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Biotechnological Approaches for the Production of Lignans

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ABSTRACT
The lignans are a large group of natural substances, which occur in a range of plant species, but only in low concentrations. Aryltetralin lignans are lead substances for the semi-synthetic anticancer derivatives etoposide, teniposide and etopophos. Their natural abundance however is scarce and their chemical synthesis is not yet economically feasible. The aim of this review is to focus on recent progress of *in vitro* production of lignans, together with the methods of increasing the levels of desired substances in plant cell and tissue cultures in general. Experience of different authors, working worldwide on plant biotechnology, has been discussed to show positive results in experiments with optimization of cultural conditions, selection of high producing cell lines, development of differentiated and transformed cultures, influence of stress factors and feeding of precursors in plant *in vitro* cultures for the enhancement of production of lignans. Knowing and evaluating the results of different factors, may serve for creating a complex impact model for increasing the production of target substances of *in vitro* cultures.
KEYWORDS: lignans, podophyllotoxin derivatives, *in vitro* production, recombinant CYP3A4

INTRODUCTION

Lignans are a large group of phenolic compounds defined as ßß'-dimers of phenylpropane (C_6C_3) units. This widely spread group of natural products possess a long and remarkable history of medicinal use in the ancient cultures of many peoples. The first unifying definition of lignans was made by R. D. Howarth in 1936, who described them as a group of plant phenols with a structure, determined by the union of two cinnamic acid residues or their biogenetic equivalents (1). According to IUPAC nomenclature, lignans are 8,8'-coupled dimmers of coniferyl alcohol or other cinnamyl alcohols (2).

Lignans have a long history of medicinal use as the first records date back over 1000 years (3). The roots of wild Chervil (*Anthriscus sylvestris* L. *Apiaceae*), containing several lignans, including deoxypodophyllotoxin, were used in a salve for treating cancer (4). Another source from 400-600 years ago reveals the use of the resin, derived from an alcoholic extract of the roots and rhizomes of *Podophyllum* perennials as a catharctic and poison, both by the natives of the Himalayas and the American Penobscot Indians of Maine (5). Throughout the years, lignan-containing plant products were used for a wide number of aliments in Chinese medicine – roots of *Kadsura coccinea*, Hance. ex Benth. *(Schizandraceae*) for treatment of rheumatoid arthritis, gastric and duodenal ulcers (6), Japanese – *Fraxinus Japonica* Blume ex K. Koch. (*Oleaceae)* (7, 8) – diuretic, antipyretic, an analgesic and antirheumatic agent. The bark of *Olea europaea* L. (*Oleaceae)* has been studied (9) for its antipyretic, antirheumatic, tonic and scrofula remedy actions.

The aryltetralin lignan podophyllotoxin (PTOX) is a natural occurring lignan derived, as previously mentioned, from the roots and rhizomes of the Himalayan *Podophyllum hexandrum* and the American *Podophyllum peltatum* L. (*Podophyllaceae ~Berberidaceae)*. It is currently being used as a lead compound for the semi-synthesis of anticancer drugs etoposide, teniposide, etopophos (fig. 1), which are used for the treatment of lung and testicular cancers and certain leukemias (10, 11). The supply of podophyllotoxin depends mainly on its extraction from roots and rhizomes of *Podophillum hexandrum* Royle (from Himalayas region) and *Podophillum peltatum* L. (North America), which contain 4% and 0.2% of the active substance on a dry mass basis, respectively. Those resources are, however limited, because of the intensive collection of the plants, lack of cultivation and the long juvenile phase and poor reproduction capacities of the plant (12). Podophyllotoxin and related compounds (fig. 2) are not only present in *Podophyllaceae*, but also in *e.g. Juniperaceae, Lamiaceae* and *Linaceae* (13). A detailed phytochemical analysis of the lignans in the *Linaceae* will be done in the groups of T.J. Schmidt (Münster, Germany) and A.W. Alfermann (Düsseldorf, Germany). Genera in which abundance of PTOX has been reproted are *Linum* (*Linaceae*) (14-18, 47, 52, 67, 69, 136, 137, 145, 153). *Juniperus* (*Cupressaceae*) (19-21), *Hyptis* (*Lamiaceae*) (22), *Thymus* (*Lamiaceae*), *Teucrium* (*Lamiaceae*), *Nepeta* (*Lamiaceae*) (23), *Dysosma* (*Berberidaceae*) (24), *Diphylleia* (*Berberidaceae*) (25), *Jeffersonia* (*Berberidaceae*) (26) *and Thuja* (*Cupressaceae*) (27). General approaches to the chemical synthesis of podophyllotoxin derivatives (28) and chemical synthesis of lignans (29) have been proposed, however, an efficient commercially viable rout to the synthesis of podophyllotoxin is still to be sought.

