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Original Article

Quantification of Aryltetralin Lignans in *Linum album* Organs and *In Vitro* Cultures

Abdolali Mohagheghzadeh[a,*], Shiva Hemmati[a], A. Wilhelm Alfermann[b]

[a] *Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Scinces Research Centre, Shiraz University of Medical Sciences, Shiraz, Iran*
[b]*Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich- Heine- Universität Düsseldorf, Universitätsstrasse 1, D-40225 Düsseldorf, Germany*

Dedicated to the memory of Professor Massoud Adrangui

Abstract

A procedure was developed for rapid *in vitro* germination of *Linum album* seeds by scarification and exposing to gibberellic acid (GA_3) and kinetin (Kn). Concentrations of podophyllotoxin (PTOX) and related aryltetralin lignans α-peltatin, β-peltatin, 5'-demethoxy-6-methoxypodophyllotoxin and 6-methoxypodophyllotoxin (MPTOX) in *L. album* fresh plant organs were determined by HPLC. A degree of variation was observed in lignan content in different plant parts and growth stages. It was found that PTOX is predominant in productive organs and leaves. The highest amount of PTOX (0.651 mg/100g dry wt.) and MPTOX (0.670 mg/100g dry wt.) and the total quantified lignans were found in the capsules. *In vitro* cultures were studied for lignans followed by high productive cell line selection. Calli cultures were more productive for MPTOX than PTOX, while suspension cells accumulate comparable amounts of PTOX and MPTOX. The highest amount of PTOX (0.301% mg/g dry wt.) was found in suspension originated immobilized cultures.

Keywords: Aryltetralin lignans; *In vitro* culture; *Linum album*; Methoxypodophyl lotoxin; Podophyllotoxin; Seed dormancy.
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1. Introduction

Plant kingdom has provided us with many important medicaments including anticancer agents. Higher plants have made important contributes in this regard [1]. Podophyllotoxin (PTOX) is a cytotoxic natural product that its availability has special limitations because of its intense collection from nature, lack of organized cultivation and few commercial exploitable sources [2, 3]. PTOX itself is used against certain viruses and skin cancers, but it is transferred to the anticancer drugs etoposide, teniposide and etopophos® by epimerization and substitution in position 4 and

*Corresponding author. A. Mohagheghzadeh, Department of Pharmacognosy, Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. P.O. Box 71345-1583.
Tel (+98)711-2425374, Fax (+98)711-2426070.
E-mail: Mohaghegh@sums.ac.ir.

	R_1	R_2	R_3	R_4
Podophyllotoxin	OH	CH_3	OCH_3	H
6-Methoxypodophyllotoxin	OH	CH_3	OCH_3	OCH_3
5'-Demethoxy-6-methoxypodophyllotoxin	OH	CH_3	H	OCH_3
α-Peltatin	H	H	OCH_3	OH
β-Peltatin	H	CH_3	OCH_3	OH

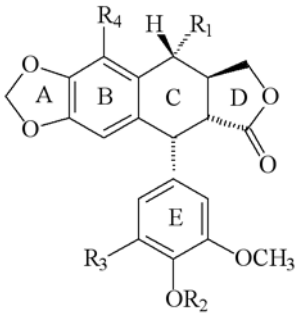

Figure 1. Podophyllotoxin and related aryltetralin lignans.

demethylation in position 4' in the pendant ring (Figure 1) [4, 5]. These drugs have been marketed commercially because of their potent effect in Hodgkin's and non-Hodgkin's lymphoma, testicular/small cell lung cancers and acute leukemia [5]. The chemical synthesis of PTOX is not commercial and rather complicated because of the presence of four chiral centers, a rigid trans lactone ring and an axially locked 1-aryl substituent [4]. Interestingly, this lignan and its related compounds are also found in plant families like Linaceae. *Linum album* Ky. ex Boiss. (Linaceae), known as 'Katan-e-Golsefid' in Persian, is an endemic herbaceous perennial plant widely distributed to mountainous areas, sandy slopes and sandy-clay soils in fields at an altitude of 1200 to 3200 m in Irano-Turanian regions [6, 7]. Its flowering time is restricted from April to June months [8]. A

