# Establishment and cryopreservation of embryogenic callus lines for clone RRIM 600 in *Hevea brasiliensis*

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**Abstract:** *Hevea brasiliensis* is cloned by grafting on non-selected rootstock material. *In-vitro* techniques such as microcutting and somatic embryogenesis were used for propagation of self-rooted plant material. However, these non-commercial techniques are still restricted to a few numbers of clones. To date, long-term somatic embryogenesis is the most promising technique for large-scale propagation. At CIRAD, successful plant regeneration and genetic modification have been obtained for clone PB 260. This paper aims at developing and cryopreserving embryogenic callus lines for clone RRIM 600. Culture conditions were modified for suitable callus growth and embryogenic induction of the low multiplication rate of RRIM600 callus compared with the clone PB 260 one. Several friable callus lines with high embryogenic potential were selected and cryopreserved in large quantity for further field test and functional genomics studies. Application of this technology to other commercial clones can be now considered based on this experience on clones PB 260 and RRIM 600.

**Keywords:** genetic modification, plant regeneration, rubber, somatic embryogenesis

## Introduction

Conventional budding is the main technique for propagation of selected rubber clones. This planting material could be improved in terms of rootstock-scion interaction and clonal root system by using self-rooted material regenerated by in vitro techniques such as somatic embryogenesis. Several research laboratories have tried to set-up somatic embryogenesis technique for main commercial clones. At CIRAD, this process was first developed for clone PB 260 then applied to several other clones (RRIM 600, PB 235, PR 107, RRIM 703, IRCA 109, PB 254 and PB 310), and in collaboration with RRIT in Thailand (clone BPM 24), and Michelin laboratory (BPM 24, PR 107, RRIM 600, PB 254, IRCA 109, IRCA 317, PB 260, RRIM 703, PB 217 and IRCA 41). Worldwide, 22 clones have been regenerated by this process at CATAS in China, RRIM in Malaysia, RRII in India and IBRIEC in Indonesia [1]. CIRAD established several field trials in partnership in Ivory Coast, Nigeria, Thailand [2]. The benefits are a higher growth compared to conventional budded plants and rejuvenation of cultivated clones. However, somatic embryogenesis should be carefully used to avoid any risk of somaclonal variation. First, CIRAD team has developed an indirect secondary somatic embryogenesis process for clone PB 260, which required short-term establishment of callus lines [3]. Second, cryopreservation is also used to limit the long-term proliferation of callus lines [4]. This paper aims at applying this procedure to the commercial clone RRIM 600. Some adaptions of culture conditions led to successful plant regeneration.

### Materials and methods

The process for indirect secondary somatic embryogenesis was described by Lardet and coll. [3]. Friable callus lines were established for clone RRIM 600 according to this procedure (Figure 1E). These lines were cryopreserved according to the protocol described by Lardet and coll. [4]. Optimization of the proliferation has been attempted as follows in Table 1.

**Table 1:** Description of growth regulator treatments in proliferation medium.

| Growth regulator | Concentration (µM) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | T1 | T2 | T3 | T4 | T5 | T6 |
| 3,4-D | 1.36 | 2.26 | 4.52 | 1.36 | 2.26 | 4.52 | 1.36 |
| BAP | 1.33 | 2.22 | 4.44 | 1.33 | 2.22 | 4.44 | 1.33 |
| ABA | 0.5 | 0.5 | 0.5 | 0 | 0 | 0 | 1 |

Effects of these treatments have been observed for 3 subcultures. Then calli were transferred on a series of media for plant regeneration according to the protocol described by Lardet and coll. This experiment was carried out with 15 initial calli at the first subculture. Morphological observations have been done such as: number of proliferating calli (Figure 1A), browning callus (Figure 1B and 1C), and callus bearing embryos during the proliferation steps (Figure 1D).

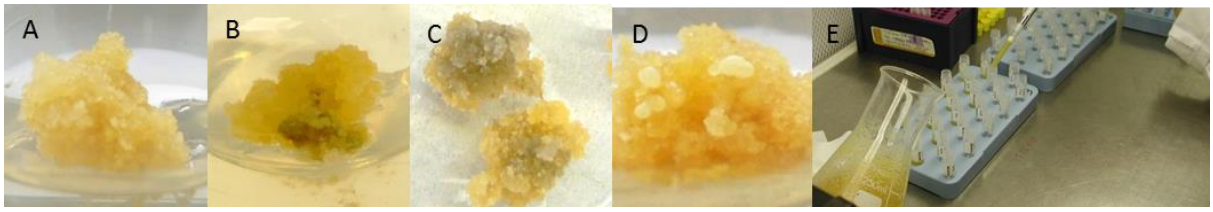


**Figure 1**: Illustration of somatic embryogenesis process for clone RRIM600: (A) regular callus, (B) brown callus (<50%), (C) very brown callus (>50%), (D) proliferating callus bearing somatic pro-embryo structures and embryos, (E) callus processing for cryopreservation.

