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Effect of screening and subculture on the production of betaxanthins in *Beta vulgaris* L. var. 'Dark Detroit' callus culture

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Abstract

Plant cell culture is an innovative technology to produce a variety of substances including natural dyes. Betaxanthins are considered food-safe nutraceutics pigments because exhibit antiradical and antioxidant activity. An important obstacle for developing large-scale production systems based in plant cells has been the instability of metabolite accumulation. In this work, a protocol was established to obtain yellow callus of *B. vulgaris* var. 'Dark Detroit'. Homogeneous and heterogeneous pigmented callus were obtained with yellow, red, orange and colorless phenotype. Particular attention was done to isolate and establish a yellow line. After continuous screening of the more intense yellow callus it was possible to increase the betaxanthins production 1.8-fold after 48 subcultures. Spectrophotometric and chromatographic analysis of the pigments, confirmed the presence of betaxanthins. HPLC analysis indicated two mainly distinct betaxanthins, vulgaxanthin I and II. *B. vulgaris* yellow callus line did not loose pigment production ability as a result of long-term subculture.

Keywords: Betaxanthins production; *Beta vulgaris*; Callus culture; Long-term subculture

Industrial relevance: The stable *B. vulgaris* cell culture technology developed could be an alternative process to obtain betaxanthins as nutraceutic pigments.

1. Introduction

Natural pigments derived from plants are valuable as color additives for foods, pharmaceutical products and cosmetics. Since color plays a decisive role in consumer acceptance of food, to meet consumer expectations numerous additives are added to restore the initial appearance or for color reinforcement. The demand of natural colors has increased because they are safe in contrast to synthetic dyes (Delgado-Vargas, Jiménez-Aparicio, & Paredes-López, 2000).

Betalains are nitrogenous plant pigments characteristic of the order Caryophyllales. The main structural characteristic common to all betalains is betalamic acid, which is condensed with *cyclo*-DOPA to form the red colored betacyanins, or with different amino acids and amines in yellow betaxanthins (Strack, Vogt, & Schliemann, 2003). Betalains are considered as food-safe colorants, water soluble and do not need chemical modification for dispersion in a wide range of products and do not provide non-desirable flavors to foods. Specifically, there is a growing interest in betaxanthins because of their antiradical and antioxidant activity (Cai, Sun, & Corke, 2003; Kanner, Harel, & Granit, 2001; Tesoriere, Butera, Allegra, Fazzari, & Livrea, 2005). Also, betaxanthins can be use as a mean of introducing essential amino acids in the diet (Delgado-Vargas et al., 2000).

Plant cell culture has been considered as an alternative to produce a wide variety of secondary metabolites which are difficult to obtain by chemical synthesis including natural dyes (Knorr et al., 1990; Zhong, 2001). There are studies on the

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production of betalains using *Beta vulgaris* varieties, i.e. 'Crosby Egyptian' (Rodriguez, Jimenez, Davila, & Sepúlveda, 1994; Trejo-Tapia et al., 1999; Trejo-Tapia, Jiménez-Aparicio, Rodríguez-Monroy, De Jesús-Sánchez, & Gutiérrez-López, 2001), 'Bikores Monogerm' (Khlebnikov, Dubuis, Kut, & Prenosil, 1995; Leathers, Davin, & Zrÿd, 1992) and 'Dark Detroit' (Akita, Hika, & Nishi, 2000). In those studies, betacyanins are reported as the principal dye while the possibility of producing betaxanthins has not been intensively studied.

A major obstacle for developing large-scale production systems based in plant cells has been the instability of metabolite accumulation. Several reports show gradual loss of secondary metabolite production ability, inconsistent production pattern or high variation in levels (Kim, Gibson & Shuler, 2004). In particular, the development of cell lines which produce betaxanthins represents a major task because these pigments are easily oxidized (Gandia-Herrero, García-Carmona & Escribano, 2006). The aim of this work was to establish a yellow callus line of *B. vulgaris* 'Dark Detroit', to analyze the produced pigments and to follow betaxanthins accumulation during long-term subculture.

2. Materials and methods

2.1. Plant material

B. vulgaris variety 'Dark Detroit' seeds were obtained from 'Semillas Battle', S. A. (Mexico). The seeds were washed with aqueous detergent for 10 min and surface sterilized with 70% ethanol for 5 min, 3% NaOCl with five drops of Tween 80 as wetting agent for 20 min, followed by three washes for 5 min each with sterile distilled water. Seeds were then germinated on hormone-free B5 culture medium (Gamborg, Miller, & Ojima, 1968) with 20 g L^{-1} sucrose and 2.0 g L^{-1} phytagel. The seedlings were grown at 25 °C and photoperiod of 16 h (at about 40 μmol m^{-2} s^{-1}).

