# Notas Científicas

# Somatic embryogenesis and plant regeneration in elite clones of *Theobroma cacao*

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Abstract – The objective of this work was to evaluated a procedure for somatic embryogenesis and regeneration of cacao (*Theobroma cacao* L.) elite clones. Petal explants from cacao clones TSH 565 and TSH 1188 were cultured on PCG and SCG-2 media, for calli growth. Somatic embryos were formed on the surface of embryogenic calli after transfer to embryo development (ED) medium. Clone TSH 565 showed a higher embryogenic potential than TSH 1188. The best combination of carbon source for embryo induction in ED medium was genotype-specific. Embryogenic callus formations increased in micropore tape-sealed Petri dishes, irrespective of cacao genotype. Mature somatic embryos were successfully converted into plantlets.

Index terms: biotechnology, cacao, gas exchange, micropropagation, morphogenesis.

## Embriogênese somática e regeneração in vitro de clones elite de *Theobroma cacao*

Resumo – O objetivo deste trabalho foi avaliar um procedimento para embriogênese somática e regeneração de clones elite de cacau. Pétalas dos clones de cacau TSH 565 e TSH 1188 foram cultivadas em meios PCG e SCG-2 para o crescimento de calos. Embriões somáticos desenvolveram-se na superfície dos calos embriogênicos, após a transferência para o meio ED. O clone TSH 565 apresentou maior potencial embriogênico do que o TSH 1188. A melhor combinação de fonte de carbono quanto à indução de embriões em meio ED foi específica do genótipo. A formação de calos embriogênicos foi superior em placas de Petri seladas com fita hipoalergênica, independentemente do genótipo. Embriões maduros de ambos os genótipos foram convertidos em plântulas.

Termos para indexação: biotecnologia, cacau, troca gasosa, micropropagação, morfogênese.

*Theobroma cacao* L. (cacao) is a tropical perennial tree, endemic to lowland rainforests of the Amazon Basin, and has been domesticated since pre-Columbian times (Hurst et al., 2002). Currently, cacao is grown throughout the humid tropics as a component of complex agroecosystems (Lobão, 2007) to supply the crescent global demand for cocoa, the processed form of cacao seeds used by the chocolate industry.

Since 1989, the witches-broom disease, caused by the hemibiotrophic fungus *Moniliophthora perniciosa* (= *Crinipellis perniciosa*), has spread throughout Brazil, destroying cacao tree (*Theobroma cacao* L.) plantations and leading to important economical and social changes in affected areas, such as the State of Bahia (Rocha et al., 1993; Purdy & Schmidt, 1996). The vegetative clonal propagation of elite cacao genotypes, resistant to *M. perniciosa*, has been used as a potential means to recover cacao production in Brazil (Rudgard et al., 1993). However, there are some disadvantages associated with the current methods for vegetative propagation of cacao, via rooting or grafting of plagiotropic cuttings, including the intensive labor and associated costs, low propagation rate, and an undesirable bush-like growth pattern (Figueira & Janick, 1995).

The clonal propagation of cacao, through modern biotechnology techniques, can be greatly facilitated by multiplying elite materials using somatic embryogenesis. The main advantages of this method include the possibility of rapidly generating asexually propagated uniform plants of high genetic value, and the clonal production of orthotropic plants with

normal dimorphic architecture and taproot formation (Maximova et al., 2002). Somatic embryo production and plantlet regeneration have been achieved in a large number of genotypes (Li et al., 1998; Maximova et al., 2002), although practical utilization of this technology for clonal propagation remains hindered by an inability to successfully induce somatic embryogenesis in many elite cacao genotypes.

The objective of this work was to evaluate a procedure for somatic embryogenesis and plant regeneration from petal tissues of the elite cacao clones TSH 565 and TSH 1188, using the basic protocol described by Li et al. (1998) and Maximova et al. (2002). The effects of genotype, carbon source, and different Petri dish sealing material on somatic embryogenesis were also assessed.

Unopened immature flower buds about 4 to 5 mm long, from field-grown cacao plants, were collected early in the morning at Cepec/Ceplac (Centro de Pesquisas da Comissão Executiva do Plano da Lavoura Cacaueira, Ilhéus, Bahia, Brazil). Immature flower buds were surface-sterilized by immersion in 70% (v/v) ethanol, for 1 min and 30 s, followed by 20 min in a 2.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20 and, then, five rinses in sterile distilled water. Explants were prepared by briefly blotting the immature flowers with sterile paper towels and slicing them perpendicular to their longitudinal axis at a position 1/3 of the flower length from the base, using a sterile scalpel blade. Petal tissues were extracted through the opening at the cut end using a sharp sterile forceps. Petal bases were used as explants source.

