Expression of Anthocyanins in Callus Cultures of Cranberry (*Vaccinium macrocarpon* Ait)

D.L. MADHAVI, M.A.L. SMITH, and M.D. BERBER-JIMÉNEZ

ABSTRACT

Expression of anthocyanins and other flavonoids in callus cultures established from different parts of cranberry plant was investigated and the effect of explant source on the *in vitro* product was determined. Callus cultures were initiated from different parts of the plant in a modified Gamborg's medium with 5.37 μM α-naphthaleneacetic acid, 0.45 μM 2,4-dichlorophenoxyacetic acid, and 2.32 μM kinetin in the dark at 25°C. Callus cultures accumulated anthocyanins only on exposure to light and maximum concentration was observed by day 12. The cultures had lower levels of anthocyanins and only cyanidin 3-galactoside, cyanidin 3-glucoside, and cyanidin 3-arabinoside were identified in all cultures regardless of source of explant. Proanthocyanidin accumulation in cultures was independent of light, and levels were higher than in mature fruit. Exposure to light induced accumulation of flavonols and enhanced activity of phenylalanine ammonia-lyase in the cultures.

Key Words: anthocyanins, cranberry, callus, cyanidin

INTRODUCTION

THE CHARACTERISTIC bright red anthocyanin pigments in cranberries (*Vaccinium macrocarpon* Ait) make them an attractive potential source for natural food colors. Consumer demand for natural products has prompted new research on development of cell culture resources for pigment extraction. *In vitro* culture of highly pigmented genotypes may circumvent seasonal and geographic restrictions of cranberry crop production, and result in more product uniformity (Ilker, 1987; Shuler et al., 1990; Stafford, 1991). Cell cultures also provide effective systems for elucidating the biochemical aspects of secondary product formation, or for exploring the physiological properties of intermediates in biosynthetic pathways. Cell cultures may accumulate different secondary metabolites from those found *in vivo*, or may produce insignificant quantities of some components (Nawa et al., 1993; Mori et al., 1993; Wilson, 1990). In other cases, product yields and/or quality traits may be enhanced through production by tissue culture (Cormier and Do, 1993; Crouch et al., 1993). Manipulation of the physical and chemical microenvironments can influence both the quality and yield of natural products (Callebaut et al., 1990; Do and Cormier, 1991).

Anthocyanin production has been reported in cell cultures of edible materials like grapes (Yamakawa et al., 1983), sweet potato (Nozue et al., 1987), carrot (Ozeki and Komamine, 1985), strawberry (Hong et al., 1989; Mori et al., 1993), and rabbiteye blueberry (Nawa et al., 1993). Cranberry has been cultured *in vitro* for micropropagation (Marcotrigiano and McGlew, 1991) and genetic transformation (Serres et al., 1992), but *in vitro* pigment production has not been reported. Our objective was to compare anthocyanins derived from cranberry fruits *in vivo*, vegetative parts, and callus cultures derived from different parts of the plant. The expression of other flavonoids like flavonols and proanthocyanidins, *in vivo* and *in vitro* were also investigated and the activity of phenylalanine ammonia-lyase, one of the early enzymes in the flavonoid biosynthetic pathway, was determined to help elucidate the anthocyanin biosynthetic potential of the cultures.

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MATERIALS & METHODS

CRANBERRY 'STEVENS' FRUITS were obtained from the Blueberry and Cranberry Research Center, Rutgers University, and stored at −20°C until assayed. Plants of the same genotype were maintained in the greenhouse and as shoot cultures in WPM medium (Lloyd and McCown, 1981) supplemented with 0.98 μM 6-(γ,γ-dimethylallylamino) purine.

