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New biotechnological systems for the research on aryltetralin lignans in *Linum Álbum*

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DOCTORAT EN BIOTECNOLOGÍA

NEW BIOTECHNOLOGICAL SYSTEMS FOR THE RESEARCH ON ARYLTETRALIN LIGNANS IN *Linum album*

Memòria presentada per Liliana Paulina Lalaleo Córdova per optar al títol de doctor per la Universitat de Barcelona

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LILIANA PAULINA LALALEO CORDOVA
2016

A mis padres, Nelson y Rosa y mis hermanas Andrea y Diana, por todo su apoyo y amor.

There is nothing to fear in life, That's the only think you need to understand.

Marie Curie

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KEY WORDS

- *Adventitious roots*
- Aryltetralin lignan
- Coronatine
- *Cytochrome B5*
- Hairy roots
- *Linum album*
- *Organogenic calli*
- Transformed cells
- Wild type cells
- *Yeast two hybrid system*

ABSTRACT

Linum album is an herbaceous plant with medical interest due to its content of podophyllotoxin (PTOX), an aryltetralin lignan with cytotoxic activity. The study of biotechnological alternatives has generated big interest. Organ and cell in *vitro* cultures offer the possibility of suppling aryltetralin lignans in accordance with a sustainable and rational utilization of biodiversity, however they need to be improved. Thus, in this work we analyzed the lignan accumulation patterns in four biotechnological systems of *L.album* in order to find alternatives of bioproduction. The four biotechnological platforms were established with wild type and transformed cell suspension cultures, adventitious roots isolated from *in vitro* plants, and hairy roots. The predominant lignan produced by wild type and transformed cells was PTOX. The main lignan produced by adventitious and hairy roots was 6-MPTOX, adventitious roots showed to be more productive, and we can infer that the transformation did not change the lignan patterns. In addition, we have studied the response to the elicitor coronatine in these systems. Transformed cells were the most sensitive after elicitation and showed an arrest of biomass growth in relation to other systems. In general, the elicitation increased the lignan content, but the more elicited route in each system resulted to be the less productive. Transcript profiling changed in elicited conditions, specially for the *PLR* gene in transformed cells. The analysis of morphogenesis development in the production of podophyllotoxin derivatives in callus cultures of *Linum album* showed that, the absence of plant growth regulators are a predominant factor, to induce the organogenic

response and the bioproduction as well. Finally, approaches aiming at increasing podophyllotoxin content require multiple enzymatic steps that facilitate the metabolic flow to the final products, most of them forming multiprotein complexes. Based on this, the Yeast two hibrid system allowed to identify CTB5 as a potential interactor of the PLR protein of *Linum album*, and evidences in other species indicate that CTB5 participates in the phenylpropanoid pathway.

Resumen

Linum album es una planta herbácea con interés farmacológico debido a su alto contenido de podofilotoxina (PTOX), el cual es un lignano de característica ariltetralin con actividad citotóxica. El estudio de las alternativas biotecnológicas ha generado gran interés. Los cultivos *in vitro* de órganos y células ofrecen la posibilidad de suministrar aryltetralin lignanos en acuerdo con una utilización sostenible y racional de la biodiversidad, sin embargo necesitan ser mejorados. Por tanto, en este trabajo se analizaron los patrones de acumulación de lignanos en cuatro sistemas biotecnológicos de *L.album* con el fin de encontrar alternativas en la bioproducción. Se establecieron cuatro plataformas biotecnológicas con cultivos en suspensión celular de tipo salvaje y transformados, raíces adventicias aisladas de plantas in vitro y raíces en cabellera. La PTOX fue el lignano predominante producido por células de tipo salvaje y transformadas. El principal lignano producido por las raíces adventicias y raíces transgénicas fue la metoxipodofilotoxina (MPTOX), en general las raíces adventicias fueron las más productivas, por lo cual podemos inferir que la transformación no afecto los patrones de bioproducción. Además, hemos estudiado la respuesta a la coronatina como elicitor en estos sistemas, donde las células transformadas fueron las más suceptibles a la elicitación mostrando una disminución en la biomasa en relación con los otros sistemas. En general, la elicitación aumentó el contenido de lignanos, aunque la ruta principal fue las menos favorecida en cada sistema. El perfil de expresión genética

