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VERBASCOSIDE PRODUCTION IN CALLUS AND SUSPENSION CULTURES OF *HYGROPHILA ERECTA*

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Abstract—Cell suspension cultures of *Hygrophila erecta* obtained from hypocotyl explants accumulate up to 2.6% of their dry wt as only one caffeic glycoside ester, verbascoside. On the contrary, different callus lines initiated from stem explants contain not more than 1% of verbascoside and a few of them produced other caffeoyl glycosidic esters.

INTRODUCTION

Caffeic glycoside esters occur in large amounts in Tubiflorae (Orobanchaceae, Scrophulariaceae, Lamiaceae, Verbenaceae and Acanthaceae) [1–6]. *Hygrophila erecta* (Burm. fil.) Hochr. is a widespread plant in tropical Asia and Indonesia. Its seeds are used in Central Europe as a cosmetic, cicatrizant (heals by forming a scar) and cell regenerating product. Our preliminary analysis on the plant showed 2–5% of verbascoside (**1**) in the leaves. Little is known about the formation of these compounds in the plant cells. Previously, Ellis [7] described cell suspension cultures of *Syringa vulgaris* accumulating up to 16% of their dry wt as a mixture of hydroxyphenylethanol glycosides where the main component is the caffeoyl ester, verbascoside (**1**). To study the metabolic conditions for the production of these compounds, we define in this preliminary paper the conditions for verbascoside production in callus and suspension cultures of *H. erecta*.

RESULTS AND DISCUSSION

Growth and verbascoside production by H. erecta *suspension cultures*

The first cell line of *H. erecta* was initiated in 1981 from hypocotyles of seedlings germinated in sterile conditions. This primary culture was obtained and subcultured on solid 1B5 medium [8] but it was also subcultured every three weeks for two years on the corresponding liquid medium to establish a continuous line before any studies were undertaken. Kinetic results of growth obtained thereafter are shown in Fig. 1. Maximal growth rate of 0.012/hr at 25° (82 hr doubling time) was observed. Stationary phase was reached in fifteen days. The cell number rose from 7×10^3 cells/ml to 1.2×10^6 cells/ml and the dry wt reached 10 g/l of cell suspension. Verbascoside (**1**) production increased during the growth period and about 2–2.6 g per 100 g of dry wt material were produced in the stationary phase. As shown in Fig. 1, the verbascoside content stayed remarkably constant in the cells during the growth phase. Moreover, verbascoside was not detected in the medium and it did not seem to diffuse out of the cells.

H. erecta cell suspension could not grow in the light. In a few hours, its creamy grey colour changed to a darkish colour and the cell growth dropped drastically. On the contrary in the dark, the cells could be maintained in the stationary phase (Fig. 1) for more than 20 days without damage. Ultraviolet light and phenoloxidases rapidly oxidize the verbascoside and give different darkish products. This phenomenon is usually observed with the

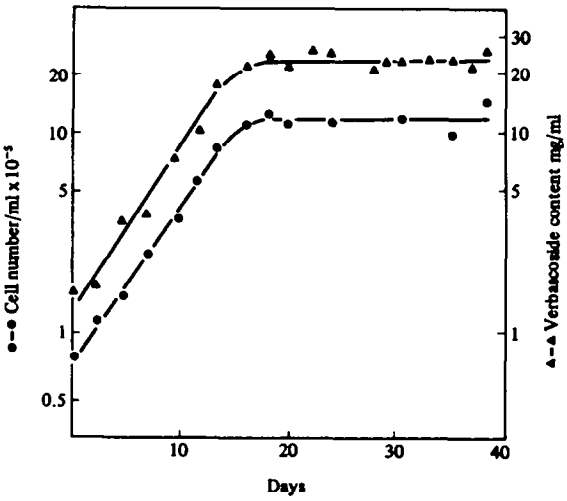

Fig. 1. Culture growth and verbascoside production. The contents of triplicate 250 ml (62 ml suspension) flasks were harvested by filtration on the days indicated. The cell number and verbascoside content were determined as in the Experimental.

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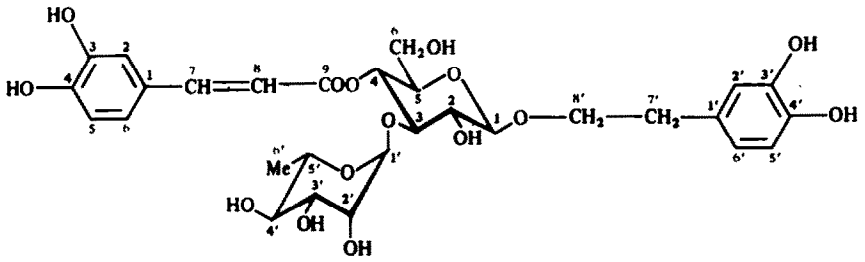

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whole plant of *H. erecta* when it is badly dried and preserved. It is interesting to note that the same phenomenon occurs with the plant cell cultures when they are exposed to white light. It is well known that ultraviolet light induces the enzymes of the pathways of general phenylpropanoid metabolism [9]. For example, the cell cultures of *Petroselinum hortense* studied by Hahlbrock *et al.* [10], respond specifically to irradiation with UV light by accumulating a number of flavone and flavonol glycosides concomitant with large and rapid increases and subsequent decreases in the enzyme activities related to their synthesis. In addition, other authors [11, 12] showed that growth and fructification of *Agaricus bisporus* and *Coprinus congregatus* primordia depend upon the action of a phenoloxidase, laccase, which is here induced by light. It would be interesting to study the relationship between the intensity of light irradiation and the phenoloxidase content which is responsible for the oxidization of verbascoside and the decrease of the cell growth.