Callus initiation and pigment production

Stem segments (1 cm) and leaves from 8 wk old shoot cultures and the mesocarp tissue of mature fruits were used for callus initiation. Explants were placed in the dark at 25°C on a callus induction medium (modified Gamborg's medium, Gamborg et al., 1968) with 8mM NO_3^- as KNO_3, 25 mM NH_4^+ as $(NH_4)_2SO_4$, 200 μM Fe as $FeNa_2EDTA$, 5.37 μM α-naphthaleneacetic acid, 0.45 μM 2,4-dichlorophenoxyacetic acid, 2.32 μM kinetin, 100 mg/L PVP (Sigma Chemical Co., St. Louis, MO), 10% coconut water, 2% sucrose, and 0.7% agar. Subcultures were derived at 3 wk intervals. After the fourth subculture, callus colonies were transferred to pigment production medium containing no coconut water, reduced NO_3^- (2mM) and increased sucrose (5%) and held under a photosynthetic photon flux of 55 μmol m^{-2} s^{-1}. Time course experiments for anthocyanin production were done both at 8mM and 2mM NO_3^- levels in the production medium. Total anthocyanins were estimated by the method of Francis (1982). Results are averages of two replicates.

HPLC analysis of anthocyanins and anthocyanidins

The samples were extracted in 1% (v/v) HCl methanol (1:10 w/v) overnight at 4°C and the extracts after filtration were evaporated to dryness at 30°C with a Buchi rotary evaporator (Switzerland). The residue was dissolved in water and extracted with ethyl acetate (1:3 v/v) three times. The aqueous fraction was evaporated to remove residual ethyl acetate and adsorbed onto an activated OnGuard-RP Sep-Pak cartridge (Dionex, CA). The cartridge was washed with water and anthocyanins were eluted with 0.01% HCl in methanol. The extract was evaporated, redissolved in 10% formic acid, and filtered through a 0.2 μm filter membrane (Phenomenex, CA). HPLC was performed using a Hitachi L-6200A Intelligent Pump (Tokyo, Japan), a Hitachi Diode Array Detector (Tokyo, Japan), and a Rheodyne (Cotiati, CA) 7125 Injector. The column was a YMC-Pack ODS-AM (250 × 4.6 mm, 5 μ) connected to a YMC-Pack S5 120A ODS-AM guard column. Solvents were 10% formic acid (A) and 100% acetonitrile (B) at 1 mL/min. The elution profile was 0–4 min, 10–14% B in A; 4–10 min, 14–16% B in A; 10–20 min, 16–18% B in A; 20–25 min, 18–30% B in A. Absorbance was monitored at 520 nm. Peak identification was done using authentic standards isolated from the fruit and other known sources.

Anthocyanins from fruit extract and callus cultures were subjected to acid hydrolysis in 2N HCl:methanol (1:1) for 30 min as described by Markham (1982). Anthocyanidins derived from acid hydrolysis were analysed using a Whatman Partisil ODS-3 (250 × 4.6 mm, 5μ) column connected to a YMC-Pack S5 120A ODS-AM guard column. Solvents were 10% formic acid (A) and 100% acetonitrile (B). Separation was obtained by an isocratic elution of 20% B in A at 1 mL/min.

HPLC analysis of other flavonoids

The ethyl acetate-extractable fraction was washed with water, passed through anhydrous sodium sulfate to remove residual moisture and evap-

