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ANTHOCYANIN RELEASE FROM GRAPE (VITIS VINIFERA L.) CELL SUSPENSION[1]

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Summary

In order to develop a protocol for the release of intravacuolarly-stored anthocyanins from grapevine (*Vitis vinifera* L.) cell suspension with preserved viability, the effects of extracellular pH modifications, of solid adsorbents in the culture medium (two-phase system) and of chemical permeabilization were investigated. The solid adsorbent Amberlite IR-120 and chemical permeabilization were efficient in enabling the release of up to 70-80% of vacuolar anthocyanins. However, the release was always at the expense of a proportionate loss in cell viability. Results demonstrate the irreversible nature of anthocyanin sequestration.

Introduction

Current interest in plant cell culture is strongly influenced by the possibility of using cells grown *in vitro* to produce secondary metabolites of value on a commercial basis. Most secondary products are localized in the vacuole and may be harvested from cells using destructive methods i.e. cell disruption. However, due to the slow growth rate of cultured plant cells and/or the minimal extent of product formation frequently exhibited by cells grown *in vitro*, use of destructive harvesting is, in many instances, economically undesirable (Kilby and Hunter, 1990). Procedures for the concurrent product release from the cells and harvesting in the medium are being developed to facilitate continuous processes that may reduce the cost of fermentation and purification and allow the use of immobilized technology (Fontanel and Tabata, 1987). The release of valuable secondary metabolites from the vacuole with preserved viability, remains the major hurdle to overcome in order to use immobilization technology for large scale plant cell based bioprocesses.

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While the dynamic behavior of secondary metabolites in plants is well recognized, mechanisms by which these compounds are sequestered into the vacuole remain poorly understood and the extent to which such mechanisms might be reverted thus enabling product release from the vacuole into the culture medium is, for now, subject to speculation.

A diffusion model called "ion trapping" has been proposed to explain the pH-regulated accumulation of secondary metabolites into the vacuole. According to this model, compounds are translocated through the biological membranes by passive diffusion in their uncharged lipophilic form. The charged form accumulates into the acidic compartment of the cell i.e. the vacuole. Furthermore, compounds accumulate to a higher level under conditions which allow the cells to reach a more acidic vacuolar pH value such as nitrate deprivation (Bouyssou *et al.*, 1987). Reversal of the ion trap mechanism by acidification of the culture medium has been efficient in promoting the release of ajmalicine from *Catharanthus roseus* cell suspensions (Bouyssou *et al.*, 1987; Renaudin, 1989; Jardin *et al.*, 1991).

Elsewhere, it has been proposed that transport mechanisms may adjust intracellular and extracellular levels of secondary metabolites in response to cellular conditions. The addition of an artificial accumulation site to a cell suspension was shown not only to promote the secondary metabolism but also increase the release of compounds into the culture medium (Strobel *et al.*, 1991). Adsorbents are thought to be efficient in increasing the productivity of a cell suspension by scavenging even trace amounts of substances from the medium thus avoiding any type of feed back inhibition which may hinder product formation (Maisch *et al.*, 1986).

Finally, procedures to induce product release into the culture medium using chemical agents have been attempted. A variety of organic solvents and detergents have been tested on various plant cell suspensions but have always shown a deleterious effect (Felix *et al.*, 1981; van Unden *et al.*, 1990; Strobel *et al.*, 1991; Brodelius, 1988). Exceptionnally, dimethyl sulfoxide (DMSO) and Triton X-100 were efficient in permeabilizing *C. roseus* cells with preserved viability (Brodelius, 1988).

Our interest in the cultivation of *Vitis vinifera* cells resides in their potential use as a source for anthocyanin pigments. Anthocyanin based colorants are used in foods and beverages and in cosmetics (Jackman *et al.*, 1987). They are also efficient in reducing serum cholesterol (Igarashi and Inagaki, 1991). In *Vitis vinifera* cell suspensions, the accumulation of anthocyanins into the vacuole is promoted in medium with low nitrate concentrations (Do and Cormier, 1991a) which agrees with the ion trapping theory. Furthermore, conditions which favor alkalinization of the vacuole such as medium containing a high concentration of ammonium, repressed anthocyanin accumulation (Do and Cormier, 1991b). Therefore, in this paper, we address the feasibility of promoting anthocyanin release into the culture medium by the following treatments: (1) reversal of ion trapping by acidification of the medium, (2) the use of solid adsorbants in the medium (two-phase culture) and, (3) chemical permeabilization.

