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Anthocyanins from *Eugenia myrtifolia* Sims

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Abstract

Eugenia myrtifolia Sims fruits have been characterized for their anthocyanin content and quality. Following the common procedures and validated methods for separation, identification and quantification of anthocyanins, we found the presence of malvidin 3,5-*O*-diglucoside as a unique anthocyanin (32,59 mg/100 g fresh weight of the fruit). An *in vitro* shoot culture of *E. myrtifolia* was established in order to explore the possibility to produce pigments *in vitro*. The presence of the only malvidin 3,5-*O*-diglucoside could be a useful feature in order to manipulate biotechnologically the anthocyanin biosynthetic pathway in the *in vitro* material (callus and suspension cultures).

Keywords: Anthocyanin; *Eugenia myrtifolia*; Australian brush cherry; *In vitro* culture

Industrial relevance: The research interest on the phenolic composition of non-traditional food plants is a leading trend in the looking for biofunctional compounds to be included in "designer foods".

Eugenia myrtifolia, a *Myrtaceae* species from the South hemisphere, produces red edible fruits, that have been characterized for their anthocyanin composition, revealing the presence of the only malvidin 3,5-*O*-diglucoside, at relatively low amounts. Since attempts for *in vitro* callus production and for anthocyanin induction from the callus of *E. myrtifolia* have been carried out, the presence of the specific anthocyanin compound could be a useful feature in order to manipulate biotechnologically the anthocyanin biosynthetic pathway in the *in vitro* material, pushing the biosynthetic pathway towards the unique final product. Since malvidin is a final product (methoxylated anthocyanin) of the anthocyanin pathway, this *in vitro* system could help to study the regulation of the pathway, altering the genetic (regulator genes) or physiological control of the pathway. This could allow to obtain better product yields even in another *in vitro* systems, possibly overcoming the key bottleneck of the secondary metabolite production by plant cell and tissue cultures.

1. Introduction

Eugenia myrtifolia Sims (also classified as *Syzygium paniculatum* Banks ex Gaertn.) is a subtropical evergreen shrub, native of the tropical area of Australia and growing to 5 m high. It has dark green ovate leaves formed in pairs. In spring, it bears small white flowers followed by red, edible fruits in autumn. The fruits are similar to sweet cherries (nearly 1.5 cm diameter) with bright-red skin, pale cream flesh and red-coated seeds; for these fruits the tree is also called Australian brush cherry. Although it is an edible fruit, so far it is reported in Australia to be used candied to decorate cakes. Instead, in USA and Europe *E. myrtifolia* is used only as an ornamental plant for hedging, limited to frost-free areas.

Some *Eugenia sp*. fruits have been characterized for their anthocyanin content (Kuskoski et al., 2003) and have also been found to have anti-diabetes properties (Pepato et al., 2005).

The production of anthocyanins from cell cultures is attractive, since it allows both continuous yield from cell biomass and the control of anthocyanin forms. Anthocyanins from plant tissue cultures have been successfully obtained in a number of plant species, and in our laboratory from sour cherry callus cultures (Blando, Scardino, De Bellis, Nicoletti, & Giovinazzo, 2005).

We report in this paper the characterization of anthocyanins in *E. myrtifolia* Sims fruits and the *in vitro* establishment of axenic cultures of the "Australian brush cherry", in order to induce the pigment production from callus cultures.

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2. Materials and methods

2.1. Pigment identification

2.1.1. Materials

The fruits of *E. myrtifolia* Sims were hand harvested in Lecce, Italy, during autumn 2005, placed in polyethylene bags and stored at −20 °C until use. Trifluoroacetic acid (TFA) (99.9%) was purchased from Romil Ltd (Cambridge, United Kingdom). Hydrochloric acid (HCl) (36–38%), potassium hydroxide (KOH), acetonitrile and water of high-performance liquid chromatography (HPLC) grade, ethanol, methanol and ethyl acetate of analytical grade were provided by J.T. Baker (Deventer, Holland). Cyanidin 3-*O*-glucoside was purchased from Extrasynthese (Genay, France). Deionized water was used to prepare all solutions, unless otherwise indicated. Sodium hypochlorite solution used for surface sterilization of plant explants was the commercial ACE (Procter & Gamble). Murashige & Skoog Medium, sucrose, agar (Plant agar) and plant growth regulators (BAP, IBA and GA_3) used in tissue culture were purchased from Duchefa Biochemie (Haarlem, The Netherlands). Polyvinylpyrrolidone (PVP) and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.2. Extraction of anthocyanins

The fruits (60 g) were cut in small pieces and extracted in the dark by stirring with 100 mL of 0.1% HCl (v/v) in methanol for 3 h at room temperature. The samples were filtered on a Buchner funnel and the solid residue washed with an additional 50 mL of 0.1% HCl (v/v) in methanol. Filtrates were combined and dried using a rotary evaporator at 30 °C. The remaining solid was dissolved in 0.01% HCl (v/v) in deionized water and successively purified.