cambió en condiciones elicitadas, especialmente para el gen PLR en las células transformadas. El análisis del desarrollo de la morfogénesis en la producción de derivados de podofilotoxina en cultivos de callos del *Linum álbum* mostró que la ausencia de reguladores del crecimiento es un factor predominante para inducir la respuesta organogénica y la bioproducción. Finalmente, las aproximaciones que apuntan a aumentar el contenido de podofilotoxina requieren múltiples pasos enzimáticos que faciliten el flujo metabólico hacia los productos finales, la mayoría de ellos formando complejos multiproteicos. Basado en lo anterior, la técnica del doble hibrido en levadura nos permitió identificar la CTB5 como un potencial interactor de la proteína PLR del *Linum álbum*, hallada en otras especies como interactor en la ruta metabolica de los fenilpropanoides.

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Abbreviations

2-4 D, 2,4-dichlorophenoxyacetic acid

3-AT, 3-aminotriazole

4CCL, 4-coumarate-CoA ligase

4-CCPU, N-2-chloro-4-pyridyl-N-phenylurea

ABA, abscisic acid

AD, activating domain

AGS, agropine synthase

AUX, auxin gene

BD, binding domain

C4H, cinnamic acid 4-hydroxylase

CAD, cinnamyl alcohol-dehydrogenase

CCMT, caffeoyl-CoA-O-Methyltransferase

CCR, cinnamoyl CoA reductase

CMT, caffeic acid 3-O-methyltransferase

CORO, coronatine

CPD, critical point dryer

CTB5, cytochrome b5

CytoY2H, cytosolic split-ubiquitin system

DO, Dropout solution

DOP6H, deoxypodophyllotoxin-6-hydroxylase

DOP7H, deoxypodophyllotoxin 7-hydroxylase.

DPO, dirigent protein oxidase

dPTOX, deoxypodophyllotoxin

DW, dry weight

ER, endoplastic reticulum

FDA fluorescein diacetate

FW, fresh biomass weight

GAL4, galactose-gene activating transcription factor

HCT,p-hydroxycinnamoyl transferase

HPLC-ESI-MS, high performance liquid chromatography electro spray mass ionization

IAA, indolacetic acid

IAA, indole-3-acetic acid

IBA, indole 3-butyric acid

IP, propidium iodide

JA, jasmonic acid

Ja-Ile, isoleucine jasmonic acid

Kin, kinetin

MAS, manopine synthase

MbY2H, membrane split- ubiquitin system

MeJA, methyl jasmomate

MPTOX, 6-metoxypodophyllotoxin

MS, Murashige and Skoog solid medium

NAA, naphtalenacetic acid

PAL,phenylalanine ammonialyase

PAM, β-peltatin-A-methylether

PAM7H,β-peltatin-A-methylether 7-hydroxylase.

PCH,p-coumarate 3-hydroxylase

PCR, polymerase chain reaction

PGR, plant growth regulators

PLR, pinoresinol lariciresinol reductase

PTOX, podophyllotoxin

qRT-PCR, quantitative real-time plimerase chain reaction

RRS, Ras recruitment system

RTA, repressed transactivator system

SA, salicylic acid

SD, secoisolariciresinol dehydrogenase

SD, yeast minimal base media

SEM, scanning electron microscopy

SRS, SOS recruitment system

TEM, transmission electron microscopy

TJOMT, thujaplicatin O- methyl transferase

WT,Wild type

Y2H, yeast two hybrid system

YPDA, yeast potato dextro agar

YT, yatein

βP6OMT, β-peltatin 6-O-methyltransferase

1. JUSTIFICATION AND AIMS

The plants have been used for centuries in traditional medicine and nowadays, their extensive use makes that are part of the quarter of all prescribed pharmaceuticals in the industry, which main compounds are derived from plants. They can act directly or as precursor via semisynthesis in the production of drugs.

