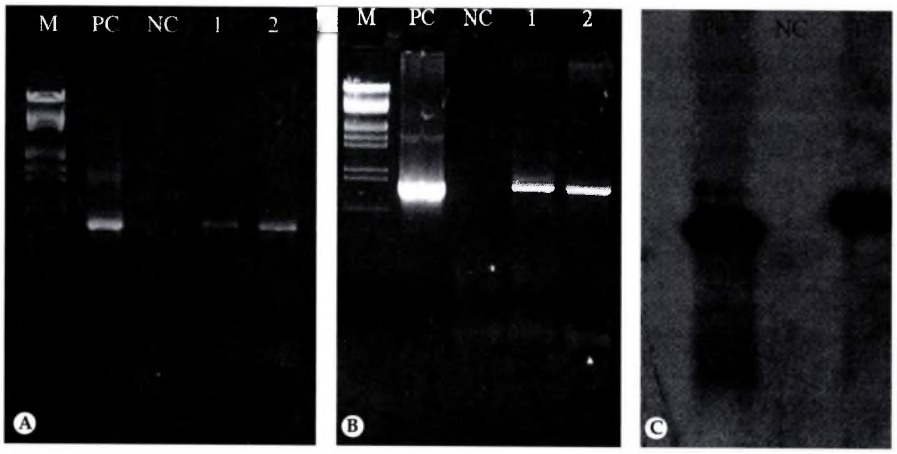

Fig. 3. Molecular confirmation of foreign gene integration
A - PCR amplification with MnSOD gene-specific primer (M: Molecular weight marker; PC: positive control (plasmid); NC: Non-transformed control plant; 1&2: Transgenic plants)
B - PCR amplification with *npt*II primer (M: Molecular weight marker; PC: positive control (plasmid); NC: Non-transformed control plant; 1&2: Transgenic plants)
C - Southern hybridization with *npt*II probe (PC: positive control (plasmid), NC: Non-transgenic control; T: Transgenic plant)

plants. The presence of *npt*II gene was confirmed by the amplification of a 800 bp single product in all the transgenic plants and in positive control. No amplification was detected in the non-transformed plant (Fig. 3B). These results confirm the presence of MnSOD and *npt*II genes in the genome of *H. brasiliensis* transgenic plants.

The presence of the T-DNA region in the genome of the plants developed from root explants was further confirmed by Southern hybridization analysis. Genomic DNA was digested with *Eco*RI to liberate the integrated *npt*II gene of 980 bp. Hybridization with ^{32}P labelled *npt*II gene probe generated a band of predicted size (980 bp) with *Eco*RI digest in the transgenic plant and in positive control (vector plasmid). No hybridization could be detected in the non-transformed plant (Fig. 3C). These results confirmed the stable integration of foreign gene in *H. brasiliensis* through *Agrobacterium*-mediated genetic transformation in repetitive embryogenesis.

This paper reports a protocol for regeneration of transgenic rubber plants in eight months through somatic embryogenesis using *in vitro*-derived root explants. The stability of foreign gene integration was ascertained by carrying out polymerase chain reaction with *npt*II and MnSOD gene-specific primers and Southern hybridization with *npt*II gene-specific probe.

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