Production of lignans from in vitro cultures

Lignans occour in many plant species, but only in low concentrations. The biotechnological part focuses on alternative production systems for these natural compounds,

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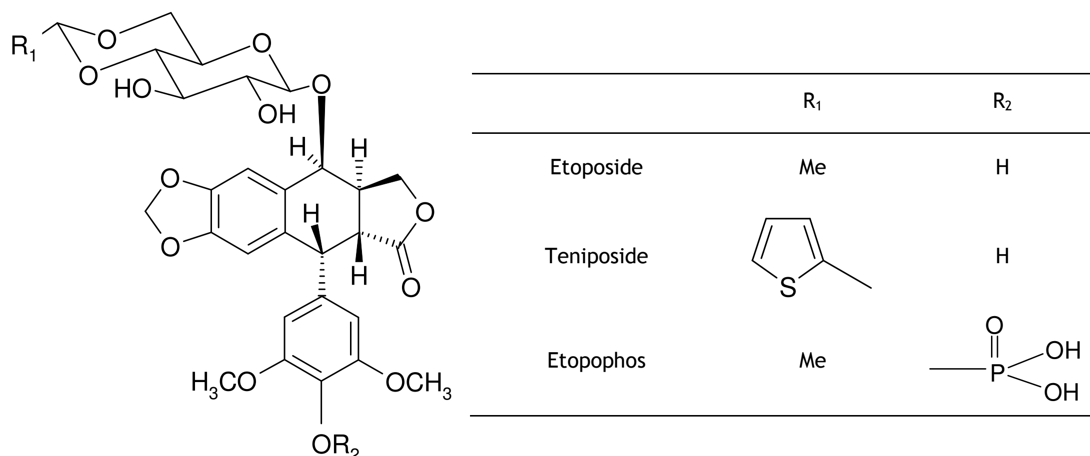


Fig. 1. Structures of Eto
poside, Teniposide and Etopophos

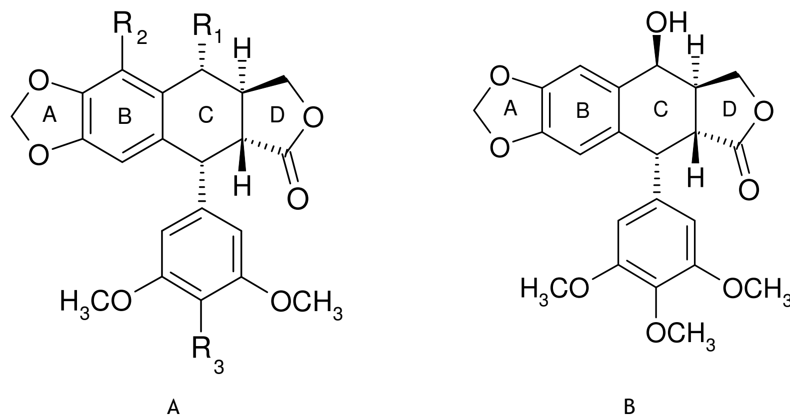
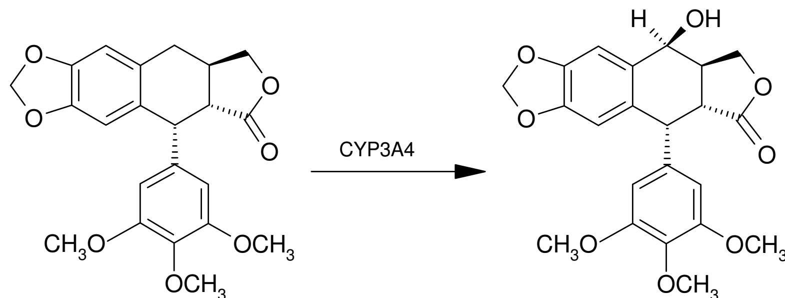


Fig. 2. Podophyllotoxin (R_1=OH, R_2=H, R_3=OCH_3), Deoxypodophyllotoxin (R_1=H, R_2=H, R_3=OCH_3), β-peltatin (R_1=H, R_2=OH, R_3=OCH_3) - A, Epipodophyllotoxin - B



Deoxypodophyllotoxin Epipodophyllotoxin

Fig. 3. Stereoselective hydroxylation of deoxypodophyllotoxin by recombinant CYP3A4 in Escherichia coli to epipodophyllotoxin

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because the plant in vitro cultivation has several advantages over collecting plants from fields (30). Growing plant cells permit a stricter control of the quality of the products as well as their regular production without dependence on the variations of natural production resulting from climate and socio-political changes in their countries of origin. Problems connected with gathering, storing (in special conditions), processing and disposal of huge amounts of biomass, connected with extraction of active substances from *in vivo* plants are also solved. Suspension cultures are of special interest due to their high growth rate and short cycle of reproduction. Another advantage is the fact that undifferentiated plant cells, maintained in a liquid medium possess a high metabolic activity due to which considerably high yields of secondary products can be achieved in short terms (from one to three weeks of cultivation). This raises the question of investigation of *in vitro* cultures of new plant species for the production of podophyllotoxin derivatives (31). In Table 1, the accumulation of lignans in plant tissue and organ cultures has been summarized.

Although there are many examples for the synthesis of PTOX and its derivatives in plant cell and tissue cultures, the *in vitro* production still has to cope with multiple tasks for the purpose of finding economically feasible paths for enhancing production. Means for increasing the productivity of in vitro cultured plant cell

Optimisation of conditions of cultivation. The most widely explored growth media compositions are those of Murashige and Skoog (84), White (85), Linsmaier and Skoog (86) and Gamborgh *et al*. (87). When discussing optimization of growth media parameters, one must keep in mind that growth and secondary product synthesis are in many cases not correlated and have different requirements. Achievement of a formula, leading to higher production of the active compound is usually empirically reached and is specific in every case. Generally speaking, factors, connected with culture media are: media components (carbon source, mineral salts, vitamins), phytohormones (growth regulators), pH, temperature, aeration, agitation, light, *etc*. Agar has been shown to stimulate shikonin accumulation in *Lithospermum erithrorizon* Sieb. et Zucc. (*Boraginaceae)* suspension cultures (88). Interactions between minerals in the medium and/or between minerals and the agar matrix have been reported to influence mineral availability and uptake (89).