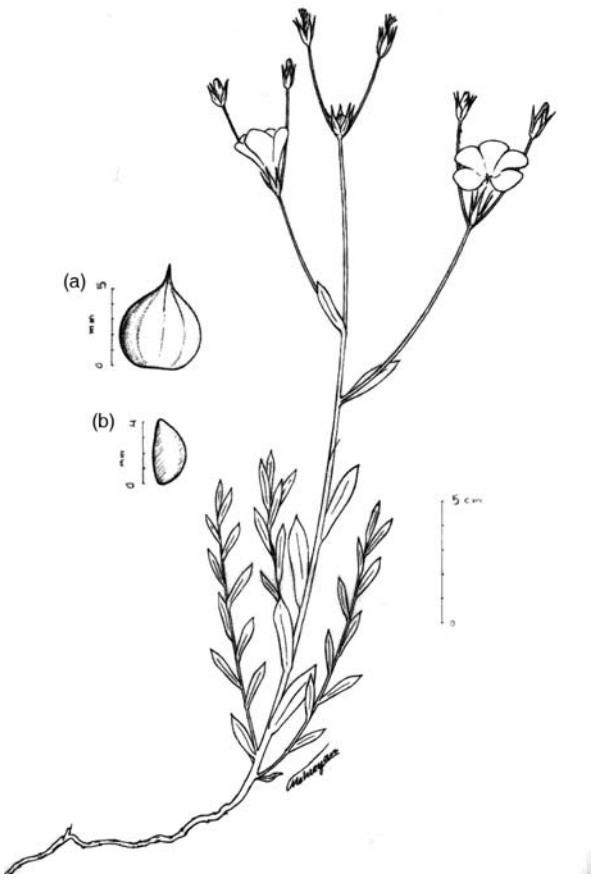

Figure 2. *Linum album* in flowering stage; (a) capsule; (b) seed.

Table 1. Content of podophyllotoxin and related aryltetralin lignans (% mg/g dry wt.) in different plant parts of *Linum album*[a].

No.	Plant part	α-PLT	PTOX	β-PLT	5'-DMPTOX	MPTOX
1	Seeds	0.140±0.009	0.120±0.013*	0.061±0.002	0.116±0.014	0.631±0.032
2	Capsules	0.051±0.311	0.651±0.050*	-	0.452±0.031	0.670±0.042*
3	Sexual organs	0.272±0.041	0.321±0.027	-	0.012±0.001	0.121±0.018*
4	Petals	0.362±0.023	0.331±0.012*	0.152±0.018	0.010±0.002	0.071±0.004*
5	Sepals	0.227±0.030	0.110±0.021	-	0.371±0.013	0.205±0.019
6	Leaves	0.341±0.012	0.312±0.011	0.130±0.012	0.051±0.003	0.042±0.002
7	Leaves of lateral branches	0.608±0.013	0.271±0.017	0.109±0.020	-	0.114±0.013
8	Branches	0.421±0.017	0.144±0.011	0.081±0.002	0.013±0.002	0.170±0.012
9	Upper stems	0.441±0.013	0.140±0.011	0.061±0.002	t	0.180±0.014
10	Middle stems	0.280±0.012	0.091±0.001	-	t	0.201±0.013
11	Lower stems	0.407±0.020	0.091±0.002	0.209±0.011	0.071±0.002	0.190±0.012
12	Upper rhizomes	0.561±0.020	0.071±0.001*	0.211±0.021	0.093±0.001	0.251±0.010*
13	Middle rhizomes	0.091±0.002	0.061±0.002	-	0.051±0.002	0.250±0.001
14	Lower rhizomes	0.124±0.025	0.031±0.001	-	0.012±0.001	0.230±0.032