## Results

Eighteen friable callus lines have been established and cryopreserved for clone RRIM 600 using standard protocol set-up for clone PB 260 (Table 2). Callus of each line was thawed and proliferated. Most of lines had a low rate of multiplication after the first cryopreservation. After several subcultures, a second cryopreservation has been carried in order to avoid long-term callus proliferation. A second thawing and proliferation revealed once again, for most lines, a low multiplication rate, which hampered plant regeneration test. Larger cryopreserved callus stocks were obtained after a third cryopreservation from four lines (CI11022, CI11054, CI11049, CI11020) using the same protocol, and for line CI14021 using a new medium from treatment T4 described below. One limitation of using standard protocol set up for clone PB 260 is the non-controlled regeneration of embryos during the callus proliferation (Figure 1D). Given the low proliferation and non-controlled production of somatic embryos, an optimization of multiplication medium was attempted.

Fifteen calli were multiplied for 3 subcultures on control medium and 6 treatments (Figure 2). Callus number dropped in the second subculture for all treatments except for control and T4 treatment. In the third subculture, the number of callus was higher than in the first subculture only for treatment T4 and T6. Multiplication rate for T4 reached 1.4 when it was 1 for control. Two types of callus were observed: regular callus without any embryos and callus bearing embryos. Embryo induction occurring in this last type of callus hampered the callus

proliferation. Calli on control and T4 treatment have a similar percentage of callus bearing embryos, 20% and 19%, respectively.

**Table 2:** Statement of cryopreserved friable callus lines for clone RRIM 600. Callus multiplication rate was assessed from 1 (no multiplication) to 5 (high rate of multiplication). *Lines created with new protocol corresponding to treatment T4. (ND): non determinated.

| First cryopreservation | | | Second cryopreservation | | | Third cryopreservation | | |
|---|---|---|---|---|---|---|---|---|
| Accession No | Cryotube No | Callus multiplication rate | Accession number | Cryotube No | Callus multiplication rate | Accession No | Cryotubes No | Callus multiplication rate |
| CI11021 | 5 | 3 | CI11040 | 7 | 1 | _ | _ | _ |
| CI11038 | 5 | 3 | CI11045 | 6 | 1 | _ | _ | _ |
| | | | CI11046 | 3 | 1 | _ | _ | _ |
| CI11002 | 7 | 3 | CI11017 | 16 | 3 | _ | _ | _ |
| CI11028 | 5 | 3 | CI11052 | 10 | 1 | _ | _ | _ |
| CI11041 | 3 | 2 | CI11051 | 18 | 3 | _ | _ | _ |
| CI11034 | 4 | 2 | CI11037 | 15 | 3 | _ | _ | _ |
| CI11027 | 4 | 2 | CI11050 | 12 | 2 | _ | _ | _ |
| CI11018 | 6 | 3 | CI11039 | 18 | 3 | _ | _ | _ |
| CI11025 | 6 | 3 | CI11029 | 18 | 3 | _ | _ | _ |
| CI11047 | 4 | 2 | CI11048 | 10 | 2 | _ | _ | _ |
| CI11019 | 4 | 2 | CI11024 | 11 | 2 | _ | _ | _ |
| CI11001 | 6 | 3 | CI11015 | 25 | 2 | _ | _ | _ |
| CI11023 | 7 | 3 | CI11035 | 13 | 2 | _ | _ | _ |
| CI11003 | 4 | 2 | CI11026 | 16 | 1 | CI14020* | 6 | ND |
| | | | CI14021* | *6* | 5 | CI14055* | *60* | 5 |
| CI11014 | 9 | 4 | CI11022 | 32 | 4 | CI14058* | *43* | ND |
| CI11053 | 5 | 3 | CI11054 | 6 | 5 | _ | _ | _ |
| CI11036 | 6 | 3 | CI11049 | 19 | 3 | _ | _ | _ |
| CI11016 | 6 | 3 | CI11020 | 32 | 4 | CI14056* | *32* | ND |