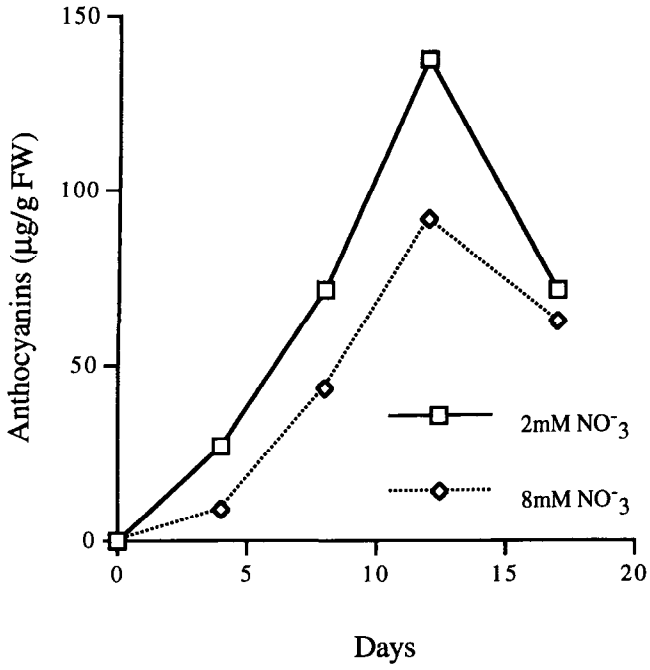

Fig. 1—Time course of anthocyanin formation in stem callus.

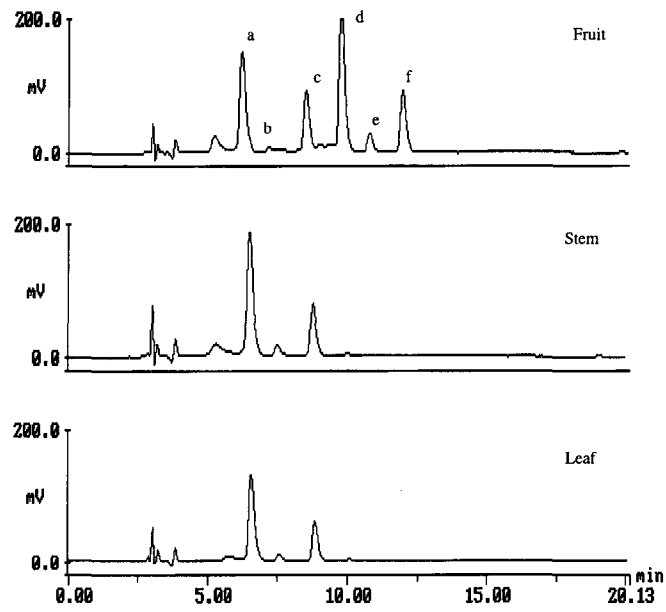

Fig. 2—Reverse phase HPLC profile of anthocyanins in cranberry fruit and vegetative tissues. Peak identification: a. Cyanidin 3-galactoside, b. Cyanidin 3-glucoside, c. Cyanidin 3-arabinoside, d. Peonidin 3-galactoside, e. Peonidin 3-glucoside, f. Peonidin 3-arabinoside

orated to dryness at 30°C *in vacuo*. The residue was dissolved in methanol. Conditions for HPLC analysis were similar to anthocyanin analysis with an altered elution profile: 0–4 min, 10–14% B in A; 4–10 min, 14–18% B in A; 10–20 min, 18–30% B in A; 20–30 min, 30–50% B in A; 30–35 min, 50–100% B in A. Absorbance was monitored at 280 nm.

Proanthocyanidins

Proanthocyanidins were estimated as anthocyanidins by the method of Nawa et al. (1993). Samples were repeatedly extracted in cold (4°C) 1% HCl methanol to remove all pigments. The residue after pigment extraction was suspended in 1% HCl methanol (1:10 w/v), incubated overnight at 50°C then filtered. Absorbance was measured at 535 nm and expressed as absorbance units (A.U.)/g fresh weight (FW). Results are averages of two replicates.

Phenylalanine ammonia-lyase (PAL) activity

Samples were ground in liquid N_2 to a fine powder and suspended in −20°C acetone for 15 min. The precipitate was collected by filtration, washed with acetone and dried at room temperature (25°C). Acetone powders were extracted with 0.1M borate buffer, pH 8.8, containing 20mM 2-mercaptoethanol, 0.5g dry Polyclar AT (ISP Technologies, Inc., Wayne, NJ), and 0.5g dry Amberlite XAD-4 (Sigma Chemical Co., St. Louis, MO). The extract was pressed through cheesecloth and centrifuged cold (4°C) at 10,000 rpm for 20 min. The supernatant was dialyzed overnight (4°C) in 0.1M borate buffer, pH 8.8, containing 20mM 2-mercaptoethanol. The dialyzate was centrifuged cold (4°C) at 10,000 rpm for 10 min and used for assay.

