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PRODUCTION OF DITERPENOIDS BY *EUPHORBIA CALYPTRATA* CELL CULTURES

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Key Word Index—*Euphorbia calyptrata*; Euphorbiaceae; diterpenoids; helioscopinolides; plant cell cultures.

Abstract—In order to identify diterpenoids (Helioscopinolides) produced in trace amounts by wild type cell cultures of *Euphorbia calyptrata*, three cell lines characterized by high production of the target metabolites have been selected. Cell lines were obtained by selection in media with different hormone composition.

INTRODUCTION

Euphorbia calyptrata, a poisonous shrub growing in the Sahara Desert, produces lactone diterpenoids which are active on the CNS [1]. In a previous study, the isolation of four diterpenoids from *E. calyptrata* cell cultures was reported [2]. They corresponded to the helioscopinolides (HELs) A and C, previously found in *E. helioscopia* [3], and to helioscopinolides E and D (see Fig. 1). These compounds, also present in plant extracts of *E. calyptrata*, represented the main metabolites produced. Some other minor compounds were detected by HPLC, but in amounts too low to be isolated. For this reason, a cell line selection was undertaken to produce them in larger amounts.

RESULTS AND DISCUSSION

A wild cell line of *E. calyptrata* was cultured in medium G5 [1] i.e. Gamborg solid medium [4] with added hormones (Table 1). Under these conditions the total production of HELs ranged from 5 to 20 $\mu g\,ml^{-1}$.

Enhancement of the total production of HELs was achieved by hormone depletion of the liquid medium. Experiments performed with decreasing amounts of hormones showed an increase from 5 to 37 $\mu g\,ml^{-1}$ in the production of the total HELs. This is in agreement with the similar observed enhancement of the benzophenantridine alkaloids produced under the same conditions by *Sanguinaria canadensis* cell cultures [5].

Based on these results, the basic medium with no hormones (G0, Table 1) was adopted in all of the experiments as 'production medium'.

However, despite the enhancement of the total productivity, the HPLC pattern of HELs was not significantly changed.

As different patterns of production of secondary metabolites depending on the mixture of hormones utilized is well documented [6–10], experiments were performed using different hormonal compositions of the medium, to change the amounts and patterns of HELs.

Five media were considered (Table 1). Wild cells were stabilized by subculturing them for at least three months on the new media, in order to ensure the depletion of the hormones present in the original G5. After this time, cells were transferred to the liquid production medium G0. Two weeks later the cell cultures were extracted and analysed.

It was observed that the best cultures for the production of HELs F and H were those stabilized on G9 and G6, respectively. No medium specifically enhanced the production of HELs B and L, which could be obtained only after the isolation of a cell line growing on the hormone-free medium G0 where a large increase of the total production of HELs occurred.

Therefore, in order to isolate high-producing cell lines with the same pattern of HELs, a selection was performed on the two media G6 and G9, as reported in the Experimental section.

This work resulted in the isolation of several hundred colonies among which three of them gave rise to three different cell lines showing an increase of the total production of HELs.

One cell line (EC6/201), grown on medium G6 produced 96 $\mu g\,ml^{-1}$ of total HELs, among which A (56 $\mu g\,ml^{-1}$) and (27 $\mu g\,ml^{-1}$) were the main products. A second cell line (EC9/115) grown on medium G9 and producing 114 $\mu g\,ml^{-1}$ of total HELs, was characterized by the presence of HELs C (63 $\mu g\,ml^{-1}$) and F (16 $\mu g\,ml^{-1}$). The third line (EC0/26) which grew in the absence of hormones, was obtained without selection. It was characterized by a total production of