[a]All values of mean ± S.D. from three measurements. α-PLT, α-peltatin; PTOX, podophyllotoxin; β-PLT, β-peltatin; 5'-DMPTOX, 5'-demethoxy-6-methoxypodophyllotoxin; MPTOX, 6-methoxypodophyllotoxin. 5'-DMPTOX and MPTOX were quantified according to PTOX and α-PLT was quantified according to β-PLT. t <0.01. -, no peak at the retention time of this compound was detected. * p <0.005; ANOVA test.

phytochemical study of *L. album* guided by Weiss *et al.* in 1975 in a random collection screening program for new anticancer agents and was bioassaied with the 9KB cell culture, resulted in the isolation of PTOX and a new lignan, 3'-demethylpodophyllotoxin and identification of α- and β-peltatin (α- and β-PLT) [9]. Derivatives of PTOX from *L. album* like 6-methoxypodophyllotoxin (MPTOX), 5'-demethoxy-6-methoxypodophyllotoxin (5'-DMPTOX), α- and β-PLT also have cytotoxic properties and thus may be an attractive alternative cytotoxic lignan of the PTOX family [10].

Sustainable bioproduction of the compounds of interest may be achieved by plant *in vitro* cultures [11]. High value and low yield of podophyllotoxin (up to 5% of dry weight in the commercial source (rhizomes of 5 years old *Podophyllum hexandrum*) has catalyzed research in the area of plant cell and tissue culture [12]. There are some reports on the accumulation of podophyllotoxin type lignans in cell cultures of *L. album* [10, 13, 14]. Cell cultures of *L. album* accumulate PTOX (up to 0.5 % of dry wt.) during a

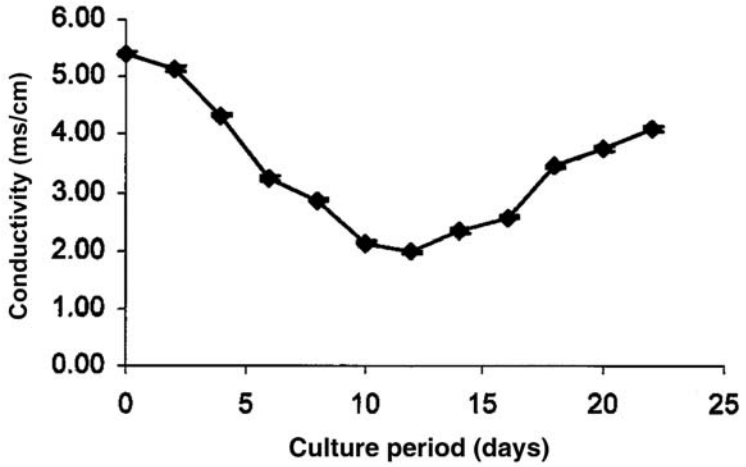

Figure 3. Change of conductivity in the medium of a suspension culture of *Linum album* during a cultivation period of 22 days. All values of mean ± S.D. from three measurements, n= 3.

