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CELL CULTURES OF *CENTAUREA CYANUS* PRODUCE MALONATED ANTHOCYANIN IN UV LIGHT

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Key Word Index—*Centaurea cyanus*; Compositae; suspension culture; induction; UV light; malonated anthocyanin.

Abstract—Callus cultures which produce anthocyanin under continuous irradiation of UV and white light were derived from the stem of blue-flowered *Centaurea cyanus*. From the callus a suspension culture, in which anthocyanin synthesis can be induced by UV light, was obtained. The pigment in the cell cultures was identified as cyanidin 3-(6″-malonylglucoside) which occurs in the leaf, but not the flowers, of the parent plant.

INTRODUCTION

Cell cultures which produce anthocyanin pigment have been isolated from *Haplopappus gracilis* [1, 2], *Daucus carota* [3–5] and *Petunia hybrida* [6], and some of these have been used successfully for studies of anthocyanin biosynthesis [7–11]. The purpose of this study was the establishment of a cell culture in which synchronous anthocyanin synthesis occurred under defined conditions and we here report isolation of cell cultures from *Centaurea cyanus* which produce malonated anthocyanin in UV light. The occurrence of malonated anthocyanins in various plant species has been reported recently[12–16].

RESULTS AND DISCUSSION

Cell cultures producing anthocyanin

The callus culture was isolated from the stem of *C. cyanus* which has blue flowers. The callus obtained did not produce anthocyanin in the dark. However, when the cultures were grown under continuous irradiation of UV and white light, anthocyanin synthesis was observed. After repeating selection of the anthocyanin-producing cells of the callus cultures for one year, an anthocyanin-synthesizing cell line was obtained. The cell cultures were maintained under continuous illumination of UV and white light. From the callus cultures, cell suspension cultures were induced by inoculation of the cells in liquid medium and grown in darkness. As the cells propagated, anthocyanin content was rapidly reduced and cell suspension cultures which lacked anthocyanin were obtained. These were subcultured every seven days.

Figure 1 shows a growth curve of the cell suspension culture in the dark. From day 3 after transfer of seven-day-old subcultures to fresh medium, the cell fresh weight increased steadily and attained a plateau after 10 days. No synthesis of anthocyanin was observed during culture in the dark. However, when the cells were irradiated with UV and white light, anthocyanin synthesis was induced. The degree of stimulation of anthocyanin synthesis by light was correlated with the growth stage of the cultures. In this cell line a maximum of stimulation was at approximately the seventh day of growth. Irradiation at earlier or later days reduced anthocyanin synthesis. When white light was used, only a trace of anthocyanin was synthesized.

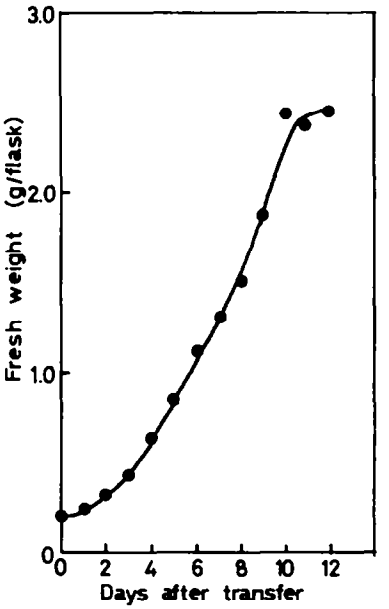

Fig. 1. Changes in the fresh weight of the suspension cultures from *C. cyanus* in the dark. Each value is the average of three measurements.

Figure 2 shows the time course for the accumulation of anthocyanin in a suspension culture after onset of irradiation with UV and white light at the seventh day of growth. Anthocyanin accumulated from 20 hr after illumination and reached a maximum at 72 hr. At the maximum, *ca* 70% of the cells synthesized anthocyanin, indicating a well synchronized cell line.

Identification of anthocyanin

On electrophoresis of the crude pigment extract from the cell cultures, the anthocyanin moved towards the

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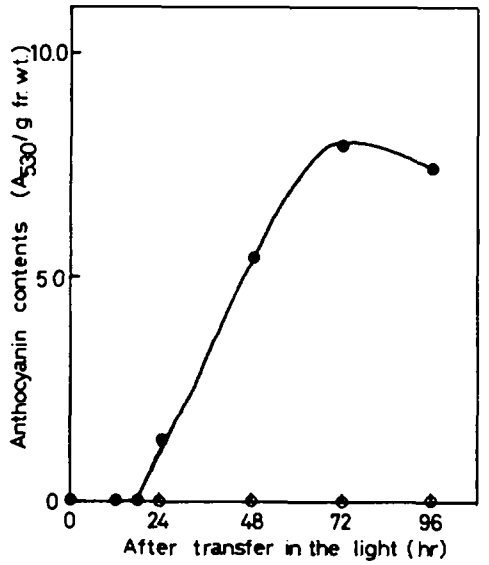

Fig. 2. Time course of anthocyanin synthesis in the suspension cultures from *C. cyanus* after transfer in the light. Each value is the average of three measurements. Light irradiation (●), dark control (○).