2.1.3. Purification of anthocyanins

The anthocyanin aqueous solutions obtained from the extraction procedure were passed through a 1 g sorbent weight C-18 Sep-Pak cartridge (Waters Corporation, Milford, MA), previously activated with methanol followed by 0.01% aqueous HCl (v/v). Anthocyanins and other polyphenolics were adsorbed onto the Sep-Pak column, while sugars, acids and other water-soluble compounds were removed by washing the cartridge with 2 volumes of 0.01% aqueous HCl (v/v). Less polar polyphenolics were subsequently eluted with 2 volumes of ethyl acetate. Anthocyanins were then eluted with 2 volumes of methanol containing 0.01% HCl (v/v). The acidified methanol solutions obtained were evaporated using a rotary evaporator at 30 °C. The remaining solid was dissolved in 0.01% HCl (v/v) aqueous solution in order to have a known concentration solution (3 mg/mL) and immediately analyzed. The solution was stored at −20 °C until used for successive acid and alkaline hydrolyses.

2.1.4. Acid hydrolysis of anthocyanins

5 mL of 2 N HCl was added to 0.5 mL of the purified anthocyanin solution (3 mg/mL) in a screw-cap test tube, flushed with nitrogen and capped. The pigments were hydrolyzed for 3 h at 100 °C; then, the solution was immediately cooled in an ice bath (Chaovanalikit, Thompson, & Wrolstad, 2004). The hydrolysate was purified by using a 500 mg sorbent weight C-18 Sep-Pak cartridge (Waters), as described in the previous section.

2.1.5. Alkaline hydrolysis of anthocyanins

1 mL of the purified anthocyanin solution (3 mg/mL) was saponified in a screw-cap test tube with 5 mL of 10% KOH for 10 min in the dark at room temperature (Chaovanalikit et al., 2004). The solution after neutralization with 2 N HCl was purified by using a 500 mg sorbent weight C-18 Sep-Pak cartridge (Waters), as described in the previous section.

2.1.6. Analytical method

HPLC analyses were performed using an Agilent 1100 Series LC/MSD system with a diode array detector (DAD) coupled to a mass spectrometer (MS) (quadrupole analyzer) equipped with an electrospray ionization interface (ESI, Agilent). Chromatographic separation was carried out using a 150×4.6 mm i.d., 5 μm SS Wakosil C18 with a 4×3 mm i.d. Phenomenex C18 guard cartridge both thermostatted at 32 °C. The mobile phase was composed of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) at a flow rate of 0.5 mL/min. The following program was utilized: 0 min, 10% B; 0–10 min, 10% B; 20 min, 20% B; 40 min, 30% B. Absorbance spectra were recorded every 2 s, between 250 and 600 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 520, 440, 310 and 280 nm. MS parameters were as follows: capillary voltage, 4000 V; fragmentor, 160 V; drying gas temperature, 350 °C; gas flow (N_2), 10 l/min; nebulizer pressure, 50 psig. The instrument was operated in positive ion mode scanning from *m/z* 100 to 800 at a scan rate of 1.43 s/cycle. The wavelength used for quantification

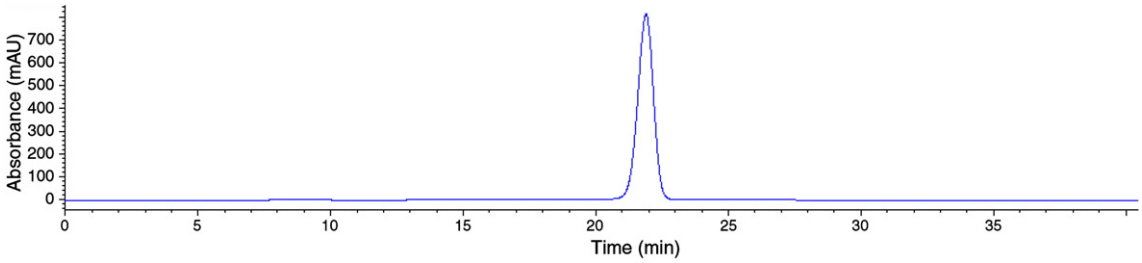

Fig. 1. HPLC-DAD chromatogram recorded at 520 nm corresponding to the purified extract of *E. myrtifolia* Sims fruits.

was 520 nm. The calibration curve was produced by the integration of absorption peaks generated from the analysis of dilution series of cyanidin 3-glucoside. Anthocyanin content was expressed as malvidin 3,5-diglucoside equivalents according to Chandra, Rana, and Li (2001).

