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Production of ^{13}C-labelled anthocyanins by *Vitis vinifera* cell suspension cultures

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Abstract

The use of plant cell cultures for producing isotopically (^{13}C) labelled phenolic substances is reported. *Vitis vinifera* cells synthesize high levels of anthocyanins when they are cultured in a polyphenol synthesis-inducing medium. Three major anthocyanin monoglucosides found in red wine were identified in grape cells: cyanidin-3-*O*-β-glucoside, peonidin-3-*O*-β-glucoside, and malvidin-3-*O*-β-glucoside. Kinetic study of the intracellular level of phenylalanine and its metabolites showed that it is preferable to add this precursor to grape cell suspensions after the 5th day of culture, i.e. at the beginning of the exponential growth phase. After adding phenylalanine to the culture medium, its uptake was complete and the accumulation of anthocyanins in grape cells was stimulated. Incorporation of [1-^{13}C]-phenylalanine into anthocyanins was measured by means of ^{13}C satellites in the proton NMR spectrum. The maximal rate of ^{13}C enrichment anthocyanins obtained with this technique reached 65%. The production of ^{13}C labelled phenolic compounds was undertaken in order to investigate their absorption and metabolism in humans.

Keywords: *Vitis vinifera*; Vitaceae; Grapevine; 13C-labelled anthocyanins; Cell suspension culture

1. Introduction

The inverse association between moderate intake of wine and coronary heart disease has been well established (Renaud & De Lorgeril, 1992; Gronbaek, Deis, Sorensen, Becker, Schnohr, & Jensen, 1995). This epidemiological finding suggests that the intake of wine counteracts the effects of a diet high in saturated fat, and prevents cardiovascular disorders in certain regions of France. This particularity is referred to as the 'French paradox'.

Compared to other alcoholic drinks, wine, notably red wine, contains a much higher level of polyphenols. It has been shown that total phenolic compounds extracted from red wine inhibit the oxidation of human LDL in vitro significantly more than α-tocopherol on a molar basis (Frankel, Kanner, German, Parks, & Kinsella, 1993; Frankel, Waterhouse, & Teissedre, 1995). These phenolic substances might therefore provide a protective effect against atherogenesis over a long period of moderate consumption of red wine (2–3 glasses per day), which corresponds to about 300–1,200 mg of red wine polyphenols (Ribereau-Gayon, 1982). This is notably greater than the recommended intake of antioxidant vitamins.

Using *Vitis vinifera* suspension cultures, we showed that these cells synthesize high levels of anthocyanins, proanthocyanidins, catechins, and stilbenes (Decendit & Mérillon, 1996; Decendit, Ramawat, Waffo Téguo, Deffieux, Badoc, & Mérillon, 1996), i.e. the major polyphenols found in red wine (Ribereau-Gayon, 1982; Goldberg, Ng, Karumanchi, Yan, Diamandis, &

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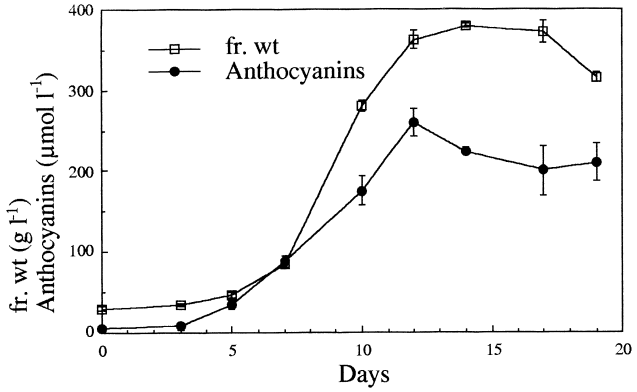


Fig. 1. Time courses of cell growth and anthocyanin production in *Vitis vinifera* cells grown in IM1 medium. Three replicates (error bars show S.D.).