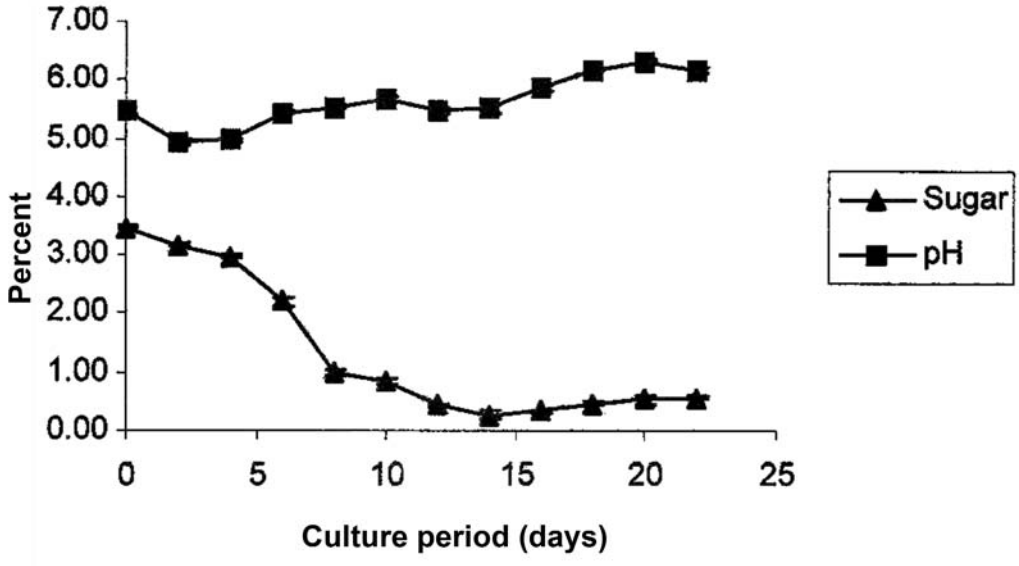

Figure 4. Change of sugar content (measured by refractive index) and pH in the medium of a suspension culture of *Linum album* during a cultivation period of 22 days. All values of mean ± S.D. from three measurements, n= 3.

cultivation period of 11 days and could, therefore, serve as an alternative source for the threatened *Podophyllum* species [13, 14]. The possibility of enhancement accumulation of PTOX has been studied in cultures of cell suspension of *L. album* [15]. Furthermore, there is a great interest to optimize the production of PTOX by selection of high producing lines for the biotechnological production. When the desired compounds are secondary metabolites then the strategy to improve natural product formation in plant cell cultures is screening for plants that highly accumulate the metabolite(s) and initiate *in vitro* cultures from selected high producing parent plants [16]. *L. album* seeds germinate only very slowly [13], which seriously impedes to use the strategy of Zenk *et al*. [16] for selecting high producing cell suspension cultures. Our study consisted of development of simple and rapid method to overcome seed dormancy, as well as spectroscopic analysis of aryltetralin lignans in different intact plant parts and *in vitro* cultures of *L. album.*

2. Materials and methods

2.1. Plant material

Seeds of *L. album* Ky. ex Boiss. and of *L. nodiflorum* L. (Linaceae) were collected from the natural location Yasuj (1650 m altitude) and Darestan forest, Roudbar (900 m altitude), Iran in August and June 2001, respectively. *L. album* plant materials were collected in June 2002 from Lashkarak road, Tehran, Iran at an altitude of 1800 m. Plant samples were identified by Iraj Mehregan, and voucher specimens were deposited at the herbarium of Faculty of Pharmacy, Shiraz University of Medical Sciences.

L. album plant parts (20-50 cm high) (Figure 2) were separated as the followings (Table 1): (1) Shiny brown mature seeds which were collected in June 2001. (2) More or less spherical capsules -containing immature seeds- are 4-6 mm long and 4-5 mm wide. (3) Inflorescence dichasium is 10-20 cm long with 5-8 pedicellate, heterostylus flowers. Sexual organs (gynoecium and androecium) were separated from the other flower parts for the analysis. (4) Unguinate white petals are 25-35 mm long. (5) Linear lanceolate sepals are 10-12 mm long and 1.5-2.5 mm wide and 2.5 times as long as capsules. Sepals are 1-veined with ciliated membranous margins. (6) Alternate 1-veined leaves that sometimes

