ANTHOCYANINS PRESENT IN CELL CULTURES OF *DAUCUS CAROTA*

JOYCE C. HEMINGSON and R. P. COLLINS

Biological Sciences Group—Botany Section, University of Connecticut, Storrs, Ct. 06268

ABSTRACT.—Four anthocyanins were found in the cell cultures of *Daucus carota*. All are glycosides of cyanidin and none appear to be acylated. Two pigments were diglycosides: cyanidin-3-glucogalactoside and cyanidin-3,5-digalactoside. The other two pigments were monoglycosides: cyanidin-3-glucoside and cyanidin-3-galactoside.

In the last decade, tissue and cell culture has had increasing use as a technique in studying metabolic pathways and the accumulation of secondary products. Differences in metabolites sometimes exist between the whole plant and its cultured cells (1). However, certain metabolites are easy to extract from cell cultures and can be obtained in large quantities. Many workers have used cell cultures of *Daucus carota* to follow flavanoid pathways, particularly that of anthocyanin synthesis. This work fully identifies the anthocyanins produced in cell cultures by *D. carota*, a member of the Umbelliferae, and makes comparisons with those reported for whole plants of the genus.

Sugano and Hayashi (2) used two carrot root tissue cultures to follow the change in synthesis of free sugars, amino acids, malic and cinnamic acids, carotenoids and anthocyanins over a 29-day period. The culture with 2,4-dichlorophenoxyacetic acid (2,4–D) instead of indole acetic acid (IAA) in the media produced no anthocyanins. The cultures with anthocyanins had pigment located in cells beneath the surface or around tracheids. They found a mixture of four anthocyanins and by paper chromatography of the hydrolysis mixture determined that the anthocyanidin was cyanidin. No analysis of the glycosides was done. Schmitz and Seitz (3) found gibberellic acid A_3 decreases the synthesis of anthocyanins in callus cultures of *D. carota* in as low a concentration as 10^{-12}M, sharply reduces it in the range of 10^{-11} to 10^{-5}M, and totally inhibits it at 10^{-4}M. They determined there were two anthocyanins present, both xyloglucosides of cyanidin. Alfermann, Merz and Reinhard (4) studied the production of anthocyanins using *D. carota* tissue cultures given various analogs of 2,4–D. They isolated a cell line which differed in the production of anthocyanins but reported no qualitative analysis.

Using whole plants, Harborne and Williams (5) identified seven flavonols or flavones in *D. carota* L. ssp. *sativa* and luteolin-7-rutinoside, luteolin-4'-glucoside, quercetin-3-glucoside, and an unidentified aglycone in *D. carota* L. ssp. *carota*. Later Harborne (6) reported a new pattern of acylated anthocyanin triglycosides in the Umbelliferae. For leaves, flowers, or roots of *D. carota* L. ssp. *sativa* and *carota*, he listed four anthocyanins: cyanidin-3-xylogalactoside, cyanidin-3-xyloglucogalactoside and the triglycoside acylated with ferulic or sinapic acid.

EXPERIMENTAL

PREPARATION OF FOUR CARROT CELL EXTRACTS.—Two carrot cell lines, WC63.3b.f and WC38–1A, were produced by a subculturing procedure (7,8) from cells originally supplied by D. F. Wetherell, The University of Connecticut, Storrs, Ct. Each line was cultured in two different media, WC–1 A2688, an unimproved or stock culture medium (9) and WC–IMP A2687, an improved medium (8,10). An extract from each cell line in each medium was then prepared by the following procedure. Each carrot cell culture was filtered through Miracloth. The anthocyanins were then extracted from the cells with 1% HCl in methanol. Each extract was filtered, and the residue was re-extracted with 1% HCl in methanol. This was repeated, and the total extract was then concentrated in a rotary evaporator at about 30°.

PREPARATION OF *D. carota* FLOWER EXTRACT.—Anthocyanins of the red flowers from the centers of *D. carota* umbels were extracted with 1% HCl in methanol. The extract was filtered and then concentrated in a rotary evaporator at about 40°.

SEPARATION OF PIGMENTS.—A narrow band of each extract was applied to Whatman No. 3 paper, and the papers were equilibrated overnight in a chromatography cabinet with both layers of 100 ml BAW (1-butanol-glacial acetic acid-water, 4:1:5). The papers were then developed in descending fashion with the upper layer of BAW. More BAW was added as needed and the total development time varied from 45–70 hours. The solvent overran the paper in all cases. After development, the papers were dried.