Carbon source. Usually sucrose or glucose is used. Examples in literature show active substances' levels enhancement when sucrose content was increased – cell suspension cultures of *Salvia officinalis (Lamiaceae)* increased rosmarinic acid yield from 0.7 to 3.5 $g.L^{-1}$ when 5% of sucrose was used, compared to 3% (90).

Macronutritiens. Generally nitrate, potassium, ammonium and phosphate are regarded to have the highest influence on cell growth. In many cases depletion of these nutritiens leads to an enhancement of secondary product formation (91).

Phytohormones. Examples have been given about auxins, having differing effects on secondary product formation. There are examples about repressing the respective synthesis by means of addition of 2,4-dichlorophenoxyacetic acid (2,4-D): for example of berberine (92), antraquinones (93), indole alkaloids (94). This might be explained by dedifferentiation of the plants to cells induced by auxins. For this reason auxins are usually added for callus induction and applied either at low concentration, or omitted for metabolite production (95). Often formulation of two-stage growth media is used – one for optimal growth and one for secondary product formation: shikonin by cell cultures of *Lithospermum erythrorhison* Sieb. et Zucc. (96), rosmarinic acid by *Coleus blumei* Benth. *(Lamiaceae)* (97), berberine by cell cultures of *Coptis japonica* (Thunb.) Makino. *(Ranunculaceae)* (98).

Temperature, light, oxygen, pH. In vitro cultures are usually maintained at a temperature range of 25-30 °C. Lowering the cultivation temperature of cell suspension cultures of *Catharanthus roseus (Apocynaceae*) resulted in increase of total fatty acid content per cell dry mass (mainly because of increase of unsaturated C18 acids); this however did not affect the content of indol alkaloids (99). Comparison of biomass to rosmarinic acid formation of cell suspension of *Lavandula vera* DC. *(Lamiaceae),* showed maximum biomass formation at 30 °C, and highest synthesis at 28 °C (100).

According to light conditions, *in vitro* cultures are photoautothrophic, (which are grown in light and thus commit photosynthesis), heterotrophic and photomixotrophic (which are grown fully or partially in the dark, and require carbohydrate source already mentioned above). The comparison of fatty acid composition of the three types of suspension cultures in *Euphorbia characias* L. *(Euphorbiaceae)* compared to those of seeds, leaves and calli, from which they were derived, showed highest values of growth during exponential phase of photosynthetic cell suspensions (101).

Proper ventilation and oxygen supply are factors, which could be modified for cell cultures maintained in a bioreactor. It is important to avoid overoxygenation of the cells. Higher oxygen supply has shown to improve alkaloid formation in *Catharanthus roseus* l. *(Apocynaceae*) (102). The pH of the medium is usually maintained in an interval between 5-6 prior to autoclaving the growth media.

Immobilization of plant cells has been explored due to the advantages of higher synthetic rates, longer production period and possible re-use of cells - for example Shikonin production by *Lithospermum erythrorizon* Sieb. et Zucc. (*Boraginaceae)* (103). This method could prove to be very effective.

Stimulation of excretion of active substances into the medium. For a limited number of active substances, which serve as defense compounds, it is natural to be excreted extracellularly in the medium, but most often they are stored within the plant cell, which is genetically determined. Excretion of the target substance into the medium could lead to improving the efficiency of plant *in vitro* cultures as a source of useful metabolites. There are reports in literature about some approaches for achieving this (91):

1. Chemical methods of permeabilization of the plant cell like the addition of dimethylsulfoxide [*Coleus blumei* Benth. *(Lamiaceae)* (104)] and other organic solvents like chloroform and propanol. This kind of treatment is most often not survived by plant cells.

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2. Solid absorbents as amberlite XAD-4 and XAD-7 resins, added to the medium act in ways of shifting the equilibrium concentrations and thus increase the overall production (105).
3. Some physical methods as higher temperatures (106), electrical permeabilization (107) and ultrasonification (108). The significant increase of extracellular taxol or taxol release, together with increase of production of taxol was reported in a *Taxus chinensis* (Pilg.) Rehder. *(Taxaceae)* cell culture due to ultrasound impact (109).
4. Formation of a two-phase medium, by means of adding water-immiscible solvents like hexadecane in medium of *Morinda citrifolia* L. *(Rubiaceae)* cultures (110) has also been reported.
5. Optimization of the factors mentioned above has proven to lead to the enhanced growth (111) and production (112) of the active compounds in many *in vitro* cultures.

Selection of high producing strains. The selection of high producing cell culture strains can be made by eye when coloured products like anthocyanins, shikonin or berberine are accumulated, or by other analytical methods. A strain of *Euphorbia milli* L. (Euphorbiaceae) was reported to accumulate seven fold higher levels of anthocyanins than the parent strain after 24 selections (113). The stability of production of red pigments was proven by means of statistical and cell-pedigree analysis. Repeated cloning using cell aggregates of *Coptis japonica* Makino. (Ranunculaceae) led to obtaining of faster growing and higher berberine producing strain which was further cultivated in a 14L bioreactor (114). It was very stable and produced high levels even after 27 generations. For selection of a free biotin producing cell line of green *Lavandula vera* DC. (Lamiaceae), pimelic acid, a precursor of biotin was used, as selecting agent, and cells were cultured in the light (115).