Hence, the possibility of producing podophyllotoxin (PTOX), which is an aryltetralin-lignan that presents anticancer properties from *Linum*, especially members of section *Syllinum* under either in vitro or ex vitro conditions are highly attractive. Comparing with other *Linum* species, *Linum album* is a promised source used as a starting material for producing various semisynthetic derivatives that are widely used in chemotherapy, such as etoposide, teniposide and etopophos.

Potent biotechnological systemshave been developed to improve the production of plant-derived compounds. These biotechnological platforms are based on plant cell and organ cultures with an optimized capacity to produce target metabolites, matching or enhancing the concentration in the mother plan. However efforts to increase the production still necessary. Currently, manipulating empirical factors related to plant cell and organ cultures it has been possible to enhance PTOX yield, with variety of inputs factors can be optimized, such as growth medium, physicochemical conditions, seed inoculum, type of reactor and processing conditions.

Additionally the “Omic” eraconstitute a potent tool for the successful elucidation of a metabolic pathway and identification of limiting

enzyme activities that control the biosynthetic process.The biosynthetic pathway leading to PTOX is not fully understood and may present some variations depending on the producing plants.

Based on this, the development of basic studies on secondary plant metabolism and studies applied to the biotechnological production of phytopharmaceuticals will give us some highlight knowledge to implement in the futurebiosustainable processes at an industrial level; This doctoral thesis has as general objective:**Study of new biotechnological systems of *Linum album* to compare the aryltetralin lignans biproduction patternand have a more deep knowledge of the biosynthesis of lignans with the purpose of applying the knowledge generated in the metabolic engineering field.**

Specific objects of third project were:

a) To compare the lignan accumulation patterns in different *in vitro* systems of *L. album,* we established four biotechnological platforms: wild type (WT) and transformed cell suspension cultures, adventitious roots and hairy roots. The study approach generates new information to improve bioproduction through empirical factors or metabolic engineering in different systems.

b) To describe the effect of coronatine and test its efficiency in lignan pattern production on four different biotechnological systems of *L. album.* We complement these results with the analysis of the expression levels of genes involved in the PTOX

pathway, and analyze potential correlations between expression and lignan production. Our results provide a detailed transcriptional and metabolic analysis that helps us to understand the underlying changes induced by coronatine elicitation that shape the bioproduction of lignans in different *Linum album* systems.

c) To study the growth, morphogenic capacity and production of PTOX, MPTOX and related compounds in *L. album* cell masses maintained in different experimental conditions. The relationship between the formation of morphogenic structures, observed both at light and at scanning electron microscope and the production of levels of these compounds has been determined. This study has permitted to gain new insights into the knowledge on the biotechnological production of these lignans and the importance of cell differentiation and organogenesis for obtaining high levels of these compounds.

d) To identify protein interactors, potentially participating in the lignan biosynthesis pathway in *Linum album,* cDNA libraries were generated for the Y2H system and PLR and TJOMT proteins used as baits in screens against the *Linum album* proteome. These approximations enable the discovery of new proteins in a potential macromolecular enzymatic complex in the lignan biosynthetic pathway.

2. INTRODUCTION

2.1.Linum species

In the last century, the *Linum* genus has a more relevance, playing an important role in the industry due to its properties. For example, flaxfiber is extracted from the stem and it is used in the textile industry. Today, the seeds of *Linum*are the more important product of the species, due to nutritionally and medicinally properties used as source of linseed oil,component of many paints, inks, varnishes, and lubricants. On the other hand, it has been reported that *Linum*species characteristically accumulate cyclolignans of the aryltetralin series as major lignan types use as important compounds in the pharmaceutical industry for cardiovascular diseases andcancer treatments, particularly breast cancer(MacRae and Towers 1984; Pujol et al. 2005; Rickard-Bon and Thompson 2003).