Soleas, 1995). We first tested the antioxidative potency of these various phenolic substances isolated and unambiguously characterized by spectrometric methods (Decendit et al., 1996; Waffo Téguo, Decendit, Vercauteren, Deffieux, & Mérillon, 1996; Waffo Téguo, Decendit, Krisa, Deffieux, & Mérillon, 1996). Our findings showed that this variety of polyphenol classes exhibits interesting radical scavenger and antioxidant properties on human LDL in vitro (Mérillon, Fauconneau, Waffo Téguo, Barrier, Decendit, & Mérillon, 1997; Fauconneau, Waffo Téguo, Huguet, Barrier, Decendit, & Mérillon, 1997). Moreover, red wine consumption seems to result in a reduced propensity of human plasma and LDL to undergo lipid peroxidation (Fuhrman, Lavy, & Aviram, 1995). However, little is known about gut absorption of phenolic compounds and their pharmacokinetics in humans. Anthocyanins have been detected in human plasma by HPLC analysis but have not been characterized (Paganga & Rice-Evans, 1997). Consequently, we undertook the production of ^{13}C-labelled polyphenols using grape cell cultures in order to solve these important problems and to assess more thoroughly the cardiovascular protective effect of wine phenolics.

In this paper, we report the experimental conditions allowing the optimal incorporation of ^{13}C-PHE into anthocyanins by *Vitis vinifera* cell suspension cultures, and the use of ^{13}C satellites in ^{1}H-NMR spectroscopy to measure the rate of ^{13}C enrichment in anthocyanins.

2. Results and discussion

2.1. Growth and anthocyanin accumulation of Vitis vinifera cells in the polyphenol synthesis-inducing medium (IM1)

To determine the optimal period for the production of anthocyanins by grape cells in IM1 medium, cultures were analyzed throughout a 19-day period (Fig. 1). Maximal production of anthocyanins (260 µmol l^{-1}) occurred on day 12, i.e. at the end of the exponential growth phase. Under these conditions, it is evident that anthocyanin production and growth are coupled. Moreover, these suspension cultures showed very good growth, about 400 g fr. wt per liter of culture. This is in contrast with observations made by other authors who found an enhanced anthocyanin accumulation in *Vitis* cell cultures associated only with a low level of final biomass, either by an osmotic stress or by phosphate deprivation (Hirose, Yamakawa, Kodama, & Komamine, 1990; Do & Cormier, 1990; Hirasuna, Shuler, Lackney, & Spanswick, 1991; Dedaldechamp, Uhel, & Macheix, 1995).

The three major anthocyanin monoglucosides in grape cells were identified by co-chromatography with authentic standards using HPLC analysis and by spectrometric methods. Our ^{1}H-NMR data are similar to those previously reported by Van Calsteren, Cormier, Do, and Laing (1991). These monoglucosides are cyanidin-3-*O*-β-glucoside (R_t 28 min), peonidin-3-*O*-β-glucoside (R_t 37 min) and malvidin-3-*O*-β-glucoside (R_t 41 min).

2.2. Changes in size of endogenous pool of phenylalanine and its metabolites

The pool of free phenylalanine in the cells is under direct competition between the phenylpropanoid pathway, which leads to anthocyanin biosynthesis and protein synthesis. Intracellular concentrations of phenylalanine, anthocyanins and proteins were therefore quantified during the culture period (Fig. 2). We expressed the content of these metabolites on a fr. wt basis, because we have previously shown that the number of grape cells is closely correlated with fr. wt (Decendit & Mérillon, 1996), so cellular metabolism may be monitored irrespective of cell division.

The phenylalanine level peaked on day 3 of culture (1 µmol g^{-1} fr. wt) and decreased sharply afterwards during the growth phase and the accumulation of proteins and anthocyanins. Thereafter, the phenylalanine concentration remained low (about 0.05 µmol g^{-1} fr. wt). Similar low endogenous levels of free phenylalanine are often observed in plant tissues (Da Cunha, 1987), for example in *Capsicum frutescens* callus (Sudhakar Johnson, Ravishankar, & Venkataraman, 1992) and *Vitis* hybrid cells (Kakegawa, Suda, Sugiyama, & Komamine, 1995).