It was reported that hypericin production was higher in liquid cultivated cell aggregates than in shoots or callus. Suspension cultures with compact globular structures were found to have a higher total content of hypericin and pseudohypericin than unorganized cell suspension cultures (116). Observations of enhanced second metabolite synthesis of compact globular structures were made on *Catharanthus roseus* L. *(Apocynaceae*) - Madagascar periwinkle and *Rhodiola sachalinensis* Boriss. (*Crassulaceae),*- arctic root (117, 118).

Use of differentiated root cultures. Although undifferentiated suspension cultures are very “convenient” for the development of secondary metabolites production, with their high growth rates and many possibilities to enhance it, mentioned above, there is one very significant drawback. Although the plant cell is considered to be totipotent, the biosynthesis of many second metabolites requires a certain level of differentiation of the tissues. Experiments have proven that although not present in an undifferentiated culture, with the inducement of somatic embryoids, roots or shoots, these compounds are detectable in the plant tissue again (119). The maintenance of a culture in a certain differentiated state is obtained through the phytohormone regime. A certain type of “hairy roots” culture can be induced by means of transformation with a specific soil *Agrobacterium rhizogenes*, and can be further maintained without phytohormones in the medium. They show a logarithmic pattern of growth with doubling times and are characterized by a high degree of branching. As a result of this transformation, bacterial genes, catalizing or interefring with the plant’s phytohormone biosynthesis are integrated into its genome, resulting in the development of specific, transformed “hairy roots”, independent of exogenous phytohormones (120). This growth is associated with the production of the characteristic secondary metabolites that resemble the parent plants. Through the described method a wide range of synthesized metabolites have been reported (120, 121, 122, 151).

Influence of stress factors. According to Brodelius, (123) stress factors exerted on plant cell cultures, influencing the accumulation of secondary metabolites can be divided into the following categories:

1. medium stress (sugar, nitrogen, phosphate, phytohormones),
2. physical stress (light, UV, nitrogen, aeration, osmolarity, pH),
3. chemical stress (heavy metals, abiotic elicitors),
4. infectional stress (pathogens, biotic elicitors).

A significant increase in intracellular accumulation of taxuyunnanine C (Tc) in *Taxus chinensis* (Pilg.) Rehder. *(Taxaceae)* suspension cultures has been reported to be enhanced by pulsed electric field (124). This is considered to be due to an induced defense response of plant cells and a possible alteration of cell membrane’s dielectric properties.

Induction of oxidative stress (as a consequence of cultivation under Fe^{2+} stress) in a *Cupressus lusitanica* Mill. *(Cupressaceae)* suspension culture has been shown to inhibit cell growth, induce lipid peroxidation and cell death and enhance ethylene and β-thujaplicin production (125).

Plants are able, in response to an external stimulus, to react by means of *de novo* synthesis of certain substances, called “phytoalexins” (process, regarded as “elicitation”). They are regarded to be defense compounds against inviding pathogens. Those could be stimulated by biotic or abiotic factors mentioned above. Compounds, already present in the plant cell, can also be stimulated by stress factors. Such a response is very fast (from a several hours to a few days), and often products are even being released into the nutrition medium (91).

Examples of biotic stress are application of pathogen extracts into the medium: increasing of rosmarinic acid production in *Orthosiphon aristatus* (Blume) Miquel. *(Lamiaceae)* suspensions after addition yeast extract (126), accumulation of extracellular capsidiol after application of cell wall fragments of *Phytophtora* spp. *(Oomycetes)* or cellulase from *Trichoderma viride* Pers. ex Fries (*Hypocreaceae)* (127).

For protection from pathogenic microorganisms and herbovours plants have developed inducible chemical defense system. In response to yeast stimulus on suspension cultures of *Rauwolfia canescens* L. *(Apocynaceae)* and *Escholtzia californica* TM.

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Table 1. Lignans in some plant in vitro cultures (31, supplemented Ionkova)