Variability of verbascoside content of different callus lines of H. erecta *compared to the whole plant*

In September 1984, 25 callus initiations were performed from stem explants of eight different plants of *H. erecta*. These initiations gave rise rapidly to undifferentiated and stable light-grey calli on 1B5 medium. Thin-layer chromatography screening of phenolics showed the production of essentially one component, verbascoside (1). In plants, leaves are generally the richest tissue in verbascoside content. On the contrary, stems are the poorest [1]. In *H. erecta*, the dried leaves showed 2.9% of verbascoside on a dry wt basis and the dried stems contained 0.85%. The verbascoside content of the different callus lines measured on the calli after three weeks of culture (Table 1) displayed only one high producing cell line with 1% of dry wt, three cell lines containing 0.1–0.2%, six containing 0.05–1% and 15 of them producing less than 0.05% of verbascoside. There was apparently no correlation between the verbascoside contents of the different cell lines and those of the leaves of the plants from which they were initiated. This is generally observed for secondary metabolite production [13]. Moreover, these different callus lines obtained from stem explants of adult plant had a lower verbascoside content than the cell suspension culture described above. The cell suspension was cultivated on the same medium 1B5 but it was obtained from hypocotyl explants and it is known that seedlings in the hypocotyl state are richer in this caffeoyl compound than the more adult plants [14]. To test the stability of verbascoside production, three assays were conducted on the same cell suspension at six month intervals but no change was observed in the verbascoside contents of the cells of the batch culture. However, among the callus lines, three of them produced other unidentified caffeoyl glycosidic esters.

Table 1. Verbascoside contents of 25 callus cell lines of *H. erecta* compared to the eight different plants from which they were initiated

Plants	Verbascoside content (g% dry wt) in the leaves	Cell lines	Verbascoside content in the cells (g% dry wt)
1	2.3	1a	0.10
		1b	0.10
		1c	0.01
		1d	0.04
2	5.2	2a	0.01
		2b	0.01
		2c	0.02
		2d	0.07
		2e	0.01
3	3.3	3a	0.07
		3b	0.17
4	2.0	4a	1.00
5	5.2	5a	0.02
		5b	0.02
6	4.4	6a	0.19
		6b	0.10
7	2.4	7a	0.09
		7b	0.04
		7c	0.20
8	2.9	8a	0.01
		8b	0.01
		8c	0.02
		8d	0.02
		8e	0.04
		8f	0.02

EXPERIMENTAL

Plant cell cultures. Seeds of *Hygrophila erecta* from Birmany were either surface-sterilized and germinated on Heller's medium (first experiment) or germinated and grown in a greenhouse (second experiment). Sterile hypocotyl explants in the first case and surface-sterilized stem segments in the second were set out on Nitsch's revised medium [15] (0.1 mg/l. 2,4-dichlorophenoxyacetic acid; 2 mg/l. adenin; pH 5.5). Primary calli formed three

weeks after, were obtained in September 1981 in the first experiment and in September 1984 in the second one. These primary cultures were transferred just after induction on Gamborg's medium 1B5 [8] and subcultured every three weeks on the same medium. Cell suspension cultures were obtained and subcultured on the same liquid medium in 250 ml flasks containing 80 ml of fresh medium and mixed on a gyratory shaker at 120 rpm. Induction, callus and suspension cultures were always performed in a dark room at 25°.

Growth measurements. Suspension cultures were vacuum filtered (200–300 mm Hg) on a glass filter and tissue fresh wts were immediately determined. Cell numbers were counted on an aliquot of fresh culture after dissociation by boiling with a mixture of 10% chromic anhydride in 2 N H_2SO_4 for 10 min. Cells were lyophilized to determine the dry wts.

Verbascoside isolation and assay. The first verbascoside assays were made after 11 subcultures, nine months after initiation, when the calli and the suspension seemed morphologically stable. Leaves and stems of the plants were oven-dried and the calli and cells of suspension were lyophilized after filtering. These different plant materials were extracted × 3 by immersion into boiling 70% MeOH. Suspension media (10 ml) free of cells were concd to dryness and added to 1 ml of warm (50°) 70% MeOH. Methanolic extracts after filtering and methanolic solns were analysed to determine the verbascoside content by densitometry or fluorescence. TLC was performed on cellulose plates (Merck, ref. 5716, Darmstadt, eluted with H_2O–AcOH, 98:2) with application of sample together with increasing vols (2, 3, 4 and 5 μl) of a standard solution of verbascoside (0.001%) in 70% MeOH. The spots were made fluorescent by spraying Neu's reagent (2-aminoethyl diphenylborinate in 1% MeOH) and the densitometric assays were conducted in a UV–Vis 2 Farrand (Optical Co. Inc., New York, USA) spectrofluorometer using excitation and emission wavelengths set at λ_{Ex} 370 nm and λ_{Em} 500 nm [1].

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