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# Lignans in plant cell and organ cultures: An overview

Elisabeth Fuss
*Heinrich-Heine-Universität Düsseldorf, Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Universitätsstr. 1, D-40225 Düsseldorf, Germany (Tel: +49-211-81-14603; Fax: +49-211-81-11466; E-mail: fuss@uni-duesseldorf.de)*

*Key words:* biosynthesis, cell suspension, elicitation, feeding, hairy roots, lignans, micropropagation, plant *in vitro* cultures, root cultures, tracer incorporation

**Abstract**

Lignans are found in a wide variety of plant species. The lignan podophyllotoxin is of special interest, since its derivatives like e.g. etopophos® are used in anticancer therapy. As chemical synthesis of podophyllotoxin is not yet economic, it still has to be isolated from wild growing *Podophyllum* species, some of which are considered to be endangered species. Therefore plant *in vitro* cultures may serve as alternative sources for podophyllotoxin and for other types of lignans as well. This review describes the establishment of plant cell and tissue cultures for lignan production and the experiments to improve product yields by changing the cultivation parameters, addition of elicitors and feeding of precursors. It also summarizes the use of plant cell and organ cultures to study the biosynthesis of lignans on enzymological level.

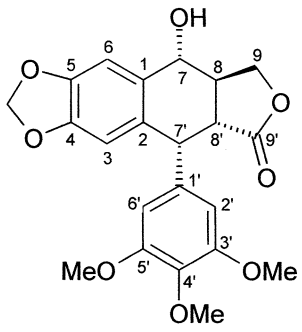
*Abbreviations:* DOP – deoxypodophyllotoxin; LARI – lariciresinol; MATAI – matairesinol; 6MPTOX – 6-methoxypodophyllotoxin; PINO – pinoresinol; PTOX – podophyllotoxin; SECO – secoisolariciresinol

## Introduction

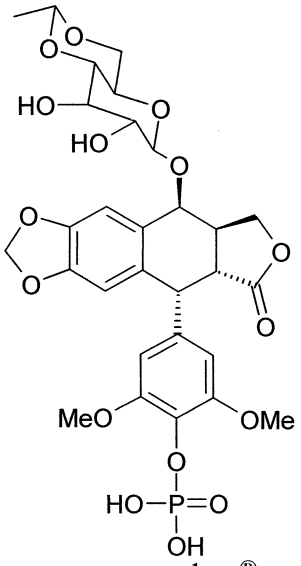
According to the IUPAC nomenclature lignans are 8,8′-coupled dimers of coniferyl alcohol or other cinnamyl alcohols (Moss, 2000). Dimers coupled otherwise are referred to neolignans. Other authors, however, use the term 'lignan' for both types of compounds as well as norlignans, a definition which is followed in this review as well. Further cyclisation or other modifications of the dimers result in a high structural diversity in this class of compounds. The numbering of the C-atoms in the structures is done according to the example of podophyllotoxin (PTOX) shown in Figure 1.

Lignans occur in a wide range of plant families from mosses to the angiosperms. They show a wide variety of biological activities. The lignans secoisolariciresinol (SECO) and matairesinol (MATAI) from e.g. rye or linseed as well as pinoresinol (PINO) and lariciresionol (LARI) are converted by intestinal microorganisms in humans into the phyto-estrogens enterodiol and enterolactone (Figure 1) which are supposed to protect against estrogen-dependent cancers (Adlercreutz, 1995; Heinonen et al., 2001). Semisynthetic derivatives of podophyllotoxin (PTOX) like etopophos® (Figure 1) are used in anticancer therapy. Chemical synthesis of PTOX is not economic on a commercial scale, therefore PTOX is extracted from *Podophyllum* roots and rhizomes. As these plant species can not be cultivated, *P. hexandrum* became an endangered species in India (Imbert, 1998; Giri and Narasu, 2000). Although strategies for the *in vitro* propagation of *Podophyllum peltatum* were established (Moraes-Cerdeira et al., 1998) plant *in vitro* cultivation systems might be an alternative source for PTOX (reviewed by Empt et al., 2000; Petersen and Alfermann, 2001; Arroo et al., 2002).

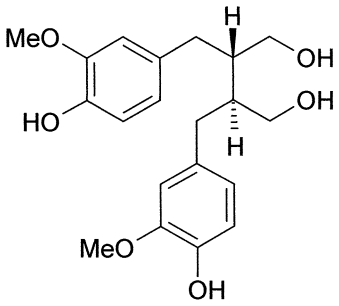
This review gives an overview about the ongoing cultivation of different plant species as cell suspension or organ cultures for the production of PTOX and other lignans. In addition, this review summarizes the elucidation of lignan biosynthesis by feeding experi-



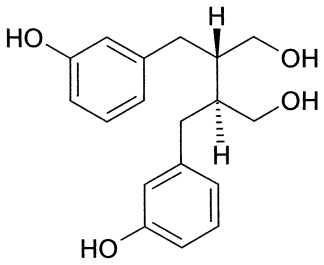

podophyllotoxin



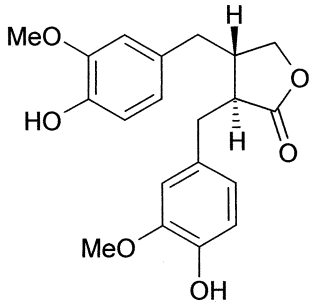

etopophos®



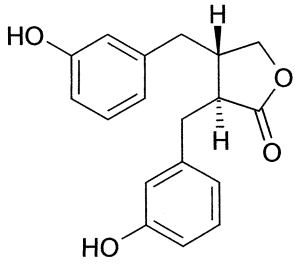

(+)-secoisolariciresinol




(+)-enterodiol




(+)-matairesinol




(+)-enterolactone

*Figure 1.* Structures of some selected lignans and their derivatives.