CONCENTRATION AND STORAGE.—When the Whatman No. 3 papers were dry, each of the bands was cut out and shredded, and the pigment was eluted with 2% glacial acetic acid in water. Each pigment was concentrated *in vacuo* at 57°. The concentrated pigments were stored in sealed vials and refrigerated.

ACID HYDROLYSIS.—The pigments were hydrolyzed in a boiling water bath with 1 N HCl and under a nitrogen atmosphere. Aliquots were removed at timed intervals and spotted on Whatman No. 1 paper. The paper was equilibrated overnight with 100 ml of the solvent glacial acetic acid-hydrochloric acid-water (15:3:82) then developed in an ascending manner. The number of spots at each time interval was noted, and R_f values were calculated.

AGLYCONE ANALYSES.—After hydrolysis, n-amyl alcohol was used to extract the aglycones. These were spotted immediately on Whatman No. 1 paper along with the following standard aglycones: cyanidin, pelargonidin, peonidin, malvidin and delphinidin. Each chromatogram was equilibrated overnight before development in an ascending manner. The solvent systems used were: glacial acid-hydrochloric acid-water (24:8:64) and propionic acid-formic acid-hydrochloric acid-water (16:40:8:48). The papers were dried and R_f values were calculated.

FORMATION OF ACETATES.—After extraction of the aglycones, the hydrolysis mixtures were washed with an equal volume of 10% di-*N*-octylmethylamine in chloroform to remove mineral acid, followed by three washes with chloroform to remove any di-*N*-octylmethylamine. The sugars in the mixtures were then reduced for one hour in 400 mg $NaBH_4$ in 10 ml of water. The reduction was ended by the addition of glacial acetic acid. The resulting sugar alcohol mixtures were passed through a small column of prewashed Dowex cation exchange resin, AG 50w-x16, 50–100 mesh, to remove sodium ions.

The mixtures were then brought to dryness *in vacuo*. To remove boron, approximately five ml of methanol was added to the residues and the solutions were heated to 64–70° in a water bath for about thirty seconds before being brought to dryness again. This procedure was repeated four times.

The sugar alcohols were next converted to acetates by refluxing with 10 ml pyridine and acetic anhydride (1:1) in a steam bath for at least one hour. The acetate mixtures were then brought to dryness *in vacuo*. Approximately 5 ml of water was added and the solutions brought to dryness again. This procedure was repeated several times to remove excess pyridine and acetic anhydride. The final residue was dissolved in methylene chloride.

GAS CHROMATOGRAPHY OF ACETATES.—Standard acetates of glucose, rhamnose, galactose, arabinose, mannose and xylose were used as references.

One column used was 1.83 m x 3.2 mm stainless steel packed with 3% ECNSS-M (a cyanoethyl silicone-ethylene succinate polymer) on 80–100 mesh acid-washed silanized Chromosorb. Operating conditions were: column 190–200°, injector 250°, detector 250°, He flow rate 30 ml/min, sample size 1–3 μl, solvent methylene chloride. A second column of mesh size 60–80 was also used.

Better resolution was obtained on a 1.83 m x 3.2 mm stainless steel column packed with 3% Sp-2340 on 80–100 mesh Chromosorb-W. Operating conditions were: column 225°, injector 250°, detector 250°, He flow rate 20 ml/min, sample size 1–2 μl, solvent methylene chloride.

VISIBLE AND ULTRAVIOLET SPECTRA OF ANTHOCYANINS.—A small amount of each concentrated pigment band was brought to dryness *in vacuo* and the residue dissolved in 0.01% methanolic HCl. The spectrum from 700–200 nm was then recorded with a Beckman DB-G spectrophotometer. The spectrum was recorded again after the addition of three drops of $AlCl_3$ solution (2.5 ml $AlCl_3$ in 47.5 ml 95% ethanol) to determine whether a bathochromic shift had occurred.

DEACYLATION.—The concentrated bands of pigment were deacylated by treatment with 2N NaOH under a nitrogen atmosphere for one hour followed by reacidification with 2N HCl. The pigments were spotted on Whatman No. 1 paper before and after deacylation along with the following standards: trans 4-hydroxycinnamic acid (*p*-coumaric acid), trans 3,4-dihydroxycinnamic acid (caffeic acid) and trans 4-hydroxy,3-methyoxycinnamic acid (ferulic acid). The papers were equilibrated overnight and developed in an ascending fashion with glacial acetic acid-10 N hydrochloric acid-water (15:0.5:82) as the solvent system. When dry, the papers were viewed under ultraviolet light to detect areas of fluorescence.