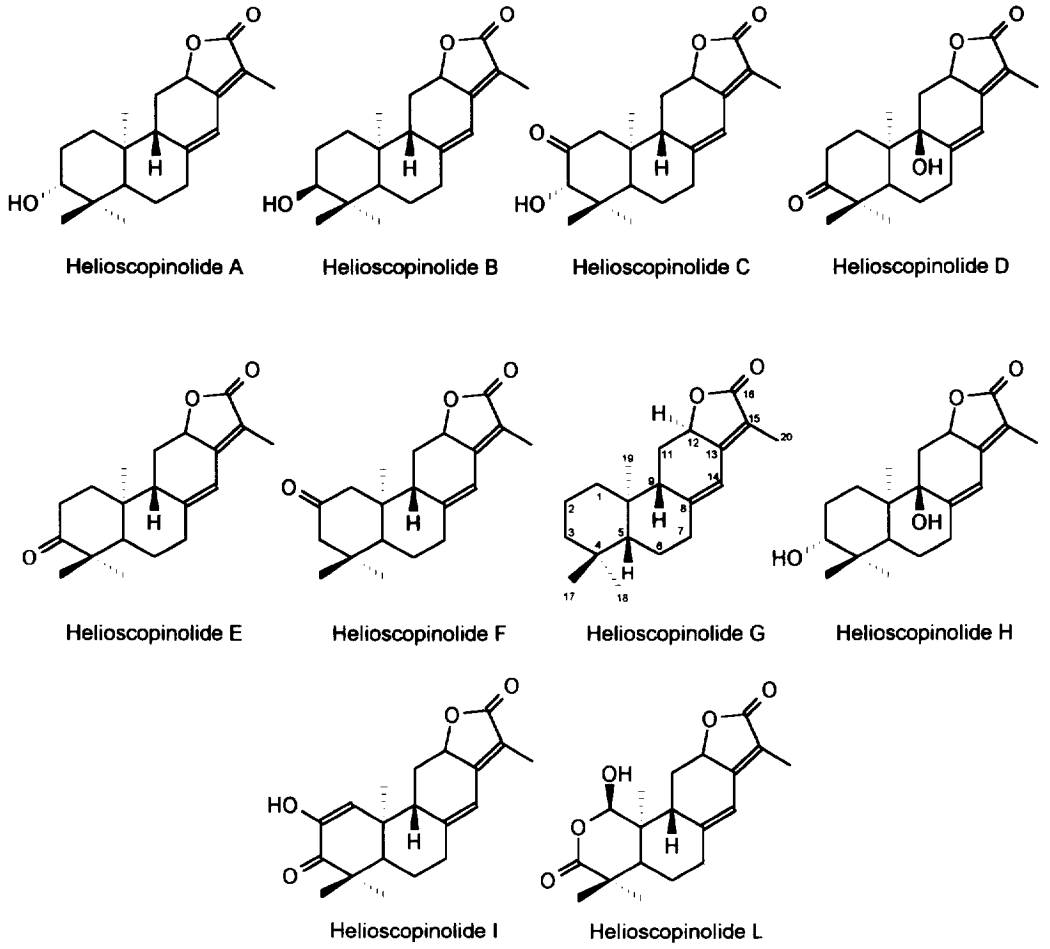

Fig. 1. Helioscopinolides found in cell cultures of *E. calyptrata*.

307 μg ml^{-1} and of 9 and 8 μg ml^{-1} of HELs B and L, respectively.

These increases allowed us to obtain HELs B, F, G, H, I and L in sufficient amounts for their isolation and identification. Among these HELs F, G, I and L (see Fig. 1) were found for the first time [11].

EXPERIMENTAL

Media. The basic medium utilized was Gamborg B5 [5]. Cells were grown in media supplemented with hormones (growth media), as reported in Table 1.

Table 1. Hormones present in solid and liquid media used for the selection of the different cell lines. Amounts are expressed as μg l^{-1} of basal medium

	Auxins		Cytokinins	
Medium	NAA	2,4-D	K	BA
G0	—	—	—	—
G5	1000	200	1000	—
G6	1000	—	1000	—
G7	1000	—	—	1000
G9	50	—	1000	—
G10	50	—	—	1000

Suspended cultures were grown in 300 ml Erlenmeyer flasks containing 50 ml of liquid medium shaken at 120 rpm at 28°.

Subculture of cell lines. Solid cultures were grown in slant tubes containing 20 ml of solid media having the same composition as the corresponding liquid media, except for the presence of 7% agar. The original cell line stabilized on medium G5 was transferred onto all of the media reported in Table 1. The cells were subcultured every 20 days for at least 3 months after which a portion of each stabilized cell line was transferred into the production liquid medium G0. After 14 days, the cultures were harvested and analysed by HPLC.

HPLC analyses. Suspended cultures were added of an equal vol. of MeCN, homogenized within Ultraturrax, centrifuged and the resultant clear supernatant taken for analysis. Analyses were performed with a Beckman System Gold apparatus equipped with a Photodiode Array Detector and two analytical columns, Hibar (Merck), assembled in series. Analytical parameters were: detection at 275 and 210 nm; flow rate 1 ml min^{-1}; solvent NaH_2PO_4 (2 g l^{-1})–MeCN (9:11).

Selection of high-producing cell lines. Small cell

aggregates were suspended in fresh liquid medium and the suspension (2×10^3 cells) transferred to Petri dishes containing the same solid medium. One month later the clone cells formed small colonies which were picked up and inoculated into tubes of solid medium of the same hormonal composition.

Twenty days later, one half of each tube was transferred to G0 liquid medium. After 14 days, the cultures were harvested and analysed by HPLC. At the same time, the second half of each tube was subcultured.

Cell lines derived from colonies giving the highest production were once more plated and selected as described above.

REFERENCES

1. Speroni, E., Coletti, B., Minghetti, A., Crespi Perellino, N., Guicciardi, A. and Vincieri, F. F. (1991) *Planta Med.* **57**, 531.
2. Borghi, D., Baumer, L., Ballabio, M., Arlandini, E., Crespi Perellino, N., Minghetti, A. and Vincieri, F. F. (1991) *J. Nat. Prod.* **54**, 1503.
3. Shizuri, Y., Kosemura, S., Yamamura, S., Ohba, S., Ito, M. and Saito, Y. (1983) *Chem. Lett.* **1**, 65.
4. Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) *Exp. Cell Res.* **50**, 151.
5. Kline, S. D., McHale, R. J. and Coscia, C. J. (1993) *J. Nat. Prod.* **56**, 19.
6. Singh, B. D., Harvey, B. L., Kao, K. N. and Miller, R. A. (1975) *Can. J. Genet. Cyt.* **17**, 109.
7. Sacchi, G. A., Morgutti, S., Abruzzese, A., Alisi, C., Cocucci, M., Espen, L., Leva, A. L., Muleo, R. Negrini, N. and Cocucci, S. M. (1995) *Plant Sci.* **106**, 107.
8. Church, D. L. and Galston, A. W. (1988) *Phytochemistry* **27**, 2435.
9. Liu, W., Hildebrand, D. F. and Collins, G. B. (1995) *Plant Sci.* **106**, 31.
10. Jaziri, M., Homes, J. and Vanhaeien, M. (1987) *Phytochemistry* **26**, 999.
11. Crespi Perellino, N., Garofano, L., Arlandini, E., Pinciroli, V., Minghetti, A., Vincieri, F. F. and Danieli, B. (1996) *J. Nat. Prod.* (in press).