

# Identification of the Major Anthocyanin of Carrot Cells in Tissue Culture as Cyanidin 3-(Sinapoylxylosylglucosylgalactoside)

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In contrast to earlier reports, the major anthocyanin of carrot cells grown in tissue culture has been identified as cyanidin 3-(sinapoylxylosylglucosylgalactoside). It is thus the same as one of the major acylated pigments in the leaf and flower of the whole plant. *Daucus carota* is therefore not anomalous among higher plants in its anthocyanin-synthesising capacity in callus or shake culture.

## Introduction

One of the very first observations of anthocyanin production in tissue culture of higher plant cells was that of Steward [1] who reported a deep red colour in callus tissues of the cultivated carrot, *Daucus carota*. Since that time, the carrot has been extensively used for examining the physiology of anthocyanin production in cultured cells [e.g. 2, 3], but remarkably the anthocyanin formed was not fully characterised. The pigment was variously reported to be a cyanidin diglycoside [3] or a cyanidin xyloglucoside [2]. Very recently, Hemingson and Collins [4] reported four pigments in tissue culture: the 3-glucosylgalactoside, 3,5-digalactoside, 3-galactoside and 3-glucoside of cyanidin. None of these four pigments however occurs in the intact carrot plant [5].

The above result was unexpected, in view of the fact that the capacity for anthocyanin synthesis in cultured plant cells usually reflects that of the intact tissue from which the callus was originally derived [6]. Thus, when proper comparison has been made, as in *Dimorphotheca* [7], in *Solanum tuberosum* [8], and in *Petunia hybrida* [9], the same pigments have

been found in culture as in the whole plant. It therefore seemed necessary to re-examine the anthocyanin profile of carrot tissue culture in order to see if it was anomalous or not.

## Results

Two-dimensional TLC of the direct pigment extract of carrot callus cells, selected for pigment production, showed the presence of a single major anthocyanin, with trace amounts of three others. This major pigment was purified by paper chromatography. In its absorption spectrum, it showed all the characteristics of an acylated anthocyanin with an intense peak at 333 nm (of 59% the intensity of the visible max.) [10] and it also exhibited the unusual spectral shift in the visible peak (max at 535 instead of at 525 nm) previously noted for the acylated cyanidin glycosides of carrot [5]. On deacylation with mild acid, it gave sinapic acid, while complete acid hydrolysis gave cyanidin and the three sugars xylose, galactose and glucose in equal amounts. It was thus identical in all its properties with cyanidin 3-(sinapoylxylosylglucosylgalactoside) previously reported in flower and leaf of the cultivated carrot and also in leaf and stem of several other related Umbelliferae [5]. This was confirmed by co-chromatography in 5 solvents and co-electrophoresis with the pigment isolated from the whole plant. The three trace pigments co-occurring with the acylated triglycoside are almost certainly breakdown products, since deacylation and loss of one or more sugar groups are likely to occur during concentration and handling of pigment extracts [10].

The erroneous results recorded earlier by Hemingson and Collins [4] are probably due to their failure to realise that hydrolysis occurred during concentration of their extracts. Indeed, these workers eluted their pigments from paper with aqueous acetic acid (instead of the recommended [10] methanol-acetic acid-water) and therefore had to heat their solutions to 57°C in order to remove the solvent in vacuo. Two of the pigments they reported, cyanidin 3-glucosylgalactoside and cyanidin 3-galactoside, are in fact breakdown products of the acylated triglycoside. The other two pigments, the 3,5-digalactoside and 3-glucoside, were probably erroneously identified because of the presence of non-cyanic impurities. Their samples were obviously

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contaminated by other UV absorbing constituents, since the  $E_{440}/E_{\max}$  ratios recorded [4] do not correspond to those of pure pigments [10].

This reanalysis shows that *Daucus carota* is not, in fact, unusual in respect of pigment synthesis in callus culture. The major pigment produced is one of the characteristic anthocyanins found in the whole plant. The acylated triglycoside undoubtedly corresponds to the cyanidin diglycoside of earlier papers on tissue culture [2, 3]. Some qualitative variation in pigment production is obviously possible in suspension culture, but this seems unlikely in that secondary product synthesis in cell culture is normally very constant and in the fact that most workers studying carrot cells have used related cell lines.

## Experimental

Carrot tissue cultures. The suspension cultures used originated from callus cultures prepared from carrot roots by Dr. J. Reinert, Free University of Berlin, and which have been used by other German workers, e.g. Alfermann and Reinhard [11] in tissue culture work. The cells were cultured in shake culture, in suspension, using the media 5B of Gamborg *et al.*

[12] with the addition of 0.2 mg/l. 2,4-dichlorophenoxyacetic acid (2,4-D). This hormone addition was needed for pigment formation, but contrary to the claim of Gregor and Reinert for other species [13], the cells grew and produced pigment equally well in light and dark.

The cells in the suspension were heterogenous for pigmentation. They were plated out to separate coloured from colourless cells. The red cells were re-cultured as callus and the callus used as the source for the suspension culture. These cells were extracted, after centrifugation, with 1% HCl in MeOH and the resulting extract freeze dried. Reversion from coloured to white cells and vice versa occurred, as also reported by Dougall *et al.* [14].

Pigment Identifications. The general procedures described earlier were used [5, 10]. Additionally, pigments were eluted from chromatograms, which had not been completely dried, in order to avoid pigment loss. Pigment solutions were carried through purification as rapidly as possible, and also concentrations were carried out on methanolic solutions at 30–35° in vacuo. Authentic cyanidin 3-(sinapoyltriglycoside) was obtained from the pigmented leaves of cultivated carrot plants, as described earlier [5].

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