The flax family is positioned in the plant kingdom as follows: Division: *Pteridophyta*; Sub-Division: *Angiospermae*; Class: *Dicotyledoneae*; Sub-class: *Rosidae*; Order: *Geraniales*. The genus *Linum* is the type genus for the flax family, *Linaceae*. It is geographically widespread with about 300 species worldwide. The genus is divided into the following five sections recognized: *Linum, Syllinum, Dasylinum, Linastrum and Cathartolinum. Linum* is the largest, exhibit a complex biogeographic distribution, but present more distribution in America, Africa and Australia. The *Syllinum* has main geographical distribution in Europe and the west of Asia, *Dasylium* are distributed in east of Europe to central Asia, North America, North Africa, Eurasia, Australia, New Zealand; *Linastrum*

present more representative in the north and south of America, Southwest of Europe and Asia. Finally, studies in the distribution of *Cathartolinum* section establish more representatively in Europe and west of Asia zones (Diederichsen and Richards 2003) (Figure 1).

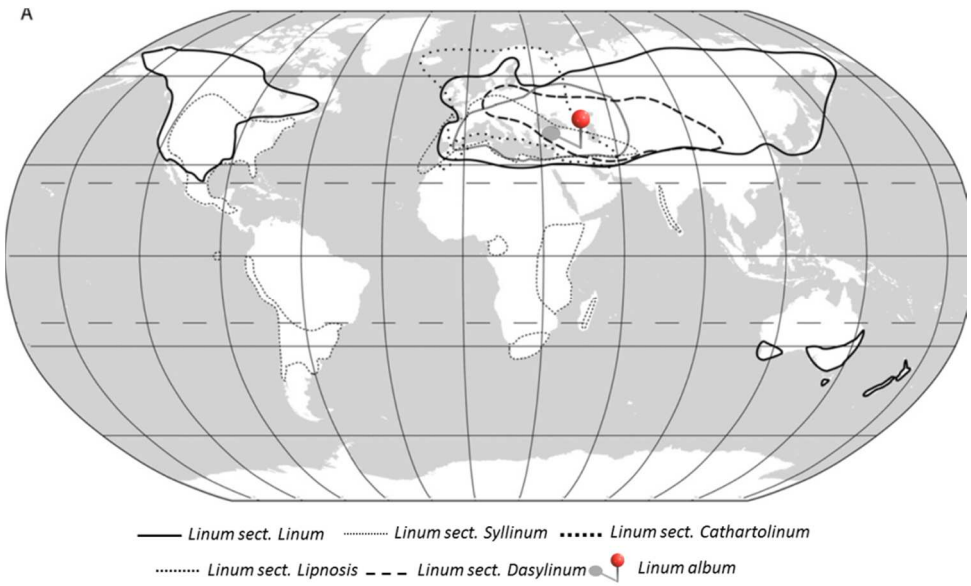


Figure 1.Geographical distributions of the section of the *Linum*

Within the *Linum* genus, *L. album* belongs to the section *Syllinum,* which is an herbaceous medicinal plant naturally distributed to mountainous areas, at an altitude of 1200 to 3200 m in Iran or Turanian regions (Konuklugil et al. 2007; Malik et al. 2014). It has petals free forming white flowers usually large, its flowering time is restricted from April to June months; fruiting pedicels elongate; sepals without obvious longitudinal veins, globose capsules containing between six to ten seeds, which contain 6% mucilage

and 20-40% oil. Stigmas are longer than wide, clavate or linear, stem with wings decurrent from leaf bases and leaves with glands at base(Figure 2). This genus can be perennials, biennials or annuals.

