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# Callus friability and somatic embryogenesis in *Hevea brasiliensis*

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**Abstract**

The influence of plant growth regulators, sucrose, calcium and various macronutrient media on callus friability and somatic embryogenesis was investigated in *Hevea brasiliensis* Müll. Arg. Friable and embryogenic calli were spontaneously formed in two rubber tree clones (PR 107 and RRIM 600) on the Medium for Hevea (MH), with 3,4-dichlorophenoxyacetic acid (3,4-D), kinetin and sucrose, while compact embryogenic calli were enhanced in three other clones (PB 260, PB 235 and GT1). Callus friability was enhanced in clone PB 260 when the concentration of one growth factor (3,4-D or kinetin) was reduced from 4.5 μM to 0.45 μM during the first culture, or when high sucrose or calcium levels 351 mM and 12 mM, respectively) were maintained during subcultures. The different macronutrient media did not alter callus texture but only use of MH and Murashige and Skoog (MS) media led to somatic embryogenesis. Friable calli obtained by modifying the auxin/cytokinin balance lost their embryogenic potential. In contrast, those obtained on media with high sucrose or calcium concentrations were mainly composed of embryogenic cells embedded in a mucilaginous matrix. Such calli could be of potential interest for establishing embryogenic cell suspension cultures.

*Abbreviations:* BC – brown calli, 3,4-D – 3,4-dichlorophenoxyacetic acid, EA – embryogenic activity, EC – callus bearing embryos, FC – friable calli, FEC – friable callus bearing embryos, MA – meristematic activity, MH – Medium for Hevea, Muc – mucilage, OP – oxidized polyphenols

## Introduction

In *Hevea brasiliensis* Müll. Arg., there is now an effective somatic embryogenesis process available to obtain somatic embryos that germinate into plants (Carron et al. 1992). However, somatic embryogenesis is still a fleeting phenomenon, with calli often becoming brown and degenerating after 2–3 months culture while embryos are developing. The use of embryogenic cell suspension cultures would probably be the most efficient mean to ensure the multiplication and continuous maintenance of embryogenic tissues. Wilson et al., (1976) obtained friable calli from stem slices led to the first *H. brasiliensis* suspension culture using an alternation of agar/liquid media. Calli from integument of seed, currently obtained on gelified medium, were compact and embryogenic; but they senesced without dissociating when transferred to agitated liquid medium.

Callus friability, which favours callus dispersion in liquid medium is sometimes obtained spontaneously due to genetic factors (Bregitzer et al. 1989). In most cases, this callus textural characteristic should be looked for relative to different culture parameters. Callus texture is sometimes dependent on the type of explant used for the initiation of callogenesis. In *Rosa hybrida* L., Noriega & Sondahl (1991) obtained

embryogenic friable calli from somatic embryos. Considering media components, plant growth regulators are quite often responsible for modifying the callus type. Excess level or the kind of synthetic auxin in the culture medium was previously found to induce callus friability in *Ginkgo biloba* L. (Carrier et al. 1990). Moreover, Lazzeri et al. (1987) obtained friable calli in *Glycine max* Merrill, on benzylaminopurine-enriched medium. The basic mineral media used can also modify callus texture. Armstrong & Green (1985) enhanced friability in *Zea mays* L. calli by adding proline to Nitsch basic medium. Indeed, the use of a reduced form of exogenous nitrogen (ammonium or amino acids) is generally recommended for dispersing *Oryza sativa* L. cells in liquid medium (Toriyama et al. 1986). Less commonly used culture medium compounds such as silver nitrate (Vain et al. 1989), or modification of the calcium/cation ratio (Yoshida & Watanabe 1971) have also been found to modify callus texture in *Z. mays* and *Nicotiana glutinosa* L. respectively.

The aim of the present study was to determine the most suitable culture conditions to obtain friable calli with a high and sustained embryogenic potential. We thus investigated the effect of various minerals and of different growth regulators, sucrose and calcium concentrations in the culture medium. The behaviour of calli issued from five different genotypes was also compared.

