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

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**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, name of “mussambê”. Recent investigation of this species led to the localization of the pigment, explants at different stages of callus formation 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° 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.

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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].

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References


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 callouses 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.