Figure 2..A) *L.album* flowers; ***B)*** plantlets and ***C)*** seeds

2.2.Chemical structure of lignans

The most important bioactive compounds isolated from *L. album* are lignans. Chemically, lignans are phenylpropanoid dimers (C6C3), where the phenylpropane units are linked by the central carbon C8 of their side chains. This nomenclature was recognized by the IUPAC in 2000 and is the basis for naming the lignan (Figure 3). The numbering begins at the benzene ring (1 to 6), and continues in the propyl group (7 to 9). The second C6C3 unit has primed numbers

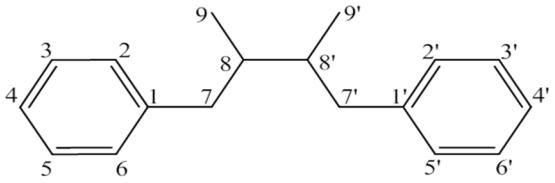


Figure 3. Chemical structure of lignans

Lignans are classified based on the way that oxygen is incorporated in the skeleton and the cyclization pattern, as following: furofuran, furan, divenzylbutane, dibenzylbutylactone, aryltetralin, arylnaphtalen, dibenzocyclooctadiene.(Umezawa 2003). The main lignan skeletons are listed in the Figure 4. Amongst them, the aryltetralin lignans are of particular interest for their remarkable anticancer activity. The most representative of this group is podophyllotoxin (PTOX), which is the most abundant lignan isolated from the resin of *Podophyllum* and *Linum* genus (Gordaliza et al 2004). PTOX has a 1-aryltetralin skeleton, containing a tetrahydronaphtalene or tetralin molecule. Also PTOX analogs, which include closely related structural compounds, such as, deoxypodophyllotoxin (DOP), 6-methoxy podophyllotoxin (6-MPTOX), 5'-demethoxy-6-podophyllotoxin (5'-d-6-MPTOX), 4'-demethyl podophyllotoxin, 4'-dimethyl podophyllotoxin, α- and β-peltatins and their D ring stereo chemical variants has been studied due to their cytotoxic effects (Zhang et al. 2014).