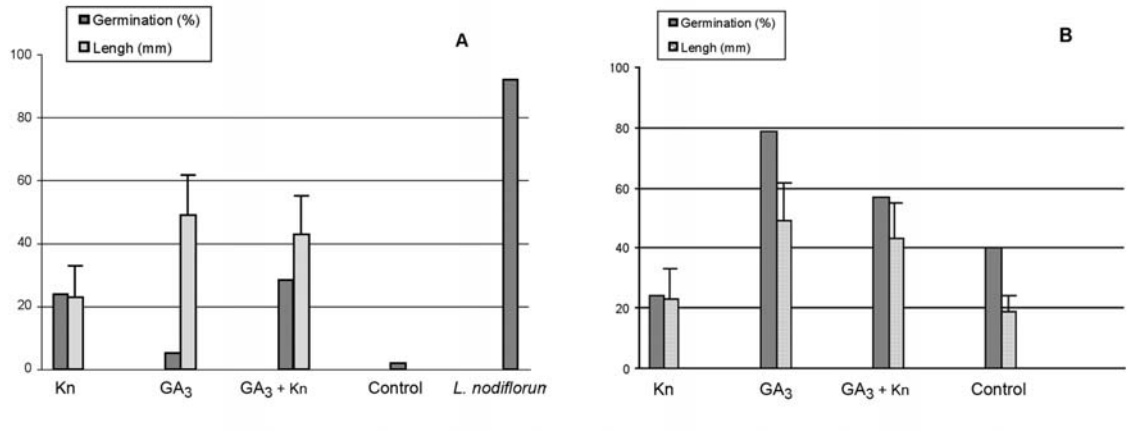

Figure 5. Effect of different treatments on *Linum album* seed germination (%) and seedling length (mm). A: Unscarified group. B: Scarified group.

have glandulas stipule are in two forms: upper leaves, 15-25 mm long and 2-5 mm wide, oblong-lanceolate, acute, with smooth cartilaginous margin and lower spatulate to lanceolate shaped leaves that is 11-15 mm long and 2-4 mm wide, with smooth and cartilaginous margins. (7) Apical leaves on the lateral branches. (8) Branches. (9-11) Stems which is white and branched at base and glaucous upwards were divided into upper, middle and lower parts. (12-14) Rhizomes were divided into upper, middle and lower parts.

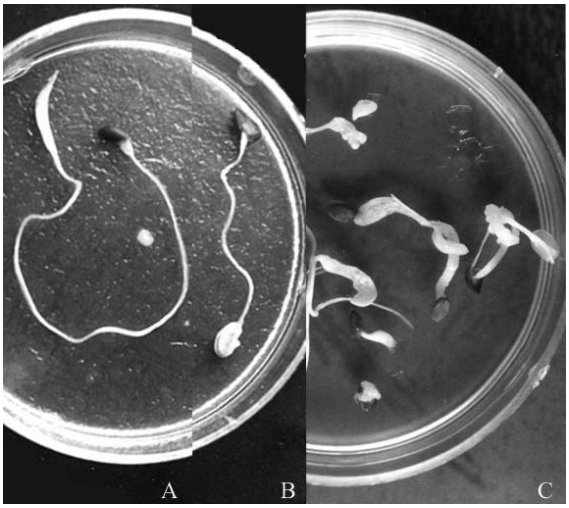

Figure 6. *Linum album* seedlings (4 weeks old). A: A fast germinated and enlarged seedling on exposure to 500 mg/l GA_3. B: A seedling on exposure to 100 mg/l Kn. C: Dwarf seedlings resulted from seed scarification.
All values of mean ± S.D. from three measurements, n= 3.

2.2. In vitro cultures

2.2.1. Seed germination

Seeds have been taken at random by mixing to avoid unreliable test results. Examined seeds were divided into two scarified and unscarified groups. Each group of seeds was treated separately for 12 h by gibberellic acid (GA_3) 500 mg/l, and kinetin (Kn) 100 mg/l and GA_3 500 mg/l + Kn 100 mg/l solutions followed by surface sterilization, using ethanol (96 %) for 1 min. and sodium hypochlorite (1 %) for 9 min.; finally rinsed three times with sterile distilled water. Two to three sterile seeds culture were put on to autoclaved (1.2 kg cm^2 pressure and 121 º C for 20 min.) agar medium (0.8 % agar w/v water containing coconut water 15 % v/v) in Petri dishes (50 mm diameter, 15 mm height). Scarified and unscarified seeds without hormonal treatment were used as controls. All cultures were incubated at 25 ± 2 ºC under dark condition. Seedling length and germination percentage were determined in all groups after 30 days. *L. nodiflorum* seeds were used as a control for germination rates of *L. album* normal germination. A minimum of 70 replicates (seeds) per treatment were raised. As the embryo is found at the distal end, mechanical scarification was achieved by abrading the lateral surface of the seeds. The results are presented as mean length ±

standard deviations (SD). Data analysis was performed using ANOVA test ($p< 0.05$).

