

Histological analysis of calluses from *in vitro* propagated plants of *Cleome spinosa* Jacq.

Norma Albarello¹, Ivan Gonçalves Ribeiro², Claudia Simões³,
Tatiana Carvalho de Castro⁴, Márcia G. Gianfaldoni⁵, Cátia H. Callado⁶,
Ricardo Machado Kuster⁷, Marsen Garcia Pinto Coelho⁸ and Elisabeth Mansur⁹

Introduction

Several compounds from plants have been related to potential applications for medicinal activities. Great interest has been paid to the beneficial effects of beta carotene (BC) mainly due its antioxidant function, showing a link with cancer prevention [1, 2].

Plant cell culture is a viable tool for production of natural products. Given the important role of carotenoids as a medicinal and biotechnological class of natural pigments [3], a continuous system of cell proliferation as calluses cultures is very useful for the study of BC biosynthesis.

C. spinosa is a shrub known in Brazil under the name of "mussambê". Recent investigation of this species led to the isolation of a rare flavone, flindulatin, and four new cembranes, which are known to display several biological function, including anti-HIV and cytotoxicity against several human cancer cell lines [4].

In order to establish callus cultures that could be used in studies of medicinal compounds of *C. spinosa* related to anticancer activity, in the present work, we report the histological analysis of calluses and BC localization at different stages of callus formation of *C. spinosa*.

Materials and methods

A. Callus culture and Pigment identification

Stem segments from *in vitro*-raised plants were placed on solid MS medium [5] supplemented with PIC or 2,4-D (0.1-1.0mg.L⁻¹). Calluses were transferred to fresh medium containing the same growth regulators concentrations at 30 days intervals and subcultured for three cycles. The cultures were incubated at 26±2°C or 36±2°C and 16-h photoperiod under an irradiance of 45µmol m⁻² s⁻¹ supplied by cool white fluorescent and GroLux lamps [6].

A lipophilic mass containing an orange pigment spread out on the callus surface was suspended in acetone and filtered through paper (Whatman n^o 2). The solution was concentrated in rotatory evaporator and solubilized in ethanol to record visible absorbance

spectra. Commercial BC standard (Sigma Chemical Co., St. Louis, MO) was used in TLC and HPLC analyses to compare with the pigment produced from callus.

B. Histological analysis

For histological studies and analysis of the cellular localization of the pigment, explants at different stages of callus development were periodically fixed during 40 days, at three days intervals, in a solution of 2.5% glutaraldehyde and 4.0 % paraformaldehyde buffered with 0.05 M cacodylate buffer at pH 7.2. Subsequently, the samples were post-fixed in 1% osmium tetroxide in the same buffer at room temperature. Then, samples were dehydrated through acetone series, infiltrated and embedded in Epon resin. Semithin sections (2 µm) of the calluses were cut and stained with toluidine blue. Lipid and starch content were identified using Sudan III and Lugol, respectively [7, 8]. Figures were obtained using a Cool Snap-PRO video camera attached to an Olympus BX 50 microscope.

Results and Discussion

Callogenesis was observed on all media tested. Shoot-forming calluses were induced on 0.1mg.L⁻¹ 2,4-D or PIC at 26±2°C (Fig. 1A). Histological examinations of this material revealed indirect development of few shoots and no evidence of somatic embryogenesis was found (Fig. 1B).

The production of beta carotene was observed in cultures maintained on media supplemented with PIC or 2,4-D, specially at 36±2 °C (Fig. 1C). Temperature has proven to be an important factor affecting the biosynthetic activity of cells in other species [9]. After seven days of culture, chloroplast and starch grains were frequently observed (Fig. 1D). Partially differentiated tracheary elements containing pigment were also found (Fig. 1E). It is possible that some morphological differentiation on callus culture has some importance for metabolite production of *C. spinosa*, as reported by other authors [10,11,12].

Orange needle-like structures were frequently observed in the callus cells after 21 days of culture.