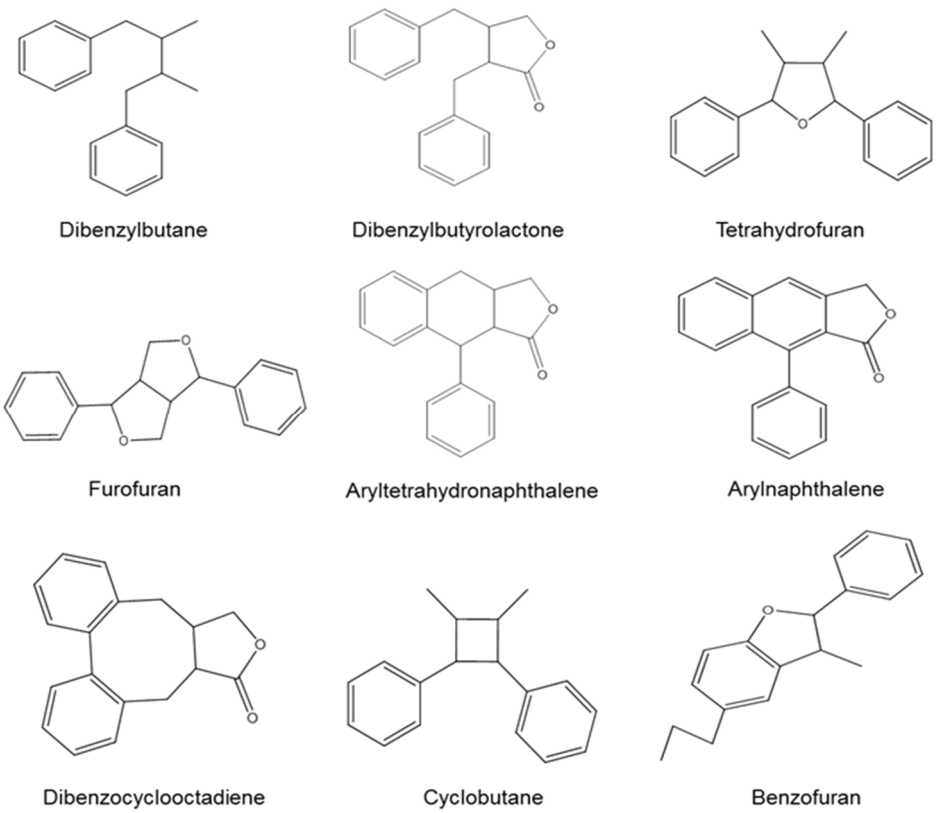


Figure 4. Representative skeletons of lignans

2.3.Biological activities of PTOX and their derivatives

The lignans are a class of natural plant compounds, which are related biochemically to the phenylalanine metabolism. Most of them are present mainly as free form in the cells, but a small proportion of them are bound with sugars as glycosides forms, they are usually present in wood and resin of plants. Lignans are known to have wide biological activities as hepatoprotective andimmunomodulatory activities, effects on cardiovascular system,anti-leishmaniosis properties, effects on high density lipoproteins and hypolipemiant, antifungal, antirheumatic, antipsoriasis and antimalarial activities. But cytotoxicity and antiviral are the more importan(Hande 2008).

The therapeutic application of the podophyllotoxin (PTOX) by itself is not possible due to its server toxicity. Also it shares the property with the anticancer drugs paclitaxel and camptothecin of being virtually insoluble in water (Farkya et al. 2004). Being more hydrophilic, the glucosides are less toxic than aglycons, but their cytostatic activity also reduces in the same degree. Thus, the derivatization of the PTOX allows obtaining analogous as: etoposide, teniposide and etopophos (Figure 5), with improved pharmacological profiles in the treatment of different types of cancer. Etoposide and tenoposide are using with effective results in gastric, testicular, ovarian and gestational carcinomas, lung cancers, leukemia, lymphomas and colon cancer. However, they are often hindered by problems of poor solubility and metabolic inactivation normally solve with c4 modified analogs in the E ring (Lee, K.H. Xiao 2003).

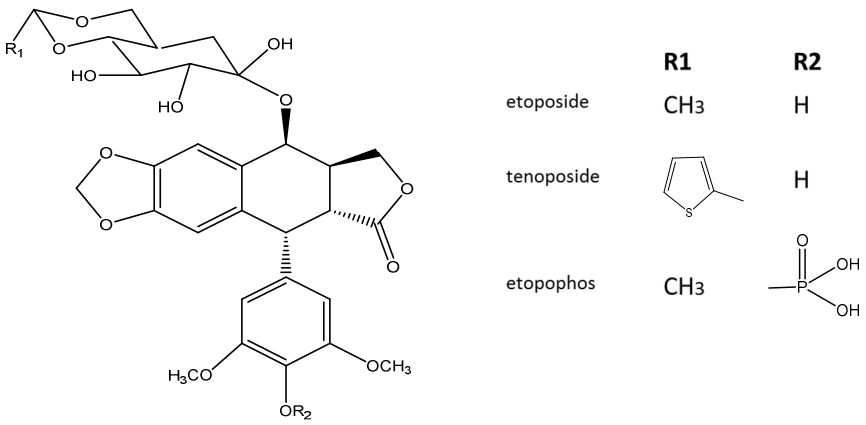


Figure 5. Structures of podophyllotoxin derivatives

As highlight of the research efforts, several semisynthetic analogs has been developed, such as etopophos carried out by Bristol-Myers company and GL-331, which has a *p*-nitro aniline group at the 4β position. GL-331 shows a potent topoisomerase II inhibitor, but also causes the apoptotic cell death by inhibiting protein tyrosine kinase activity. NK 611 is other etoposide derivative perfomed in Nippom-Kayaku Company, which has a demethylamino-side chain instead a hydroxyl group at the 2' position of the ethylidene glucoside moiety. Thus, makes it improve water solubility by forming salts. Another derivative, TOP53

developed by Taiho Pharmaceuticals has a carbon chain containing different functional groups such as, hydroxyl-, amino- or amino groups instead of the glycoside group of the etoposide. All of them are chemotherapeutic candidates for cancer treatments and are represented in the Figure 6.