Species	In vitro culture	Lignans synthesized	Ref.
Callitris drummondii	Callus, Suspension	PTOX	32, 33
Daphne odora	Callus, Suspension	Matairesinol, Lariciresinol, Pinoresinol, Secoisolariciresinol, Wikstromol	34
Forsythia x intermedia	Callus, Suspension	Epipinoresinol	35
Forsythia x intermedia	Callus, Suspension	Matairesinol	36
Forsythia x intermedia	Suspension	Pinoresinol, Matairesinol	37, 38
Forsythia spec.	Callus, Suspension	Matairesinol, Epipinoresinol, Phillyrin, Arctigenin	39
Haplophyllum patavinum	Callus	Justicidin B, Diphyllin, Tuberculation, Arctigenin	40
Ipomea cairica	Callus	Trachelogenin, Arctigenin	41, 42
Ipomea cairica	Callus	Pinoresinol	43
Jamesoniella autumnalis	Gametophyte	8', 8,2'-Tricarboxy-5,4-dihydroxy-7'(5')-6'-pyranonyl-7',8'-dihydronaphtalene and its two monomethylesters	44
Juniperus chinensis	Callus	PTOX	27
Larix leptolepis	Callus	Pinoresinol; 2,3-dihydro-2-(4-hydroxy-3-metoxyphenyl)-3-hydroxymethyl-5-(ω-hydroxypropyl)-7-metoxybenzofuran, Lariciresinol, Secoisolariciresinol, Iso-lariciresinol	45, 46
Linum album	Suspension	PTOX, 6MPTOX, DPTOX, Pinoresinol, Matairesinol, Lariciresinol, β-peltatin, α-peltatin	47
Linum altaicum	Cell cultures	Justicidin B Isojusticidin B	17
Linum austriacum	Callus, Suspension, Root, Hairy root	Justicidin B, Isojusticidin B	48
Linum austriacum ssp. *euxinum*	Cell cultures	Justicidin B, Iisojusticidin B	17
Linum africanum	Callus, Suspension	PTOX, DPTOX	50
Linum campanulatum	Callus, Suspension	Justicidin B	51, 52
Linum cariense	Suspension	6MPTOX 5'-demethoxy-6-methoxypodophyllotoxin, and the corresponding 8'-epimers 6-methoxypicropodophyllin, 5'-demethoxy-6-methoxypicropodophyllin	17
Linum flavum	Root	6MPTOX	53, 54
Linum flavum	Suspension, Embryogenic Suspension	6MPTOX	55, 56
Linum flavum	Suspension, Root like tissue	6MPTOX, 5'-demethoxy-6-methoxy-PTOX	57, 58
Linum flavum	Hairy roots	6MPTOX	59
Linum flavum	Hairy roots	Coniferin	60
Linum leonii	Callus,	Justicidin B	51, 61, 62,
Linum leonii	Hairy roots	Justicidin B	151
Linum lewisii	Cell cultures	Justicidin B, Isojusticidin B	17
Linum mucronatum ssp. *armenum*	Shoot, Suspension	6MPTOX, PTOX	63
Linum narbonese	Callus	Justicidin B	51, 61, 62
Linum nodiflorum	Suspension	6MPTOX	65
Linum nodiflorum	Suspension	6-MPTOX, DPTOX, PTOX	66
Linum nodiflorum	Suspension	6MPTOX, PTOX, DPTOX	67
Linum persicum	Callus, Cell cultures	PTOX, 6MPTOX, α- and β-peltatin	68
Linum tauricum ssp. *tauricum*	Callus, Suspension, Shoots	6MPTOX 4'-demethyl-6MPTOX	69, 154

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Picea glechni	Suspension	Pinoresinol, Dihydrodehydrodiconiferil alcohol	70
Podophyllum hexandrum	Callus, Suspension	PTOX	71-75
Podophyllum hexandrum	Embryogenic callus	PTOX	76
Podophyllum peltatum	Callus	PTOX	77, 78
Podophyllum peltatum	Embryogenic suspension	PTOX, DPTOX, 4'- DPTOX	79, 80
Podophyllum species	Callus	PTOX	78
Sesamum indicum	Callus, Suspension, Hairy roots	Sesamin, Sesamolin	81-83

Table 2. Optimisation of lignan in vitro production (31, supplemented Ionkova)

Species, (In vitro culture)	Lignans synthesized	Varying factors with impact on the culture	Results of optimization	Ref.
Callitris drummondii (Suspension)	PTOX-beta-D-glucoside	Illumination	0.02 % in the dark 0.11 % in light	32
Forsythia x intermedia (Suspension)	Pinoresinol Matairesinol	Carbon source	2 % succrose - 0.001 % lignans 6 % succrose - 0.07 % pinoresinol; 0.1 % matairesinol	37
Ipomoea cairica (Callus)	Arctigenin, trace-Logenin	Phytohormones, Carbon source	4 mg/L 2,4-D, 3% maltose, pH 6.4 - 0.03 % lignans	41, 42
Juniperus chinensis (Immobilized cells)	PTOX	Various calcium alginate concentrations	1.8 % Ca-alginate gel - 5-fold increased levels, compared to free cell suspension; maximum excretion of PTOX in the medium *3 % Ca-alginate gel* - (0.21-0.025 $mg.g^{-1}$ dw) 4-5-fold increase 6 % Ca-alginate gel - small amount	134
Linum africanum (Suspension)	PTOX DPTOX	Source of explant material, Light, IAA, NAA, 2,4-D, Cytokinin/Kinetin ratios	Highest synthesis - Kinetin $10ml.L^{-1}$, IAA - $0.4ml.L^{-1}$, 2,4-D - $0.2ml.L^{-1}$; PTOX - increases in dark conditions (no difference between callus and suspension) DPTOX - increases when cultivated in the dark and has higher levels in callus than suspension.	135137
Linum album (Suspension)	PTOX and related lignans	Illumination	0.2 % - dark 0.5 % - light	47
Linum album (Suspension, shake-flasks: 1000ml - 50mL medium, 300mL - 50 mL medium, establishment of bioreactor)	PTOX	Oxygen supply Increasing of shaker speed	Enhancement of PTOX accumulation.	136
L. altaicum (Callus, Suspension)	Justicidin B Isojusticidin B	Dark	justicidin B between 0.92 – 0.96%.	17
L. austriacum ssp. euxinum (Callus, suspension)	Justicidin B	Dark	justicidin B between 0.50 and 0.96%	17
Linum campanulatum (Callus, Suspension)	Justicidin B	Illumination, Kinetin, IAA, 2,4-D, Succrose	Higher lignan content - dark conditions 1.41 % in the dark 0.40 % in light	52
L. lewisii (Callus, Suspension)	Justicidin B	Dark	justicidin B between 0.16 – 0.30%	17
Linum nodiflorum (Suspension)	6MPTOX	Illumination	Light - 0.6 % Dark - trace amounts	67
Linum tauricum *ssp.* tauricum *(Suspension, Shoots, Callus)*	4'-Demethyl-6-MPTOX 6MPTOX	Phytohormones	Suspension - 4 $mg.L^{-1}$ NAA, maximal production of 4'-demethyl-6-methoxy-podophyllotoxin (2.16 $mg.g^{-1}$ dw) Callus - 0.1mg/L 2,4-D, 0.2mg/L	69, 154