2.2.2. Calli and cell suspension cultures

Plant leaves or capsules were surface sterilized using ethanol (96 %) for 30 min., sodium hypochlorite (1 %) for 7 min. and rinsed three times with sterile distilled water. Calli cultures were initiated from plant leaves, capsules, seedling's hypocotyl or stem on MS (Murashige and Skoog) basal medium [17] supplemented with sucrose 3%, α-naphthaleneacetic acid (NAA) 1 mg/l, Kn 0.5 mg/l, 2,4-dichlorophenoxyacetic acid (2,4-D) 0.5 mg/l and were solidified with agar 0.9% at pH 5.6 before autoclaving. Calli cultures were subcultured every 4 weeks. Suspension culture was initiated by transfer of callus tissues into liquid MS medium (as above, but without agar; 50 ml in 300 ml Erlenmeyer flasks) and wasere incubated on a rotary shaker (120 rpm). Cells (5 g) were subcultured in the same medium every 9-10 days and cultures were maintained at 25 ± 2 °C under permanent darkness. For the characterization of cell suspension cultures, parallel flasks of the cultures were inoculated and cultivated as above. Samples were taken during a cultivation period of 22 days. In the cell-free medium, conductivity (Figure 3), pH and sugar content (determined by refractive index) (Figure 4) were measured with a refractometer and the appropriate electrodes, respectively.

2.2.3. Cell immobilization

L. album biomass was immobilized within luffa sponge disc obtained from matured dried fruit of *Luffa cylindrica*. The sponge was cut into discs of 2-5 cm diameter, 2–3 mm thick, soaked in boiling water for 30 min., thoroughly washed under distilled water, changed three to four times. The luffa discs were put in MS medium including hormones (as above, 50 ml in 300 ml Erlenmeyer flasks) and autoclaved. Each of these flasks was inoculated with 5 g, 2 weeks old callus or cell cultures of *L. album* and incubated for 3 weeks on a rotary shaker (90 rpm). Cultures were maintained at 25 ± 2 °C under permanent darkness.

2.3. Extraction and determination of lignans

Separated explants, calli tissues, suspension or immobilized cells were freezed immediately and then lyophilized by freeze dryer. Fine powders (200 mg) were extracted with ethanol (2 ml) in an ultrasonic bath (two times for 30 seconds with cooling ice for 90 seconds in between). Distilled water (6 ml) was added and pH was adjusted to 5.0 by *o*-phosphoric acid. After adding β-glucosidase (1 mg), the samples were incubated at 35 °C for 1 h. Ethanol was added and the mixture incubated for another 10 min. at 70 °C in an ultrasonic bath [18]. After centrifugation the supernatant was used for HPLC analysis using Waters instruments equipped with a UV detector in 290 nm, a column of 250 mm long and 4.6 mm inner diameter filled with Nucleosil 100 C_{18}, and a particle size of 5 μm. The flow of the solvent was 0.8 ml/min. and an isocratic system was with water (A) and acetonitril (C) as eluents by the ratio 55:45. α-PLT, PTOX, β-PLT, 5'-DMPTOX and MPTOX were determined at R_t 5.98, 8,35, 9.20, 11.32 and 11.73 min., respectively. Calibration curve for PTOX and β-PLT were drawn in the range of 0.3-300 μg/ml and 6.4-64 μg/ml, respectively. 5'-DMPTOX and MPTOX were quantified according to PTOX standard (Roth 3946.1) [19], and α-PLT was quantified according to β-PLT standard. Accuracy of each quantified lignan was admitted by retention time, co-chromatography with standard samples [10], and measuring on-line UV spectra using a Thermo Quest HPLC system (Egelsbach, Germany) equipped with a spectra system KO 6000 LP photodiode array detector.