Enzyme activity was measured by the method of Havir and Hanson (1971). The assay mixture contained 0.5 mL of 0.2M borate buffer, pH 8.8, 0.2 mL enzyme solution, and water to a final volume of 2.8 mL. Control assays contained buffer or water in place of enzyme solution or L-phenylalanine. The mixture was incubated at 30°C for 30 min. The reaction was initiated by addition of 0.2 mL of 0.1M L-phenylalanine. The absorbance of the reaction mixtures and blanks was recorded at 280 nm at 15 min intervals using a Beckman DU-65 spectrophotometer (Fullerton, CA) with a temperature controlled auto-6 sampler, at 30°C. One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 µmole cinnamate/min at 30°C. Results are averages of three replicates. Protein was estimated by Bradford's method (Bradford, 1976).

RESULTS

Callus cultures and anthocyanin formation

A yellow colored callus was obtained in the dark in callus induction medium. Modification of Gamborg's medium was necessary to enhance growth and reduce callus culture browning. An increase in NH_4^+ level and a decrease in NO_3^- level with a 3:1 NH_4^+ and NO_3^- ratio was essential to reduce browning and induce friability of the cultures. Addition of coconut water enhanced callus growth. On transferring cultures to light in the production medium, intense pigmentation was observed. Maximum anthocyanin formation was observed by day 12 (Fig. 1) after transferring cultures to light. The level of NO_3^- in the production medium had an effect on anthocyanin concentration. At the 2 mM level, a higher anthocyanin accumulation was observed on all days.

Anthocyanins and anthocyanidins

Reverse phase HPLC chromatograms of the anthocyanins *in vivo* from fruits and from stem and leaves (Fig. 2) showed fruit extract had four major pigments comprised of cyanidin and peonidin 3-galactosides and 3-arabinosides. Cyanidin and peonidin 3-glucosides were the minor pigments. Stems and leaf tissues from the greenhouse and shoot cultures had only cyanidin 3-galactoside, 3-glucoside, and 3-arabinoside, with the glucoside as the minor fraction. The anthocyanin profiles (Fig. 3) showed simple profiles consisting of mainly cyanidin 3-galactoside, 3-glucoside, and 3-arabinoside in all pigmented callus cultures regardless of source of explant. Anthocyanins were not detected in dark grown callus cultures. On acid hydrolysis of the anthocyanins, cyanidin and peonidin were the aglycones in the fruit extract and cyanidin was the only aglycone in the callus cultures (Fig. 4).

Proanthocyanidins

Incubation of the tissue residues in methanol-HCl at 50°C for 18 hr resulted in the release of intense red pigments from all samples with absorbance maximum at 535 nm. The pigments were not extractable by cold methanol-HCl. The A.U./g FW from anthocyanins and anthocyanidins derived from proantho-

