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ENHANCEMENT OF ANTHOCYANIN SYNTHESIS AND DIHYDROFLAVONOL REDUCTASE (DFR) ACTIVITY IN RESPONSE TO PHOSPHATE DEPRIVATION IN GRAPE CELL SUSPENSIONS

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Key Word Index—*Vitis vinifera*; Vitaceae; cell suspensions; anthocyanins; dihydroflavonol reductase; phosphate deprivation.

Abstract—Anthocyanin synthesis occurred early during the culture cycle of grape cell suspensions. Dihydroflavonol reductase (DFR) activity and anthocyanin synthesis were found well correlated within this period. Phosphate deprivation leads to a large enhancement in DFR activity with a maximum which was observed earlier than in control medium. An increase in anthocyanin accumulation was also observed in association with a higher degree of peonidin 3-glucoside acylation.

INTRODUCTION

Inorganic phosphate (Pi) is one of the most effective nutrient factors for the growth of suspension cell cultures, and a linear correlation was reported between the number of cells which had entered the stationary phase and the initial medium phosphate concentration [1]. Nutrient culture conditions for anthocyanin formation have been investigated [2], but few studies have been conducted on the relation between anthocyanin synthesis and Pi availability [3]. In cell suspensions issued from *Vitis* hybrids, cell biomass was decreased with Pi concentration in the 4.5–0.15 mM range, and anthocyanin production was increased only in the narrow 1.25–0.15 mM range [4]. On a general assumption, the regulatory mechanism which has been evoked is a possible competition for phenylalanine as a substrate, for both protein synthesis and secondary metabolite production [5, 6].

It has been demonstrated that anthocyanin biosynthesis is not regulated at the phenylalanine-ammonia lyase level, but rather through chalcone synthase activity [7]. Furthermore, the involvement of dihydroflavonol reductase (DFR) in anthocyanin biosynthesis has been established through genetic studies with defined flower lines [8] or barley seed mutants [9] and it is responsible for the loss of anthocyanin synthetic capacity in some mature leaves [10].

The present work's main objective was to investigate the effects of different phosphate concentrations on both anthocyanin synthesis and DFR activity in grape cell suspensions prepared from a Teinturier red cultivar which accumulate high levels of anthocyanins in the berry pulp at maturity.

RESULTS AND DISCUSSION

Effects of Pi medium on grape cell suspension growth

During the first three days, the cell number in the control medium (P1) changed slightly (Fig. 1), then

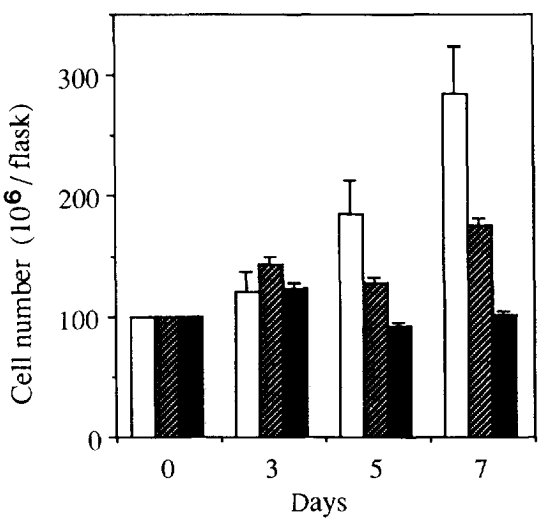

Fig. 1. Effect of different phosphate concentrations on cell number in grape cell suspensions (± S.E. for four replications). P1 medium (1.1 mM Pi), □; P2 medium (0.25 mM Pi), ■; P3 medium (0.008 mM), ■.