3. Results and discussion

3.1. Seed germination

Germination of *L. album* seeds was examined using a medium solidified with agar and supplemented with coconut water. Under these conditions germination of *L. album* seeds was negligible, even after months. Seeds of *L. nodiflorum* which belong to the same section (*Syllinum*) as *L. album* [6] were used as control. Under the same conditions up to 92 % of these seeds germinated. In order to overcome seed dormancy, hormonal treatment and scarification were examined. *L. album* seeds were divided into two groups (unscarified and scarified) and each group of seeds was treated by GA_3, Kn and their combination. Untreated seeds were included as a control in each group. Germination rate (%), average seedling length and its morphology were measured [20]. The germination rates of unscarified seeds in presence of GA_3 and GA_3 + Kn were 5% and 28.5%, respectively. Pretreatment by scarification resulted in the maximum germination rates of 78.6% and 57%, respectively (Figure 5 B). Obviously, the seed coat of *L. album* presents a barrier to the absorption of GA_3 that the role of GA_3 by itself in such a case was not significant (Figure 5 A). Scarification, however, significantly enhanced the GA_3 effect, indicating that the effectiveness of GA_3 was related to its ability to penetrate the seed coat and overcoming dormancy (Figure 5 B) as reported earlier [20, 21]. It appears that treatments could be used truly in succession to overcome dormancy. Seedlings originating from the seeds which received GA_3 treatment grew more rapidly and were larger (Figure 6 A). The important role of plant growth regulators such as GA_3 in promoting seed germination has been indicated by several observations [20]. The germination rates in exposure to Kn with and without scarification were 23.8 % and 24 %, respectively. It has been reported that cytokinins facilitate germination and has a permissive role in GA_3 activation, in combination treating [22]. However the seedlings were significantly smaller than those in the presence of GA_3. Scarification without hormonal treatment permitted *L. album* seeds to germinate (up to 40 %, see Figure 5 B). Under this condition the minimum length was *ca* 1.9 mm. Unscarified control seeds did not show any significant response even after months. One may say, the lower germination yield (23.7%) in scarified seeds treated with Kn in comparison to the control (40%), would be due to the natural tendency to forming callus. Morphological manifestation showed some dwarf seedlings in the scarified control group. Furthermore pricking the seeds from the middle part resulted in hypocotyls protrusion only (Figure 6 C). The radical began to protrude at the apical region approximately 72 h and 48 h after cultivating time for unscarified and scarified groups, respectively, and the residual seeds not germinated did so within the next 72 h. Our best findings for *L. album* seed germination was a treatment comprising scarification followed by 12 h 500 mg/l GA_3 exposure (Figure 5 B). The results suggest that at least 2 factors will accelerate the germination of *L. album* seeds: treatment with GA_3 and scarification of impervious seed coat which may have a role to overcome chemical and mechanical dormancy. Root lengths in all hormonally treated groups were shorter than normal roots. The simple treatment protocol described here will reduce *L. album* seed germination starting time and increase germination yield, seedling length and available biomass which in turn will facilitate biotechnological works on *Linum* species as a source of lignans used in medicine [3, 18].

3.2. Lignans in L. album organs

The percentage content of PTOX and related lignans in different parts and growth stages of *L. album* are summarized in Table 1. The results show a variation in the distribution and relative abundance of lignans extracted from plant parts. α-PLT, PTOX and MPTOX appear to be common to all parts studied. Their concentrations, however, varied in plant tissue parts. Upper plant parts, petals, leaves and productive organs showed to be more productive for PTOX (0.271-0.331%) than MPTOX (0.042-0.121%). In

Table 2. Content of podophyllotoxin and related aryltetralin lignans (% mg/g dry wt.) in *Linum album in vitro* cultures [a].