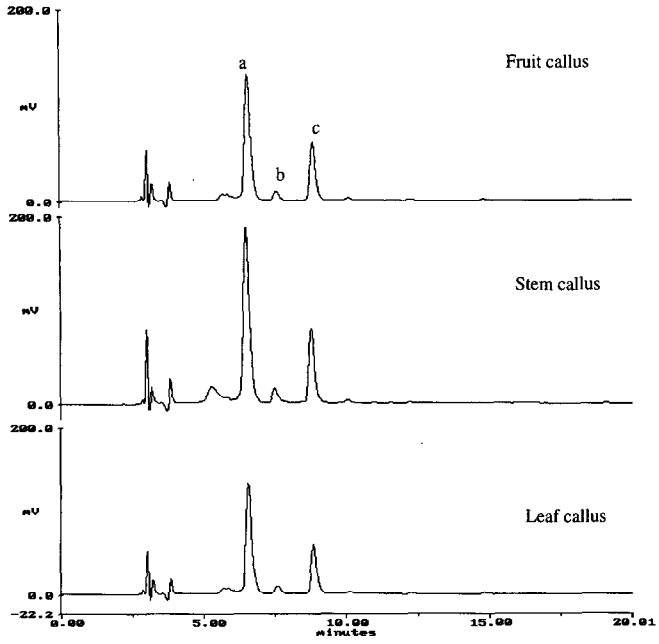

Fig. 3—Reverse phase HPLC profile of anthocyanins in cranberry callus cultures. Peak identification: a. Cyanidin 3-galactoside, b. Cyanidin 3-glucoside, c. Cyanidin 3-arabinoside

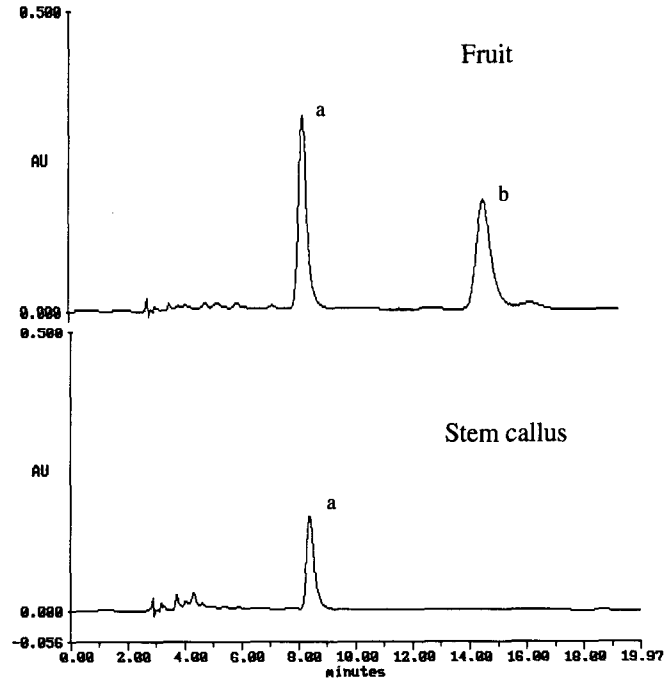

Fig. 4—Reverse phase HPLC profile of anthocyanidins from cranberry fruit (*in vivo*) and stem callus (*in vitro*). Peak identification: a. Cyanidin, b. Peonidin.

cyanidins in vivo and in vitro (Table 1) showed fruit extract had higher absorption due to anthocyanins whereas extracts of vegetative explants had a very high absorption due to proanthocyanidins. Fruit callus culture extracts had a very low level of anthocyanins and a higher proanthocyanidin absorption. Pigmented callus cultures from stems and leaves had a higher anthocyanin level and a lower absorption due to proanthocyanidins. Callus cultures in the dark accumulated proanthocyanidins at comparable levels to cultures under light.

Table 1—Absorbance due to anthocyanins and proanthocyanidins in cranberry *in vivo* and *in vitro*

Source	Anthocyanins (A.U./g FW)	Proanthocyanidins (A.U./g FW)
Fruit (*in vivo*)	29.62	21.46
Stem (from microcultures)	1.04	82.59
Leaf (from microcultures)	0.61	85.31
Stem Callus (colored)	2.52	27.39
Stem Callus (colorless)	0.112	23.5
Leaf Callus (colored)	3.31	49.2
Fruit Callus (colored)	2.19	57.31
Fruit Callus (colorless)	0.254	59.5

Table 2—Percentage of total peak area of compounds in ethyl acetate-extractable fraction from cranberry *in vivo* and *in vitro*