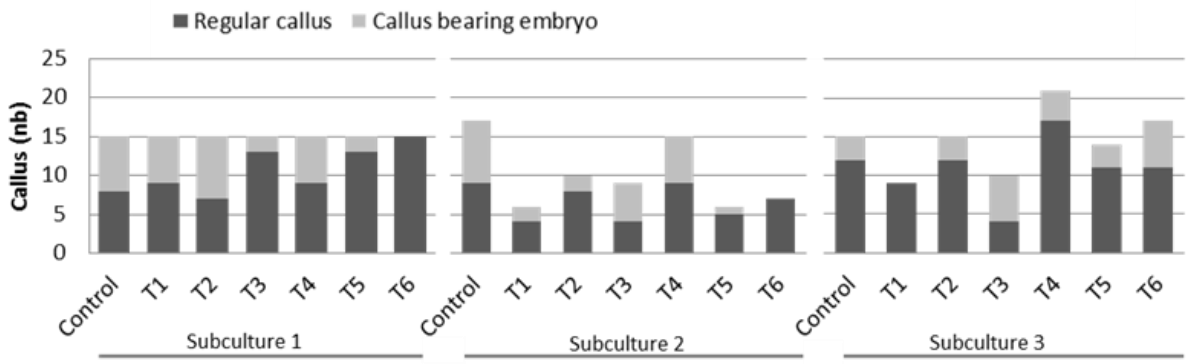


**Figure 2**: Morphological observations of callus from clone RRIM 600 during 3 cycles of multiplication.

Modification of callus multiplication medium induced more callus browning except for treatment T1 (Fig. 3A). Callus growing on Control medium, T1, T4 and T5 treatments had a low percentage of brown callus. Thus, T4 showed to be the best treatment for callus multiplication, control of embryo induction and low browning.

## Discussion and conclusions

Several friable and embryogenic callus lines have been established and cryopreserved for clone RRIM 600 using a procedure established for clone PB 260. Successive cycles of callus cryopreservation were shown to improve the quality of callus by selecting active cells [5]. Low

callus multiplication rate and early embryo induction hampered establishment of large cryopreserved callus stock. Higher concentration in 3,4-D and BAP and depletion in ABA (treatment T4) allowed both improving callus multiplication with low browning and controlling embryo induction. Although the multiplication phase was improved, plant regeneration steps should be optimized to better synchronized embryo development. Anyway, this current protocol led to regenerate more than 150 plantlets (Fig.3C), which were acclimatized in greenhouse. Field trial is now necessary to evaluate the quality of this new planting material. This successful adaptation of the indirect secondary somatic embryogenesis procedure paves the way to the establishment of this technique to other commercial clones to which primary somatic embryogenesis was obtained. Finally, the availability of friable and embryogenic callus lines will allow application to genetic engineering as was reported for the modification of antioxidant enzyme [6].

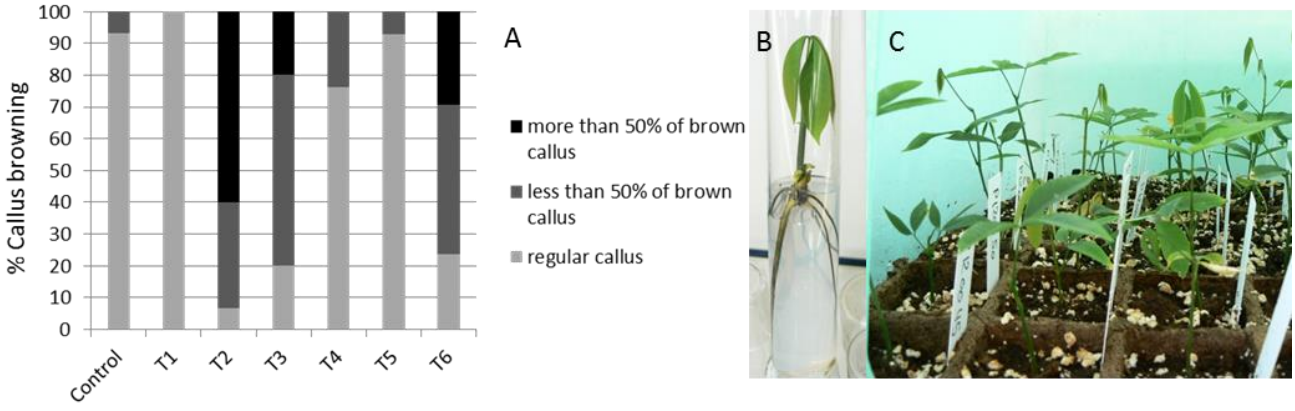


**Figure 3**: (A) Observations of callus browning for clone RRIM 600 after 3 cycles of multiplication. (B) In vitro plantlet. (C) Plantlet acclimatization in greenhouse.

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