*Table 1.* Accumulation of lignans in plant cell and organ cultures of different plant species 'without feeding and elicitation experiments'

| Species | Type of culture | Lignans | Reference |
|---|---|---|---|
| *Callitris drummondii* | Callus, suspension | PTOX | Van Uden et al. 1990c; Van Uden and Pras 1993 |
| *Daphne odora* | Callus, suspension | MATAI, LARI, PINO, SECO, wikstromol | Okunishi et al. 2002 |
| *Forsythia* × *intermedia* | Callus, suspension | Epi-PINO | Rahman et al. 1986 |
| *Forsythia* × *intermedia* | Callus, suspension | MATAI | Rahman et al. 1990b |
| *Forsythia* × *intermedia* | Suspension | PINO, MATAI | Schmitt and Petersen 2002a, b |
| *Forsythia* spec. | Callus, suspension | MATAI, epi-PINO, phillyrin, arctigenin | Dewick 1994 |
| *Haplophyllum patavinum* | Callus | Justicidin B, diphyllin, tuberculatin, arctigenin | Puricelli et al. 2002 |
| *Ipomea cairica* | Callus | Trachelogenin, arctigenin | Páska et al. 1998, 1999 |
| *Ipomea cairica* | Callus | PINO | Páska et al. 2002 |
| *Jamesoniella autumnalis* | Gametophyte | 8′,8,2′-Tricarboxy-5,4-dihydroxy-7′(5′)-6′-pyranonyl-7′,8′-dihydronaphtalene and its two monomethylesters | Tazaki et al. 1995 |
| *Juniperus chinensis* | Callus | PTOX | Muranaka 1998 |
| *Larix leptolepis* | Callus | PINO; 2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-methyl-5-(ω-hydroxypropyl)-7-methoxybenzofuran, LARI, SECO, iso-LARI | Nabeta et al. 1991; Nabeta 1994 |
| *Linum album* | Suspension | PTOX, 6MPTOX, DOP, PINO, MATAI, LARI, β-PELT, α-PELT | Smollny et al. 1998 |
| *Linum austriacum* | Callus, suspension, root, hairy root | Justicidin B, isojusticidin B | Mohagheghzadeh et al. 2002 |
| *Linum flavum* | root | 6MPTOX (by an error indicated as β-PELT-A methyl ether) | Berlin et al. 1986, 1988 |
| *Linum flavum* | Suspension, embryogenic suspension | 6MPTOX | Van Uden et al. 1990b, d |
| *Linum flavum* | Suspension, root-like tissue | 6MPTOX, 5′-demethoxy 6-methoxy-PTOX | Wichers et al. 1990, 1991 |
| *Linum flavum* | Hairy roots | 6MPTOX | Oostdam et al. 1993 |
| *Linum mucronatum spp. armenum* | Shooty suspension | 6MPTOX, PTOX | Konuklugil et al. 2001 |
| *Linum nodiflorum* | Suspension | 6MPTOX, PTOX, DOP | Konuklugil et al. 1999 |
| *Picea glehni* | Suspension | PINO, dihydro-dehydrodiconiferylalcohol | Nabeta et al. 1994 |
| *Podophyllum hexandrum* | Callus, suspension | PTOX | Van Uden et al. 1989, 1990a; Giri and Narasu 2000, 2001; Chattopadhyay et al. 2001 |
| *Podophyllum hexandrum* | Embryogenic callus | PTOX | Sharma et al. 2000 |
| *Podophyllum peltatum* | Callus | PTOX | Kadkade 1982, Fujii 1991 |
| *Podophyllum peltatum* | Embryogenic suspension | PTOX, DOP, 4′-demethoxypodophyllotoxin, podophyllotoxone | Kutney et al. 1991, 1993 |
| *Podophyllum* spec. | Callus | PTOX | Fujii 1991 |
| *Sesamum indicum* | Callus, suspension, hairy root | Sesamin, sesamolin | Khanna and Jain 1973; Mimura et al. 1987; Ogasawara et al. 1998 |

*Table 2.* Lignan content (mg/g dry weight) in cultures of *Linum austriacum* (Mohagheghzadeh et al., 2002)

| Compound | Callus tissue | Cell suspension | Root | Hairy root |
|---|---|---|---|---|
| Justicidin B | 2.9 | 6.7 | 12.5 | 16.9 |
| Isojusticidin B | 0.4 | 1.3 | 7.4 | 2.5 |

