



ACCUMULATION OF PODOPHYLLOTOXIN AND RELATED LIGNANS IN CELL SUSPENSION CULTURES OF *LINUM ALBUM*

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Key Word Index—*Linum album*; Linaceae; white flax; biosynthesis; lignans; podophyllotoxin; 5-methoxypodophyllotoxin; coniferin; plant cell cultures.

Abstract—Cell suspension cultures of *Linum album* were established, which were able to synthesize and accumulate lignans. Podophyllotoxin and 5-methoxypodophyllotoxin were the main products and were present as glycosides, together with small amounts of deoxypodophyllotoxin, 5'-demethoxy-5-methoxypodophyllotoxin, lariciresinol, pinoresinol, matairesinol, α - and β -peltatin, as well as the monolignol glucoside, coniferin. In dark and light grown cultures, maximal product yields of lignans of up to 0.2% and 0.5% of the dry weight, respectively, were achieved, mainly consisting of podophyllotoxin. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Lignans, long known as natural products, are distributed widely in the plant kingdom. Several hundreds of compounds in this general class have been identified, which are derived from two phenylpropanoid units. From a medicinal point of view, the most important compounds today are etoposide and teniposide, semisynthetic derivatives of podophyllotoxin (Fig. 1), which are used in cancer chemotherapy. Total chemical synthesis of both compounds is possible, but is not an economic proposition. Therefore, podophyllotoxin (Ptox) still serves as the starting material for semisynthesis of both compounds [1]. It is extracted from rhizomes of *Podophyllum hexandrum* and *P. peltatum*, which contain ca 4 and 0.2% of Ptox, respectively, calculated on a dry basis [2]. There is an increasing demand for podophyllotoxin due to its medicinal importance. However, a sufficient supply of *Podophyllum* plants is rather limited, since the occurrence of these plant species is scarce and the plants need a growth period of 5 to 7 years, before harvesting of the rhizomes is convenient. *Podophyllum peltatum*, moreover, has poor reproduction capacities [3]. *Pod-*

phyllum hexandrum, which is the species in most demand, originates from Himalaya. There it even became a threatened species due to the intensive collection of this plant. Nowadays, additional sources for podophyllotoxin and related lignans in other plant species (for a review, see [4]) are identified and *in vitro* propagation methods for *Podophyllum* species [5, 6, 24, 25] are being established. Attempts to produce podophyllotoxin by cell cultures of *P. hexandrum* and *Callitris drummondii* have been reported [7–10], but the yields obtained were low and the cultures grew only slowly. Cell and root cultures of *Linum flavum* grow much better, but these cultures accumulate only 5-methoxypodophyllotoxin (5-Mptox) in substantial amounts [11–15]. In 1975, Weiss *et al.* [16] reported that plants of *L. album* contained podophyllotoxin besides some other lignans.

Recently, we initiated callus and cell suspension cultures of this species, which accumulate podophyllotoxin besides small amounts of 5-Mptox, as well as traces of some other lignans [17]. Herein we describe more detailed experiments about the accumulation of Ptox, 5-Mptox and a possible biosynthetic precursor, coniferin, during the culture cycle of cell suspension cultures of *Linum album* grown in the dark and light.

RESULTS AND DISCUSSION

Shoot cultures were established from seeds, which sometimes needed several weeks to germinate. From

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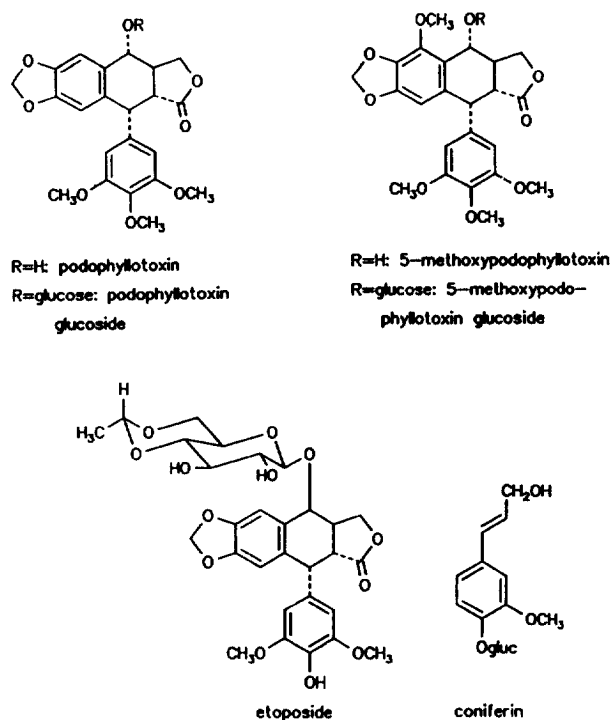


Fig. 1. Structural formulae of some lignans.