Maximal accumulation of proteins and anthocyanins was observed on days 5 and 7, respectively (Fig. 2). These concentrations then decreased gradually as the culture grew. To obtain an optimal incorporation of

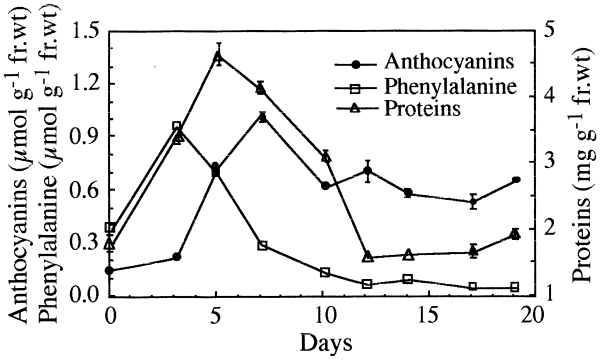


Fig. 2. Time courses of anthocyanin, phenylalanine, and protein levels in *Vitis vinifera* cells grown in IM1 medium. Three replicates (error bars show S.D.).

^{13}C-PHE into anthocyanins, this precursor must therefore be added after the 5th day of culture.

2.3. Effect of phenylalanine addition on anthocyanin production and on grape cell growth

Previous investigations of phenylalanine addition on plant cell cultures reported the use of concentrations between 1 and 5 mM (Kakegawa et al., 1995; Smith, Slywka, & Krueger, 1981; Sudhakar Johnson & Ravishankar, 1996). We examined whether supplied non-labelled phenylalanine (1–10 mM, final concentration) modified grape cell growth and anthocyanin production (Fig. 3). When phenylalanine was added at day 8 at a concentration superior to 4 mM, we observed a browning of the suspension cultures of grape cells.

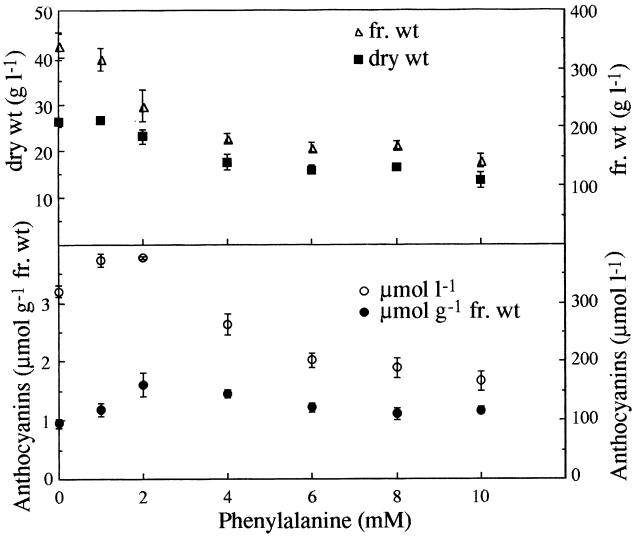


Fig. 3. Effect of addition of phenylalanine on growth and anthocyanin content of *Vitis vinifera* cells grown in IM1 medium. Phenylalanine was added at day 8 and cells were harvested at day 12. Four replicates (error bars show S.D.).

A decrease in fresh and dry biomasses occurred for a phenylalanine concentration superior to 1 mM. However, anthocyanin content expressed as μmol g^{-1} fr. wt increased 1.7-fold from zero to 2 mM added phenylalanine, therefore compensating highly the loss of biomass. Indeed, in terms of production (μmol l^{-1}) the anthocyanin content was 1.2 times greater in the presence of 2 mM phenylalanine compared to the culture without added precursor. These results indicate that the accumulation of anthocyanins in *Vitis vinifera* cells is stimulated by phenylalanine.