Petal base explants were first cultured on primary callus growth (PCG) medium (Li et al., 1998), with the abaxial surface in contact with the medium. Cultures were maintained in the dark at 27±2°C for 2 weeks, then transferred for another 2 weeks in a secondary callus growth (SCG-2) medium (Maximova et al., 2002). Somatic embryos were induced by transfer of floral tissue-derived calli from SCG-2 medium to embryo development (ED) medium (Maximova et al., 2002). Cultures were subcultured every 2 weeks in ED medium, under the same incubation conditions described above.

Various concentrations of sucrose, in combination or not with glucose in ED medium, were evaluated for their ability to stimulate somatic embryo production in the two cacao genotypes, as follows: ED4w/g–ED4w/g (first subculture in ED medium, supplemented with 4% sucrose and 0.1% glucose – subsequent subcultures in same ED medium), ED4w/g–ED3w/g (first subculture in ED medium supplemented with 4% sucrose and 0.1% glucose – subsequent subcultures in ED medium supplemented with 3% sucrose and 0.1% glucose), ED3w/g–ED3w/g (first subculture in ED medium supplemented with 3% sucrose and 0.1% glucose – subsequent subcultures in same ED medium), ED4–ED4 (first subculture in ED medium supplemented with 4% sucrose – subsequent subcultures in same ED medium), ED4–ED3 (first subculture in ED medium supplemented with 4% sucrose – subsequent subcultures in ED medium supplemented with 3% sucrose), and ED3–ED3 (first subculture in ED medium supplemented with 3% sucrose – subsequent subcultures in same ED medium).

In order to evaluate the effects of Petri dish sealing material on frequency of somatic embryos production, petal base explants from both cacao genotypes were cultured in Petri dishes containing PCG, SCG-2, and ED (ED3w/g–ED3w/g) media, as describe above, sealed with two rounds of parafilm (American National, USA), polyvinyl chloride (PVC) transparent film, or hypoallergenic micropore tape (Micropore 3 M, Brazil).

Experiments were performed with 25 explants per Petri dish, five Petri dishes per treatment, and repeated once at least. The percentage of embryogenic and rhizogenic calli over the total number of cultured explants, representing the frequency of embryogenic and rhizogenic calli, respectively, and the average number of embryos per explant were determined two months after the culture initiation. Statistical analysis was performed with the software BIOESTAT (Universidade Federal do Pará, Brazil), which tested the experiments as a completely randomized design. Data of frequencies (%) were arc sen $x^{0.5}$ transformed prior to statistical analysis. Analysis of variance was applied and, for means comparison, Bonferroni's test was used, with a critical value at 5% probability.

The clone TSH 565 presented significantly higher frequency of embryogenic calli (42%) than the TSH 1188 (4.3%). The frequency of rhyzogenic calli in TSH 1188 was as twice as higher than the frequency of embryogenic calli, which indicates a need of further protocol improvement. The clone TSH 565 also produced significantly more embryos per explant (4.8) compared to TSH 1188 (1.6). However, the milky to

translucent rate of embryos was lower in TSH 565 (ca. 45%) than in TSH 1188 (ca. 70%). Milky embryos present higher conversion rates into plantlets, as compared to translucent embryos (Teixeira et al., 2002). Furthermore, they have cotyledons with higher embryogenic potential for secondary embryogenesis, increasing the efficiency and quality of embryos produced in vitro (Maximova et al., 2002).

Carbon source in ED medium significantly affected the rhyzogenic calli frequency, the average number of embryos per explant, and the average number of milky embryos per explant produced by both cacao clones (Table 1). In general, rhyzogenic calli were produced in higher frequencies, when explants from both clones were cultured on ED4–ED4 medium, although these differences were not significant for TSH 1188. A high number of embryos per explant was obtained in ED4w/g–ED4w/g and ED4w/g–ED3w/g media for TSH 565. For TSH 1188, more embryos per explant were produced in ED4w/g–ED3w/g, ED3w/g–ED3w/g, and ED3–ED3 media. The clone TSH 565 produced significantly more milky embryos per explant, when cultured in ED4w/g–ED4w/g and ED4–ED3 media, while the best responses for TSH 1188 were obtained in ED4w/g–ED3w/g and ED3–ED3 media. Sucrose appears to be the most effective carbon source for somatic embryogenesis in general, and its combination with glucose (ED3w/g–ED3w/g) has been reported to be beneficial in cacao (Li et al., 1998). This study demonstrates that glucose is not considered to be an essential requirement for milky embryo production in TSH 565 and TSH 1188, since similar results can be obtained in the absence of this sugar, through variation or maintenance of sucrose levels in ED medium, depending on cacao genotype.