Materials and methods

Cell suspension culture. Cell suspension cultures of *Vitis vinifera* cv Gamay Fréaux were grown in B5 medium (Gamborg *et al.*, 1968) containing per litre 30 g sucrose, 250 mg casein hydrolysate, 0.1 mg α-naphthaleneacetic acid and 0.2 mg kinetin. Stock culture maintenance and routine transfers were performed as described elsewhere (Cormier *et al.*, 1990). Cell viability was determined using the double staining

procedure with fluorescein diacetate and propidium iodide (Huang *et al.*, 1986).

Anthocyanins determination. Anthocyanins were recovered from the culture medium by passing an aliquot of filtered medium through a Prep-Sep C18 cartridge and eluting the pigments from the column using ethanol-1% HCl (85:15). Anthocyanins were extracted from fresh cells overnight with ethanol-1% HCl (85:15) at 5°C. Anthocyanin concentration was determined using the extinction coefficient, $E^{1\%}=982$ at 535 nm (Francis, 1982).

Anthocyanin release experiments. The following assays were conducted using 7 day old cultures adjusted to a packed cell density of 40% (v/v):

- **Reversal of ion trapping.** These assays were conducted by acidifying the culture medium with a solution of hydrochloric acid prior to autoclaving. The cell suspension was washed three times in this medium before adjusting the cell density to 40% (v/v). Anthocyanin release and viability of the cells was performed thereafter.
- **Adsorbent culture system.** Amberlite IR-120, Amberlite IRC-50, Duolite C-433 (mesh size 16-50 from Rohm and Haas), Amberlite XAD-7 (mesh size 20-50 from Rohm and Haas) and, Lichroprep RP-18 (25-40 μm from Merck) were used as adsorbents. Amberlite IR-120 and IRC-50 and Duolite C-433 resins were regenerated to H^+ form according to the manufacturer's procedure. Amberlite XAD-7 and Lichroprep RP-18 were washed with methanol and water prior to use. Except for Lichroprep RP-18, all adsorbents were easily separated from the cells by decantation. Lichroprep RP-18 was entrapped in alginate beads to enable separation from the cells by sieving. Ten grams dry Lichroprep RP-18 were suspended in 200 ml of 1% (w/v) alginic acid and added dropwise to 0.1 M $CaCl_2$ to form beads.
- **Chemical permeabilization of cells.** Chemical agents listed in Table 2 were added to the cell suspension.

Results

Techniques used to promote the release of intravacuolarly-stored anthocyanins included acidification of the culture medium, addition of solid adsorbents to the suspension and, use of permeabilizing agents (Tables 1 and 2). The various treatments were applied either alone or in combination. Acidification of the culture medium and addition of polymeric adsorbent Amberlite XAD-7 were applied together to the suspension. The permeabilizing agent geraniol was used in combination with two cation exchange resins. Results show that none of the treatments induced the discharge of anthocyanins into the medium while preserving the viability of the cell suspension (Table 1). Release of anthocyanins was always accompanied by a loss of cell viability.

A wide array of monoterpenes and fatty acids was used to permeabilize cells (Table 2). Among these, geraniol, nerol, neomenthol and caprylic acid were effective at concentrations lower than those commonly used for DMSO. Also, their action was faster than that of DMSO. However, these concentrations caused an irreversible loss of membrane integrity which led to cell death. Additionally, treatments which led to a total loss of viability resulted in the release of 70-80% of intracellularly-stored anthocyanins.

Table 1. Percentage of intracellular anthocyanins released from a grape cell suspension (40% v/v) after 30 min incubation under various conditions. Adsorbent concentration (wet wt/l): a, Amberlite IR-120; b, Duolite C-433; c, Amberlite IRC-50; d, calcium alginate beads containing Lichroprep RP18; e, Amberlite XAD-7.

Exogeneous geraniol	pH of medium	Adsorbent[1]	Mortality (%)	Anthocyanins released (%)
-	5.8	-	< 5	0
-	2.5	-	< 5	0
-	2.0	-	< 5	0
-	5.8	a (10 g/l)	70	80
-	5.8	a (20 g/l)	90	80
-	5.8	b (10 g/l)	< 5	0
-	5.8	b (20 g/l)	< 5	0
-	5.8	b (40 g/l)	< 5	0
100 µl/l	5.8	b (10 g/l)	< 5	0
100 µl/l	5.8	b (20 g/l)	< 5	0
100 µl/l	5.8	b (40 g/l)	< 5	0
200 µl/l	5.8	b (10 g/l)	100	70
200 µl/l	5.8	b (20 g/l)	100	70
200 µl/l	5.8	b (40 g/l)	100	70
-	5.8	c (10 g/l)	< 5	0
-	5.8	c (20 g/l)	< 5	0
-	5.8	c (40 g/l)	< 5	0
200 µl/l	5.8	c (10 g/l)	100	70
200 µl/l	5.8	c (20 g/l)	100	70
200 µl/l	5.8	c (40 g/l)	100	70
-	5.8	d (100 g/l)	< 5	0
-	5.8	e (10 g/l)	< 5	0
-	5.8	e (20 g/l)	< 5	0
-	5.8	e (40 g/l)	< 5	0
-	2.0	e (40 g/l)	< 5	0

Table 2. Permeabilizing agents and concentration beyond which anthocyanin release from a grape cell suspension was observed. N.D., not determined (no anthocyanin release at concentrations up to 600 µl/l).