Phenylalanine may act directly on anthocyanin biosynthesis by induction of the transcription of genes that encode enzymes of the anthocyanin biosynthetic pathway such as chalcone synthase (Kakegawa et al., 1995). Anthocyanins may be synthesized from this larger pool of available phenylalanine.

Similar results were observed when phenylalanine was added on day 6 of culture (harvest at day 12). By contrast, the stimulating effect of phenylalanine added at day 10 on anthocyanin accumulation was not significant and biomass was not affected up to 4 mM (results not shown).

2.4. Incorporation of ^{13}C-phenylalanine into anthocyanins

[1-^{13}C]-L-phenylalanine feeding experiments were

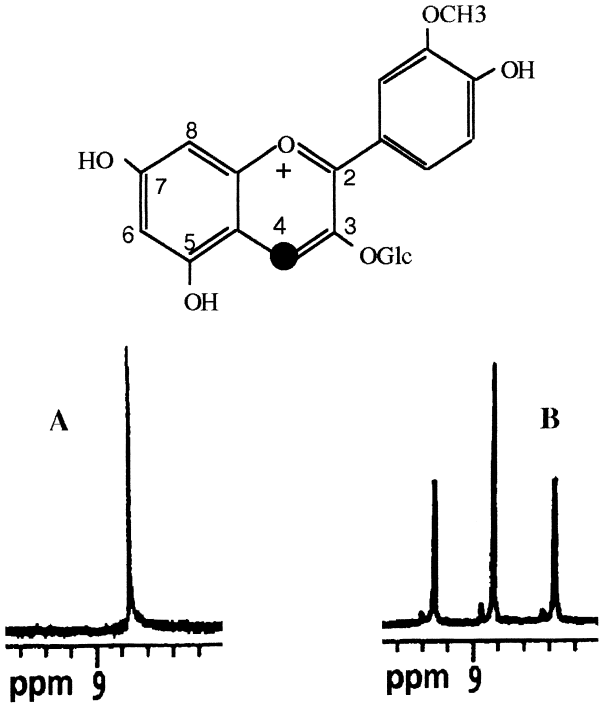


Fig. 4. Excerpts of ^{1}H NMR spectra of peonidin-3-*O*-β-glucoside. A: unlabelled reference, B: biosynthesized from [1-^{13}C] phenylalanine in *Vitis vinifera* suspension cell culture.

Table 1
Production of anthocyanins and rate of ^{13}C enrichment of peonidin-3-*O*-β-glucoside after incorporation of [1-^{13}C] PHE by *Vitis vinifera* cells grown in IM1 medium. Cells were harvested at day 12. Data are the mean of 2 or 3 independent experiments for 1 and 2.5 mM, respectively

[1-^{13}C] PHE (mM)	Day of addition	Anthocyanins (μmol l^{-1})	Rate of ^{13}C enrichment (E, %)
0	–	296	–
2.5	6	361	55
2.5	8	369	43
2.5	10	302	33
2.5 (×2)	6, 8	298	50
2.5 (×2)	6, 10	341	57
2.5 (×2)	8, 10	374	52
1	6	359	48
1 (×2)	6, 8	469	56
1 (×3)	6, 8, 10	482	65

done to optimize its incorporation into anthocyanins. We investigated the time of addition of phenylalanine (days 6, 8 and 10) and the final concentration of this added precursor (1 and 2.5 mM). Grape cells were harvested at day 12, when anthocyanin production was maximal (Fig. 1).

Compounds 1–3 were purified by a combination of chromatography techniques and subjected to ^{1}H-NMR. Fig. 4 shows a signal at δ 8.98 assignable to H-4 and symmetrically two signals (^{13}C satellites, $J = 170.7$ Hz), indicating that C-4 was labelled. The percentages of anthocyanin enrichment are calculated and presented for peonidin-3-*O*-β-glucoside in Table 1. Indeed, similar results were found for the three anthocyanins.