## Materials and methods

### *Standard culture conditions*

Inner integument of immature seeds from maternal origin was used. Callogenesis was initiated for 25 days on Medium for Hevea (MH) described by Carron et al. (1989), modified with 30 μM $AgNO_3$, 4.5 μM kinetin, 4.5 μM 3,4-dichlorophenoxyacetic acid (3,4-D), 234 mM sucrose and 2 $g\,l^{-1}$ gelrite (Etienne et al. 1991). After completion of this callogenesis phase, calli were subcultured for 25 days on an embryogenesis expression medium having the same composition, supplemented with spermidin (50 μM), but with reduced 3,4-D content (1.35 μM) and with benzylaminopurine (BAP at 1.35 μM) replacing kinetin. Subculturing was carried out in the dark, at 27°C, in glass culture tubes (150 × 25 mm) closed with polycarbonate plugs. Each treatment and standard culture was initiated with 144 explants. Test described by Goodman (1965) for multinomial proportions has been used for statistical analyse of results.

### *Genotypic comparisons*

Five industrial clones calli (PB 260, PB 235, PR 107, RRIM 600 and GT1) were cultured on the same standard MH medium and compared for their ability to form friable and/or embryogenic calli. Clones PB 260 and GT1 which developed compact calli on standard culture medium have been used to investigate modifications of the medium relative to the enhancement of friability.

### *Modification of growth regulators supply*

Growth regulator concentrations were modified in the culture medium during callogenesis (the first 25 day culture). We compared the effects of a range of 3,4-D concentrations (0.45, 2.25, 4.5, 6.75 and 9 μM) with the kinetin concentration remaining constant at 4.5 μM and, conversely, of a range of kinetin concentrations (0.45, 2.25, 4.5, 6.75 and 9 μM) with 3,4-D at 4.5 μM.

### *Modification of the sucrose supply*

A range of sucrose concentrations (58, 117, 175, 234, 292 and 351 mM), maintained throughout the all culture period, were compared in terms of their effect on callus morphogenesis.

### *Modification of the calcium supply*

This experiment was designed to assess the impact of calcium concentration on callus morphogenesis. The calcium concentrations tested were: 0, 1, 3, 6, 9, 12 and 24 mM, and maintained throughout the all culture period.

### *Morphological parameters*

Callus morphology was assessed at the end of the second subculture (50 days), as calli bearing

clearly visible proembryos (EC), brown or necrotic calli (BC) and friable calli (FC).

*Histological parameters*

Histological slices were cut from representative zones of the callus showing no browning. Specimens were fixed in a solution containing 1% glutaraldehyde, 2% paraformaldehyde, 1% caffeine in 0.2 M phosphate buffer, pH 7.2, for 24 h. After progressive dehydration, specimens were embedded in Kulzer 7100 resin and cut into 3 μm thick sections. Sections were double-stained with PAS (periodic acid-Schiff) – NBB (napthol blue-black). PAS specifically strained polysaccharides (i.e. walls and starch), while NBB specifically revealed soluble and storage proteins.

Four histological parameters were assessed to determine the major differences between compact and friable calli:
- clumps of meristematic cells (MA),
- embryogenic activity (cells and globular proembryos, EA),
- mucilage (Muc),
- oxidized polyphenols (OP).

These parameters were quantified as follows (−): absent, (+): rare, (+ +): common (+ + +): abundant.

## Results

*Genotypic effect*

The *in vitro* behaviour of the industrial *H. brasiliensis* clones differed under the standard culturing conditions tested. Only two out of the five studied-clones (PR 107 and RRIM 600) were able to spontaneously form a substantial quantity of friable-embryogenic calli (Table 1, Fig. 1). Two others (PB 235 and PB 260) formed compact calli (Fig. 2) with high embryogenic expression. The last clone (GT1) formed compact calli, but few were embryogenic. Although only compact calli were formed with clone PB 260 under the current culture conditions, it had the highest embryogenic potential. This clone (PB 260) was thus the obvious choice for investigating the role of specific medium components in view of obtaining a friable and embryogenic callus structure.