single seedlings we established calli and, subsequently, suspension cultures (lines 2-1, 2-3 and 2-8). About two years after establishment of the cultures in a preliminary screening, the medium [11] was modified using as single growth hormone 0.4 mg l^{-1} and 0.8 mg l^{-1} NAA, respectively, and the cells investigated for the presence of lignans and monolignols after a subculture time of 1-3 weeks. The presence of Ptox and 5-Mptox was verified using HPLC, mass spectroscopy and ^1H NMR, that of 5'-demethoxy-5-methoxypodophyllotoxin and lariciresinol by HPLC and mass spectroscopy, α - and β -peltatin, matairesinol, deoxypodophyllotoxin, as well as coniferin and coniferyl

alcohol, were identified by HPLC. HPLC analyses showed that podophyllotoxin is the main lignan. The amounts of 5-Mptox are substantially lower, but the Ptox:5-Mptox ratio differs in the three cell lines (Table 1). The other lignans mentioned above are found only in trace amounts. Line 2-1 had the highest production of podophyllotoxin, with only very low amounts of 5-Mptox. The amounts of both lignans were routinely calculated as the aglycones Ptox and 5-Mptox after enzymatic hydrolysis of the corresponding glycosides. Extraction by the procedure of Berlin *et al.* [18] avoiding hydrolysis of the glycosides showed, however, that both lignans are present in the cells

Table 1. Content of podophyllotoxin and 5-methoxypodophyllotoxin in cell cultures of *Linum album* calculated on an aglycone basis (mg per culture vessel, 50 ml medium) after 1, 2 and 3 weeks of culture in the dark in a medium containing 0.4 mg and 0.8 mg l^{-1} NAA

	Podophyllotoxin			5-Methoxypodophyllotoxin		
	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
0.4 mg l^{-1} NAA						
Line 2-1	1.87	2.46	2.44	0.16	0.25	0.27
Line 2-3	0.77	1.29	0.79	0.49	0.59	0.69
Line 2-8	0.40	0.75	0.77	0.12	0.18	0.41
0.8 mg l^{-1} NAA						
Line 2-1	1.23	1.83	0.90	0.09	0.13	0.10
Line 2-3	0.40	0.89	0.69	0.11	0.18	0.47
Line 2-8	0.66	0.90	1.03	0.10	0.92	0.70

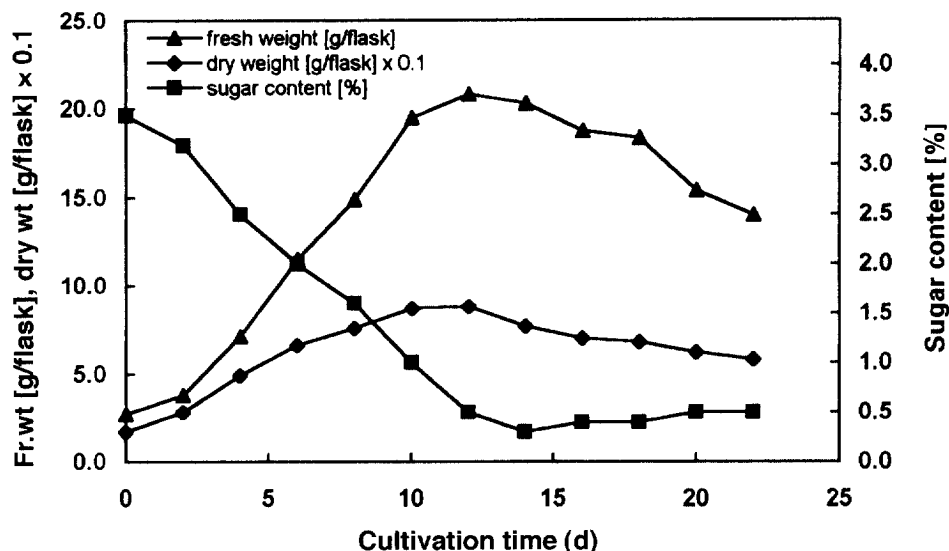


Fig. 2. Growth curve (fr. wt, dry wt: g per flask (50 ml medium); residual sugar in %) of cell suspension cultures of *Linum album* strain 2-1 during a culture period of 22 d.

genuinely as glycosides. Only up to ca 2% of the total podophyllotoxin was identified as the aglycone (data not shown).

Line 2-1 was used to examine cell growth and production of lignans during a culture period of 22 days. Maximum biomass (fr. and dry wt) was achieved after 10 to 12 days (Fig. 2), together with maximal uptake of sucrose and mineral ions, the latter indicated by a decrease in conductivity. The increase in conductivity indicated cell lysis after day 14. This is accompanied by a substantial increase in the pH of the medium (data not shown). Production of Ptox as well as of 5-Mptox parallels growth reaching a maximum yield at day 10 to 12 (Fig. 3). Calculation of the relative amounts of lignans as mg g^{-1} dry wt, shows first that the lignan content decreases until day 2 and then increases again until day 18 (data not shown). Interestingly, maximal accumulation of coniferin, a probable precursor of Ptox and 5-Mptox [13, 19-21], can be observed some days before maximal accumulation of both of these lignans (Fig. 3).