1. Professor Adjunto do Departamento de Biologia Vegetal, Instituto de Biologia Roberto de Alcântara Gomes, Universidade do Estado do Rio de Janeiro. Rua São Francisco Xavier, 524, PHLC, sala 509, Laboratório de Biotecnologia de Plantas (LABPLAN), Maracanã, Rio de Janeiro, RJ, CEP 20550-013. E-mail: labplan_uerj@yahoo.com.br

2. Aluno de Mestrado do Programa de Pós-graduação em Biologia, UERJ.

3. Bióloga do Laboratório de Biotecnologia de Plantas (LABPLAN), UERJ.

4. Técnica em Biotecnologia do Laboratório de Biotecnologia de Plantas (LABPLAN), UERJ.

5. Bióloga - Bolsista PROATEC do Departamento de Biologia Vegetal, Instituto de Biologia Roberto de Alcântara Gomes, UERJ.

6. Professor Adjunto do Departamento de Biologia Vegetal, Instituto de Biologia Roberto de Alcântara Gomes, UERJ.

7. Professor Adjunto do Núcleo de Pesquisas em Produtos Naturais (NPPN), UFRJ.

8. Professor Adjunto do Departamento de Bioquímica, Instituto de Biologia Roberto de Alcântara Gomes, UERJ.

9. Professor Titular do Departamento de Biologia Celular e Genética, Instituto de Biologia Roberto de Alcântara Gomes, UERJ.

Financiamento: FAPERJ.

These structures resemble the carotene bodies named “crystalline cromoplasts”, which are found in *Daucus carota* roots [13]. Accumulation of orange bodies was found in the apoplast within 30 days (Fig. 1F) and cells also presented lipid bodies (Fig. 1G), which were confirmed by Sudan III reagent (Fig. 1H). BC production could be seen on the apoplast about three weeks in culture, while the pigment spread on the callus surface was observed with naked eye only after one month. During callus development, chloroplasts became scarce and the lipid bodies became more present. It is possible that the lipid bodies could constitute plastoglobuli, since *in vitro* culture conditions are related to stress situations and changes in the ultrastructure of chloroplasts as well as increase in the number and size of plastoglobuli and lipid bodies are usually the earlier symptoms of stress-induced injury [14,15,16,17,18].

Acknowledgements

The authors are grateful to Dra. Maria Bernadete Costa e Silva, Herbarium IPA - Pernambuco, Brazil, for the identification of plant material. This work was supported by FAPERJ.

