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INDUCTION OF ANTHOCYANIN FORMATION AND OF ENZYMES RELATED TO ITS BIOSYNTHESIS BY UV LIGHT IN CELL CULTURES OF *HAPLOPAPPUS GRACILIS*

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Key Word Index—*Haplopappus gracilis*; Compositae; tissue culture; biosynthesis; flavonoids; anthocyanin.

Abstract—Only UV light below 345 nm stimulates anthocyanin formation in dark grown cell suspension cultures of *Haplopappus gracilis*. A linear relationship between UV dose and flavonoid accumulation, as found previously with parsley cell cultures, was not observed with the *H. gracilis* cells. Only continuous irradiation with high doses of UV was effective. Drastic increases in the activities of the enzymes phenylalanine ammonia-lyase, chalcone isomerase and flavanone synthase were observed under continuous UV light. The increase in enzyme activities paralleled anthocyanin formation.

INTRODUCTION

It has been shown in a number of cases that induction of flavonoid formation by light is accompanied by an increase in the activity of enzymes involved in their biosynthesis [1]. Large increases in the activities of all enzymes involved in the accumulation of flavone and flavonol glycosides were shown to occur upon illumination of previously dark-grown parsley cell cultures [2, 3]. It was later demonstrated that UV with a wavelength below 350 nm triggers flavonoid synthesis in these cultures [4, 5] and that the induction of flavonoids and related enzymes is UV dose-dependent [6].

In callus cultures of *Haplopappus gracilis*, anthocyanin formation can be induced by blue and near UV light and the action spectrum was found to have two peaks, one at 438 nm and the other at 372 nm [7]. Cell suspension cultures of *H. gracilis* have been used successfully for studies of anthocyanin biosynthesis [8, 9]. In order to find reproducible conditions for such experiments we have now investigated the UV-dependent induction of anthocyanin biosynthesis and of related enzymes in these cultures. We also report for the first time the detection of flavanone synthase [10] in the *H. gracilis* cultures.

RESULTS AND DISCUSSION

Detection of flavanone synthase in H. gracilis *cell cultures*

When cell free extracts of *H. gracilis* cells in the anthocyanin production phase were incubated with *p*-coumaryl-CoA and [^{14}C]-malonyl-CoA, a radioactive compound which cochromatographed with naringenin (5,7,4′-trihydroxyflavanone) was present on paper chromatograms run in 45% EtOH. The identity of this compound with naringenin was established by cochromatography in three other solvents [11].

An intensely fluorescing spot near the origin of the chromatogram was also present in some incubations. It corresponded in its R_f to bis-noryangonin (4-hydroxy-6[4-hydroxystyryl] 2-pyrone) which has been identified as a release product of the flavanone synthase from parsley cultures [12].

Effect of UV irradiation on anthocyanin formation and activity of related enzymes

By illumination of the cell cultures with white light in the presence of UV absorbing filters, it could be shown that only UV light below 345 nm can stimulate anthocyanin synthesis under our conditions (Table 1).

A linear relationship between UV dose and flavonoid accumulation, as noted in parsley cell cultures [6], was not observed with the *H. gracilis* cells. Only continuous irradiation with high doses of UV was effective (Table 2). Cells transferred to fresh medium prior to irradiation gave a much larger UV response (Table 2).

The cells were exposed to continuous irradiation with high intensity UV light. Anthocyanin accumulation, which was thereby promoted, as well as changes in the fresh weight of the cells and in the activity of the enzymes

Table 1. Estimation of the spectral range active in stimulation of anthocyanin synthesis by means of UV absorbing filters

Filter used λ_H*	Anthocyanin (10^{-7} mol/g fr. wt of cells)	
	I†	II
420	0.51	0.23
375	0.62	0.38
345	0.71	0.56
Without filter ($\lambda > 290$ nm)	1.05	1.80
Dark control	0.49	0.19

The samples were irradiated with white light (xenon arc) for 48 hr. * Wavelength at which transmittance of filter is 50%. † Two independent expts.