WHATMAN NO. 3 PAPER.—To determine whether the Whatman paper used in the chromatographic separation of the pigments was producing free sugars, two pieces of Whatman No. 3 paper were equilibrated in the chromatography cabinet and developed for three days with BAW, upper layer. The papers were then dried under a hood and the lower two-thirds of the papers shredded and soaked in 2% acetic acid for three hours. The solution was then filtered, taken to dryness *in vacuo* at 57° and redissolved in a small amount of water. The solution was next given the same treatment used for the reduction of sugars and their conversion to acetates, mentioned above. The final residue was dissolved in methylene chloride for gas chromatography.

RESULTS AND DISCUSSION

In *D. carota* flowers, the pigments of bands 2 and 4 are produced in the greatest amounts, followed by the pigments of bands 3 and 5. In the cell cultures of *D. carota*, the pigments of bands 1 and 3 are produced in the greatest amounts in the unimproved medium, WC–1 A2688, and the pigments of bands 1 and 4 are produced the most in the improved medium, WC–IMP A2687. The improved medium contains ammonium as the sole nitrogen source. It also has succinate, which gives the greatest accumulation of anthocyanin of fifteen organic acids that were tested (8). When co-chromatographed in descending fashion with BAW, bands 2–5 of *D. carota* flowers roughly correspond to bands 1–4 of the cell culture extracts.

The anthocyanidin found in the cell cultures is cyanidin. The R_f values for the anthocyanidin are those of cyanidin when co-chromatographed with authentic anthocyanidins. The visible maximum of the anthocyanidin of the cell cultures also undergoes a bathochromic shift (a shift to longer wavelengths) upon the addition of $AlCl_3$ solution (table 1). This shift characterizes a phenolic ring with ortho hydroxy groups, such as is found in cyanidin.

TABLE 1. Data from the visible spectrum of pigments from cell cultures, *D. carota*, and cyanidin.

Medium/cell line	Band	Max vis	$E_{max\ vis}$	E_{440}	$E_{440}/E_{max\ vis}$ %	$AlCl_3$ shift
WC–IMP A2687/ WC38–1A 10.5	1	532	0.19	0.06	31	+
	2	540	0.16	0.15	94	+
	3	540	0.16	0.005	31	+
	4	530	0.15	0.11	72	+
WC–IMP A2687/ WC63.3b.f. 10.5	1	530	0.84	0.33	38	+
	2	530	0.13	0.20	160	+
	3	530	0.36	0.38	106	+
	4	530	0.20	0.26	128	+
WC–1 A2688/ WC38–1A 10.5	1	530	0.85	0.26	31	+
	2	530	0.83	0.86	104	+
	3	540	0.49	0.38	78	+
	4	540	0.38	0.45	118	+
WC–1 A2688/ WC63.3b.f 10.5	1	530	0.26	0.065	25	+
	2	535	0.13	0.06	48	+
	3	537	0.15	0.14	93	+
	4	530	0.06	0.045	75	+
D. carota flowers	1	535	0.28	0.13	46	+
	2	530	1.3	0.32	26	+
	3	540	0.67	0.22	33	+
	4	540	0.61	0.17	28	+
	5	530	0.36	0.15	41	+
Cyanidin	—	535–540	0.14	0.11	79	+

In all four cell cultures, the pigment of band 1 is a 3-substituted diglycoside, the pigments of bands 2 and 3 are 3-substituted monoglycosides and that of band 4 is a 3,5-substituted diglycoside. This assignment of position of sugar attachment is based on the R_f values of each pigment (table 2) and on Harborne's (11) observation that 3-substituted compounds have a shoulder in their visible spectrum around 440 nm, whereas those which are substituted at both the 3 and 5 positions do not. The pigments in cell culture bands 1–3 have a shoulder or broad slope around 479–500 nm and band 4 does not. This method works, although no one has explained why. Harborne has said that the choice of 440 nm was an arbitrary one (11). Cyanidin itself has a maximum near 470 nm and thus a shoulder at 440 nm. The flavylium cation has a maximum at 468 nm

TABLE 2. R_f values from chromatograms of acid hydrolysis of pigments from cell cultures. Solvent: glacial acetic acid-10 N hydrochloric acid-H_2O (15:3:82).