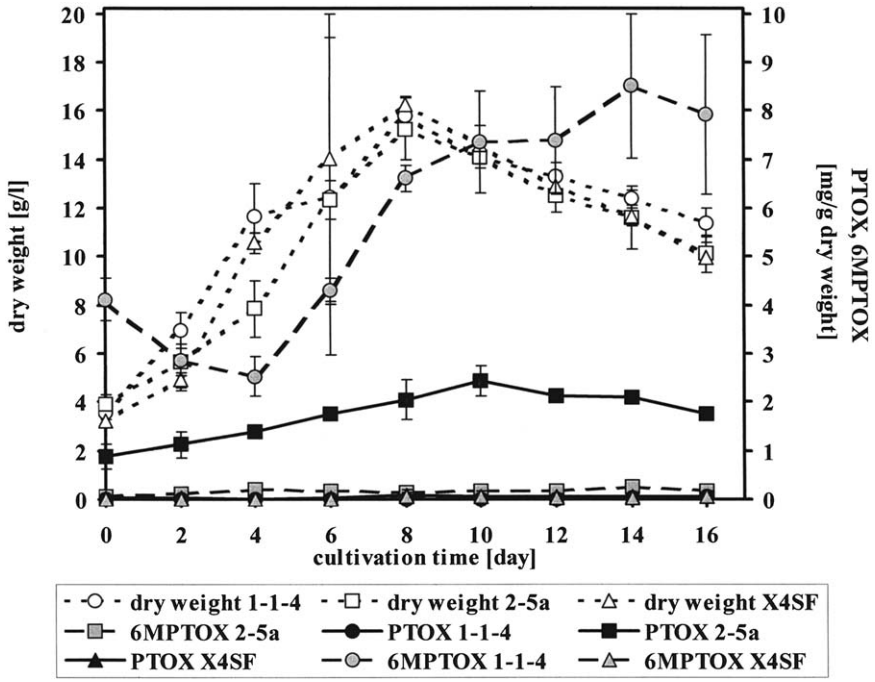



*Figure 2.* Growth (measured as dry weight) and accumulation of podophyllotoxin (PTOX) and 6-methoxypodophyllotoxin (6MPTOX) in three different cell suspension lines of *Linum album* (1-1-4, 2-5a, X4SF) during a cultivation period of 16 days. (The data are the average of two to three experiments. The bars show the standard deviations.)

ments, elicitation and biochemical approaches using plant *in vitro* cultures.

## Lignan accumulating plant cell and organ cultures

Plant *in vitro* cultivation may have several advantages over collecting plants from wild or cultivating them on fields (Alfermann et al., 2003). Metabolites like lignans can be produced under controlled and reproducible conditions, independent of e.g. geographical and climatic factors. It is not necessary to use herbicides or insecticides. Especially cell suspension cultures can show high growth rates combined with high accumulation of the desired metabolite in short time.

One of the basic plant *in vitro* techniques is micropropagation. With this technique it is possible to produce virus-free plant clones in high numbers. Examples for the use of this technique are the establishment of a micropropagation protocol for *Podophyllum peltatum* by Moraes-Cerdeira et al. (1998), for *P. hexandrum* by Nadeem et al. (2000) and for *Rollinia mucosa* by Figueiredo et al. (1999). Whereas *P. peltatum* and *P. hexandrum* plants accumulate the cytotoxic lignan PTOX, *R. mucosa* produces furafuran lignans like magnolin, epiyangambin and yangambin, which are antagonists of platelet-activating factor.

In 1982, Kadkade was the first to initiate callus cultures of *Podophyllum peltatum* to overcome the problems of PTOX supply from natural resources. These cultures accumulated up to 0.38% PTOX in their dry mass. Since then many experiments followed

