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# Efficient transformation and regeneration of PB 260 *Hevea* clone mediated by *Agrobacterium tumefaciens*.

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A procedure has been established for *Agrobacterium tumefaciens*-mediated genetic transformation of *Hevea brasiliensis* embryogenic friable calli and the plantlet regeneration by somatic embryogenesis.
Precultivation of tissues on a $CaCl_2$-free maintenance medium is required for enhancing the efficiency of *Agrobacterium tumefaciens*-mediated gene transfer assessed by GUS activity (Montoro et al., 2000). Following steps of the procedure have been optimized (Rattana et al., 2001). Transient GUS activity was more dependent on the duration of the co-cultivation step than the inoculum concentration. Washing of the inoculated tissues in a solution supplemented with tetracycline reduced GUS activity, probably reflecting the lower number of transformation events occurring after co-cultivation. Replacement of cefotaxime by ticarcillin in the decontamination medium delayed tissue browning and allowed part of the inoculated tissue to proliferate. Growth recovery of the whole inoculated tissue was stimulated when higher concentrations of growth regulators were introduced in the culture medium following co-cultivation. However stability of the GUS activity was adversely affected by these modifications in growth regulator concentration and timing of application. The transfer of smaller pieces of non-washed inoculated tissues onto the decontamination medium further improved growth recovery and allowed maintenance of the GUS activity. Paromomycin was proved more effective than kanamycin for the selection of transformed cells, as it inhibits the growth of non-transformed cells more radically. Five transgenic callus lines were established (Montoro et al., 2003). Afterwards, low consistency of this result was attributed to a variation or a loss in embryogenic capacity of callus lines.

Recently, using embryogenic callus tissues after one or two cycles of cryopreservation showed enhancement in transformation efficiency (GUS activity at day 23) and consequently allowed obtaining isolated transgenic callus lines (Table 1). Although only one cryopreservation did not change the GUS activity, the capability of cryopreserved callus to produce transgenic lines might be attributed to the better growth recovery, 2.77 for cryopreservation instead of 0.95 mg/aggregate, and the larger number of growing aggregates after transformation.

Twenty-four paromomycin-resistant callus lines were isolated from three independent experiments. The presence of Transferred-DNA in the plant genome was confirmed by Southern hybridization on 19 lines. Eleven out of 19 lines bear a single T-DNA copy, the higher number being 4 copies. Regeneration was carried out according to the standard

conditions for somatic embryo production. Thereby, more than 200 transgenic plants were acclimated from 1,000 embryos.

These results confirmed that *A. tumefaciens* is an effective system for mediating stable transformation of rubber tree calli with a low copy number of transgenes. Regeneration capacity was not affected by stresses occurring upon transformation. Additionally, establishment of transgenic callus lines enables unlimited production of plantlets. Further experiments on functional analysis of promoters isolated from genes encoding hevein and glutamine synthetase is under way. Transgenic callus lines also constitute a useful tool for studying genes of interest at cellular level since friable calli were shown expressing a large range of genes (Montoro et al., 1997).

**Table 1.** Effect of the cryopreservation on the transient GUS of PB 260 VP10 line. GUS activity at day 23 consists of the number of blue spots and cell clusters per gram of fresh matter of callus.

| | | Growth | | | | |
|---|---|---|---|---|---|---|
| Cryopreservation | PM | FM | Growing aggregates | GUS activity at day 23 | | Transgenic callus lines |
| | CaCl2 (mM) | (mg.agg$^{-1}$) | (nb) | (TU agg$^{-1}$) | (TU.g-$^{1}$) | (nb) |
| No* | 9 | 2.61±0.47$^{a}$ | 24.6±2.5$^{a}$ | 0 | 0 | 0 |
| No | 9 | 0.95±0.10$^{d}$ | 5.20±0.83$^{c}$ | 1.86±0.65$^{b}$ | 1942±681$^{b}$ | 0 |
| Once | 9 | 2.77±0.27$^{ab}$ | 19.00±1.00$^{b}$ | 4.76±1.41$^{b}$ | 1716±462$^{b}$ | 4 |
| Twice | 1 | 3.23±0.42$^{a}$ | 17.40±1.67$^{b}$ | 11.78±2.94$^{a}$ | 3728±1259$^{a}$ | 3 |

* Not inoculated with agrobacteria suspensions.
Statistical analysis (ANOVA) showed score of P<0.01. Mean comparison performed with Tukey test. Identical letters correspond to non significantly different treatments.

**Table 2.** Molecular analysis of resistant callus lines by Southern hybridization: characterization of the T-DNA copy number and the identical lines.

| Transgenic lines | Number of copies | Identical lines |
|---|---|---|
| Control (-) | 0 | |
| Control (+) | 2 | |
| 1 | 1 | |
| 4 | 0 | |
| 8 | 2 | |
| 13 | 1 | |
| 15 | 3 | =27,29 |
| 18 | 0 | |
| 20 | 1 | |
| 21 | 1 | |
| 23 | 4 | |
| 25 | 1 | |
| 27 | 3 | =15,29 |
| 29 | 3 | =27,15 |
| 31 | 3 | |
| 33 | 2 | |
| 35 | 1 | |
| 36 | 1 | |
| 38 | 1 | |
| 41 | 1 | |
| 42 | 0 | |
| 44 | 2 | |
| 46 | 2 | |
| 47 | 1 | |
| 56 | 1 | |
| 61 | 2 | |

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