Source	Absorbance 270-290 nm[a] 280-320 nm[b]	Absorbance 280, 350-380 nm[c]
Fruit (*in vivo*)	33.7	66.2
Stem (from microcultures)	68.1	31.9
Leaf (from microcultures)	70.1	29.9
Stem Callus (colored)	79.8	20.2
Stem Callus (colorless)	100	—
Leaf Callus (colored)	73.0	27
Fruit Callus (colored)	93.0	7.0
Fruit Callus (colorless)	100	—

[a] Compounds with absorption spectra of catechins, flavanones
[b] Compounds with absorption spectra of cinnamic acids
[c] Compounds with absorption spectra of flavonols and their glycosides

Other flavonoids

Peaks from reverse phase HPLC chromatograms of the ethyl acetate-extractable fractions were classified tentatively into two major groups of compounds based on spectral properties. Group 1 was comprised of compounds absorbing mainly at 270–290 nm, indicative of catechins, flavanones, and those absorbing at 280–330 nm, indicative of cinnamic acids. Group 2 was comprised of compounds absorbing at 280, 350–375 nm, indicative of flavonols and their glycosides (Table 2). Fruit extract had a higher percentage of group 2 compounds whereas vegetative explants had a higher percentage of group 1 compounds. Pigmented callus cultures from vegetative explants were similar to explants. However, pigmented fruit callus culture had a much lower percentage of group 2 compounds in contrast to the fruit. Dark-grown callus cultures (colorless) had mainly group 1 compounds, and flavonols and their derivatives were not detected (Fig. 5).

Phenylalanine ammonia-lyase activity

Sample enzyme activity (Table 3) showed dark-grown callus cultures had a lower activity. A threefold increase in activity was observed on exposure to light. However, all cultures had lower levels of activity compared to explants.

DISCUSSION

OUR MAIN OBJECTIVE was to establish and compare cell cultures from different parts of cranberry plants for their potential to accumulate anthocyanins and other flavonoids and to determine the influence of explant source on composition of in vitro products. Anthocyanins in cranberry fruits are well characterized and our results confirmed previous reports (Hong and Wrolstad, 1990; Fuleki and Francis, 1967). Callus cultures resembled other anthocyanin producing systems in many properties. Callus cultures accumulated anthocyanins only on exposure to light. Anthocyanin level was lower as in other cell culture systems like rabbiteye blueberry (Nawa et al., 1993). Higher sucrose levels and lower nitrate levels enhanced anthocyanin accumulation in parallel with other cell culture systems like grapes (Do and Cormier, 1991; Hirasuna et al., 1991). Previous studies in our lab-

