Production of secondary metabolite (anthocyanin) from callus culture of Daucus Carota.

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Abstract
Anthocyanins are naturally occurring polyphenols that impart bright color to fruits, vegetables and plants. The bright red anthocyanin pigmentation was stimulated in callus cultures of Daucus carota. Anthocyanin production was affected strongly by auxins. Callus establishment was achieved by culturing seed and taproot explants on MS medium supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), indole acetic acid (IAA) with kinetin (KIN).The highest callus growth were recorded on MS medium contained 3.0mg/L 2,4-D with 0.1mg/L KIN and 3.0mg/l IAA with 0.2mg/l KIN for both explants. Seed callus produced higher anthocyanin than taproot callus. Anthocyanin appeared as light brown on MS medium. Extraction solvent used was 70% methanol containing 1% (v/v) hydrochloric acid and UV Spectrophometer at 535 nm, identified the presence of anthocyanin. Further, the results of high-performance liquid chromatography (HPLC) analysis of the anthocyanins extracted from the callus culture of Daucus carota revealed the presence of anthocyanin components, which were tentatively identified as anthocyanidin (Delphinidin).

Keywords- D.Carota, anthocyanin, Callus, flavonoids, cell suspension, HPLC.

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Introduction

Plants have been the source of many compounds like primary and secondary metabolite that are useful to food and medicine industry. Over 85 % of the approximately 30,000 known natural products are of plant origin [1]. After chlorophyll, anthocyanins are the most ubiquitous pigments seen in nature. Widely distributed in the pericarps of several fruits, flowers, and vegetables, these are glycosylated polyhydroxy and polymethoxy derivatives of flavylium (2- phenylbenzopyrylium) salts and belong to a group of compounds generically called flavonoids. Structurally, they are made up of two or three portions, the aglycone base, a group of sugars, and a group of acyl acids. The aglycone moiety is

referred to as anthocyanindin. There are more than 15 anthocyanidins, [3] of which only 6 are common, pelargonidin, cyanidin, delphinidin, peonidin, petuinidin, malvidin. The biosynthesis of anthocyanins follows the general phenylpropanoid pathway [2]. While the anthocyanins are thought to have a limited physiological role in pollination and seed dispersal, they are commercially very important pigments. They are used for coloring a variety of food products including beverages. Anthocyanins are known to have several pharmacological attributes such as anti-inflammatory, anti-ulcer and wound healing properties [13]. The commercial source of these pigments is the grape peel. In view of the quoted price of US $ 1200 - 1500 kg-1, and the estimated market of US $ 135 million [4], plant cell culture as an alternative source of supply of these pigments has been investigated all over the world. Combination of high 2, 4-D with low Kinetin in B5 medium also increased anthocyanin pigmentation without affecting cell growth in *Aralia cordata* cultures [10].

Anthocyanins are glycosides of anthocyanidins and may have different sugars bonded to their ringed structure. They are classified as mono, di or triglycosides, and the diglycoside and triglycoside forms are more stable than the monoglycoside forms. They display color variations according to their structural forms, pH value, number of hydroxyls and methoxyls and temperature. pH level usually used in developmental stage in plant tissue culture in-vitro, is generally between 4 and 6 [12].

Flavonoids are able to absorb light over a wide range of the light spectrum. Their absorbance shifts towards longer wavelengths as the conjugation of the 3 planar ring structures increases and saturation decreases. The most highly modified forms are the anthocyanins, which have maximum absorbance across the visible spectrum (500 - 550 nm). Further maximum modification by the effect of pH and interactions with metal ions and co-pigments brings forth visual cues that undoubtedly promote the primary functions of flavonoids in flowers, seeds and fruits being the recruitment of pollinators and seed dispersers [11]. Plant coloration is also of great aesthetic value to humans and is therefore the encouragement for using conventional breeding, as well as biotechnology to create novel colors in flowers.

The present investigation dealt with the studies on production of anthocyanin from Daucus carota cell cultures from callus culture of carrot followed by identification of anthocyanin by High performance liquid chromatography (HPLC).