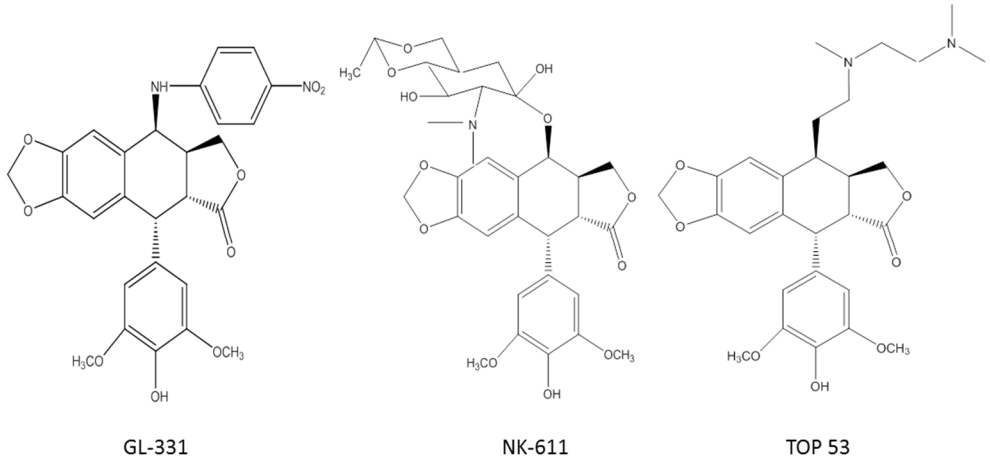


Figure 6. Representative of podophyllotoxin analogs

The biological activity of PTOX is based in inhibiting the assembly of the mitotic spindle, and consequently inducing the arrest of the cell cycle at the mitosis by binding to the monomeric subunits of microtubules. The same biological activity presents colchicine, but the difference between them is that the PTOX has a rapid effect and does not have a reversible effect.The ability to bind tubulin, makes to study in inhibition of reverse transcriptase, which may be exploited to selectively combat RNA viruses, also not only cancer treatments(MacRae and Towers 1984)

In contrast, the mechanism of the podophyllotoxin analogs consist in stabilizing the DNA-enzyme cleavable complex and inhibiting the strand rejoining activity to the topoisomerase II, so the double strand DNA breaks(Hande 2008). Figure 7 summarized the biological action of PTOX and its analogs.

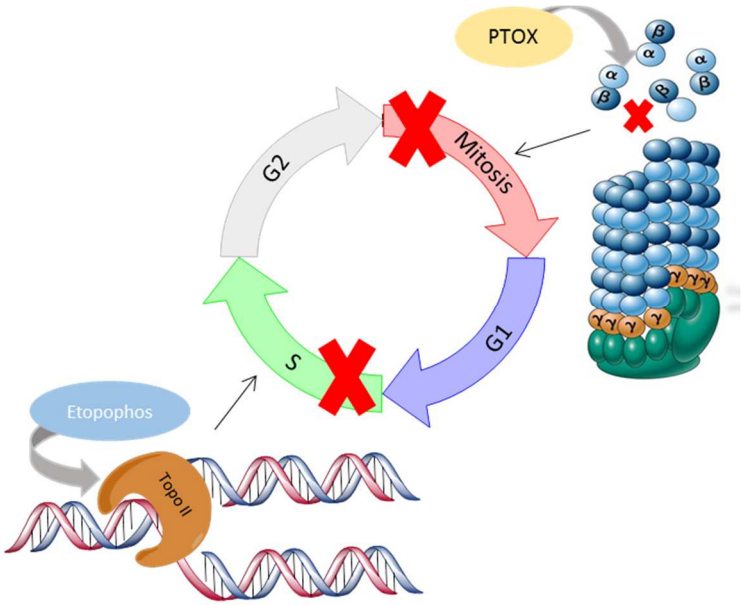


Figure 7. Action of PTOX and derivatives etopophos. Mechanism for PTOX compound effect on microtubule formation and etopophos on the topoisomerase II activity.