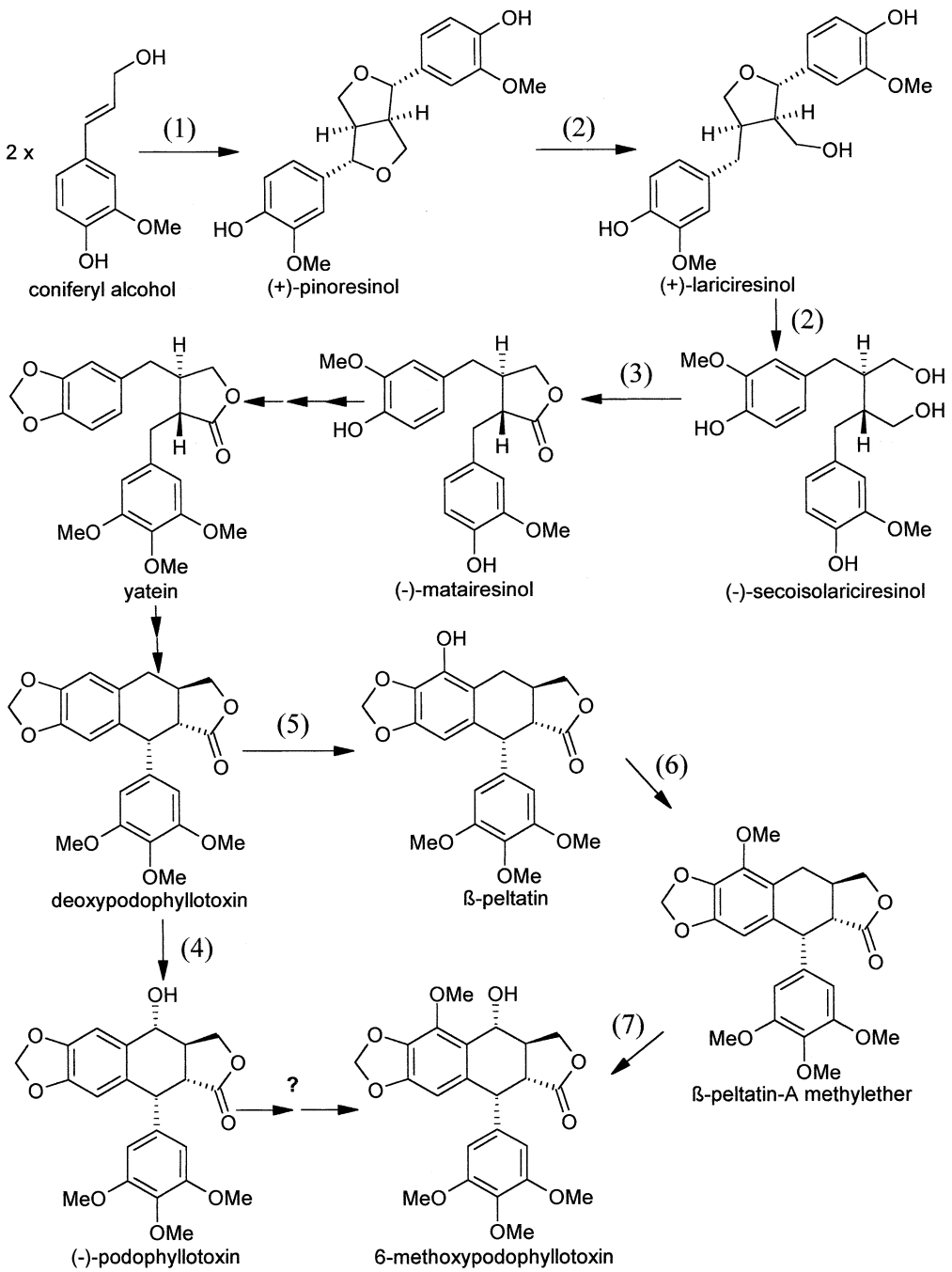



*Figure 3.* Hypothetical biosynthetic pathway leading from coniferylalcohol to podophyllotoxin and 6-methoxypodophyllotoxin. 1=pinoresinol synthase, 2=pinoresinol-lariciresinol reductase, 3=secoisolariciresinol dehydrogenase, 4=deoxypodophyllotoxin 7-hydroxylase, 5=deoxypodophyllotoxin 6-hydroxylase, 6=$\beta$-peltatin 6-*O*-methyltransferase, 7=$\beta$-peltatin-A methylether 7-hydroxylase.

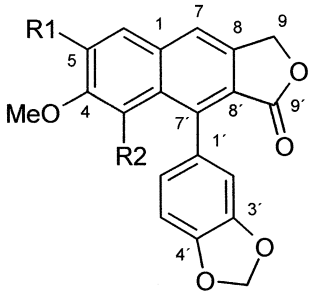
to establish plant *in vitro* cultures from different plant species not only accumulating PTOX but also other lignans (Table 1).

The main focus was to find well growing cultures for the production of PTOX. The highest amounts of PTOX (up to 0.38% on dry weight basis) in plant *in vitro* cultures could be detected by Kadkade (1982) in callus cultures of *Podophyllum peltatum*. But cultures of *Podophyllum* species showed browning and grew only slowly (Fujii, 1991; Kutney et al., 1991, 1993; van Uden et al., 1989; Giri and Narasu, 2000; Chattopadhay et al., 2003). Cultures of other PTOX accumulating plant species showed only minor amounts of PTOX (less than 0.1% on dry weight basis). We established fast growing cell suspension cultures of *Linum album* which accumulate up to 0.3% PTOX on a dry weight basis together with minor amounts of other lignans (Smollny et al., 1992, 1998; Empt et al., 2000). Since then, by screening cell suspension cultures initiated from different seedlings or genotypes of *Linum album* we could establish cultures accumulating the PTOX derivative 6-methoxypodophyllotoxin (6MPTOX) as the main lignan besides cultures with only trace amounts of PTOX or 6MPTOX (Figure 2).

The PTOX accumulation in some cell suspension lines of *Linum album* seems to be an exception among *in vitro* cultures of *Linum* species because most *Linum* cultures accumulating cytotoxic lignans, either cell suspensions or roots and hairy roots, accumulate 6MPTOX as the main lignan sometimes in quite high amounts (Table 1). For example cell suspension cultures of *Linum nodiflorum* accumulate 0.18% PTOX and 0.6% 6MPTOX both on a dry weight basis besides traces of deoxypodophyllotoxin (DOP) (Konuklugil et al., 1999). The highest yields of a cytotoxic lignan (6MPTOX) in plant *in vitro* cultures was found in a root-like suspension culture of *Linum flavum* (0.7% of the dry weight 6MPTOX) (Van Uden et al., 1991) and in cell suspension cultures of *Linum nodiflorum* (1.7% of the dry weight 6MPTOX) (Kuhlmann et al., 2002).

Lignans like pinoresinol (PINO), lariciresinol (LARI), secoisolariciresinol (SECO) and matairesinol (MATAI) which serve as intermediates in the biosynthetic pathway leading to PTOX and its derivatives can be found in many different cultures from several plant species (Table 1, Figure 3).