We found that the rate of ^{13}C enrichment of peonidin-3-*O*-β-glucoside was higher when phenylalanine (2.5 mM) was added at the beginning (at day 6) of the exponential growth phase compared to the middle (at day 8) and the end (at day 10) of this phase. After two applications of phenylalanine (2×2.5 mM), the rate of enrichment (50–57%) had not increased. Analysis of phenylalanine (Table 2) showed a very low concentration of 13 μmol l^{-1} medium in grape cell cultures supplied with [1-^{13}C] PHE 2.5 mM. This concentration was similar to that of cells cultured without added phenylalanine (16 μmol l^{-1} medium). At two-fold concentration, there was also a large uptake of phenylalanine into grape cells (extracellular concentration of 37 μmol l^{-1} medium). We observed that the endogenous pool of free phenylalanine remained high in the [1-^{13}C] PHE treated cultures: 620 (2.5 mM added) and 1503 μmol l^{-1} (2×2.5 mM added). Thus, we tested the treatment of grape cell cultures with a lower concentration of [1-^{13}C] PHE (1 mM), which led to a better rate of ^{13}C enrichment of anthocyanins (65%) after three additions (at day 6, 8, 10). It also appears that anthocyanin production (μmol l^{-1}) was stimulated about 1.6-fold.

Table 2
Effect of [1-^{13}C] PHE on phenylalanine level in *Vitis vinifera* cells grown in IM1 medium. Cells were harvested at day 12. Two replicates

[1-^{13}C] PHE (mM)	Day of addition	Phenylalanine (μM)	
		medium	cells
0	–	16	19
2.5	8	13	620
2.5 (×2)	6, 8	37	1503

To obtain rapidly sufficient quantities of labelled anthocyanins, cultures in a bioreactor will be performed. We have previously shown that polyphenol production by grape cells in a stirred fermenter is similar to that in shake flasks (Decendit et al., 1996).

Plant cell cultures have been studied as a means of producing plant secondary metabolites for use in medicine or in the food industry. With the help of this technique, we now demonstrate that it is possible to produce isotopically (^{13}C) labelled anthocyanins found in red wine in order to investigate their absorption, in vivo metabolism, and pharmacokinetics in humans. A 10 l-bioreactor culture is now required.

3. Experimental

3.1. General procedures

^{1}H NMR spectra were recorded on a 500 MHz Bruker, CD3OH/TFA was used as solvent. Mass spectra were recorded with VG Autospec-Q in the FAB+ Mode.

3.2. Cell culture

Cell suspension cultures of *Vitis vinifera* (L.) cv Gamay Fréaux var. Teinturier were maintained as previously described (Decendit & Mérillon, 1996). The ex-

perimental cultures were performed by inoculating a 7-day-old cell suspension at a 1:8 (v:v) ratio into a polyphenol synthesis-inducing medium (IM1), for one transfer (Decendit & Mérillon, 1996). Cells were harvested at different times by filtration under partial vacuum, rapidly washed with cold distilled water, weighed and then frozen until analysis.

3.3. Precursor feeding

For feeding experiments, *Vitis vinifera* cell cultures were treated with phenylalanine after dissolution in DMSO–H_2O (15:85) and filter sterilization: L-phenylalanine [1-^{13}C] (purchased from Eurisotop, CEA, France) or non-labelled L-phenylalanine (from Sigma, France). The final DMSO concentration did not exceed 0.5% (v:v); controls contained the same concentration of DMSO. More details are included in the results section.