*Table 1.* Morphological parameters (calli bearing embryos, EC; brown calli, BC; friable calli, FC) of different clone calli cultured on standard Medium Hevea (MH).

| Clone | Morphological parameters (%) | | |
|---|---|---|---|
| | EC | BC | FC |
| PB 260 | 64.3[a] | 10.1[a] | 0[a] |
| PB 235 | 36.7[b] | 80.5[b] | 0[a] |
| GT1 | 1.0[c] | 26.0[c] | 12b[b] |
| PR 107 | 40.8[b] | 11.2[a] | 100[c] |
| RRIM 600 | 31.4[b] | 6.5[a] | 100[c] |

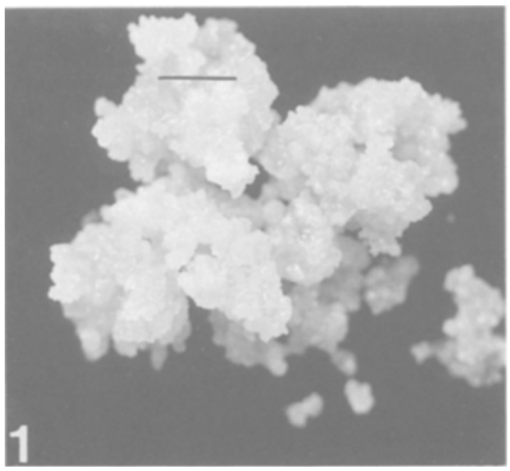
Percentages followed by the same letter are not significantly different according to the test of Goodman (1965) at the 5% level.
Data were collected at the end of the second subculture (day 50). 144 initial explants were cultivated for each clone.

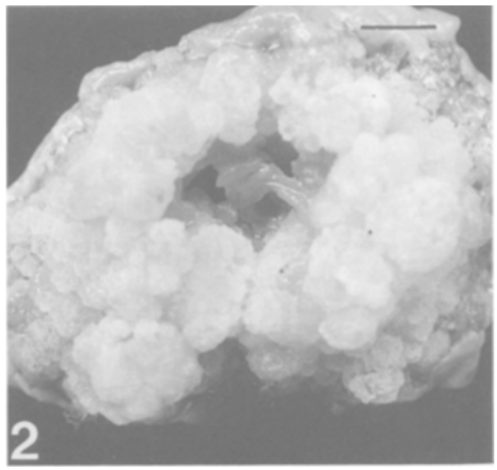
*Plant growth regulators effect*

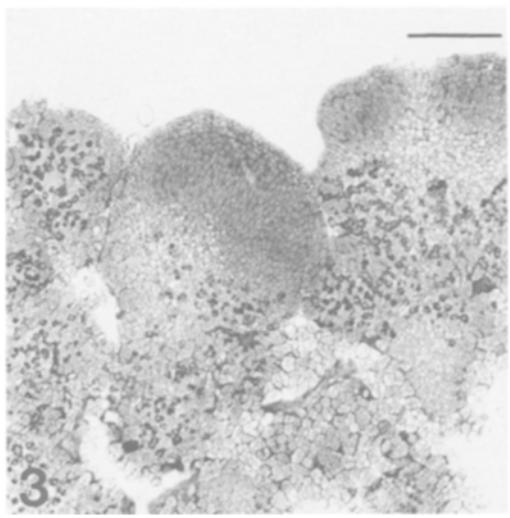
There was little variation in the percentage of EC (32 to 48%) increasing 3,4-D or kinetin supply from 2.25 to 9 μM in clone PB 260 (Table 2). Conversely, when the concentration of one of these growth factors was 10-fold less than the standard concentration (0.45 μM), the embryogenic potential dropped sharply with a concomitant rise in the percentage of FC. The histological observations confirmed that these calli had lower MA or EA than compact calli. Indeed, these compact calli are composed of highly meristematic sites from which embryos will subsequently develop (Fig. 3). Callus browning was found to be correlated with an increase in the kinetin supply. Moreover, histological sections of callus zones not yet showing browning revealed high polyphenol oxidation and an absence of mucilage surrounding these parenchymatous like cells (Fig. 4).