Petal explants cultured in Petri dishes, sealed with micropore tape produced significantly more embryogenic calli than those cultured in Petri dishes sealed with PVC or parafilm, irrespective of cacao genotype (Table 2). However, the differences were not

**Table 1.** Effect of different carbon sources on rhyzogenic calli frequency, average number of embryos per explant, and average number of milky embryos per explant of petal explants, from TSH 565 and TSH 1188 cultured in embryo development (ED) medium[(1)].

| Cacao clone | Carbon source (ED medium) | Rhyzogenic calli frequency (%)[(2)] | Average number of embryos per explant | Average number of milky embryos per explant |
|---|---|---|---|---|
| TSH 565 | ED4w/g–ED4w/g | 13b | 5.53a | 3.18a |
| | ED4w/g–ED3w/g | 6b | 5.55a | 2.01ab |
| | ED3w/g–ED3w/g | 13b | 3.49c | 1.39b |
| | ED4–ED4 | 26a | 3.52c | 1.36b |
| | ED4–ED3 | 8b | 5.44ab | 3.49a |
| | ED3–ED3 | 3b | 4.74b | 1.56b |
| TSH 1188 | ED4w/g–ED4w/g | 12[ns] | 0.04b | 0.04b |
| | ED4w/g–ED3w/g | 9 | 1.60a | 1.60a |
| | ED3w/g–ED3w/g | 10 | 1.03a | 0.47b |
| | ED4–ED4 | 12 | 0.00c | 0.00b |
| | ED4–ED3 | 6 | 1.00ab | 0.20b |
| | ED3–ED3 | 9 | 1.60a | 1.40a |

[(1)]Data from two independent experiments; means followed by the same letters, for each cacao clone, are not statistically different by Bonferroni's test, at 5% probability. [(2)]Data of rhyzogenic calli frequency (%) were transformed (arc sen $x^{0.5}$) prior to statistical analysis.

**Table 2.** Effect of different Petri dish sealing materials on embryogenic calli frequency and average number of embryos per explant of petal explants, from TSH 565 and TSH 1188 cultured in embryo development (ED) medium[(1)].

| Cacao clone | Petri dish sealing material | Embryogenic calli frequency (%)[(2)] | Average number of embryos per explant |
|---|---|---|---|
| TSH 565 | PVC | 19c | 1.93[ns] |
| | Parafilm | 45b | 1.80 |
| | Micropore tape | 65a | 1.96 |
| TSH 1188 | PVC | 2c | 0.17[ns] |
| | Parafilm | 9b | 0.12 |
| | Micropore tape | 13a | 0.19 |

[(1)]Data from two independent experiments; means followed by the same letters, for each cacao clone, are not statistically different by Bonferroni's test, at 5% probability. [(2)]Data of embryogenic calli frequency (%) were transformed (arc sen $x^{0.5}$) prior to statistical analysis.

significant, when the average number of embryos and milky embryos per explant were evaluated. Carbon dioxide and ethylene are produced by all plant tissues cultured in vitro, they may accumulate to high levels inside culture vessels, under low light and reduced gas exchanges with the external environment, and may be detrimental or beneficial for cell growth and regeneration (Marino & Berardi, 2004). Micropore tape allows better gaseous exchange with the external environment, reducing carbon dioxide and ethylene accumulation inside the culture vessels.

Mature somatic embryos were selected for conversion into plantlets. It was observed that the conversion rate varied between the two cacao clones, and TSH 1188 showed higher rates than TSH 565. This observation could be related to morphological characteristics of the embryos. Besides more milky embryos, TSH 1188 presented higher rates of embryos with well-defined hypocotyl and cotyledons (normal embryos), while most of the embryos produced by TSH 565 presented fused hypocotyls and underdeveloped or extra cotyledons (abnormal embryos). About six months after culture initiation, plantlets of TSH 565 and TSH 1188, with more than three leaves and with healthy root systems, were successfully established in greenhouse.

Collectively, these data indicate that the cacao elite clones TSH 565 and TSH 1188 can be successfully regenerated from petal tissues, by using the somatic embryogenesis methods described by Li et al. (1998) and Maximova et al. (2002). Modifications of carbon source in ED medium and Petri dish sealing material improve the efficiency of the method. This procedure may allow the practical use of somatic embryogenesis for clonal propagation and genetic transformation of these important elite clones.

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