anode, suggesting that the pigment is zwitterionic [14]. Accordingly the anthocyanin was isolated by extraction into methanol–acetic acid–water (10:1:9) and solvents containing mineral acid were avoided. The purified pigment showed one spot on electrophoresis and thin-layer chromatography using various solvents. On complete acid hydrolysis, the anthocyanin gave cyanidin and glucose. By alkaline hydrolysis, the pigment yielded cyanidin 3-monoglucoside and malonic acid which were identified by thin-layer chromatography. Additionally, hydrogen peroxide oxidation of the pigment yielded 6-malonylglucose, identified by co-chromatography with an authentic sample prepared by similar oxidation of cyanidin 3-(6″-malonylglucoside) [15]. That the anthocyanin is cyanidin 3-(6″-malonyl)glucoside was confirmed by co-electrophoresis and co-chromatography with an authentic sample, and by absorption spectrophotometry. Moreover FAB-MS of the anthocyanin gave the M_r as 535, with fragments corresponding to cyanidin 3-glucoside (*m/z*) 449 and cyanidin (*m/z*) 287 [13].

The pigment in the cell cultures is thus different from the anthocyanin in the blue flowers of the parent plant, which is cyanidin 3-(6″-succinyl glucoside)-5-glucoside [17, 18]. Though anthocyanin is not generally found in the leaves or the stems of *C. cyanus*, red pigment is synthesized occasionally in the epidermis of these organs in autumn or winter. This was isolated and found to be the same as the pigment from the cell cultures.

EXPERIMENTAL

Plant material and cell cultures. The callus culture was isolated from the stem of *C. cyanus* which had blue flowers. The culture was maintained on Murashige and Skoog medium [19] containing 2,4-D (1 mg/l), kinetin (0.1 mg/l) and 0.9% agar in the dark at 25°. The pH of the medium was adjusted to 5.7. The cells were subcultured every two weeks for more than five years. For the suspension culture, a liquid medium of the same composition but without agar was used. The cells (10 ml of the suspension) were transferred to 50 ml of the fresh medium in 300 ml flasks every seven days and grown on a reciprocal shaker (100 strokes/min) in the dark at 25°.

Illumination conditions. The cultures were illuminated with white light (*ca* 6 W/m^2) from Toshiba fluorescent lamps (FL20SW) and UV light (*ca* 0.5 W/m^2) from Toshiba lamps (FL20SBL, 310–410 nm, λ_{max} 350 nm and FL20SE, 270–410 nm, λ_{max} 310 nm). Since the glass of flask used for the culture absorbed UV light below 280 nm, the cells were irradiated with UV light in the range 280 nm–410 nm.

Isolation of anthocyanin. Cells were harvested by centrifugation and stored at −30°. Pigment was extracted from the frozen cells with MeOH–HOAc–H_2O (10:1:9, MAW) and the filtered extracts were evaporated to dryness *in vacuo* at 30°. The residue, after dissolution in MAW, was passed through a Sephadex LH-20 column (2.5 × 25 cm) in the same solvent. The pigment fraction was evaporated to dryness and further purified by prep. PC using the solvents *n*-BuOH–HOAc–H_2O (4:1:2, BAW) and 30% HOAc.

Anthocyanin identification. Acid hydrolysis, saponification and H_2O_2 degradation were carried out by standard procedures. The products obtained were identified by TLC and PC. The following solvents were used; HOAc–HCl–H_2O (30:3:10), *n*-BuOH–HCl–H_2O (7:2:5, top, BHW) and HCO_2H–HCl–H_2O (5:2:3) for aglycone; *n*-BuOH–pyridine–H_2O (6:3:1) and BAW for sugar; *n*-BuOH–toluene–pyridine–H_2O (5:1:3:3) and *n*-BuOH–EtOH–H_2O (4:1:2.2) for acyl sugar; BAW, EtOAc–HOAc–H_2O (3:1:1) and EtOH–H_2O–NH_4OH (16:3:1) for organic acid. For detection, aniline hydrogen phthalate was used for sugars and xylose–aniline for organic acids. The pure pigment was subjected to absorption spectrometry in 0.1% MeOH–HCl, TLC on microcrystalline cellulose in four solvents. HOAc–HCl–H_2O (15:3:82, AHW), BAW, BHW and 1% HCl, electrophoresis at pH 4.8 on Toyo 51B filter paper, 200 V, 0.4 mA/cm for 2 hr, and FAB-MS. FAB-MS spectrum was obtained by use of a JEOL JMS-HX100 tandem mass spectrometer. Xenon was used as the source of the fast atom beam (6 keV). The mass spectrometer was operated at 5 kV accelerating voltage. R_fs (× 100) of the pigment, cyanidin 3-(6″-malonylglucoside), cyanidin 3-glucoside and cyanidin 3,5-diglucoside were as follows: 24, 24, 17 and 34 in AHW, 49, 49, 39 and 30 in BAW, 40, 40, 26 and 14 in BHW, 5, 5, 4 and 11 in 1% HCl.

Anthocyanin content. Cells were collected by vacuum filtration of 1 ml cell suspension and weighed. Pigment was then thoroughly extracted with 1% MeOH–HCl, the total volume was adjusted to 10 ml and absorbance at 530 nm was measured.

Fresh weight of cultures. For determination of changes in fr. wt of the cultures, 2 ml of a seven-day-old subculture was transferred to 10 ml of the fresh medium in 50 ml flask and grown in the same condition as above. Then the cells were collected by vacuum filtration and weighed.

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