Besides the cytotoxic lignans PTOX and 6MPTOX and their precursors also other lignans were detected in plant *in vitro* cultures (Table 1). Mohagheghzadeh et al. (2002) recently found in different cultures




justicidin B:
R1: OMe R2: H

isojusticidin B
(3-methoxy-5-demethoxy-justicidin B):
R1: H R2: OMe

*Figure 4.* Structures of justicidins isolated from cell and hairy root cultures of *Linum austriacum* (Mohagheghzadeh et al. 2002).

of *Linum austriacum* justicidin B which is new for the genus *Linum* and the new lignan isojusticidin B (Figure 4). The comparison of the content of the two substances in different types of culture shows the importance of selecting the appropriate culture regime (Table 2). It is clearly visible that the more differentiated tissues produce more justicidins than the undifferentiated ones. This is a good example to show that more differentiated cells often produce a secondary compound in higher amounts than undifferentiated ones.

In conclusion the presented data show that by selection of the appropriate plant species and type of culture a lot of effort was made in the last 10–15 years to establish high producing and well growing plant *in vitro* cultures with the main goal to overcome problems with PTOX supply. The highest amounts of PTOX in cell suspension cultures grown in an airlift bioreactor ever seen were detected by Garden (2003) with 130 mg PTOX per litre culture volume reached in 10 days. Verpoorte et al. (1999) calculated that a production rate of 300 mg/l within 14 days results in a price of $ 1500/kg. A Chinese company is offering PTOX for $ 150/kg. These data clearly show that the amounts of PTOX are still not high enough to justify a commercial biotechnological production. Therefore the next paragraphs will deal with the possibilities to enhance the product yield in plant cell and organ cultures.

*Table 3.* Optimisation of plant *in vitro* cultures for the production of lignans (without feeding and elicitation experiments)

| Plant species | Type of culture | Lignan | Optimisation parameter → lignan content [% on dry weight basis] | Reference |
|---|---|---|---|---|
| *Podophyllum peltatum* | Callus | PTOX | - carbon source:<br>sucrose → 0.57<br>maltose → 0.023<br>- optimal phytohormones:<br>2,4-D + kinetin → 0.57 | Kadkade 1982 |
| *Podophyllum hexandrum* | Cell suspension | PTOX | light → 0.03<br>dark → 0.09 | Van Uden et al. 1989 |
| *Podophyllum hexandrum* | Callus | PTOX | - optimal phytohormones:<br>2,4-D + kinetin → 0.077 | Heyenga et al. 1990 |
| *Podophyllum hexandrum* | Cell suspension | PTOX | pH 6.0, 1.25 mg/l of IAA, 72 g/l glucose, 8 g/l inoculum → 0.075 | Chattopadhay 2002, 2003 |
| *Forsythia* × *intermedia* | Cell suspension | PINO<br>MATAI | 2% sucrose → < 0.001<br>PINO + MATAI<br>6% sucrose → 0.07<br>PINO + 0.1 MATAI | Schmitt and Petersen 2002a |
| *Ipomoea cairica* | Callus | Arctigenin, trachelogenin (T) | 4 mg/l 2,4-D, 3% maltose, pH 6.4 → 0.03 T | Páska et al. 1998, 1999 |
| *Linum nodiflorum* | Cell suspension | 6MPTOX | light → 0.6<br>dark → trace amounts | Konuklugil et al. 1999 |
| *Linum album* | Cell suspension | PTOX | light → 0.5<br>dark → 0.2 | Smollny et al. 1998 |
| *Linum album* | Cell suspension | PTOX | light → 0.028<br>dark → 0.22 | Fuss et al., unpublished |

*Variation of the cultivation conditions and the medium composition*

Product yields in plant tissue culture can be improved by optimisation of the culture process. This can be done either by selecting a high-producing cell line or optimisation of the medium composition or by changing other cultivation parameters like the light conditions. A summary is given in Table 3.

Cell suspension cultures which are releasing the desired compound into the medium are preferred for biotechnological processes. Therefore it is of interest to discuss this possibility in the case of lignans. Some lignans especially the PTOX from which the semisynthetic derivatives are used in anticancer therapy show phytotoxicity (Arimoto et al., 1994; Oliva et al., 2002). Therefore the PTOX producing plant cell has to store the compound in the vacuole or release it into the medium to avoid its own intoxication. Up to now lignans could not be found in the medium. We analysed the medium in different cultures of *Linum album* producing PTOX and/or 6MPTOX and we could detect only trace amounts of these compounds in the medium probably due to cell lysis. Henges et al. (publication in preparation) could show that in *Linum album* cell suspension cultures PTOX and 6MPTOX are stored in the vacuoles as glucosides. Since our current knowledge on the compartimentation of lignan biosynthesis and the transport of lignans like PTOX into the vacuole is very low, we first have to investigate the production and intracellular storage of lignans.