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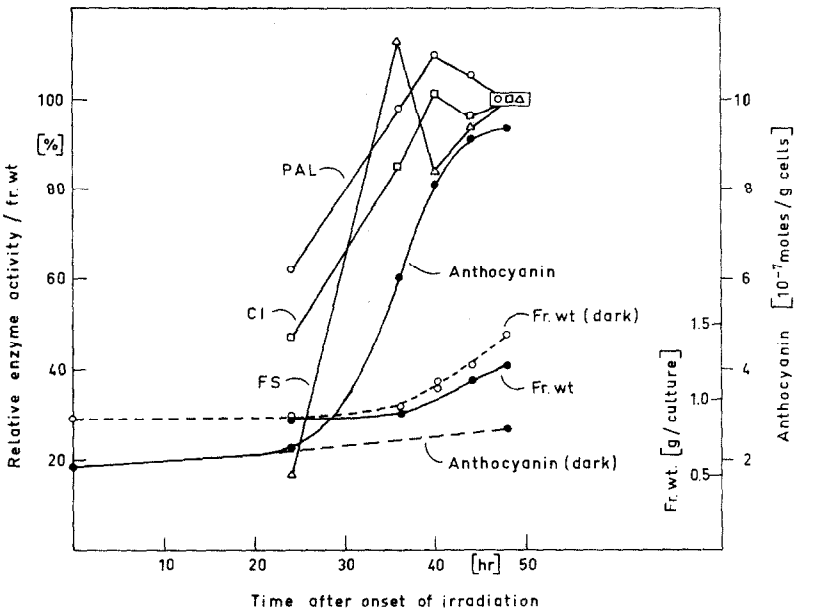

Fig. 1. Anthocyanin accumulation and activities of phenylalanine ammonia-lyase (PAL), chalcone isomerase (CI) and flavanone synthase (FS) and changes in fr. wt in response to continuous irradiation with UV. Enzyme activities of the dark controls have been substracted previously. The enzyme activities in the dark controls were less than 20% of induced maximum enzyme activities. Dark controls of all three enzymes remained unchanged during the period of UV-induced increase in enzyme activities and were not different from the enzyme activities present at the onset of irradiation.

phenylalanine ammonia-lyase (PAL, E.C.4.3.1.5), chalcone isomerase (E.C.5.5.1.5) and flavanone synthase [10] were monitored. Whereas the cell fr. wt was only slightly reduced in irradiated cultures, large increases in activity of all 3 enzymes were observed under UV light (Fig. 1). The enzymes reached maximal activity between 33 and 44 hr whereas maximal anthocyanin accumulation occurred somewhat later. However, a correlation between enzyme activity and anthocyanin biosynthesis would necessitate more extended kinetic investigations.

It is hoped that the results presented in this paper will facilitate the isolation of enzymes involved in flavonoid biosynthesis in *H. gracilis* cell cultures.

Table 2. Stimulation of anthocyanin formation under different growth and light conditions

Irradiation program	Anthocyanin (10^{-7}mol/g fr. wt of cells)
I. 6 day old cells in original medium	
48 hr UV	0.45
dark control	0.23
II. Cells transferred prior to irradiation to fresh medium	
48 hr UV	1.76
48 hr UV 1/3 intensity	0.78
48 hr UV 1/10 intensity	0.32
3 hr UV + 45 hr dark	0.30
24 hr dark + 3 hr UV + 21 hr dark	0.34
Dark control	0.28

EXPERIMENTAL

PC was carried out on Whatman 3 MM paper using 45% EtOH, 15% HOAc, *t*-BuOH–HOAc–H_2O (3:1:1) and C_6H_6–HOAc–H_2O (115:72:3).

Cell cultures and illumination conditions. *H. gracilis* cells were grown in the dark at 25–27° for 6–7 days as described previously [8]. White light was obtained from a high pressure Xe arc (*ca* 50 $W \cdot m^{-2}$) and UV light (1.2 $W \cdot m^{-2}$) from Osram L 40 W/73 lamps (310–410 nm, λ_{max} 355 nm). Cells were collected on a fritted glass filter and 0.8 g (fr. wt) each transferred to 5 × 5 cm glass petri dishes containing 15 ml fresh medium. The dishes were covered with plastic lids which had good transmission in the near UV. Irradiations were performed under aseptic conditions at 25° with continuous shaking.

Anthocyanin determination. For quantitative determination of anthocyanin, 0.3–1.0 g cells (fr. wt) were extracted for 5 min at room temp. with 50 ml MeOH–HCl (0.01%). After filtration the absorbance at 520 nm was measured immediately. An ϵ of 33000 [15] was used for calculation of the concentration.

Enzyme assays. 0.5 g cells were ground in a chilled mortar with 0.3 g PVP and 0.3 g quartz sand in 1 ml 0.02 M Pi buffer pH 5.5 (containing 1.4 mM mercaptoethanol). Cell debris was removed by low speed centrifugation. 10–20 μl of the clear supernatant liquid were used immediately for the PAL assay [13] and 50–100 μl for the chalcone isomerase assay [14]. The remaining supernatant was adjusted to pH 8.0 with 6 N KOH and then treated for 20 min with Dowex 1 (equilibrated with pH 8.0 Pi buffer). After removal of the Dowex by centrifugation, 50 μl of the extract was incubated with 50 μl 0.2 M Pi buffer, pH 8.0, 5 μl *p*-coumaryl CoA (1 nmol) and 5 μl 2-^{14}C malonyl CoA (2.44 nmol, 10^5 cpm) for the naringenin synthase assay [10].

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