2.2. In vitro culture

Buds were taken from growing shoots of *E. myrtifolia* plants in the spring period. The explants were washed in 70% ethanol solution for a few seconds, followed by 50% ACE solution for 30 min; then at least three rinses in sterile water. The sterilized buds were put in culture in vials with 10 ml of AM (Apex Medium) medium containing MS mineral salts and vitamins (Murashige & Skoog, 1962) at half concentration formula, 3% sucrose, 0.7% agar, PVP 1g/L, BAP 0.5 mg/L, IBA 0.01 mg/L and GA_3 0.05 mg/L; filter-sterilized ascorbic acid (10 mg/L) was added after autoclaving the medium at 120 °C for 20′. Developing buds were then transferred on SM (Shoot Medium) medium, differing from AM in mineral salt and vitamin composition (MS-full formula).

3. Results and discussion

3.1. Pigment identification

The anthocyanin content of *E. myrtifolia* Sims fruits was determined by means of HPLC-DAD-MS analysis. The chromatogram of the purified anthocyanin extract recorded at 520 nm is shown in Fig. 1. As can be seen, there is only one peak in the chromatogram and it was identified as malvidin 3,5-*O*-diglucoside on the basis of its λ_{max} of 526 nm and a mass spectrum comprising a M^+ at *m*/*z* 655 and a fragment ion at *m*/*z* 331 (M^+ — 324). The chemical structure of this compound is shown in Fig. 2.

$Abs_{440}/Abs_{\lambda max}$ ratio (15%) calculated for the anthocyanin found in *E. myrtifolia* fruits indicated the glycosidic substitution at positions 3 and 5 of the flavylium ring (Kuskoski et al., 2003; Giusti, Rodrìguez-Saona, & Wrolstad, 1999). In addition, $Abs_{280}/Abs_{\lambda max}$ (94%) and $Abs_{310}/Abs_{\lambda max}$ (25%) ratios confirmed that *E. myrtifolia* anthocyanin was not acylated with aromatic acids (Mozetic, Trebse, & Hribar, 2002).

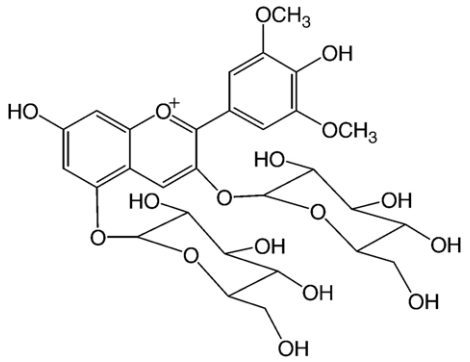

Fig. 2. Chemical structure of malvidin 3,5-*O*-diglucoside.

Fig. 3. *In vitro* shoot of *E. myrtifolia* Sims.

Alkaline hydrolysis of the purified extract produced a chromatographic profile similar to the one reported in Fig. 1, confirming that *E. myrtifolia* anthocyanin was not acylated (Hong & Wrolstad, 1990), while acid hydrolysis of the purified extract produced one peak corresponding to malvidin.

Quantitative analysis showed that the amount of malvidin 3,5-*O*-diglucoside in the fruits of *E. myrtifolia* was 32.59 mg/100 g of fresh berries.

In other *Eugenia* species (ex *E. umbelliflora* Berg) six major anthocyanins (covering all the six major aglycones) have been isolated (Kuskoski et al., 2003), revealing that, as is often found, the metabolic profile is a typical feature of a species, not always common to the botanical genus.

3.2. In vitro culture

After growing in AM medium for 10 days, buds developed small shoots (Fig. 3). It was quite difficult to establish a sterile culture, since microbiological problems occurred very often. Finally, few sterile plants were obtained using high concentration ethanol and sodium hypochlorite (see Section 2.2). The medium used for the bud development (AM) was characterized by a low concentration of mineral salts and vitamins (half-strength MS), low concentration of plant growth regulators, in a balanced composition suitable for the apex development. In this medium, buds from internode cuttings developed in nearly 20 days, then they were excised and were subcultured on SM medium. On this medium the shoots developed and proliferated (quite low proliferation), and some pigment accumulation on leaves was observed (Fig. 3). At the moment we are proliferating the *in vitro* shoots in order to have sufficient plant material for the induction of callus cultures from leaf sections. Consequently, we plan to investigate the callus production and the anthocyanin induction from the established callus, testing several media and growth regulator composition, as we had already done in another plant system (Blando et al., 2005).

4. Conclusions

This is the first report on the anthocyanin composition of *E. myrtifolia* Sims fruits. The amount of the only anthocyanin present in these fruits is not high compared to other characteristic "red fruits", but the fact that only one molecular form of anthocyanin is produced (malvidin 3,5-diglucoside) could represent an interesting aspect for biotechnological exploitation. In fact, since malvidin is a final product (methoxylated anthocyanin) of the anthocyanin pathway, this could be a useful system to study the regulation of the pathway, altering the genetic (regulator genes) or physiological control of the pathway.

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