Cells cultivated in continuous light form chlorophyll, but besides that behave similarly to those grown in darkness. Maximal biomass, as well as lignan accumulation, is achieved at day 12, together with depletion of sugar from the medium. The biomass produced is not significantly different from that produced in darkness. The yields of Ptox and 5-Mptox, however, are higher, reaching almost 60 mg l^{-1} Ptox and 40 mg l^{-1} 5-Mptox or a total of more than 0.5% of these lignans on a dry wt basis.

Our results show, that cell suspension cultures of *L. album* are able to accumulate podophyllotoxin, a valuable precursor for the chemical synthesis of the anticancer drugs, etoposide and teniposide. As stressed by Zenk *et al.* [22], it is important to select

the appropriate genotype in order to obtain high production of a desired natural product in plant cell cultures. Whereas cell and root cultures of *L. flavum* only produce 5-Mptox [9, 11, 14, 15], cell suspensions of *L. album* accumulate Ptox as the main product, whereas 5-Mptox is only a by-product. The amount of 5-Mptox, however, depends on the genotype used for the initiation of the cultures (cf Table 1). Coniferyl alcohol is supposed to be a precursor of lignan biosynthesis [19-21]. The increase of coniferin content and the decrease during lignan accumulation in *Linum* cells might indicate the utilization of coniferin, the glycoside of coniferyl alcohol, as a lignan precursor. Compared with rhizomes of *Podophyllum* plants, which are the commercial source of podophyllotoxin, the amounts of lignans, and especially those of Ptox found in our *Linum* cell cultures are quite high. They are the highest yields of podophyllotoxin that have been reported for tissue cultures. Experiments are in progress to initiate cell cultures from other genotypes of *L. album* and to improve the product yield by variation of the culture conditions.

EXPERIMENTAL

Seeds of *L. album* Kotschy were collected in 1988 near Teheran and germinated under sterile conditions on hormone-free MS-medium [23] at 25° in continuous light ($50 \mu\text{E m}^{-2} \text{s}^{-1}$ photosynthetic active radiation, Philips 18 W fluorescent tubes). Shoot cultures from single seedlings were established on the same medium. These were used to initiate calli and, subsequently, cell suspension cultures in MS-medium with various amounts of auxins. MS-medium with 0.4 mg l^{-1} NAA proved to be best for good growth of the cultures. Cells (5 g fr. wt) were transferred every 7

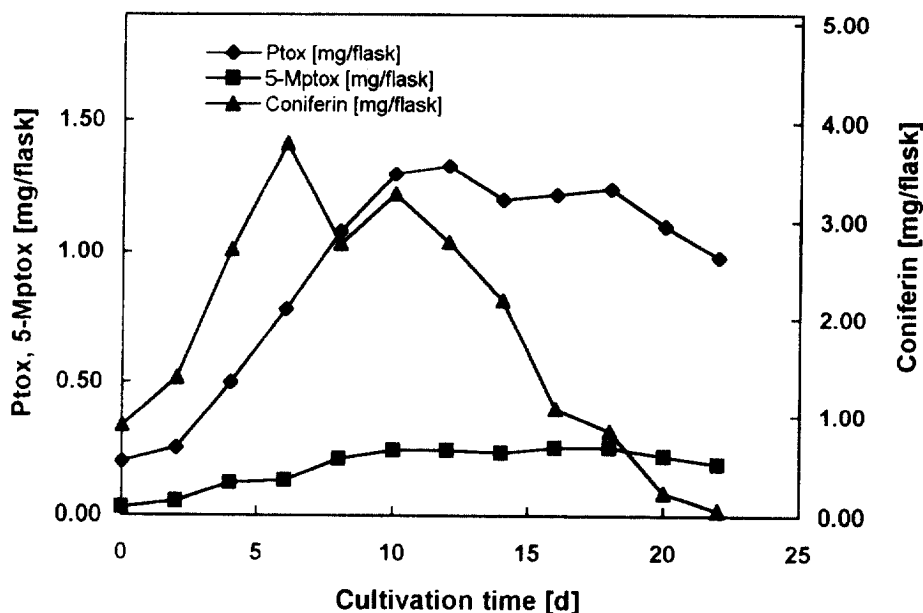


Fig. 3. Formation of lignans and coniferin by cell cultures (mg per flask, 50 ml medium) of *Linum album*.

days into 50 ml of fr. medium in 300 ml Erlenmeyer flasks and incubated on a gyratory shaker at 120 rpm in the darkness or continuous light at 25°. Fresh wts of cells were measured after suction filtration, dry wt after lyophilization. Conductivity and sugar content of the medium were measured using a conductometer and refractometer, respectively. The methods used for identification and quantitation of lignans by MS, as well as by HPLC, are given in ref. [17]. Ptox and 5-Mptox were isolated by HPLC and their identity verified by comparing their ¹H NMR spectra with those of authentic compounds [15]. Additionally, lignans were extracted according to Ref. [18] in order to determine whether lignan aglycones were genuine products of the *Linum* cells or were artefacts produced during the extraction procedure.

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