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Condensed tannin and anthocyanin production in *Vitis vinifera* cell suspension cultures

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ABSTRACT

Suspension cultures of *Vitis vinifera* were cultured in different media in order to establish a model system for promoting high levels of phenolic substances identical with those found in wine. These media were: a low sucrose maintenance medium (MM) and four high sucrose media (differing mainly in sucrose and mineral contents) which were shown to induce secondary metabolism. In MM medium, polyphenol accumulation in the cells was low, and concentrations of 0.1 mg/gfw for condensed tannins and 0.3 mg/gfw for anthocyanins were reached within two weeks of cultivation. Values of 1.4 and 6.4 mg/gfw, respectively, were obtained with a low nitrate and high sucrose medium (HM1), but cell proliferation was reduced. To obtain a maximal production of polyphenols, we investigated the most effective conditions for cell growth and polyphenol production (a high mineral and high sucrose medium, IM1; inoculum dilution of 1.25:10). Under these conditions, the cells produced mainly anthocyanins (1100 mg/l), proanthocyanidins (300 mg/l) and catechins (25 mg/l).

ABBREVIATIONS: BuOH, n-butanol; dw, dry weight; fw, fresh weight.

INTRODUCTION

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 envisaged producing isotopically (^{13}C) labelled phenolic substances found in red wine, i.e. anthocyanins, catechins and condensed tannins. Indeed we need to know much more about gut absorption, *in-vivo* metabolism and pharmacokinetics of wine phenolics.

These compounds have potent antioxidant properties towards human low-density lipoprotein in *in-vitro* studies (Mangiapane *et al.* 1992; Frankel *et al.* 1993), providing a plausible explanation for the "French paradox", i.e. the epidemiological evidence showing that in France consumption of red wine may counteract the effects of satured fats and reduce the risk of coronary heart disease (Renaud and de Lorgeril 1992).

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Plant cell cultures often need optimization of the culture conditions for growth and metabolite production (Berlin *et al.* 1987; Moreno *et al.* 1995), and may have a higher rate of metabolism than differentiated plants (Zenk 1991). For example, Kobayashi *et al.* (1993) reported an anthocyanin yield of 17% in callus of *Aralia cordata*. Cell suspension cultures of *Vitis vinifera* have been the subject of some investigations focused on stimulating anthocyanin production. A medium with reduced nitrate and elevated sucrose, and an increase in the length of the initial lag-growth phase induced very high accumulation of anthocyanins (Hirose *et al.* 1990; Hirasuna *et al.* 1991; Cormier *et al.* 1994; Iborra *et al.* 1994). Since the biosynthetic pathway of catechins and proanthocyanidins is closely related to that of anthocyanins (Heller and Forkmann 1993), it may be presumed that tannin accumulation was enhanced by the same factors. This paper describes the establishment of a model system for promoting high levels of condensed tannin and of anthocyanin production. This is the first report demonstrating the presence of condensed tannins in *Vitis vinifera* cell suspension cultures.

MATERIAL and METHODS

Cell Culture

In January 1994 cell suspension cultures of *Vitis vinifera* (L.) cv. Gamay Fréaux var. Teinturier were established from callus provided by C. Ambid (ENSA, Toulouse). They were maintained under continuous fluorescent light (5000 lux) at 25± 1°C in 250 ml Erlenmeyer flasks containing 50 ml of cell suspension on an orbital shaker (100 rpm). The maintenance medium (MM) contained macroelements (Gamborg *et al.* 1968), microelements (Murashige and Skoog 1962) and vitamins (Morel 1970) supplemented with 58 mM sucrose, 250 mg/l casein hydrolysate, 0.54 µM 1-naphthaleneacetic acid and 0.93 µM kinetin. Concentrations of components in MM differing in modified media were: 25 mM nitrate, 1 mM magnesium, 1.1 mM phosphate, and 1 mM ammonium. The pH was set to 5.8 before sterilization. Cells were subcultured every seventh day with an inoculum dilution of 1:5 (ca 2.7 gdw/l; 64 gfw/l; 1.8 x 10^7 cells/l).