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			IAA, 2.0mg/L Kinetin - maximal production of 6-methoxy-podophyllotoxin (3.99 mg/g dw)	
Podophyllum hexandrum (Suspension)	PTOX	Illumination	Light - 0.03 % Dark - 0.09%	71
Podophyllum hexandrum (Callus)	PTOX	Phytohormones	2,4-D + Kinetin - 0.077%	138
Podophyllum hexandrum (Suspension, 3L-stirred-tank bioreactor)	PTOX	Mathematical model for developing nutritient-feeding strategies, low shear conditions in fed-batch modes of operation, prolonging the productive log-phase of cultivation.	Improvement to 48.8 $mg.L^{-1}$ PTOX, corresponing volumetric productivity of 0.80 $mg.L^{-1}$ per day	139
Podophyllum hexandrum (Suspension)	PTOX	pH, Phytohormones, Carbon source, Inoculum	pH 6.0 IAA - 1.25 $mg.L^{-1}$ Glucose 72 $g.L^{-1}$ Inoculum level - 8 $g.L^{-1}$	140
Podophyllum hexandrum (Suspension)	PTOX	Sugar, Nitrogen source, Phosphate	MS medium, NH_4+Salts:Nitrate - 1:2, 60 $g.L^{-1}$ Glucose - highest growth and PTOX accumulation	141
Podophyllum peltatum (Callus)	PTOX	Red light (660 nm) Carbon source Phytohormones	Inhancement of production in red light Sucrose - 0.057 % Maltose - 0.023 % 2,4-D + Kinetin - 0.57 %	77
Rollinia mucosa (Jacq.) Bail. (Callus).	Furofuranic lignans: Epigambin, Magnolin, Epiyangambin	Origin of plant material, Explant type, Growth regulators (2,4-D, NAA, BA, PIC)	Foliar blade explants - biomass, synthesis enhancement; PIC - best biomass production; NAA, 2,4-D - Epiyangambin, Magnolin (dependent on explant source); PIC - Epiyangambin - Calli from foliar blade	142

(*Papaveraceae)* a rapid synthesis of jasmonic acid and methyl jasmonate is observed. Various species tested in cell suspension culture could be elicited with respect to the accumulation of secondary metabolites by exogenously applied methyl jasmonate. Its addition initiates *de novo* transcription of genes, such as phenylalanine ammonia lyase, which is involved in the chemical defense mechanisms of plants (128). Fluoro- and hydroxyl-containing jasmonate derivatives have been reported to enhance production of taxuyunnanine C up to 60% more than by methyl jasmonate in *Taxus chinensis* (Pilg.) Rehder *(Taxaceae)* cell cultures (129). The successful application of those phenomena to *in vitro* cultures for enhancing or inducing the synthesis of useful metabolites requires a precise analysis of the plant cells' response to biotic and abiotic stress (130).

The production of important secondary metabolites of plant *in vitro* cultures is a promising source of their supply. In order to make this feasible, however, problems with the low or even missing production of important metabolites in culture have to be solved. When a certain metabolite is produced *in vitro,* the screening, selection and medium optimization may lead to 20-to 30-fold increase of its levels (131). When the compound of interest is a phytoalexin, different methods of elicitation are applicable. Development of differentiated or modified ("hairy roots") cultures is a solution if the desired metabolite is not inducible in undifferentiated cultures of the respective species. The scale-up of such cultures is not as feasible, however. So another approach to utilize opportunities of plant cell cultures is the feeding of precursors and biotransformation.

Addition of precursors and biotransformation.

As plant cell culture represents a metabolic "factory", with functioning enzymatic systems, cheaper precursors of valuable second metabolites, typical for the given species can be fed to it, resulting in the enhanced synthesis of the desired substance.

One of the first to show feeding of precursors, either primary metabolites or intermediary metabolites of biosynthetic pathways was the group of Zenk (93, 94, 132). Tryptophan was fed as a precursor for indole alkaloids to cell cultures of *Catharanthus roseus* (L.) G. Don, (94) or phenylalanine to cell cultures of *Coleus blumei* Benth. (132). As a result

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enhanced rosmarinic acid byosynthesis was observed. Plant cell cultures are a convenient source of enzymes, which are much more easily extractable and with less risk of denaturation than from the intact plant. This gives the opportunity for performing biosynthetic and/or biotransformation experiments for metabolite production (133). Once extracted, the enzyme can be included in a biochemical reaction.

Increasing the yields of plant cell cultures for the productions of lignans

The above-described methods and approaches have been applied to *in vitro* cultures producing PTOX, its derivatives and other lignans.

Optimization of conditions of culturing, of plant in vitro cultures, for enhancement of lignan production. *Multiple experiments from different working groups have been done for the enhancement of lignan production from in vitro cultures with established synthesis (Table 2).*

Selection of high producing strains. A cell line of *Linum album*, accumulating mainly PTOX at a level of 0.2% on dry mass basis was reported by Empt et al. (143). With this cell line, about 28 mg PTOX can be produced in 1L of culture medium in 11 days (13).

Development of differentiated and transformed cultures.