2.1.1. Callus induction and cell line establishment

Callus induction was carried out on B5 culture medium supplemented with 20 g L^{-1} sucrose, 2 g L^{-1} phytagel and different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D; 0.5, 1.0 and 1.5 mg L^{-1}) and kinetin (0.05, 0.10 and 0.20 mg L^{-1}). Hypocotyl and leaf (*ca*. 1 cm long) explants were excised from 20-day-old seedlings and plated on 60×10 mm Petri dishes with 10 mL of the corresponding culture medium. The pH of the medium was adjusted to 5.5 prior to the addition of gelling agent and autoclaving at 121 °C for 15 min. The plant material was incubated as described above.

Induced callus were separated in accordance to their color and transferred every 14 days to the same culture medium for growth and pigment analysis. Every subculture, cells of different color were eliminated. After the yellow callus line was established, the kinetical growth (dry and fresh weight) and the concentration of betaxanthins were evaluated every three days for 16 days. In all cases, five samples per each day were analyzed. The doubling time dt (days) between two sampling points were calculated according to the following equation (King & Street, 1977):

$$dt = (\ln 2/\mu) \quad (1)$$

2.1.2. Spectrophotometric analysis of pigments

Pigments were extracted by the method proposed by Rodriguez et al. (1994) which consists in an aqueous extraction and enzymatic inactivation at 80 °C for 2 min, before filtration and centrifugation (Beckman, Avanti J-25, USA) at 10,000 min^{-1} for 30 min, followed by freeze-driyng (LabConCo Freezone 16, USA). The visible spectra of a sample containing 1 mg mL^{-1} of pigment prepared in de-ionized water was recorded between λ=400–700 nm using a spectrophotometer (Shimadzu UV-1000, Japan). Pigment content was determined as previously described by Nilson (1970).

2.1.3. Separation of pigments by TLC

Separation of betalains was accomplished by a preparative TLC procedure (Bilyk, 1981), using 0.5 mm thick cellulose (MERCK) hand-coated TLC plates (20×20 cm). These plates were activated in a convection oven (Lab-Line 3481, USA) at 50 °C for 24 h before use. The aqueous extract of yellow callus was re-suspended in 1 mL de-ionized water, centrifuged at 5000 min^{-1} for 2 min for analysis. Samples of 4 μL were applied on the plates. Red beet-powder solution (Redibeets E-162, Boca Raton, Fl.) in a concentration of 10% (p/v) was used as a standard. The samples were developed with isopropanol: ethanol:water:acetic acid (30:35:30:5, v/v) as mobile phase. After the solvent moved 15 cm, the plate was dried at ambient temperature protected from light. Each fraction was carefully separated from the plate, re-suspended in 1 mL de-ionized water and centrifuged at 5000 min^{-1} for 2 min. Finally, a visible spectrum for the supernatant was obtained in a spectrophotometer and its concentration was determined using the above-mentioned methodology.

2.1.4. Betaxanthins analysis by HPLC

Prior to HPLC analysis, aqueous extracts of the pigments were desalted in a Sephadex G-25 column (Pharmacia, Sweden) and treated in a 20×1.6 cm Dowex 50 W-X2 ion-exchange column. The yellow fraction was evaporated to dryness under vacuum employing a Büchi rotary evaporator (Laboratoriums-Technick, Schweiz, Switzerland). The residue was dissolved in 100 μL of a solution of 0.2% TFA and 10% of formic acid (65:35 v/v), centrifuged and filtered through 0.2 μm membranes (SupelCo, USA) to avoid impurities. A Beckman Golden System 60 (USA) liquid chromatograph equipped with a diode photo array detector and a binary pump was used for the analysis of the various betaxanthins. HPLC analysis was carried out using the method reported by Hempel and Böhm (1997). Sample (20 μL) was injected into a 250×4.6 mm Waters Spherisorb ODS-2 C_{18} reverse phase column (5 μm particle size). The elution was performed at a flow rate of 1.0 mL $min^{-1}$with solvent A composed of 0.2% TFA and 10% of HCOOH (65:35 v/v), and eluent B was