For experimental purposes seven day-old cultures were transferred to other media formulated by changing different levels of components in the maintenance medium : HM1, a medium identical to the MM, except that the nitrate content was 2.5 mM and the sucrose content 234 mM; S80, an aqueous solution of 234 mM sucrose; CM, a medium identical to the MM, but containing 6.25 mM nitrate and 132 mM sucrose; IM1, a medium which was as MM, but containing 2 mM ammonium, 2.2 mM phosphate, 2 mM magnesium and 175 mM sucrose. We also investigated the effects of inoculum level on polyphenol production. More details are included in the results and discussion section. Cells were harvested at different times by filtration under partial vacuum (nylon cloth, 30 µm), rapidly washed with cold distilled water, weighed and then stored at -20°C until analysis.

Quantification of anthocyanins.

Anthocyanins were extracted from the frozen cells (150 mg) overnight with ethanol - 0.32 M HCl (85:15, v/v) at +4°C. Absorbance of the anthocyanin extract was measured at 535 nm. The extinction coefficient ($E^{1\%}$ = 98.2 at this wavelength) of cranberry anthocyanin extracted in the same solvent, was used to calculate the anthocyanin content (Francis 1982).

Quantification of condensed tannins.

Frozen cells (300 mg) were homogenized and extracted with 60% (v/v) aqueous acetone for 1 hour. Acetone was removed from the extract *in vacuo*. The aqueous extract was chromatographed on a cation-exchange resin column (0.6 cm X 4 cm) and eluted with 50% (v/v) aqueous methanol to obtain condensed tannins. Proanthocyanidin analysis was based on the formation of anthocyanidins upon acid hydrolysis. A 0.5 ml aliquot of methanolic eluate plus 2 ml of BuOH - 12 M HCl (4:1, v/v) reagent was kept in a water bath at 95°C for 20 min. Estimates of the proanthocyanidins as mg/g fw of cells were made at 550 nm using an $E^{1\%}$ value of 150 (Stafford and Cheng 1980).

Analysis of catechins

In some experiments, catechins were extracted by diethyl ether from aqueous extracts as obtained above. Diethyl ether was evaporated to dryness, 1 ml of distilled water and 2 ml of a freshly prepared solution of 66 mM vanillin in 13 M sulfuric acid added, and the mixture incubated at 20°C for 15 min. Absorbance was read at 500 nm against a blank prepared without sample. Total concentration of catechins including mainly (+)-catechin and (-)-epicatechin (Waffo, unpublished), was calculated from a standard curve obtained with (+)-catechin.

RESULTS and DISCUSSION

Condensed tannins were quantified by a method based on oxidative depolymerization in BuOH/HCl which forms anthocyanidins (Bate-Smith 1973; Scalbert 1992). This technique required separation of tannins from anthocyanins by cation exchange chromatography before assaying (unpublished results).

Growth and polyphenol accumulation of *V. vinifera* cells in the maintenance medium.

To study the optimal period of polyphenol accumulation in relation to growth, *V. vinifera* cell suspension cultures were analysed during the growth period in MM (Fig.1 A, B).

Both growth criteria used, fresh and dry biomass, increased steadily up to day 7. Optimal production was observed at day 7 for anthocyanins (120 mg/l), and day 14 for condensed tannins (40 mg/l). Levels of anthocyanins or condensed tannins expressed per mg/gfw or mg/gdw increased 3-fold from day 3 to day 5. It is evident that the polyphenol accumulation in grape cells increased markedly during the exponential growth phase. Neither anthocyanins nor condensed tannins were detected in the culture medium.

