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Lignan accumulation in cell cultures of *Linum strictum* ssp. *strictum* L.

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Shoot, callus and hairy root cultures of *Linum strictum* ssp. *strictum* L. were initiated. The lignan 6-methoxypodophyllotoxin was detected and quantified.

Keywords: Linum strictum ssp. *strictum* L. *(Linaceae)*, plant cell cultures, lignans, 6-methoxypodophyllotoxin

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Linum strictum ssp. *strictum* L. is an annual plant, widespread in Southern Europe (1). It has robust stems, 10–45 cm high, which are seldom branched. Its leaves are 1.5–5 mm wide, with margins that are minutely serrulate, very rough and often inrolled. The inflorescence is a dense, spike-like cyme or a corymb. The flowers are sessile or subsessile with thick pedicels, rarely longer than the calyx. The sepals are 4–6 mm long, ovate-lanceolate, long-acuminate, minutely serrulate and glandular-ciliate. The petals are 6–12 mm long and yellow. The fruit is a 2–2.5 mm long subglobose capsule.

Cell cultures of *Linum* species accumulate considerable amounts of aryltetralin lignans (2). Production of podophyllotoxin and its derivatives by means of cell cultures from *Linum* species might thus be an alternative to the supply from natural sources (3). Aryltetralin lignans are used after chemical derivatization in the treatment of malignancies such as Hodgkin's disease and small cell anaplastic lung carcinoma (4). The pharmacological effects of lignans also include antiviral, antifungal, immunosuppressive, hypolipidemic, antiasthmatic and antiplatelet activities (5, 6).

The objective of this study was to quantify lignan accumulation in cell cultures of *Linum strictum* ssp. *strictum* L., which were initiated by us for the first time. To our knowledge this is the first phytochemical examination of podophyllotoxin-like compounds in cell cultures of *Linum strictum* ssp. *strictum* L.

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EXPERIMENTAL

Plant material

The seeds of *Linum strictum* ssp. *strictum* L. *(Linaceae)* were kindly provided by the botanical garden of Musèum d'Histoire Naturelle, Paris, France.

Plant cultures

Seeds of *Linum strictum* ssp. *strictum* L. were germinated in the light, under sterile conditions, on a hormone free Murashige and Skoog (MS) medium (7). Sterile grown seedlings were used to initiate shoot and callus cultures.

Shoot cultures were developed on a half-concentration MS solid medium containing 6-benzylamino purine (BAP, 0.22 mg L^{-1}) and indole-acetic acid (IAA, 0.1 mg L^{-1}). Shoots were transferred every 4 weeks into 100 mL of fresh medium.

The standard medium for callus cultures was MS-medium containing 6-furfurylaminopurine (kinetin) (2 mg L^{-1}), 2,4-dichlorophenoxyacetic acid (0.1 mg L^{-1}) and IAA (0.2 mg L^{-1}). Calli were incubated under permanent light and they were transferred every 3 weeks into 100 mL of fresh medium.

Hairy roots were induced by direct incubation of segments from sterile grown plants with *Agrobacterium rhizogenes* strain TR 105 cultured in the yeast mannitol broth (YMB) medium (in g L^{-1}: K_2HPO_4 – 0.5, $MgSO_4 \cdot 7\ H_2O$ – 2.0, NaCl – 0.1, mannitol – 10.0, yeast extract – 0.4, and agar – 15.0, pH 7.0). After 2 days, the explants were transferred to MS solid medium without phytohormones, supplemented with 500 mg L^{-1} antibiotic cefotaxim-natrium (Claforan, Hoechst Marion Roussel, Germany). The obtained hairy roots were transferred after 2–3 weeks into a liquid MS medium without phytochoromones, containing cefotaxim, and the cultures were subsequently maintained without cefotaxim. Hairy roots were grown in the dark on a rotary shaker (80 rpm) and refreshed with a new medium every 2 weeks (8).

Extraction and isolation of lignans

A fine powder (200 mg) of lyophilized cells was extracted with methanol (2 mL). The mixture was further homogenized in an ultrasonic bath (2 × 30 s) with intermediate cooling on ice. Distilled water (6 mL) was added and the pH was adjusted to 5.0 by adding a few drops of 5% phosphoric acid. After adding β-glucosidase (1 mg), the sample was incubated at 35 °C for 1 h in a water bath. MeOH (12 mL) was added and the mixture was incubated for another 10 min at 70 °C in an ultrasonic bath. After centrifugation for 7 min at 4500 rpm, the volume of supernatant was determined. The supernatant (1 mL) was taken and centrifuged at 13 000 rpm for 5 min at 25 °C. This final supernatant was used for the HPLC analysis.

Determination of lignans

The HPLC analysis was performed on a Thermo Quest (Analytische Systeme GmbH, Germany) equipped with a Spectra SYSTEM UV6000LP detector. The separation column

was a GROM-SIL 120 ODS-5 ST (250 × 4 mm, particle size 5 μm) supplied with a precolumn (20 × 4 mm, particle size 5 μm). The gradient system used water with 0.01% phosphoric acid (85%) (A) and acetonitrile (B) as follows: 0 to 17 min from 40% to 67% B, from 17 to 18 min back to 40% B. The flow rate was 0.8 mL min^{-1} between 0 and 17 min, 1 mL min^{-1} between 17 and 24 min and again 0.8 mL min^{-1} after 24 min. Detection was performed at 290 nm against reference standards. The retention time of 6-methoxypodophyllotoxin (6-MPTOX) was 11.71 min.