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Table 1. Effects of various concentrations of phosphate in medium on growth, protein content and intracellular phosphate in cell suspensions of *Vitis vinifera*. The initial concentrations in phosphate were P1: 1.10 mM; P2: 0.25 mM; P3: 0.008 mM

Medium	Days	Fr. wt (g flask^{-1})	Proteins (mg g^{-1} fr. wt)	Intracellular Pi (mM)	Medium Pi level (mM)
P1	0	7.04 ± 1.26	7.48 ± 0.12	3.50 ± 0.22	1.10 ± 0.08
	3	9.93 ± 1.70	9.06 ± 0.46	11.16 ± 1.09	0.25 ± 0.12
	5	19.89 ± 4.40	8.85 ± 0.95	5.25 ± 0.89	0.21 ± 0.06
	7	28.35 ± 4.13	7.98 ± 0.89	3.61 ± 0.28	0.19 ± 0.07
P2	0	8.73 ± 0.40	7.48 ± 0.12	3.50 ± 0.22	0.26 ± 0.02
	3	10.24 ± 1.62	8.73 ± 0.40	4.20 ± 1.41	0.20 ± 0.04
	5	15.60 ± 3.99	7.15 ± 2.12	4.15 ± 0.49	0.23 ± 0.04
	7	21.56 ± 6.22	8.52 ± 0.18	3.40 ± 0.28	0.19 ± 0.06
P3	0	7.02 ± 0.94	7.48 ± 0.12	3.50 ± 0.22	trace
	3	10.15 ± 3.12	8.70 ± 0.36	3.17 ± 0.78	trace
	5	9.77 ± 2.25	6.47 ± 0.29	3.35 ± 0.63	trace
	7	9.71 ± 2.03	6.42 ± 0.55	3.23 ± 0.47	trace

Each value represent the mean ± S.E. for three replications.

increased considerably between days 3 and 7. The cell number rate was 0.31 per day and the doubling time was 3.2 days. Thus, grape cell suspensions divided slowly compared with other cells grown with 2,4-D [11]. Under Pi-deprivation conditions, growth was greatly disturbed. On day 7, the cell number decreased by 38% in P2 medium, whereas no variation in cell number was observed in P3 medium.

About 80% of the initial Pi was absorbed during the first three days (Table 1); this period corresponded to an energetic phase or lag phase as generally observed with other cell suspensions [2]. Parallel to this, cellular Pi concentration (vacuolar Pi) reached a maximum of 11.6 mM on day 3. In P2 and P3 media, the Pi level in cells was, respectively, 2.5- and 3.5-fold lower than in the control on day 3. Simultaneously, cell biomass was reduced by 39 and 86%. Thus, the low phosphate concentrations acted as a limiting factor for growth of grape cell suspensions [3].

Changes in anthocyanin synthesis

Under the three conditions studied, anthocyanin synthesis occurred mainly during the first three days (Fig. 2), with a maximum accumulation on day 5. This maximum was enhanced by 32 and 46% in P2 and P3 media, respectively. Although anthocyanin synthesis appears early in *Vitis* cell suspensions, the antagonistic relation between anthocyanin synthesis and cell division, as previously shown with *Vitis* cells [3], is found here when modifying Pi concentrations. Under the three Pi conditions, a decrease in anthocyanin content was observed between days 5 and 7 (Fig. 2). It was likely due to a degradative mechanism [12, 13].

Cyanidin 3-glucoside, peonidin 3-glucoside and their acylated derivatives are the main anthocyanins present in our *Vitis* cell suspensions [14]. Although this has been confirmed at all growth stages and for the three Pi

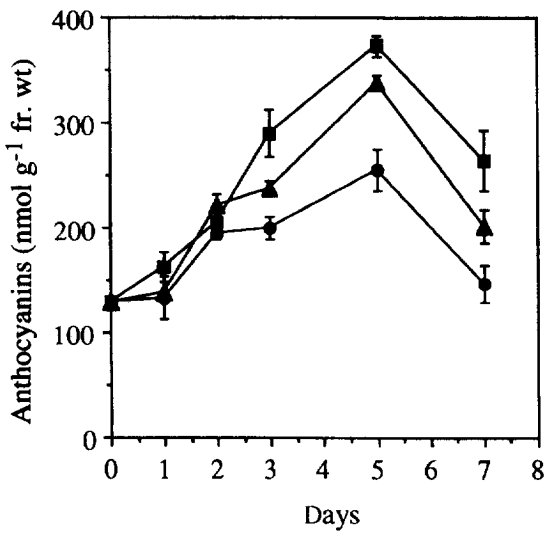

Fig. 2. Effect of different phosphate concentrations on anthocyanins accumulation in grape cell suspensions (± S.E. for four replications). P1 medium (1.1 mM Pi), —●—; P2 medium (0.25 mM Pi), —▲—; P3 medium (0.008 mM), —■—.

concentrations (Table 2), a higher proportion of acylated forms was observed in Pi-deprived cell suspensions (Table 2). In any case, peonidin 3-glucoside was the major acylated anthocyanin. Since it was shown that acylation facilitates transtonoplastic transport [15], it is possible for Pi deprivation to lead both to an early and a better storage of anthocyanins in grape cell suspensions.