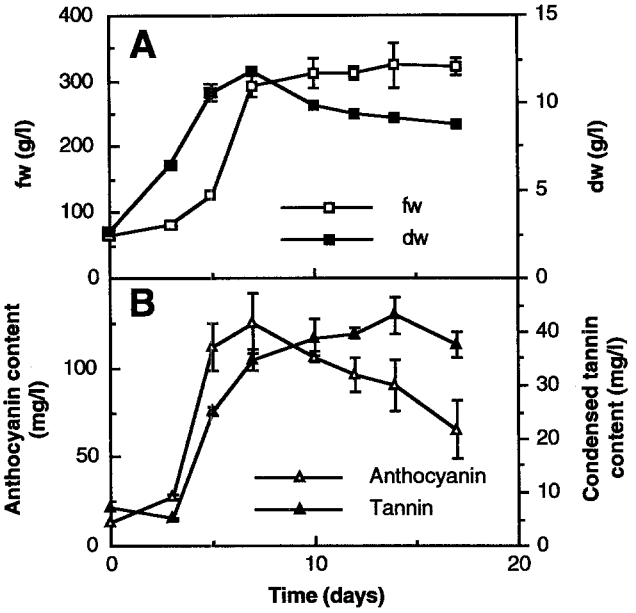

Fig. 1 : Time courses of growth (A) and polyphenol contents (B) in *Vitis vinifera* cells grown in the maintenance medium (MM) with an inoculum dilution of 1:5. Three replicates (error bars show SD).

Enhanced production of polyphenols in *V. vinifera* cells.

Optimal biosynthetic capabilities depended on the nutritional and environmental conditions. We tested different media with reduced nitrate and elevated sucrose contents for stimulated anthocyanin accumulation. CM and HM1 media were developed according to Cormier *et al.* (1994) and Hirasuna *et al.* (1991), respectively. We also used an aqueous solution of sucrose (S80 medium) which induced secondary metabolism in *Catharanthus roseus* cells (Mérillon *et al.* 1986).

Growth and polyphenol production of *V. vinifera* cells were examined in these media at day 4, 7, 12, 14 and 18. Maximal anthocyanin and condensed tannin levels were recorded at day 12 and 14. The data for these days were

used for further interpretation (Table 1). The reduction of the inoculum from 1:5 (v/v) to 1:10 (v/v) led to a 3-fold increase in the condensed tannin and anthocyanin production in the cells grown in MM medium. Concomitantly the lag phase increased from 24 h to about 72 h, but the same biomass level was reached at the end of the growth phase. This is in contrast to observations made by other authors who obtained an enhanced anthocyanin production in *V. vinifera* cell cultures only associated with a low level of final biomass (Hirose *et al.* 1990, Hirasuna *et al.* 1991). In all three production media tested, drastic reduction in growth on a fresh weight basis was recorded. Fair accumulation of condensed tannins and anthocyanins was found with HM1 medium only. The dry weight of cells in HM1 medium (inoculum dilution of 1:5) was higher than that of cells grown in MM medium. The presence of a high concentration of sucrose in the culture medium could induce a considerable increase in the carbohydrate content of the cells, as has been shown by Mérillon *et al.* (1984) and Schlatmann *et al.* (1994) with *Catharanthus roseus* cells. The cell density in HM1 medium (1.3 x 10^6/ml) was 2.5-fold lower than in MM medium (3.4 x 10^6/ml). Fresh weight is therefore a better estimate for cell proliferation than dry weight, if media with different sucrose concentration are to be compared. Levels of anthocyanins or condensed tannins expressed per mg/gdw have the same magnitude in both media (HM1 and MM), but expressed in mg/gfw they are notably higher in HM1 medium compared with those in MM medium (Table 1). Therefore, the rate of biosynthesis of polyphenols in *V. vinifera* cells was much higher in HM1 compared to MM medium. This is supported by observations made by Petersen *et al.* (1994) who found a very high activity of phenylalanine ammonia-lyase in suspension cultures of *Coleus blumei* grown in media containing elevated sucrose concentrations. To obtain a maximal production of condensed tannins and anthocyanins, we optimized conditions for cell proliferation and biosynthetic efficiency.