The highest enhancement in percent by variation of the cultivation parameters was achieved by Konuklugil et al. (1999) with a cell suspension culture of *Linum nodiflorum*. This culture accumulated only trace amounts of 6MPTOX when it was grown in the dark in comparison to 0.6% of 6MPTOX on a dry weight basis when grown under permanent illumin-

*Table 4.* Elicitation experiments

| Plant species | Type of culture | Lignan | Elicitor | Enhancement | Reference |
|---|---|---|---|---|---|
| *Juniperus chinensis* | Callus | PTOX | a) 1 mg chito-oligosaccharides b) laminaran enzyme-hydrolysates | a) 15-fold b) 3.5-fold | Muranaka et al. 1998 |
| *Forsythia* × *intermedia* | Cell suspension | a) PINO b) MATAI | 100 $\mu$M methyl jasmonate | a) 3-fold b) 7-fold | Schmitt and Petersen 2002b |
| *Linum album* | Cell suspension | PTOX | 100 $\mu$M methyl jasmonate | 10-fold | Fuss et al., unpublished |

ation. Whereas Smollny et al. (1998) found similar results with cell suspension cultures of *Linum album* we could show a broad spectrum of adaptation to light conditions (permanent light, 150 $\mu Em^{-2}$ $s^{-1}$, 50% Philips TLD18W/25 and 50% TLB18W/83) for other cell lines of *Linum album*. Two lines accumulate about 10 times more PTOX when grown in the dark in comparison to cultivation in light. One line accumulated almost no PTOX when grown in darkness and trace amounts of PTOX after growth under light conditions. Other lines show no difference in PTOX accumulation after growth in light or darkness. This shows that light does not directly influence the accumulation of PTOX. It may have an effect via selecting the better growing cells during establishment of a new cell line. Therefore the optimisation has to be done for each new cell line.

Whereas most authors changed only one or a few cultivation parameters Chattopadhay et al. (2002, 2003) choose a complex set of parameters and a statistical approach to combine their results concerning the optimisation of the medium composition (concentration of sugar, phytohormones and phosphate, pH and nitrogen source) and the amount of inoculum. They could experimentally confirm the feasibility of a statistical approach leading to the optimal parameters for growth and PTOX accumulation in a cell suspension culture of *Podophyllum hexandrum*. The optimum values of different parameters were: pH 6.0, 1.25 mg/l IAA, 72 g/l glucose and 8 g/l inoculum on day zero which resulted in 22.7 g/l cell dry weight and 0.075% of the dry weight PTOX after a cultivation period of 30 days.

Like all other authors Chattopadhay tried to optimise one medium for growth and accumulation of lignans instead of development of a two stage cultivation regime with a medium for growth followed by a production phase in a specially optimised medium. The establishment of such a two stage process only makes sense when the compound of interest is accumulated non-growth-related e.g. in the stationary phase. This is not the case for the accumulation of PINO and MATAI in cell suspension cultures of *Forsythia* × *intermedia* (Schmitt and Petersen, 2002b) where the main accumulation takes place in the second half of the linear growth phase. The accumulation of PTOX and 6MPTOX in *Linum album* suspension cultures occurs during late linear phase and stationary phase (Figure 2). Therefore the accumulation is neither growth-related nor non-growth related. Furthermore the stationary phase is extremely short, not more than one day. This would make it very difficult to find the time point when the medium has to be changed.

*Elicitation*

Elicitation either with biotic or abiotic elicitors is widely used in plant *in vitro* cultures to enhance the yield of compounds. The attempts performed with lignan-accumulating cultures are shown in Table 4. Unfortunately our results concerning the elicitation of several cell suspension lines of *Linum album* by addition of methyl jasmonate to the culture medium showed only in one line a tenfold increase in PTOX content up to 0.2% of PTOX on a dry weight basis. No further increase of the PTOX content could be observed in cultures with already quite high accumulation of PTOX. The same seems to be true for the experiments with cultures of *Juniperus chinensis* and *Forsythia* × *intermedia* (Muranaka et al., 1998; Schmitt and Petersen, 2002b). Also these cultures were producing only minor amounts of lignans before

*Table 5.* Feeding experiments (# complexed with cyclodextrin)

| Substrate | Product | Plant species | Reference |
|---|---|---|---|
| L-phenylalanine | 6MPTOX | *Linum flavum* | Van Uden et al. 1990d |
| Coniferyl alcohol | PINO | *Forsythia* × *intermedia* | Schmitt and Petersen 2002b |
| Coniferin | PTOX | *Podophyllum hexandrum* | Van Uden et al. 1990a |
| Coniferyl alcohol, coniferyl alcohol #, coniferin | PTOX | *Podophyllum hexandrum* | Woerdenbag et al. 1990 |
| DOP | 6MPTOX | *Linum flavum* | Van Uden et al. 1997 |
| PTOX # | PTOX $\beta$-D-glucoside | *Linum flavum* | Van Uden et al. 1993 |
| DOP # | 1) PTOX<br>2) 6MPTOX | 1) *Podophyllum hexandrum*<br>2) *Linum flavum* | Van Uden et al. 1995 |
| DOP' PTOX | Podophyllo-toxone | *Forsythia* × *intermedia* | Broomhead and Dewick 1991 |
| Mixture of $R^*S^*$-diastereomers | Pure dia-stereomers | several | Takemoto et al. 2000, 2001 |
| [U-$^{14}$C] phenylalanine | $^{14}$C labelled MATAI | *Forsythia* × *intermedia* | Rahman et al. 1990a |
| [2-$^{13}$C] methylene-dioxycinnamic acid' [2-$^{13}$C] ferulic acid' [2,3-$^{13}$C] ferulic acid | DOP<br>PTOX | *Linum album* | Seidel et al. 2002 |

elicitation which led to a significant enhancement of lignan accumulation. However up to now only a few trials to enhance lignan accumulation in *in vitro* cultures by elicitation are published. Therefore it would still be interesting to perform more experiments with a broader range of elicitors in lignan producing cultures.