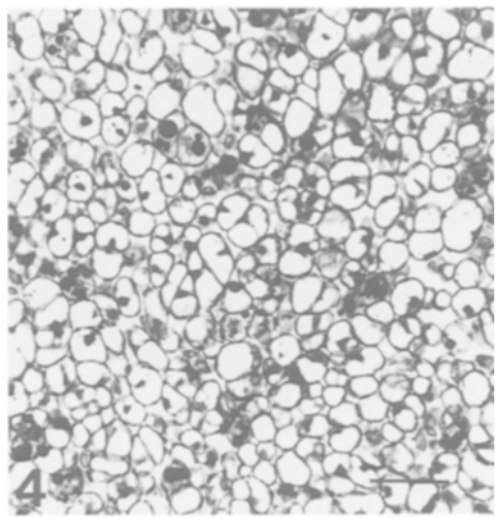
*Sucrose effect*

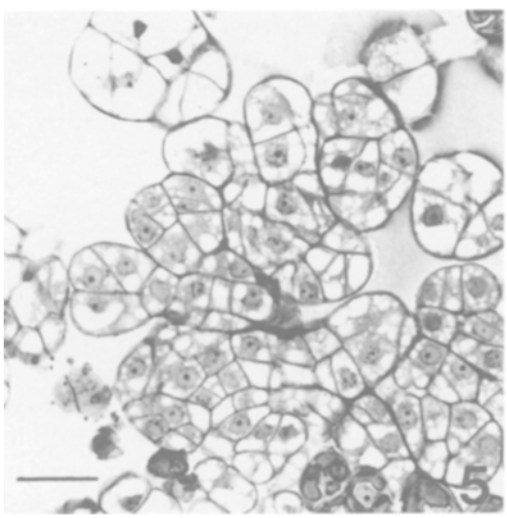
Sucrose level in the MH culture medium had an even more marked effect on the texture and the embryogenic potential of calli (Table 3). The percentage of EC peaked with 292 mM sucrose, accompanied by a marked decrease in callus browning. It should be noted that beyond this concentration (351 mM) many calli lost their compact structure. These FC produced no visible somatic embryos even though the histological investigations indicated that they were composed




*Figs. 1–5.* Morphological aspects of a friable (Fig. 1) and a compact callus (Fig. 2), scale bar = 2.5 mm. Histological aspect of meristematic clusters in a compact callus, scale bar = 210 μm (Fig. 3), parenchymateous tissue and phenolic cells, scale bar = 21 μm (Fig. 4) and embryogenic cells and proembryos in mucilagineous matrix, scale bar = 21 μm (Fig. 5).

*Table 2.* Effect of plant growth regulators (PGR) concentration, 3,4-D or kinetin, in the first culture (day 0 to day 50), compared to the standard MH medium (*), on morphological (embryogenic calli, EC; brown calli, BC; friable calli, FC) and histological (meristematic activity, MA; embryogenic activity, EA; mucilage, Muc; oxidized polyphenols, OP; quantified as follows: absent (−), rare (+), common (+ +), abundant (+ + +)) parameters of calli of *H. brasiliensis* clone PB 260.

| PGR (μM) | Morphological parameters (%) | | | | Histological parameters | | | |
|---|---|---|---|---|---|---|---|---|
| | EC | BC | FC | FEC | MA | EA | Muc | OP |
| 3,4-D | | | | | | | | |
| 0.45 | $0^{a}$ | $0^{a}$ | $100.0^{a}$ | 0 | − | + | − | + + + |
| 2.25 | $36.0^{b}$ | $2.9^{a}$ | $12.9^{b}$ | 0 | + | + | + | + + + |
| *4.5 | $36.4^{b}$ | $0.7^{a}$ | $7.0^{b}$ | 0 | + | + + | + | + |
| 6.75 | $42.0^{b}$ | $0^{a}$ | $5.6^{b}$ | 0 | + | + + | + | + |
| 9 | $37.3^{b}$ | $2.1^{a}$ | $8.5^{b}$ | 0 | + + | + + | + + | + |
| Kinetin | | | | | | | | |
| 0.45 | $4.3^{a}$ | $14.4^{a}$ | $28.4^{a}$ | 0 | + | + | + | + + + |
| 2.25 | $36.7^{bc}$ | $24.5^{ab}$ | $1.8^{b}$ | 0 | + + | + + | + | + + |
| *4.5 | $38.8^{b}$ | $23.7^{ab}$ | $3.6^{b}$ | 0 | + | + + | + | + + |
| 6.75 | $47.9^{c}$ | $27.5^{ab}$ | $0.7^{b}$ | 0 | + | + + | + | + + |
| 9 | $31.7^{b}$ | $37.3^{b}$ | $0.4^{b}$ | 0 | + | + | + | + + |

Percentages followed by the same letter are not significantly different according to the test of Goodman (1965) at the 5% level. Observations at the end of the second subculture (day 50). 144 initial explants were cultivated for each clone.