A fourteen days old hairy root culture of Linum album Kotschy. (Linaceae) was reported to produce about 2,5% of the dry mass basis 6MPTOX (144). A hairy root culture of L. austriacum L. was reported by Mohagheghzadeh et al. (48), which synthesized the arylnaphthalene lignans justicidin B and isojusticidin B. Hairy root cultures of Linum leonii, obatained by genetic transformation using the agropine-type strain Agrobacterium rhizogenes 15834, accumulate justicidin B (151). The products encoded by rol A and rol C genes were found to have a synergistic effect on root induction and to induce increased sensitivity to auxin. The transformation of these genes from A. rhizogenes into the hairy root was checked by PCR (polymerase chain reaction). Proof of transformation was given by the PCR products showing that rol A and rol C genes were present in the hairy roots of L. leonii. Genetically modified hairy roots produced 5-fold higher yields of justicidin B (10.8 mg g-1 DW) compared to untreated callus (61) and show that differentiated roots produce higher amount of secondary compound. This suggests that this technique may be used to enhance the accumulation of justicidin B. In addition to the production, we investigated the cytotoxic effect of justicidin B in three chronic human myeloid leukemia-derived cell lines (LAMA-84, K-562 and SKW-3), that show a lower responsiveness to cytotoxic drugs due to a strong expression of the fusion oncoprotein BCR-ABL (a non-receptor tyrosine kinase). IC50 values of justicidin B were 1.1, 6.1 and 1.5 μM for the chronic myeloid leukemia (LAMA-84), pre-B-cell lymphoma (K-562) and chronic lymphoid leukemia (SKW) cell lines respectively. These IC50 were comparable to the anticancer drug etoposide (a semi-syntehetic lignan derivative).

Influence of stress factors.

The influence of methyl jasmonate has been reported on pinoresinol and matairesinol production in Forsythia x intermedia Zab. (Oleaceae) suspension culture (38). In a medium, containing 2% sucrose, the amounts of pinoresinol was increased eightfold (0.058 ± 0.015mg.g^{-1} dry mass), matairesinol about fivefold (0.029 ± 0.026mg.g^{-1} dry mass). In a medium containing 6% sucrose, pinoresinol was enhanced to 0.086±0.19 mg.g^{-1} dry mass and matairesinol – to 2.24±1.00mg.g^{-1} dry mass. The accumulation of PTOX and 6MPTOX was enhanced about twofold, expressed on dry mass basis in a given cell line of suspension culture of Linum album after the addition of methyl jasmonate - 7.69±1.45mg.g^{-1} dry mass and 1.11±0.09mg.g^{-1} dry mass respectively (145).The PTOX production of Juniperus chinensis L. (Cupressaceae) callus culture was stimulated to 6.4 fold by addition of COS (chito-oligosaccharides), a biotic elicitor with most active component chitopentaose, detected by HPLC (146). The concentration 100μM of the elicitor methyl jasmonate in the nutrition medium of suspension of Linum tauricum ssp. tauricum, leads to substantial increase of the levels of lignans 4'DM-6MPTOX and 6MPTOX from traces, reaching a maximum of 0.1180mg/g dw and 0.1250mg/g dw respectively. The results (154) indicate that addition of extracellular methyl jasmonate can not only increase the biosynthesis of both 4'DM-6MPTOX and 6MPTOX in a L. tauricum ssp. tauricum grown suspension culture, but also change the ratio of both compounds, in comparison with the intact plant and callus cultures, towards the more pharmacologically valuable 4'-DM-6MPTOX (16).

Feeding of precursors and/or biotransformation. Coniferin feeding to in vitro culture to produce PTOX derivatives is a method, which has been reported in literature (147, 148). Levels of PTOX increased 13 to 56-fold after application of coniferin. The initiation of *Linum flavum* L. hairy roots was reported by Han-Wei Lin et al. (149) as a source of coniferin. Significant variation was reported for its accumulation between hairy root lines, originating from different L. flavum seedlings and/or *A. rhizogenes* strains. After culturing the roots in Linsmaier and Skoog medium (LS) with 2,4-D (2,4-dichlorophenoxyacetic acid) and NAA (naphthalenacetic acid) as growth regulators coniferin reached 58 mg.g^{-1} dry mass (60). Another experiment is the cross species co-culture of *L. album* hairy roots, as a source of coniferin, and *Podophyllum hexandrum* cell suspensions (150). Increasing of PTOX concentrations in coculture was observed.

Feeding of coniferin has led to fast increase of pinoresinol content, and no influence on matairesinol synthesis in *Forsythia x intermedia* suspension culture (38). Feeding of phenylalanine and coniferil alcohol in *Juniperus chinensis* callus culture, led to 3.6 and 2.2 fold increase of PTOX, respectively (146). Certain enzymes of podophyllotoxin biosynthetic pathway have been isolated and characterized and podophyllotoxin and 6MPTOX have been established to be stored in the vacuole (66). Elucidation in details of the biosynthetic pathway of PTOX, isolating all enzymes and genes, responsible for their encoding, would lead to new strategies for enhancement of the yields of this valuable substance by means of combination of biotechnological and biochemical methods.