Materials and Methods

Seeds of Daucus carota (carrot) and tap root were purchase from local market at Janakpuri, New Delhi, India and Standard compounds, Hormone (IAA, 2, 4-D, sodium hypochlorite, mercuric chloride and all constituents of MS media were purchased from (Merck India). All other chemicals and solvents used were analytical grade (AR), unless mentioned otherwise.

The nutrient medium used for the present study was MS media [7]. Stock solution of macronutrient, micronutrient, iron, vitamins and hormones were prepared in double distilled water and stored in a refrigerator at 4ºC. Sucrose was used as carbohydrate source. The pH of the medium was adjusted 5.8 using 1 N NaOH/ HCl. Agar (10 %), used as a gelling agent, was dissolved in the medium by boiling. The medium was then dispended in to pre-sterilized conical flask. The medium was autoclaved at 121ºC, 15 lbs pressure for 20 min, the medium was transferred in to cultures jars, test tubes and Petri plates dishes under a laminar air flow and kept for cooling and stored in a sterile condition, till inoculated.

Initiation of callus cultures from seedling plant.

Selected seeds were primarily surface sterilized with mercuric chloride solution (0.1%, w/v) for four min and washed several times with sterile distilled water to remove the traces of mercuric chloride. Seeds were then aseptically transferred in to the MS media and allowed to germinate in dark condition for 8-10 days. The 10 days old seedlings were then transferred aseptically onto semi solid nutrient medium containing salts supplemented with sucrose (3%, w/v), myo-inositol (100 mg/L), 2,4-Dichlorophenoxyacetic acid (2,4D) and IAA (indole acetic acid) (1.0mg/L, 2.0mg/L, 3.0mg/L, 4.0mg/L) and kinetin (0.1mg/L, 0.2mg/L, 0.3mg/L, 0.4mg/L), in case of D. carota, solidified with agar (1%, w/w). The pH of the medium was adjusted to 5.8 before autoclaving at 1.3 kg cm-2 for 25 min. The cultures were incubated at 24 ± 2 °C under 10 hr. photoperiod light at 3000 Lux using 40 W tubular fluorescent lamps (Philips India Ltd., Calcutta) as the source of light. Callus which appeared after 2 week of transferring to the above medium was allowed to grow for 6 weeks.

Initiation and maintenance of cell suspension cultures

Callus tissues (approx.1g) were transferred to 10 mL of liquid medium of the same composition described the above, but without the gelling agent in 50 mL Erlenmeyer flasks. They were incubated on a rotary shaker (New Brunswick Scientific Co., Inc., New Jersey) at 90 rpm under 10 hr. photoperiod light (3000 lux). The cell suspensions were filtered through appropriate sieves to obtain single cells and few celled aggregates which were used as inoculum for subculture and some sample took for the check anthocyanin pigmentation spectrophotometrically in supernatant after centrifugation.

Estimation of anthocyanin

Anthocyanin was extracted from the cells by using acidified methanol (HC1 1 %, v/v) and estimated by UV-visible spectrophotometrically (Cary 50 Bio, Varian) at 535 nm and followed by Qualitative analysis of anthocyanins by HPLC. The HPLC (Shimadzu, Japan) system consisted of UV–Vis photodiode array detector. The chromatographic separations were achieved on a ODS Hypersil (Thermo-hypersil- Keystone, USA) C18 reverse-phase column (150 mm x 4.6 mm, 5 microns ID).The mobile phase consisted of methanol, acetic acid and water (70:10:20, v/v/v). Flow rate was maintained at 1 mL/min and absorbance was measured at 530 nm. Anthocyanin in the samples was identified by comparing their relative retention times with that of authentic standard.

RESULT AND DISCUSSION

Seeds of Daucus carota germinated within ten days upon implanting on MS medium. The excised seedlings gave rise to callus upon transferring into MS medium supplemented with 2, 4 D and IAA along with kinetin. Maximum calli growth was observed in 2, 4 D kinetin at 3 mg/l and 0.2 mg/l and IAA-kinetin combinations showed less callus growth compared to 2, 4 D – kinetin. Callus of D. carota was friable and light brownish in color with zones of pigmented cells. Suspensions of cells were obtained by transferring the calli of D. carota into the liquid medium of the same composition as the semi-solid medium except the gelling agent, agar. Fine cell suspension cultures were established by repeated selection and sub culturing of small cell aggregates.