3.4. Extraction, isolation and identification of anthocyanins

Freeze-dried cells were extracted (×2) with 1% TFA in MeOH. The filtered extracts were combined and concd. The aq. solution remaining after concentration of the combined filtrates in vacuo at minus 40° was applied to an Amberlite XAD-7 column chromatography (Hosokawa, Fukunaga, Fukushi, & Kawabata, 1995) and washed with 1% aq. TFA. Anthocyanins were eluted by 50% MeOH containing 1% aq. TFA. For further purification, the crude anthocyanins were separated by semiprep. HPLC on an Ultrasep reversed-phase C18 column (6 μm packing, 20 mm i.d. × 250 mm) protected with a guard column of the same material. The elution prog. at 7 ml min^{-1} was as follows: solvent A, 0.15% TFA in H_2O; solvent B, H_2O–1% TFA–MeCN (55: 15: 30); linear gradient from 30 to 100% B in 80 min. The eluate was monitored at 280 and 521 nm. These compounds were compared with authentic material by analytical HPLC and identified by NMR and MS. Three major anthocyanins together accounting for 90% of the total anthocyanins are: *Cyanidin-3-O-β-glucoside* (17%), HPLC R_t 28 min, FAB–MS m/z: 449 $[M+1]^+$, ^{1}H NMR δ 8.87 (1H, s, H-4); *Peonidin-3-O-β-glucoside* (70%), HPLC R_t 37 min, FAB–MS m/z: 463 $[M+1]^+$, ^{1}H NMR δ 8.98 (1H, s, H-4); *Malvidin-3-O-β-glucoside* (13%), HPLC R_t 41 min, FAB–MS m/z: 493 $[M+1]^+$, ^{1}H NMR δ 9.03 (1H, s, H-4).

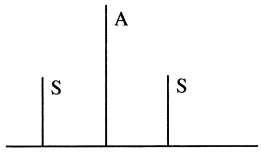
3.5. Measurement of rate of ^{13}C enrichment by means of ^{13}C satellites

^{1}H-NMR spectra were obtained at 303°K in the Fourier-transform mode at 500 MHZ on a Bruker spectrometer (AMX 500) equipped with a broad band 20-mm probe, using a spectral width of 20 ppm (16 K memory size), a 90° pulse angle with an interpulse delay (D_1) of 1.5 s. Chemical shifts were expressed as ppm relative to the MeOH (3.3 ppm) resonance.

Each organic compound contained 1.1% of the stable isotope carbon-13 in natural abundance. Since carbon-13 has a nuclear spin of $I = 1/2$ these molecules show a spin–spin interaction ^{13}C–^{1}H that leads to doublet splitting in the proton NMR spectrum. If the resonance signal is recorded at high gain, one observes a low intensity singlet on both sides of the main signal. The position of these weak signals is not dependent upon the spinning rate of the sample cell, so they are not spinning side bands. Instead, the signals are the so-called ^{13}C satellites.

These signals can be used to measure the rate of ^{13}C enrichment in anthocyanins.

The rate of enrichment is calculated from the equation below:



$$E = \frac{2S}{2S + A} \times 100$$

A: integral of the main signal
E : rate of ^{13}C enrichment
S : integral of ^{13}C satellite

3.6. Phenylalanine quantification

Phenylalanine was extracted from freeze-dried cells (50 mg) with EtOH–H_2O (70:30) for 6 h at 20°. After centrifugation (2.700 × *g*, 10 min), the supernatant was used to analyze phenylalanine following treatment with ninhydrine or in an automatic amino acid analyzer.

3.7. Protein quantification

Frozen cells (1 g) were extracted with 0.3 M KOH for 3 h at 20°. After centrifugation (2,700 × *g*, 10 min), protein concentration was determined in the supernatant according to the method of Bradford (1976), with bovine serum albumin as standard.

3.8. Anthocyanin quantification

To estimate total anthocyanins, freeze-dried cells (40 mg) were extracted with MeOH–0.32 M HCl (85:15) overnight at 4° and the absorbance of the anthocyanin extract was measured at 535 nm. Anthocyanins were estimated according to the molar extinction coefficient ($\log \varepsilon$ 4.53) of peonidin-3-glucoside purified from cultured grape cells.

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