*Table 3.* Effect of sucrose and calcium concentrations, maintained throughout the all culture period (day 0 to day 50), compared to the standard MH medium (*), on morphological (embryogenic calli, EC; brown calli, BC; friable calli, FC) and histological (meristematic activity, MA; embryogenic activity, EA; mucilage, Muc; oxidized polyphenols, OP; quantified as follows: absent (−), rare (+), common (+ +), abundant (+ + +)) parameters of calli of *H. brasiliensis* clone PB 260.

| Medium (mM) | Morphological parameters (%) | | | | Histological parameters | | | |
|---|---|---|---|---|---|---|---|---|
| | EC | BC | FC | FEC | MA | EA | Muc | OP |
| Sucrose | | | | | | | | |
| 58 | $1.4^{a}$ | $51.8^{a}$ | $1.8^{a}$ | 0 | − | + | − | + |
| 117 | $2.6^{a}$ | $39.7^{ab}$ | $2.6^{a}$ | 0 | − | + | − | + |
| 175 | $43.4^{b}$ | $34.9^{b}$ | $7.8^{a}$ | 0 | + | + | − | + |
| *234 | $36.2^{bd}$ | $12.3^{c}$ | $2.6^{a}$ | 0 | + | + + | + | + + |
| 292 | $61.1^{c}$ | $11.1^{c}$ | $4.8^{a}$ | 0 | + + | + + | + | + + |
| 351 | $25.3^{d}$ | $16.5^{c}$ | $42.4^{b}$ | 0 | + + | + + + | + + | + + |
| Calcium | | | | | | | | |
| 0 | $62.9^{a}$ | $37.7^{a}$ | $3.0^{ab}$ | 0 | + | + | + | + + |
| 1 | $62.3^{a}$ | $28.5^{ab}$ | 0 | 0 | − | + | + | + |
| *3 | $51^{ab}$ | $25.8^{ab}$ | $1.0^{a}$ | 0 | + | + + | + | + + |
| 6 | $58.4^{a}$ | $15.4^{bc}$ | $7.7^{b}$ | 0 | − | + + | + + | + |
| 9 | $42.9^{b}$ | $20.1^{b}$ | $20.1^{c}$ | 0 | + | + + + | + + | + |
| 12 | $2.1^{c}$ | $5.7^{c}$ | $80.1^{d}$ | 0 | + | + + | + + | + |
| 24 | 0 | $100^{d}$ | 0 | 0 | − | − | − | + + + |

Percentages followed by the same letter are not significantly different according to the test of Goodman (1965) at the 5% level. Observations at the end of the second subculture (day 50). 144 initial explants were cultivated for each clone.

of many embryogenic cells and globular proembryos embedded in mucilaginous matrix, and displayed low oxidized polyphenols content (Fig. 5).

*Calcium effect*

The modification of calcium level in the MH basic medium was the most effective mean to

*Table 4.* Effects of two calcium concentrations (3 and 12 mM) in the medium, maintained throughout the all culture period (day 0 to day 50), on morphological parameters (embryogenic calli, EC; brown calli, BC; friable calli, FC) in two *H. brasiliensis* clones (PB 260) and GT1).

| Clone | Calcium (mM) | Morphological parameters (%) | | | |
|---|---|---|---|---|---|
| | | EC | BC | FC | FEC |
| PB 260 | 3 | 51.0[a] | 25.8[a] | 1.0[a] | 0 |
| | 12 | 2.1[b] | 5.7[b] | 80.1[b] | 0 |
| GT 1 | 3 | 1.0[b] | 97.4[c] | 8.7[c] | 0 |
| | 12 | 1.0[b] | 37.9[d] | 56.2[d] | 0 |

Percentages followed by the same letter are not significantly different according to the test of Goodman (1965) at the 5% level. Observations at the end of the second subculture (day 50). 144 initial explants were cultivated for each clone.

induce callus friability (Table 3). The percentage of FC was increased with calcium concentrations above 6 mM up to 12 mM and spectacular rise to 80% friable calli showing no browning was obtained at the latter concentration but dropped to 0 at 24 mM calcium. However, as with sucrose, the texture change occurred at the expense of the embryogenic expression. Embryos were only observed on compact calli, although the histological analysis showed intense EA in this new type of friable callus (Fig. 5). Friable callus on high calcium level were consisted mostly of embryogenic cells and proembryos at an early development stage. We noted an abundance of mucilage in this type of callus, as also observed in calli formed on medium containing 351 mM sucrose. The enhancement of callus friability and limitation of browning at increased calcium supply was confirmed with clone GT1 (Table 4).