Medium	Cell line	Band	R_f		
			Aglycone	Monoglycoside	Diglycoside
WC-1 A2688.....	WC63.3b.f 10.5	1	7	20	40
		3	6.4	22	
WC-1 A2688.....	WC38-1A 10.5	1	7.2	20	42
		3	6.4	25	
WC-IMP A2687.....	WC63.3b.f 10.5	1	7.2	19	40
		4	6.5	19,29	56
WC-IMP A2687.....	WC38-1A 10.5	1	5.6	19	39
		3	4	19	
		4	5.7	29	

whose intensity decreases as the pH is raised and accordingly the absorbance at 373 nm increases because the chalcone forms (12). It has been suggested that the 3,5-substituted anthocyanins have less of an absorption at 440 nm because they favor the carbinol side of the flavylium cation⇌carbinol equilibrium (13,14). The ratios of absorption at the visible maximum to that at 440 nm have also been used to assign the attachment of the sugar moiety (11), but the present data show no consistent ratios (table 1).

Linear triglycosides of cyanidin have been reported as the predominant anthocyanins of the Umbelliferae (6). The present analysis shows no triglycosides in cell cultures of *D. carota*.

It was shown by gas chromatography (table 4) of the sugars which were removed from the anthocyanins by acid hydrolysis and then converted to alditol acetates that the pigment of band 1 contains galactose and glucose, band 2 contains glucose, band 3 contains galactose and band 4 contains galactose.

The R_f values of band 1 (table 2) agree closely with those previously reported for a cyanidin-3-glucosylgalactoside ($R_f=39$) in the Umbelliferae (6). The R_f value of band 2, 26, also agrees with the value widely reported for cyanidin-3-

TABLE 3. R_f values from chromatogram of pigment of cell culture Band 2 and pigment of *D. carota* flower Band 3. Solvent: glacial acetic acid-10 N hydrochloric acid-H_2O (15:3:82).

Medium	Cell line	R_f
WC-1 A2688.....	WC38-1A 10.5	26
WC-IMP A2687.....	WC38-1A 10.5	26
WC-1 A2688.....	WC63.3b.f 10.5	26
WC-IMP A2687.....	WC63.3b.f 10.5	25
D. carota.....		29

TABLE 4. R_f values from deacylation chromatograms of cell culture pigments. Solvent: glacial acetic acid-10 N hydrochloric acid-H_2O (15:0.5:82).

Medium	Cell line	Band	R_f	
			Before deacylation	After deacylation
WC-1 A2688.....	WC38-1A 10.5	1	39	40
		2	26	28
		3	23	23
		4	24,51	23,51
WC-IMP A2687.....	WC63.3b.f 10.5	1	37	36
		2	24	24

glucoside (R_f=26) (table 3). The R_f value of band 3 is 19–25 and the attached sugar is galactose. Harborne (6) was able to separate cyanidin-3-glucoside from cyanidin-3-galactoside by two-day paper chromatography and found the latter has a lower R_f value, but did not report them. The R_f value of band 4 is 54–56. No R_f values for 3,5-digalactosides have been given in the literature. Sugano and Hayashi (2) reported four unidentified pigments in *D. carota* cell cultures. Schmitz and Seitz (3) reported two cyanidin-xyloglucosides.

One disadvantage of using Whatman paper for the separation of pigments is contamination by extraneous sugars. When an extract of Whatman paper was made and then subjected to reduction, acetylation and gas chromatography, five sugars were found (table 5): rhamnose, arabinose, mannose, glucose and an unknown, probably ribose. The same procedure has been used to analyze the primary walls of sycamore cells, which are predominantly cellulose (15). The first four sugars were reported, plus galactose, galacturonic acid, xylose and fucose. Prewashing the Whatman papers with 2% acetic acid did reduce the extraneous sugars, however.

TABLE 5. Sugars identified by gas chromatography of their alditol acetates.

Medium/cell line	Band	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Unknown**
WC-IMP A2687/								
WC38-1A 10.5	1	X	X	X		X	X	X
	2	X	X				X	X
	3	X	X					
	4		X				X	X
WC-IMP* A2687/								
WC63.3b.f 10.5	1	X	X			X		
	4		X		X	X		
WC-1* A2688/								
WC38-1A 10.5	3		X		X	X		
WC-1 A2688/								
WC63.3b.f 10.5	1		X	X			X	X
	3		X		X		X	
Whatman No. 3								
Paper		X	X		X		X	X

*Acid-washed Whatman No. 3 paper used for chromatographic separation; **Probably ribose.

The chromatograms of the deacylated compounds of the cell culture pigments showed no significant differences in R_f values before and after treatment with base and no new uv absorbing areas were generated on the chromatograms. Therefore, these pigments appear not to be acylated.

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