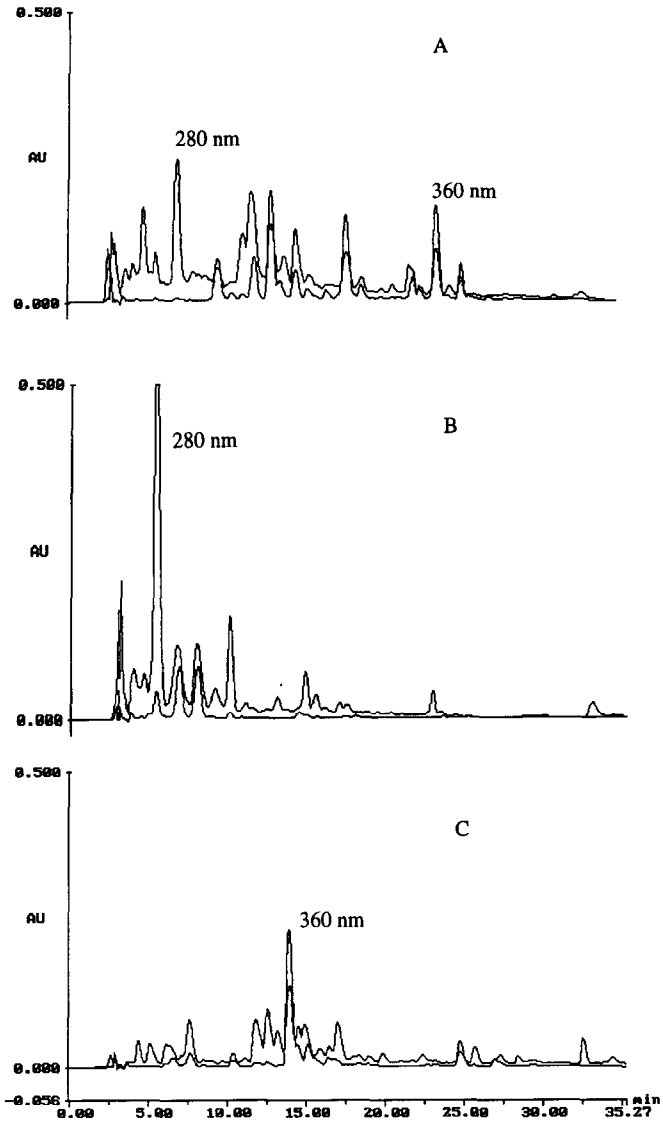

Fig. 5—Reverse phase HPLC profile of the ethyl acetate-extractable fraction. A. Stem, B. Stem Callus (colorless), C. Stem Callus (colored).

Table 3—Phenylalanine ammonia-lyase activity from cranberry *in vivo* and *in vitro*

Source	Units × 10^{-3}/g FW	Units × 10^{-3}/mg protein
Stem (from microcultures)	58.21	18.0
Leaf (from microcultures)	42.98	5.4
Stem Callus (colored)	4.69	4.27
Stem Callus (colorless)	0.813	1.56
Leaf Callus (colored)	4.60	3.96
Leaf Callus (colorless)	1.89 1.39	

oratory have indicated that higher sucrose levels were necessary to induce anthocyanin production in cranberry cultures (Madhavi et al., 1993). Total anthocyanin levels increased from 0–3.0% on increasing sucrose levels from 2–5% in the production medium. Anthocyanin profiles were simpler, similar to other *in vitro* systems like rabbiteye blueberry (Nawa et al., 1993) and grapes (Yamakawa et al., 1983). The decline in anthocyanin concentration after 12 days could be mainly due to depletion of nutrients in the culture medium and has been reported in rabbiteye blueberry cell cultures (Nawa et al., 1993). It was interesting to note that fruit callus had low levels of anthocyanins and a similar anthocyanin profile to callus cultures derived from vegetative explants, indicating that fruit callus was physiologically distinct from differentiated fruit tissue.

Qualitative and quantitative differences in anthocyanin accumulation in fruits *in vivo* and callus cultures *in vitro* could be due to the physiological nature of explant tissues used for callus induction. In cranberry fruits, anthocyanins are localized in the epidermis and the fruit callus culture was derived from mesocarp tissue, devoid of anthocyanins. Vegetative tissues both in the greenhouse and microculture had traces of anthocyanins and qualitatively only cyanidin glycosides. Peonidins differ from cyanidins by a single methyl group substitution at the 3' position in the B ring and are generally considered to be derived from cyanidins by a methylation reaction. Methylation reactions in the flavonoid biosynthetic pathway are catalysed by specific *O*-methyltransferases with S-adenosyl-L-methionine as the methyl donor (Poulton, 1981). *O*-methyltransferases specific for glucosylated and acylated anthocyanidins have been demonstrated in *Petunia hybrida* (Jonsson et al., 1982).

No information is available on properties of methyltransferases in cranberries. Our results, however, indicated that the callus cultures and vegetative tissues may lack methyltransferases specific for anthocyanins or the methyl donor. In general, the metabolic constraints physiological regulations operative in the callus cultures seemed to be similar to the source explants. Our results also indicated that some constraints could be overcome by manipulation of cultural conditions. Anthocyanin accumulation was lower *in vitro* as compared to the fruit extract but was clearly higher than explant tissues (Table 1). Establishment of suspension cultures and use of selection protocols may help in developing intensely pigmented cell lines as with *Vitis hybrida* (Yamakawa et al., 1983), *Ajuga reptans* (Callebaut et al., 1988), and *Daucus carota* (Kinnersley and Dougall, 1980).