## Discussion

Results of the present study confirmed the genotype/medium effects on callus texture in *H. brasiliensis*. On standard MH medium for somatic embryogenesis induction, two clones (PR 107 and RRIM 600) developed friable-embryogenic calli while three others (PB 260, GT1 and PB 235) formed very compact calli highly different in their EC percentage.

Different culture parameters were found to efficiently modify callus texture and to induce friability. Indeed, friability was obtained when auxin or cytokinin concentrations were reduced, thus substantially upsetting of the auxin/cytokinin balance during callogenesis, and when high sucrose (351 mM) or calcium (12 mM) concentrations were maintained throughout the two subcultures. Sucrose and calcium concentrations had to be kept stable to avoid modifying the osmotic potential of the medium, which otherwise would be detrimental to callus development (Etienne et al. 1991). The effects of sucrose and calcium are relatively less known than those of growth regulators. Moreover, in clone PB 260, callus texture could only be modified at extreme culture parameter values, thus confirming the stability of the compact callus texture in this clone.

The histological observations revealed that the different considered parameters did not affect calli in the same ways. FC obtained by modifying the growth regulator balance of the medium had weak or no EA, as they were mainly composed of parenchymatous like cells containing oxidized polyphenols. In contrast, calli obtained in the presence of high calcium or sucrose concentrations were very active and mainly composed of embryogenic cells and globular proembryos embedded in a mucilaginous matrix. The lack of any further development of these proembryos was certainly due to the inadequacy of the culture system. We previously demonstrated, in compact embryogenic calli of clone PB 260 under our culture conditions, the coexistence of two distinct ways for somatic embryogenesis. One from unicellular origin terminated prematurely while the other from multicellular origin lead to the formation of developed embryos (Michaux-Ferrière et al. 1992). Nevertheless, our observations on these friable calli indicated a

high embryogenic potential which could be expressed by modifying the culture conditions.

Different mineral compositions reported to favour callus friability or cellular dispersion in other plant species had no effect on callus texture in *H. brasiliensis* (results not shown): N6 supplemented with 25 mM proline of Armstrong & Green (1985) and AA of Muller & Grafe (1978).

In compact *H. brasiliensis* calli, meristematic sites are focused at callus periphery, whereas the centre of the callus seems to be quite inactive and composed of parenchymatous-like cells saturated with polyphenols (Michaux-Ferrière et al. 1992). This structure promotes gradual browning and then necrosis of all tissues. FC obtained with high level of calcium or sucrose, were composed of embryogenic sites separated from one another and active enough to avoid browning. Hence, browning at one site did not automatically spread to neighbouring cell sites. These calli were thus suitable basic material to obtain long-term cultures on liquid or gelified medium.

It would be interesting to examine the specific effects of these factors on plant material, leading to the formation of two very different types of friable calli. The biochemical mechanism of friability in parenchymatous calli formed as a result of a hormonal imbalance undoubtedly differs from that of mucilaginous embryogenic calli obtained on medium with high calcium or sucrose concentrations. Culture conditions could then be defined in a less empirical way and conclusions extended to other plants through a better overall understanding of these biochemical mechanisms.

Friable embryogenic calli were obtained in four out of five tested *H. brasiliensis* clones. They were formed spontaneously in two clones (RRIM 600 and PR 107) and after modification of the culture medium in the other two (PB 260 and GT1). This suggests that such calli could be obtained in most *H. brasiliensis* clones. These results should signal the way to establish suspensions of embryogenic cell clusters or protoplasts. Indeed, through selection of friable embryogenic calli, Vasil & Vasil (1986) in maize and Gray & Mortensen (1987) in grape were able to successfully establish continuous cultures in liquid medium and agar, respectively.

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