*Feeding of precursors*

Precursor feeding can be done in three different ways (Table 5). First, feeding of biosynthetic intermediates may enhance the product yield in the case that this intermediate was rate-limiting. Here the main goal is to feed an easily available and cheap precursor to get a high amount of the desired compound. Van Uden et al. (1990d) fed the commercially available L-phenylalanine to cell suspension cultures of *Linum flavum* and could enhance the yield of 6MPTOX from 0.004% to 0.02% on dry weight basis. The also commercially available but more expensive lignan precursor coniferyl alcohol was fed to cell suspension cultures of *Forsythia* × *intermedia* to increase the PINO yield (Schmitt and Petersen, 2002b) and to cell suspension cultures of *Podophyllum hexandrum* leading to a slightly higher PTOX accumulation (Woerdenbag et al., 1990). In the latter case the yield of PTOX could be enhanced further when the poorly water-soluble coniferyl alcohol was complexed with $\beta$-cyclodextrin which leads to a higher solubility of coniferyl alcohol in the medium. Feeding of $\beta$-cyclodextrin complexed coniferin, the glucoside of coniferyl alcohol, gave even better results, but this precursor is not commercially available.

*In vitro* cultivated cells of *Forsythia* × *intermedia* converted fed DOP via PTOX into podophyllotoxone (a podophyllotoxin with a keto group at C7 instead of an OH group) which was not expected because in this plant species neither DOP nor PTOX or podophyllotoxone can be found (Broomhead and Dewick 1991). This leads to the second aim of feeding experiments: the bioconversion of substances which is sometimes difficult for chemists. By using this approach one can utilise biosynthetic activities for the desired conversions which are in the plant material normally involved in other biosynthetic pathways. A second example for such conversions are the dediastereomerisation experiments done with cultures of different plant species which are not necessarily accumulating lignans without feeding (Takemoto et al., 2000, 2001). When the dibenzylbutanolide $4R^*S^*-1$ was fed to *Catharanthus roseus* cells in B5 medium,

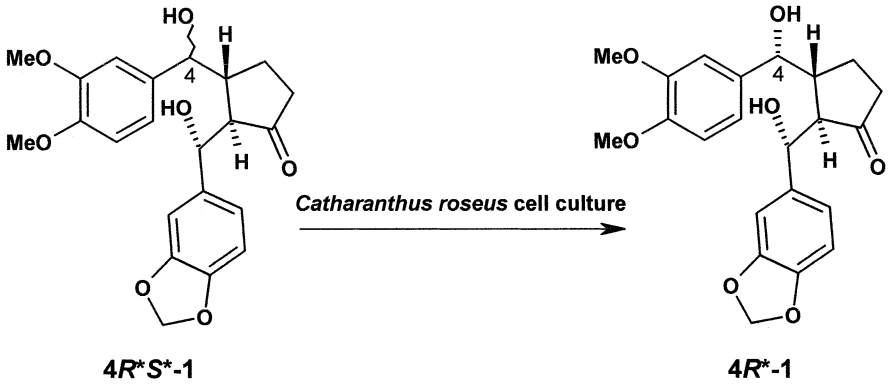



*Figure 5.* Dediastereomerization of 4$R^*S^*-1$ by cell cultures of *Catharanthus roseus* (Takemoto et al. 2000).

4$R^*S^*$-1[5] was converted into 4$R^*$-1[6] with 80% chemical yield, 100% dediastereomeric excess and 0% enantiomeric excess (Figure 5). The authors could show that 4$R^*-1$ was resistant to the reaction and was recovered unchanged whereas 4$S^*-1$ was inverted to 4$R^*-1$.

The third aim of feeding experiments is to get insights into biosynthetic pathways which will be discussed in detail in the next paragraph.

## Biosynthesis

Most work was done to elucidate the biosynthetic pathway leading to PTOX and 6MPTOX (Figure 3). The first steps until MATAI were investigated by Lewis and Davin and their coworkers in *Forsythia* species (for a review see Lewis and Davin, 1999). Two molecules of coniferyl alcohol derived from the general phenylpropanoid pathway and the monolignol biosynthesis are coupled stereospecifically to (+)-PINO via an oxidase with help of a dirigent protein which has no catalytical activity itself. The following two reactions to form SECO via LARI are performed by pinoresinol-lariciresinol reductase. SECO is oxidized to MATAI by secoisolariciresinol dehydrogenase. On genetic level only for these first steps cDNAs could be isolated up to now. The first cDNAs encoding dirigent proteins and also cDNAs encoding pinoresinol-lariciresinol reductase were isolated from *Forsythia* × *intermedia* (Dinkova-Kostova et al., 1996; Gang et al., 1999). cDNAs encoding secoisolariciresinol reductase were isolated not only from *Forsythia* × *intermedia* but also from *Podophyllum peltatum* at the same time (Xia et al., 2001). In addition Xia et al. (2000) could isolate cDNAs encoding a dirigent protein involved in the stereospecific coupling of coniferyl alcohol from *Podophyllum peltatum*. In our laboratory we could isolate a cDNA encoding a pinoresinol-lariciresinol reductase from a PTOX-producing cell suspension culture of *L. album* (von Heimendahl, Schäfer, Fuss, unpublished results).