Auxins used 2,4 D, IAA and cytokinin used kinetin, were studied to determine the optimum level of hormones for the maximum production of anthocyanin. IAA at 3.0 mg/L and kinetin at 0.2 mg/L was found to be the optimum hormonal supplementation required for the maximum anthocyanin production. The cell suspension cultures were maintained for 10 days and then used for qualitative analysis for pigments.

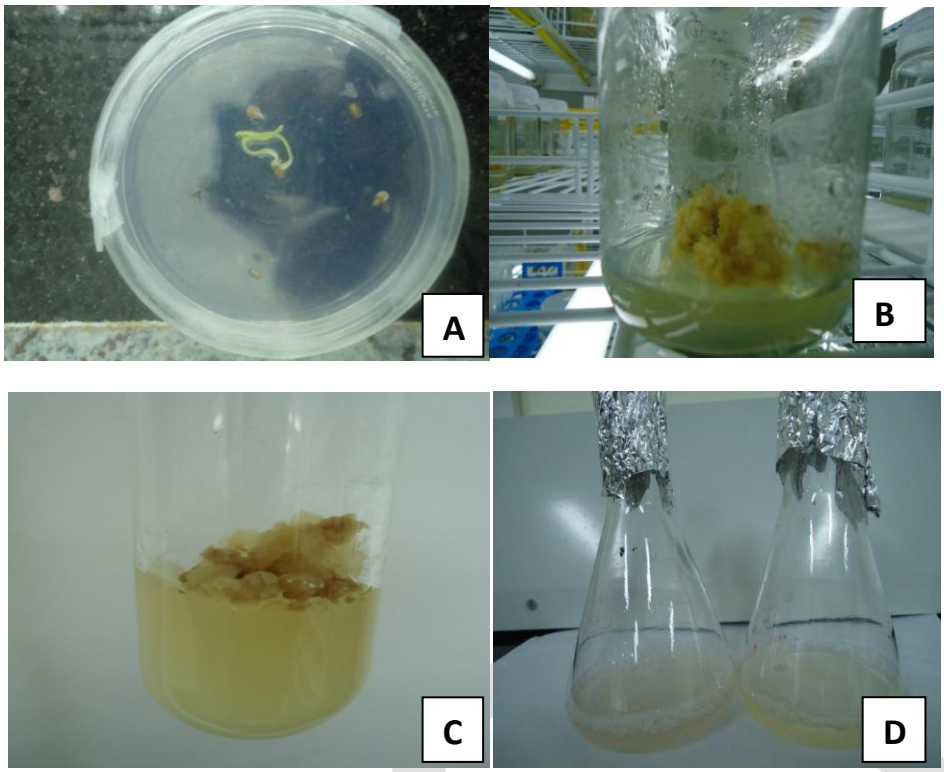

Fig1. Explant's Seedling(A), Callus of D. carota with combination of 2,4 D and Kin(B), Callus of D. carota with combination of IAA and Kin(C), Cell suspension culture of IAA + Kin (l) and 2,4 D + Kin (r)(D).

Anthocyanins were extracted from the suspension cells by using acidified methanol (HC1, 1 %, v/v) and estimated by UV-visible spectrophotometrically (Cary 50 Bio, Varian) at 535 nm, using double distilled water as a blank. Maximum absorbance was absorbed for calli grown in IAA (3.0 mg/mL) and kinetin (0.2 mg/mL) (Table.1, Fig.1C) and less absorbance was occur for calli grown in 2, 4D and kinetin combination (Table.2, Fig.1B).

Table 1: Different combination of (IAA + Kin) and abs. at 535 nm

S.No.	IAA(mg/L)	Kinetin (mg/L)	Absorbance (535nm)
1	2	0.2	0.215
2	3	0.1	0.884
3	2	0.1	0.765
4	3	0.2	1.234
5	4	0.1	0.896
6	3	0.3	0.365
7	4	0.3	0.388

Table 2: Different combination of (2, 4 D + Kin) and abs. at 535 nm

S.No.	2,4 D (mg/L)	Kinetin (mg/L)	Absorbance (535nm)
1	2	0.2	0.215
2	2	0.1	0.765
3	3	0.1	0.875
4	4	0.2	0.277
5	4	0.1	0.766
6	3	0.2	0.356
7	4	0.3	0.233

Determination of anthocyanidin.