DFR activity

DFR activity was reported here for the first time in a fruit material (Fig. 3). In P1 medium, it was easily

Table 2. Effects of various concentrations of phosphate on anthocyanins. Cyanidin 3-glucoside (Cy), peonidin 3-glucoside (Pn) and acylated forms are expressed in % of total anthocyanins. Pn acylated forms in % of acylated pool. The initial concentrations in phosphate were P1: 1.10 mM; P2: 0.25 mM; P3: 0 mM

Medium	Days	Cy	Pn	Acylated forms	Pn acylated forms
P1	3	14.6 ± 3.8	55.6 ± 13.1	17.4 ± 1.9	54.0 ± 2.9
	5	11.5 ± 0.3	40.6 ± 3.2	34.6 ± 1.7	71.3 ± 2.0
	7	12.9 ± 0.1	40.2 ± 0.5	33.9 ± 2.6	72.9 ± 0.3
P2	3	11.2 ± 1.1	41.9 ± 3.1	24.1 ± 2.9	72.7 ± 1.2
	5	11.7 ± 0.4	37.6 ± 0.1	40.4 ± 1.0	72.9 ± 11.0
	7	13.9 ± 0.7	45.1 ± 0.8	33.9 ± 2.1	73.7 ± 2.1
P3	3	11.6 ± 0.2	32.0 ± 0.4	47.7 ± 1.9	75.9 ± 8.1
	5	14.6 ± 0.3	34.1 ± 0.2	39.9 ± 1.9	69.6 ± 10.9
	7	12.7 ± 0.5	35.2 ± 1.2	43.4 ± 2.1	73.9 ± 4.7

Each value represents the mean ± S.E. for three replications.

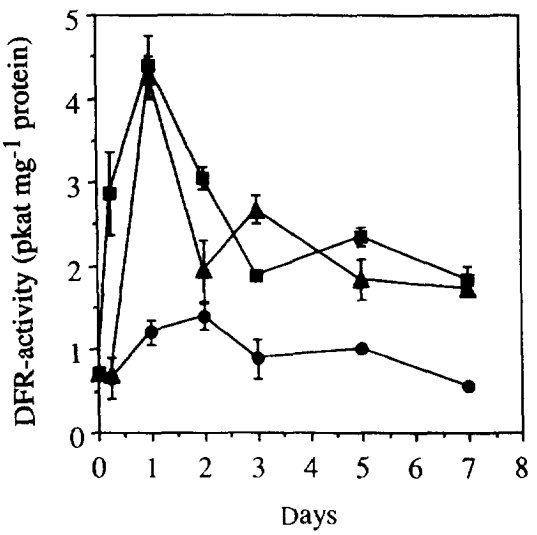

Fig. 3. Effect of different phosphate concentrations on DFR activity in grape cell suspensions (± S.E. for three replications). P1 medium (1.1 mM Pi), —●—; P2 medium (0.25 mM. Pi), —▲—; P3 medium (0.008 mM), —■—.

detected with a maximal value of 1.39 pkat mg^{-1} protein after 2 days. Specific activity was lower than in *Matthiola incana* flowers [16], but it was similar to the values obtained on *Dianthus* [17]. As of day 3, DFR activity slowly decreased, whereas anthocyanin still accumulated up to day 5 (see Fig. 2). A similar evolution was noted when DFR activity was expressed, as nmoles of cyanidin formed either per mg of proteins (specific activity) or per g fresh weight (data not shown). In P2 or P3 medium, DFR activity was about 3.1-fold higher than in the control (Fig. 3). Moreover, it reached its maximum earlier than in the control, *ca* 24 hr after cell inoculation. Later on, DFR activity was still higher compared that to in P1 medium.