Plant part	α-PLT	PTOX	β-PLT	5'-DMPTOX	MPTOX
Calli cultures					
LO	0.121±0.021	0.081±0.003	-	t	0.091±0.004
CO	1.310±0.300*	0.281±0.032	-	0.331±0.021*	0.370±0.031*
SHO	0.251±0.030	t	-	0.051±0.002	0.290±0.033*
SSOS	0.081±0.003	-	-	t	t
Suspension culture	0.931±0.041	0.190±0.032	0.061±0.002	0.028±0.003	0.170±0.045
Immobilized cultures					
CAO	1.410±0.302*	0.280±0.022	0.093±0.003	0.041±0.002	0.213±0.050
SO	1.660±0.421*	0.301±0.022*	0.043±0.002	0.061±0.002	0.213±0.034

[a]All values of mean ± S.D. from three measurements. α-PLT, α-peltatin; PTOX, podophyllotoxin; β-PLT, β-peltatin; 5'-DMPTOX, 5'-demethoxy-6-methoxypodophyllotoxin; MPTOX, 6-methoxypodophyllotoxin. LO, Leaf originated; CO, Capsule originated; SHO, Seedling's hypocotyl originated; SSO, Seedling's stem originated; CAO, Callus originated; SO, Suspension originated. 5'-DMPTOX and MPTOX were quantified according to PTOX and α-PLT was quantified according to β-PLT. t <0.01. -, no peak at the retention time of this compound was detected. * p <0.005; ANOVA test.

contrast, underground parts and stems yielded MPTOX (0.180-0.251 %) more than PTOX (0.031-0.140 %). It has been concluded for *L. flavum* that roots and stems are important accumulation sites for MPTOX [19].

Green capsules contained the highest amount of PTOX (0.651 %), MPTOX (0.670 %) and 5'-DMPTOX (0.452 %) despite mature seeds which is mainly rich in MPTOX (0.631%). On the whole, the plant parts yielded β-PLT (up to 0.211 %) lower than α-PLT. Differences in the state of maturation of the plants at the time of harvesting may have played a role in the lignan contents [19]. For *Podophyllum peltatum* it has reported that PTOX content strongly depends on the harvesting time of the samples [23]. As it has reported for *P. peltatum*, such diversity of results suggests that the presence of podophyllotoxin and related compounds in the various parts of *Linum album* is influenced by complex biological, geographical, and environmental variables as yet unstudied [24]. *L. album* as well as *L. persicum* species belong to the *Syllinum* section produce and accumulate aryltetralin lignans [25-27]. Besides, it has been reported that *L. mucronatum* belonging to the subgroup b of the section *Syllinum* plant is rich in MPTOX [28]. Some other *Linum* species, however, have been shown to accumulate lignans of different classes [18].

Finding the high yielding tissues for PTOX and its congeners will be useful to observe responsible genes encoding these compounds and to find the main production and storage sites of metabolites; optimizing the production of podophyllotoxin by selecting explants with high producing lines.

3.3. Lignans in L. album in vitro cultures

Suitable organs for callus initiation which accumulate high levels of PTOX or MPTOX (Table 1), i.e. capsules and leaves were selected. Besides, calli cultures were established from seedling's hypocotyl and stems (Table 2). HPLC analysis showed a variation in the lignan pattern of the different lines of calli cultures. However, such cultures were more productive for MPTOX than PTOX.

Cell suspension culture was established from high productive calli tissues. A general view of the changes of medium is monitored by determination of pH, and conductivity as a measure of all ionic components of the medium (Figures 3 and 4). The sucrose fed to the suspension cultures is completely consumed within 14 days (Figure 4); the refractive index never reaches zero, since other medium components interfere with the measurement. The increase of conductivity after day 12 of the culture period is caused by the release of internal substances from cells

(Figure 3). The uptake of ions from the medium as well as the release of intracellular substances to the medium causes a continuous change of the medium pH (Figure 4) [14]. The resulting cells accumulate comparable amounts of PTOX and MPTOX. Furthermore, callus as well as suspension originated immobilized cultures were established. The resulting cultures were more productive for PTOX than MPTOX (Table 2). The highest amount of PTOX (0.301% dry wt.) was found in suspension originated immobilized cells. Considerable amounts of α-PLT were found in all *in vitro* cultures.

Oriented organ and corresponding *in vitro* culture screening for lignans followed by high productive cell line selection, will guide us to suitable *in vitro* culture for the production of aryltetralin lignans in the large scale.

Acknowledgements

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