The acid treatment of samples resulted in the hydrolytic separation of anthcyanidins from the sugar and acyl moieties. Identification of the anthocyanidin moiety was done based on the retention time (RT in HPLC with methanol-acetic acid-water (70:10:20) as the mobile phase. The elution profile of internal standard anthocyanidins reflected their polarity i.e. anthocyanidins with more hydroxyl was delphinidin, cyanidin and peonidin, Based on the retention time, gave a single peak at a retention time in the range of 3.5 - 4.0 min indicating that the delphinidin was the only anthocyanidin present in the high yielding cell line of D. carota. (Fig.2) shows the HPLC profile of D. carota cell extract.

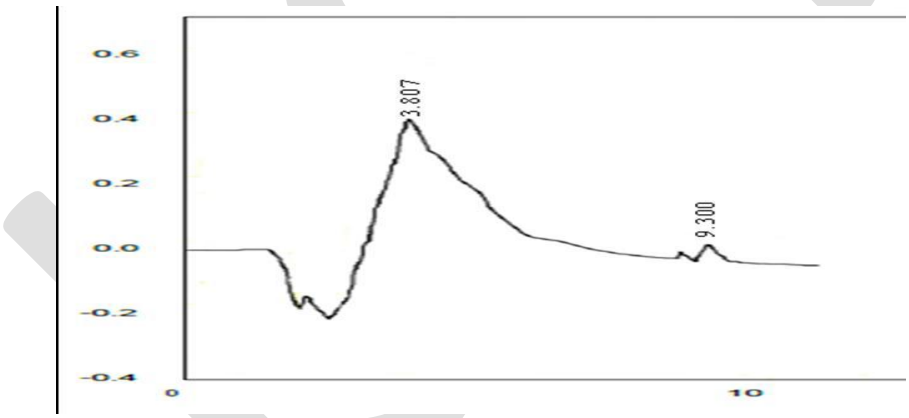

Fig 2: HPLC profile of Daucus carota.

These results suggest that sample present with the delphinidin-3-glucoside. The HPLC profile showed that the delphinidin was the major anthocyanin in D. carota cells.

The present study found that, from the balance of growth and anthocyanin content, the combination of IAA and Kinetin (3.0 mg/L, and 0.2 mg/L, respectively) was optimum for the maximum production of anthocyanin from D. carota cells. The highest callus fresh weight and callus moisture content were obtained in solid medium supplemented with 2,4-D type of growth regulator (S.J**.** [6] was more effective than medium type on growth of carrot callus, [8]**.** Cytokinins, singly, never influence the production of anthocyanin, its need to combination of IAA and kinetin resulted in the maximum production of anthocyanin, [5]. In medium supplemented with 2, 4-D, higher amounts of anthocyanin obtained. Anthocyanins are naturally occurring polyphenols that impart bright color to

fruits, vegetables and plants. The bright red anthocyanin pigmentation was stimulated in a callus culture of Daucus carota. Anthocyanin production was affected by auxins concentration Callus establishment was achieved by culturing seed and taproot explants on MS medium supplemented with different combinations of 2,4-D, IAA and kinetin. The highest callus growth was recorded on MS medium with 3.0mg/L 2,4-D with 0.1mg/L KIN and 3.0mg/l IAA with 0.2mg/L KIN for both explants. Seed callus produced higher anthocyanin than taproot callus. Anthocyanin appeared as light brown on MS medium. Pigments were extracted in acidified Methanol (70% with 1% HCl) and their absorbance was recorded at 535 nm using UV- Visible spectrophotometer. The results showed the presence of anthocyanin compound. This was further confirmed by HPLC analysis. In addition, [9] reported that salicylic acid like 2, 4-D also increased the anthocyanin pigment in vitro plants significantly.

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