RESULTS AND DISCUSSION

Sterile grown seedlings of *Linum strictum* ssp. *strictum* L. were initiated, and were then used for the development of shoot, callus and genetically transformed (hairy roots) cultures.

6-MPTOX was identified by HPLC equipped with DAD UV-detector (Fig. 1) as the only lignan in the developed cultures. 6-MPTOX is known to be of the same cytotoxic potency as podophyllotoxin (9). The amount of 6-MPTOX was determined by HPLC as an aglycone after enzymatic hydrolysis with β-glucosidase. The results are given in Table I. The shoot cultures of *Linum strictum* ssp. *strictum* L. yielded higher levels of 6-MPTOX (1.03 ± 0.11 mg g^{-1} dry mass) than the callus and hairy-root cultures (0.60 ± 0.14 and 0.57 ± 0.09 mg g^{-1} dry mass, respectively). The higher yields of 6-MPTOX in shoot cultures are probably due to the differentiation of the shoot and specific phytohormone present in the medium with BAP and IAA.

The results we present from *Linum strictum* ssp. *strictum* L. are to our knowledge the first reports of the production of aryltetralin lignans in the *Linastrum* section (10). According to our data, cultures of *Linum strictum* ssp. *strictum* L. produced only 6-MPTOX, similarly to cell cultures of *L. flavum* from the *Syllinum* section (11).

The search for 6-MPTOX high-producing species is of major importance in the development of systems producing high amounts of podophyllotoxin. To obtain an increase in podophyllotoxin production, it seems most useful to block the 6-hydroxylation and prevent the formation of 6-MPTOX. To date, the highest productivities of 6-MPTOX were reached in *Linum nodiflorum* suspension cultures: 17 mg g^{-1} dry mass after 9 days (12). The cultures of *Linum strictum ssp. strictum* L., developed by us, did not produce such

Table I. Lignan content in cultures of Linum strictum *ssp.* strictum *L.*

Culture type of *L. strictum* ssp. *strictum*	6-MPTOX (mg g^{-1} dry mass)[c]
Shoots[a]	1.03 ± 0.11
Callus[b]	0.60 ± 0.14
Hairy roots[a]	0.57 ± 0.09

[a] After 30 days of cultivation.
[b] After 21 days of cultivation.
[c] Values are averages ± SD of triplicate determinations.

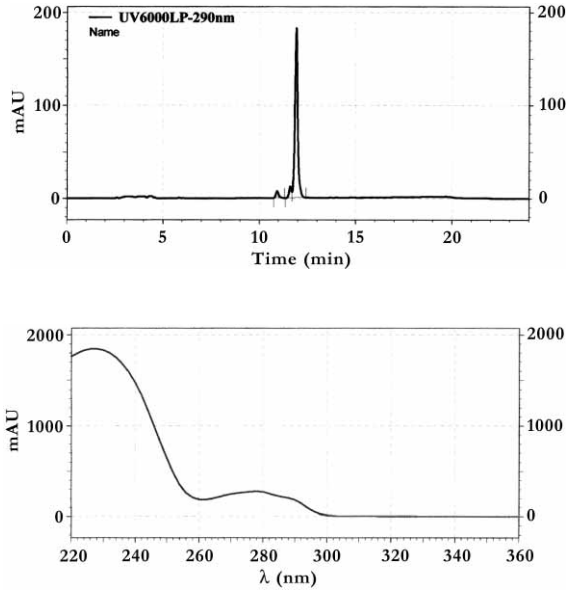

Fig. 1 A typical HPLC and UV-spectrum of the 6-MPTOX from the extracts of shoot cultures of *Linum strictum ssp. strictum* L.

high amounts of 6-MPTOX. This can be explained by the fact that L. *nodiflorum* and *L. strictum* ssp. *strictum* L. belong to the sections *Syllinum* and *Linastrum*, respectively, and this difference results in different biosynthetic capacities for 6-MPTOX.

CONCLUSIONS

6-MPTOX appears to be the only lignan in cell cultures of *Linum strictum*. Quantitative analysis has shown that shoot cultures produced the highest content of 6-MPTOX. Therefore, we believe that the cultures of *Linum strictum* ssp. *strictum* L., especially the shoots, are a useful system for studying the biosynthesis of 6-MPTOX.

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S A Ž E T A K

Akumulacija lignana u staničnoj kulturi biljke *Linum strictum* ssp. *strictum* L.

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Uzgojena je stanična kultura mladica, kalusa i čupavog korijenja biljke *Linum strictum* ssp. *strictum* L. U njima je određena količina lignana 6-metoksipodofilotoksina.

Ključne riječi: Linum strictum ssp. *strictum* L. *(Linaceae)*, kultura biljnih stanica, lignani, 6-metoksipodofilotoksin

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