Compounds	Concentration for anthocyanin release	Compounds	Concentration for anthocyanin release
Monoterpenes:		Fatty acids:	
geraniol	150 µl/l	caprilate	200 µl/l
nerol	150 µl/l	heptanoate	400 µl/l
neomenthol	150 µl/l		
citral	600 µl/l	Other:	
menthol	600 mg/l	dimethyl sulfoxide	400 µl/l
linalool	600 µl/l		
α-terpineol	600 µl/l		
menthone	N.D.		
cineol	N.D.		
ß-myrcene	N.D.		
α-terpinene	N.D.		
p-cymene	N.D.		

Discussion

Failure to revert "ion trapping" by acidification of the medium suggests that this mechanism may not play a direct role in the sequestration of anthocyanins in the vacuole. Indeed, the ion trapping model presupposes that accumulation of a product is dependent on its pK, e.g. the more alkaline the pK the higher the accumulation ratio (Ci/Ce). Monoglucosidic anthocyanins such as those found in *Vitis vinifera* cell cultures i.e. cyanidin 3-glucoside, peonidin 3-glucoside and peonidin 3-p-coumaroylglucoside (Van Calsteren *et al.*, 1991), possess relatively low pK in the 4 - 4.3 range (Brouillard, 1982). Therefore, other mechanisms such as complexation of anthocyanins to other constituents of the vacuole may play a critical role in the accumulation of anthocyanins and may also explain the irreversibility of the process. Observations by Brouillard *et al.* (1990) supplement this view. They proposed that anthocyanins are accumulated into the vacuole principally in colourless forms and are converted to a stabilized colored form by complexation with other vacuolar constituents. In the case of *C. roseus* cell suspension culture, there is some evidence that only alkaloids of the cytosolic compartment and not those in the storage vacuole, are diffusible and can be efficiently secreted by acidification of the culture medium (Bouyssou *et al.*, 1987). Again, complexation of alkaloids with phenolics and other components of the vacuolar sap is believed to contribute to their sequestration in the vacuole (Renaudin, 1989).

While DMSO was efficient in permeabilizing *C. roseus* cells with preserved viability (Brodelius, 1988), this treatment had a deleterious effect in *Vitis vinifera* cell cultures. Similarly, DMSO treatment led to the death of *Thalictrum rugosum*, *Chenopodium rubrum* (Brodelius, 1988), *Cinchona ledgeriana* (Parr *et al.*, 1984) and, *Digitalis lanata* cell cultures (Kreis *et al.*, 1990). Thus efficient use of DMSO as a physiologically acceptable permeabilizing agent is questionable. Furthermore, use of this compound in a plant cell based process may be confronted with regulatory issues since it is not listed as a carrier or extraction solvent permitted by regulatory agencies (Food and Drugs Administration, 1988).

Essential oils, oleoresins (solvent-free) and natural extractives (including distillates) are, on the other hand, classified as substances "generally recognized as safe" (GRAS) and may constitute efficient natural permeabilizing agents. The results presented in this study demonstrate that some monoterpenes and fatty acids were effective permeabilizing agents at concentrations lower than for DMSO. However, their action on the grape cell suspension inevitably led to cell death.

Results show that conditions which induced the release of 70-80% of anthocyanins resulted in total loss of cell viability after treatment. Microscopic observation of permeabilized cells revealed that the anthocyanins remaining in the cells were adsorbed to a solid matrix (inclusion) within the vacuole (Cormier and Do, in press). Similarly, the release of ajmalicine from *C. roseus* cell cultures promoted by extensive washing of the suspensions could only enable the release of 72% ajmalicine with the remaining 28% being strongly retained in the vacuole (Renaudin, 1989).

Conclusion

Failure to promote the release of vacuolarly-stored anthocyanins under physiologically-acceptable conditions using three different methods strongly suggests that vacuolar sequestration of anthocyanin is irreversible. It appears that anthocyanins can only be

recovered from plant cell suspension cultures using destructive methods.

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