Pi deprivation led both to an increase in anthocyanin content and in DFR activity in grape cell suspensions. Whatever the Pi concentration, the maximum of DFR activity always preceeded anthocyanin accumulation, in agreement with its presumed role in anthocyanin synthesis [18]. However, it clearly appeared that changes in DFR activity and anthocyanin synthesis in Pi deprivation were not of the same magnitude. The magnitude of enhancement was higher for DFR activity (× 6) than for anthocyanin (× 3). In fact, DFR is not the final enzyme involved in anthocyanin biosynthesis and other enzyme(s) lead from leucocyanidin to cyanidin [19] and, with DFR, likely contribute to the regulation of anthocyanin synthesis.

Phosphate deprivation leads classically to a decrease in primary metabolism and in growth parameters [20], associated with a reorientation towards secondary metabolism [6]. Although one of the possible regulatory mechanisms likely concerns substrate availability and energetic aspects [5], a direct action on some key enzymes of secondary metabolism is not excluded. In the case of grape cell suspensions, it clearly appears that Pi-deprivation acts on DFR activity, which then increases quickly. This suggests either a *de novo* synthesis or an activation of the enzyme, which may be considered as part of a rapid adaptative response of secondary metabolism to Pi deprivation [5, 21]. Furthermore, grape cell suspensions are known to contain both red cells which accumulate anthocyanins and non pigmented cells [22] and investigations are presently being conducted in our laboratory to investigate the effects of phosphate depletion on the two types of cells.

EXPERIMENTAL

Plant material. Grape cell suspensions derived from calli generated from berry pulp fragments of a Teinturier variety (*V. vinifera* Gamay Fréaux). Calli cultures were maintained on a solidified medium (0.7% agar)

containing the Gamborg macro-elements [23], the Murashige and Skoog micro-elements [24] and the Morel vitamins [25]. It was supplemented with 2% sucrose and 0.025% casein hydrolysate. Kinetin (0.2 mg l^{-1}) and NAA (0.1 mg l^{-1}) were used as growth factors. The pH was adjusted to 6.0 before autoclaving. Cell suspension cultures were conducted in 250 ml flasks (100 ml), in a giratory shaker (120 rpm), under low, continuous irradiation (40 μE m^{-2} sec^{-1}) at 23° on the same medium without agar. The initial concn of Pi was 1.1 mM in control medium (P1). Experiments were carried out either by lowering the initial Pi concn to 0.25 mM (P2 medium) or by omitting Pi in fresh medium at subculturing (P3 medium). Cells were routinely subcultured by transferring an inoculum of about 7 g of cells in fresh medium every 7 days. Harvesting was made at different time intervals. Cells were collected through filtration under red. pres. on to a nylon net (60 μm). Cells of an aliquot of suspension (5 ml) were counted with an haematocymeter (Malassez) after aggregates dissociation. Viability tests were made according to ref. [26].

Anthocyanin extraction and analytical procedure Anthocyanins were thoroughly extracted from fresh material with 0.1% HCl MeOH. After homogenization with an Ultra Turrax (for 20 sec), extraction was carried out by stirring in the dark for 15 min. The extract was clarified by passing through a 0.45 μm Millipore filter. The absorbance was measured at 530 nm, and converted into nmoles (mM extinction coefficient = 48.4 with a peonidin 3-glucoside soln as reference). HPLC analysis was performed according to ref. [14]. MeOH was substituted for anthocyanin extraction, and the extract was acidified with $HClO_4$ prior to injection. Water-soluble phosphate was extracted at 4° from fresh material with a diluted acid soln (0.01 M HCl) in a 1:20 ratio (w/v) for 16 hr in the dark. An aliquot of the supernatant (10 000 *g* for 5 min) was assayed according to ref. [27], using K_2HPO_4 as standard and sulphomolybdate as specific reagent. Protein content determination was made according to ref. [28], using BSA as standard.

Enzyme extraction and assay. Cells were frozen under liquid N_2. All steps were carried out at 0°–4°. Me_2CO powders were prepd and extraction was performed with 0.1 M borate–HCl buffer (pH 8.8), containing 10% glycerol, 0.3 g Polyclar AT, 0.5 g XAD-4 and 20 mM Na ascorbate. The supernatant (38 000 *g* for 10 min) was passed through a 1 × 5 Sephadex G-25 (fine) column, equilibrated with 0.1 M Tris–HCl buffer (pH 7.4), containing 20 mM Na ascorbate. This desalted extract was used for DFR assay (90 min, 30°), using dihydroquercetin as substrate and NADPH as reducing agent [29].

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