The ethyl acetate-extractable fraction was analysed mainly to get an indication of the presence of flavonoids other than anthocyanins. The chromatograms were monitored at 280 nm since all compounds absorbed at that wavelength and the area percentage was also expressed in the same wavelength (Table 2). This slightly underestimates flavonols and their derivatives which absorb more strongly at 350–370 nm. However, this method of representation based on spectral properties gave a good indication of differences *in vivo* and *in vitro*. Cranberry fruits are a rich source of flavonols (Bilyk and Sapers, 1986) and our results indicated a higher percentage of flavonols. Callus cultures from vegetative explants were similar to the explants and had a higher percentage of cinnamic acids and catechins. Fruit callus differed from fruit in that it had a lower percentage of flavonols. Callus cultures accumulated flavonols only on exposure to light (Fig. 5).

Proanthocyanidins are condensation products of monomeric leucoanthocyanidins and catechins and include dimers, oligomers, and polymers. Those with molecular weights >7000 become insoluble in organic and aqueous solvents (Czochanska et al., 1979; Haslam, 1980). Proanthocyanidins extractable in cold organic solvents have been reported in cranberry fruits (Wang et al., 1978; Foo and Porter, 1981). We have demonstrated the presence of insoluble proanthocyanidins both *in vivo* and *in vitro* indirectly by hydrolysis in HCl methanol. *In vivo,* fruits seemed to have a low level of insoluble proanthocyanidins which could be mainly related to the degree of fruit ripening (Haslam, 1977). *In vitro*, callus cultures from vegetative explants showed lower accumulation of insoluble proanthocyanidins as compared to explants. Fruit callus again differed from fruit and had a higher level of insoluble proanthocyanidins and was similar to cultures from vegetative explants. Light was not a limiting factor for proanthocyanidin accumulation in our cultures (Table 1). Light independent accumulation of high molecular weight proanthocyanidins has been reported in other cell culture systems like *Cryptomeria japonica* (Ishikura and Teramoto, 1983), *Fagopyrum esculentum* (Moumou et al., 1992) and *Vaccinium ashei* (Nawa et al., 1993). Cultural conditions also reportedly influence

the accumulation of proanthocyanidins in cell cultures (Zaprometov, 1988). Proanthocyanidins and anthocyanins share common intermediates in the biosynthetic pathway. Hence, inhibition of proanthocyanidin synthesis by manipulating cultural conditions may enhance anthocyanin accumulation.

Phenylalanine ammonia-lyase catalyzes the formation of *trans*-cinnamic acid, the first phenylpropanoid, by the deamination of L-phenylalanine and provides the link between primary metabolism and the phenylpropanoid pathways (Ebel and Hahlbrock, 1982). The enzyme may also have a regulatory effect in anthocyanin accumulation (Hahlbrock, 1981). Sapers et al. (1987) demonstrated PAL activity in the skin of cranberry fruits and reported that the activity did not correlate with anthocyanin accumulation. Our studies indicated that the observation could be extended to vegetative parts of the plant. In vitro, dark grown cultures had a basal level of activity which was enhanced by illumination (Table 3). Exposure to light induced the formation of anthocyanins and flavonols. PAL and other enzymes in the flavonoid biosynthetic pathway are highly light inducible (McClure, 1975) and our results confirmed this. However, cultures also accumulated proanthocyanidins in the absence of light, which implied the synthesis of dihydroflavonols and leucoanthocyanidins, common intermediates for anthocyanins, flavonols, and proanthocyanidins in the biosynthetic pathway. This indicated that the increase in PAL activity could be responsible for an enhanced precursor pool in the biosynthetic pathway.

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