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A novel system for the production of 2,7'-cyclolignans was recently demonstrated (152). Deoxypodophyllotoxin is stereoselectively converted into epipodophyllotoxin by recombinant human cytochrome P450 3A4 (CYP3A4). Cytochrome P450 3A4 (CYP3A4) is the main human metabolizing enzyme. As a new biotechnological alternative, recently we described the successful stereoselective hydroxylation of deoxypodophyllotoxin by recombinant CYP3A4 in *Escherichia coli*. Epipodophyllotoxin (Fig.3) has been detected as the only metabolite in yields up to 90%. (152). Therefore, the heterologous expression of CYP3A4 in *E. coli* presents an interesting alternative for a large-scale production of epipodophyllotoxin.

DISCUSSION

Results, summarized in Table 1 and Table 2, show that a broad range of experiments have been carried out, resulting in enhancement of lignan production of *in vitro* cultures. Cell cultures of different Linum species of section Syllinum are shown to produce considerable amounts of lignans, mainly MPTOX. Although the both PTOX and MPTOX have comparable cytotoxic activity, due to the different substitution in position 6, MPTOX is not used for the production of anticancer drugs (66). Since PTOX is the preferred precursor for the semi-synthesis of anti-cancer drugs like etoposide and etopophos®, the accumulation of predominantly PTOX is especially interesting. *L. tauricum* ssp. *linearifolium* is now beside *L. album and L. persicum* the third *Linum* species of section Syllinum with PTOX as the main lignan (153). Generally increasing of sucrose level has generally shown to increase accumulation of active substances. Auxins, although usually suppressing production might actually enhance it in particular cases as suspension cultures of *Linum tauricum* ssp. *tauricum*. When analyzing influence of different phytohormones one must have in mind that agar in solid media might also play the role of an elicitor for the synthesis of certain compounds as it was discussed above.

Light has shown to improve production in suspension cultures of *Callitris drummondii* (Parl.) F. Muell*., (Cupressaceae) Linum album, L. nodiflorum*, while cultivation in the dark has enhanced production in *L. africanum* (as levels were higher in callus than suspension), *L. bienne, L. campanulatum, Podophyllum hexandrum*. The difference in accumulation shows that light may have an effect for selecting the better growing cells and optimization is need for each new cell line (31). Although plant cells are considered to be totipotent, as every plant cell of a species contains the same genetic information, there are certain experimental data, which show that the choice of an explant might play a differing role on the production of active compounds of the respective *in vitro* culture as in *Rollinia mucosa* Baill. *(Annonaceae)*.

Development of differentiated cultures as a general rule results in higher production of active substances. This approach however is not economically feasible for scale-up of production, as it encounters problems of *in vitro* cultivation and processing of great biomass and longer growth periods than undifferentiated cultures. For this reason it is not focused for *in vitro* cultures producing lignans. An efficient alternative of differentiated cultures are the genetically transformed, hairy roots cultures. Their advantage is the shorter cycle (10 - 14 day), combined with the state of differentiation which makes possible the stable production of active substances.

Stress factors as biotic [Chito-oligosaccharides (COS) in *Juniperus chinensis*] and abiotic elicitors (methyl jasmonate in *Forsythia x intermedia*. *Linum tauricum ssp tauricum*) have been demonstrated to enhance production of lignans.

The feeding of a cheaper precursor as phenylalanine, coniferin and coniferil alcohol in plant cell and tissue cultures of lignan producing plants, results in higher levels of production, as plant cells represent a “ready” and organized system for bioprocessing and synthesis of target compounds.

As a new biotechnological alternative is the successful stereoselective hydroxylation of deoxypodophyllotoxin by recombinant CYP3A4 in *Escherichia coli* to epipodophyllotoxin (152), the direct precursor for the semi-synthesis of anti-cancer drugs etoposide (VP-16) and teniposide (VM-26).

A summary of literature data on effects of different factors on *in vitro* cultures shows that a precise analysis should be carried out and an empirical model created for each concrete species.

CONCLUSION

Due to the pharmaceutical importance and the low content in the plants the present review focuses on alternative production systems for lignans. By this review we do not aim at complete analysis of the *in vitro* production of lignans. We concern mainly the problem of optimization of lignans accumulation in *in vitro* cultures. Accumulation of lignans in plant tissue and organ cultures has been discussed based on the work of different authors. A survey of literature data has shown positive results in experiments with optimization of conditions of culturing, selection of high producing cell lines, influence of stress factors and feeding of precursors in plant *in vitro* cultures for the enhancement of production of lignans. Medium factors as phytohormones, carbon source, macro- and micronutritiens, light or dark conditions, oxygen supply and pH values have been reported to play decisive role in production of lignans from *in vitro* cultures. The influence of each factor on the production of the desired compounds has to be determined empirically and could vary in different cases. The knowing and precise monitoring of the results of the above mentioned experiments could lead to developing of a mathematical model of the factors resulting in enhancement of production of lignans, which is however strictly specific for each plant species. During the last years, experiments of optimization of growth conditions, physical and chemical elicitation, and selection are being carried out in the authors' working group in Bulgaria. Subject of investigation are different *Linum* species with a special focus on Balkan endemits. A model for complex impact is being sought, based on achieved results and current research on cell and tissue cultures.

Abbreviations. - PTOX – podophyllotoxin; 6MPTOX – 6-methoxypodophyllotoxin; DPTOX – deoxypodophyllotoxin; 4'DM-6MPTOX -4'-demethyl-6-MPTOX, 2,4-D – 2,4-dichlorophenoxyacetic acid; NAA – naphthalenacetic acid; BA

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- 6-benzyladenine; PIC – picloram; IAA – indole-3-acetic acid, LS - Linsmaier and Skoog medium; COS - chito-oligosaccharides;

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