Rahman et al. (1990a) produced $^{14}$C-labelled MATAI by feeding of [U-$^{14}$C] phenylalanine to cultures of *Forsythia* × *intermedia* which showed that phenylalanine is a precursor of MATAI. This labelled MATAI was fed to cultures of *Forsythia* × *intermedia* and incorporated into arctigenin (Rahman et al., 1990a). In addition it was fed to cultures of *Podophyllum hexandrum*, *P. peltatum* and *Diphylleia cymosa* and was incorporated into the aryltetralin lactone lignans PTOX, 4′-demethyl-PTOX, $\beta$-peltatin, $\alpha$-peltatin and 4′-demethyl-DOP suggesting that MATAI is the precursor of the 3′,4′,5′-trimethoxy and 4′-hydroxy-3′,5′-dimethoxy groups of *Podophyllum* lignans (Broomhead et al., 1991). Seidel et al. (2002) could show the incorporation of [2-$^{13}$C] methylenedioxycinnamic acid, [2-$^{13}$C] ferulic acid and [2,3-$^{13}$C] ferulic acid into DOP and PTOX after feeding to a PTOX-producing cell suspension line of *Linum album*. These experiments led to discussions about the substitution patterns at the aromatic rings of the precursors and lignans necessary or acceptable for lignan biosynthesis. If really the methylenedioxycinnamic acid is incorporated as such the bridge formation would be very early in the pathway changing the view which is shown in Figure 3. But it still has to be shown whether or not the methylenedioxy bridge of the [2-$^{13}$C] methylenedioxycinnamic acid was first cleaved and the usual pathway was followed via ferulic acid, especially because of the results of Stöckigt and Klischies (1977) who could not find an incorpor-

ation of methylenedioxycinnamic acid into lignans in *Forsythia suspensa*.

The feeding of DOP to cell cultures of *Podophyllum hexandrum* producing PTOX and to cell cultures of *Linum flavum* producing 6MPTOX showed that DOP is the precursor for both lignans (Van Uden et al., 1995). However, PTOX added to cell cultures of *Linum flavum* was not converted to 6MPTOX, but glycosylated indicating that PTOX is not a precursor for 6MPTOX.

The disadvantage of such feeding experiments for biosynthetic studies is already obvious regarding the data discussed in the previous chapters. After addition of a possible precursor or intermediate of a biosynthetic pathway to cell cultures or plants one never knows whether the observed conversions are real biosynthetic steps or results of aberrant synthesis in cell cultures. In most cases it is not known whether the externally applied compound reached the cellular site of biosynthesis. Even high incorporation rates like those found by Seidel et al. (2002), 13.8% for the incorporation of methylenedioxycinnamic acid into PTOX, are no guarantee that the incorporation is based on the natural biosynthetic pathway which is indicated by other results and the possible degradation of methylenedioxycinnamic acid before incorporation, as discussed before. Therefore the results of feeding experiments have to be supported by other experimental approaches like enzymatic studies.

In enzymatic studies using *Linum* cell suspension cultures as protein source the activities of DOP 7-hydroxylase (*L. album*, Henges unpublished results), DOP 6-hydroxylase (*L. album*, Federolf, unpublished results; *L. flavum*, Molog et al., 2001; *L. nodiflorum*, Kuhlmann et al., 2002) and S-adenosylmethionine: $\beta$-peltatin 6-*O*-methyltransferase (*L. nodiflorum*, Kranz and Petersen, 2003) were demonstrated confirming the results by van Uden et al. (1995) described before (see Kuhlmann et al., 2002 for a recent review). The advantages of using plant cell suspension cultures instead of whole plants or plant parts are the availability of the plant material everywhere and all over the year and the often easier preparation of protein extracts (Zenk, 1990).

In conclusion, not all biosynthetic steps leading to PTOX are yet known, especially those between DOP and PTOX and the hydroxylation reaction from $\beta$-peltatin A-methylether to 6MPTOX still have to be investigated. In addition, little is known about the regulation of the lignan biosynthetic pathways. First experiments to figure out possible rate limiting steps in the monolignol formation were done using a real-time-PCR approach by Anterola et al. (2002) in cell suspension cultures of *Pinus taeda*, but the authors did not follow lignan accumulation in these cultures.

## Conclusion

Plant cell cultures have been shown to accumulate lignans of different chemical types. Great effort was made to study different types of plant *in vitro* cultivation for the accumulation of cytotoxic lignans like (−)-podophyllotoxin. But also after enhancement of the lignan accumulation by elicitation and feeding of precursors the lignan levels might not be sufficient to use these cell cultures as biotechnological production systems. Several cultures already served as useful experimental systems to study the biosynthetic pathway leading to lignans like PTOX. Feeding of non-labelled or labelled precursors to cell cultures helped to find intermediates and biosynthetic steps of this pathway. *Linum* cell suspension cultures serve as a source to identify and characterise biosynthetic enzymes like deoxypodophyllotoxin 7-hydroxylase, deoxypodophyllotoxin 6-hydroxylase and S-adenosylmethionine: $\beta$-peltatin 6-*O*-methyltransferase. In addition, *Linum album* cell suspension cultures with different lignan accumulation patterns and levels can be used in future to isolate structural and regulatory genes of the biosynthetic pathway with the aim to manipulate this species genetically to enhance lignan accumulation.

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