References

- [1] FINLEY, J.W. 2005. Proposed criteria for assessing the efficacy of cancer reduction by plant foods enriched in carotenoids, glucosinolates, polyphenols and selenocompounds. *Annals of Botany*, 95: 1075-1096.
- [2] PEREZ-GALVEZ, A.; RIOS, J.J. & MINGUEZ-MOSQUERA, M.I. 2005. Thermal degradation products formed from carotenoids during a heat-induced degradation process of paprika oleoresins (*Capsicum annuum* L.). *Journal of Agricultural and Food Chemistry*, 53: 4820-4826.
- [3] LEE, P.C. & SCHIMDT-DANNERT, C. 2002. Metabolic engineering towards biotechnological production of carotenoids in microorganisms. *Applied Microbiology and Biotechnology*, 60: 1-11.
- [4] COLLINS, D. O.; REYNOLDS, W. F.; REESE, P.B. 2004. New cembranes from *Cleome spinosa*. *Journal of Natural Products*, 67:179-183.
- [5] MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15:473-497.
- [6] ALBARELLO, N. *Aplicação de métodos biotecnológicos para estudos fitoquímicos e farmacológicos de Cleome spinosa Jacq. (Capparaceae)*. 2005. Tese de Doutorado, Programa de Pós-graduação em Biologia, UERJ, Rio de Janeiro.
- [7] SASS, J. E. 1951. *Botanical microtechnique*. 2a Ed. Ames, Iowa State College Press, 391 p.
- [8] JENSEN, W. A. 1962. *Botanical histochemistry, principles and practice*. San Francisco, W. H. Freeman, 409 p.
- [9] AKSU, Z. & EREN, A.T. 2005. Carotenoids production by the yeast *Rhodotorula mucilaginosa*: use of agricultural wastes as a carbon source. *Process Biochemistry*, 40: 2985-2991.
- [10] YOSHIKAWA, T. & FURUYA, T. 1985. Morphinan alkaloid production by tissues differentiated from cultured cells of *Papaver somniferum*. *Planta Medica*. 51:110-113.
- [11] BAIS, H.P.; SUDHA, G. & RAVISHANKAR, G.A. 1999. Putrescine influences growth and production of coumarines in hairy root cultures of *Cichorium intybus*, L. cv. Lucknow local (Witloof Chicory). *Journal of Plant Growth Regulators*, 18: 159-165.
- [12] ZHAO, J.; HU, Q.; GUO, Y-Q. & ZHU, W-H. 2001. Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. *Applied Microbiology and Biotechnology*, 55: 693-698.
- [13] CAMARA, B.; HUGUENEY, P.; BOUVIER, F.; KUNTZ, M. & MONEGER, R. 1995. Biochemistry and molecular biology of chromoplast development. *International Review of Cytology*, 63: 175-247.
- [14] BONDADA, B.R. & OOSTERHUIS, D.M. 2002. Ontogenic changes in epicuticular wax and chloroplast integrity of cotton (*Gossypium hirsutum* L.) leaf. *Photosynthetica*, 40: 431-436.
- [15] KAUP, M.T.; FROESE, C.D. & THOMPSON, J.E. 2002. A role for diacylglycerol acyltransferase during leaf senescence. *Plant Physiology*, 129:1616-1626.
- [16] MERZLYAK, M.N. & SOLOVCHENKO, A.E. 2002. Photostability of pigments in ripening apple fruit: a possible photoprotective role of carotenoids during plant senescence. *Plant Science*, 163: 881-888.
- [17] KUTÍK, J.; HOLÁ, D.; KOCOVÁ, M.; ROTHOVÁ, O.; HASEL, D. & WILHELMOVÁ, N. 2004. Ultrastructure and dimensions of chloroplasts in leaves of three maize (*Zea mays* L.) inbred lines and their F1 hybrids grown under moderate chilling stress. *Photosynthetica*, 42:447-455.
- [18] PAPADAKIS, I. E.; DIMASSI, K. N.; BOSABALIDIS, A. M.; THERIOS, I. N.; PATAKAS, A. & GIANNAKOULA, A. 2004. Effects of B excess on some physiological and anatomical parameters of “Navelina” orange plants grafted on two root stocks. *Environmental and Experimental Botany*, 51:247-257.

Figure 1. Callus cultures derived from stem explants of *in vitro* propagated of *C. spinosa*. (A) Adventitious bud formation on callus cultured on medium supplemented with 0.1 mg.L⁻¹ 2,4-D. (B) Section of organogenic callus showing bud development. (C) Pigmented calluses obtained on medium supplemented with 1.0 mg.L⁻¹ 2,4-D (left) and 1.0 mg.L⁻¹ PIC (right). (D) Light microscopy of 14-day-old callus cultured on medium supplemented with 1 mg.L⁻¹ PIC showing chloroplasts (arrow) and starch grains (asterisk). (E) Detail of tracheary elements in the inner part of callus showing the pigment content. (F) Light microscopy of callus showing orange needle-like structures after 21 days of culture on medium supplemented with 1 mg.L⁻¹ PIC. (G) Section of pigmented callus showing lipid bodies (asterisk) 28 days after culture initiation on medium supplemented with 1 mg.L⁻¹ PIC. (H) Lipid bodies (arrow) stained in Sudan III. Bars: Fig. 1A, 1 mm; Fig. 1B, 40 µm; Fig. 1C, 0.67 cm; Fig. 1D, 50 µm; Fig. 1E, 50 µm; Fig. 1F, 10 µm; Fig. 1G, 20 µm; Fig. 1H, 20 µm.