Promotion of cell growth was observed if the sucrose concentration of the production medium was reduced from 234 to 175 mM, and some major inorganic nutrients were increased (data not shown). This study resulted in the formulation of IM1 medium. The importance of the inoculum dilution can be seen in Table 2. A minimum of 1.25:10 is necessary to obtain optimal cell proliferation and condensed tannin production (368 mg/l) in IM1 medium. Increasing the cell density of the inoculum did not influence growth, but accumulation of condensed tannins sharply decreased. A possible explanation for this reduced tannin accumulation may be O_2 limitation during the first few days of the culture, as was assumed by Moreno *et al.* (1993) who observed reduced formation of ajmalicine in dense cultures of *Catharanthus roseus*.

To determine the period optimal for the production of condensed tannins and anthocyanins in IM1 medium, cultures were analysed over 17 days (Fig. 2).

Optimal production was observed between day 12 and 14 for catechins (25 mg/l), and between day 14 and 17 for condensed tannins (300 mg/l) and anthocyanins (1100 mg/l). A similar magnitude of anthocyanin production has been reported for *Aralia cordata* cells (Kobayashi *et al.* 1993) and for *Vitis* hybrid cells (Hirasuna *et al.* 1991). The production rates for catechins and condensed tannins were not appreciably different from those of *Cinnamomum cassia* suspension cultures (Yazaki and Okuda 1990), but were lower than those found by Stafford *et al.* (1986) in *Pseudotsuga* cell cultures.

Table 1. Growth and polyphenols production of grape cells cultured in different media

		condensed tannins mg/l (mg/gfw)		anthocyanins mg/l (mg/gfw)		fresh weight (dry weight) g/l	
		day of harvest					
Medium	Inoculum dilution	12	14	12	14	12	14
MM	1:5	40 (0.1)	30 (0.1)	99 (0.3)	112 (0.3)	310 (9.4)	330 (9.1)
	1:10	100 (0.4)	90 (0.3)	317 (1.2)	277 (0.8)	264 (10.1)	334 (9.4)
HM1	1:5	63 (0.7)	69 (0.6)	341 (3.5)	392 (3.5)	96 (13.0)	112 (14.7)
	1:10	69 (1.4)	40 (0.9)	241 (5.0)	280 (6.4)	48 (6.5)	44 (5.8)
S80	1:5	20	23	39	55	42	70
	1:10	11	10	18	22	18	24
CM	1:5	38	22	94	93	84	70
	1:10	23	33	95	183	38	60

Standard deviations of the mean were < 5% (three replicates)

Table 2. Effects of inoculum dilution on growth and condensed tannins production of grape cells cultured in IM1 medium, at day 12.

Inoculum dilution	Condensed tannins mg/gfw	Condensed tannins mg/l	Fresh weight g/l
1:5	0.20	79	393
1.5:10	0.27	112	408
1.25:10	0.96	368	383
1:10	1.17	197	168
0.75:10	1.32	120	91

Standard deviations of the mean were < 5% (three replicates).

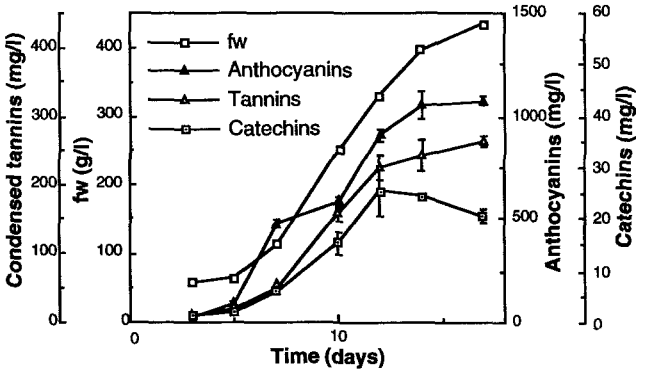

Fig.2 : Time courses of growth and polyphenol contents in *Vitis vinifera* cells grown in IM1 medium with an inoculum dilution of 1.25:10. Three replicates (error bars show SD).

Our results show that suspension cultures of *Vitis vinifera* synthesize high levels of anthocyanins, catechins and condensed tannins, while they show reasonably good growth. Further investigations are in progress to characterize these main polyphenols in *Vitis vinifera* cell suspension cultures.

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