2.4.PTOX Sources

PTOX was isolated for first time from the genus *Podophyllum* composed byperennial rhizomatous herbs growing in the understored of subalpine forests in the Himalayas (Farkya et al. 2004). The main source of this lignan is the roots/rhizomes of *P.*

hexandrum and *P. peltatum*, whichcontain 4.3 and 0.25% PTOX, respectively, whereas the content is almost negligible in shoots of *P. hexandrum* (0.005%) compared to *P. peltatum* (2.05%) (Yousefzadi et al. 2010 b;Kumar et al. 2015; Baldi et al. 2007). The availability of the plant sources is limited because this genus have a long juvenile phase, poor fruit-setting ability, limited habitat and slow regeneration. Thus, have been generated the overexploitation of this genus.

To cover the high demand of PTOX , alternatives sources of this compound have been explored, founding PTOX production in other genera such as: *Jeffersonia*, *Diphylleia* and *Dysosma* (Berberidaceae), *Catharanthus* (Apocynaceae), *Polygala* (Polygalaceae), *Anthriscus* (Apiaceae), *Linun* (Linaceae), *Hyptis* (Verbenaceae), *Teucrium*, *Nepeta* and *Thymus* (Labiaceae), *Thuja*, *Juniperus*, *Callitris* and *Thujopsis* (Cupressaceae), *Cassia* (Fabaceae), *Haplophyllum* (Rutaceae), *Commiphora* (Burseraceae) and *Hernandia* (Hernandiaceae) (Gordaliza. et al. 2004).

2.5.Biosynthesis of podophyllotoxin

The biosynthetic pathway leading to PTOX is not fully understood and may present some variations depending on the producing plants. PTOX and related compounds are derived from the shikimic acid/phenylpropanoid pathway through several metabolic steps which have not yet been completely elucidated (Jackson and Dewick 1984 a; b; c; Xia et al. 2000). So far, only 33 steps are known up to the formation of the intermediate pluviatolide;

thereafter, 36 possible steps have been predicted but not validated either for the structures of intermediates or for genes/proteins/enzymes involved (Wankhede et al. 2013; Kumar et al. 2016). From these 33 steps, a total of 26 genes encoding podophyllotoxin pathway enzymes are known, starting from D-erythrose-4-phosphate to intermediate compound pluviatolide (Scheme 1). Out of these 26 genes, only 12 genes, namely *PHENYLALANINE AMMONIALYASE (PAL), CINNAMIC ACID 4-HYDROXYLASE (C4H), p-COUMARATE 3-HYDROXYLASE (PCH), p-HYDROXYCINNAMOYL TRANSFERASE (HCT), 4-COUMARATE-COA LIGASE (4CCL), CAFFEIC ACID 3-O-METHYLTRANSFERASE (CMT), CAFFEOYL-COA-O-METHYLTRANSFERASE (CCMT), CINNAMOYL COA REDUCTASE (CCR), CINNAMYL ALCOHOL-DEHYDROGENASE (CAD), DIRIGENT PROTEIN OXIDASE (DPO), PINORESINOL LARICIRESINOL REDUCTASE (PLR), and SECOISOLARICIRESINOL DEHYDROGENASE (SD)*, have been characterized in *Podophyllum spp*. and their role in PTOX biosynthesis has been shown through studies on expression analysis (Kumar et al. 2016; Wankhede et al. 2013; Lan et al. 2010; Yousefzadi et al. 2010 b, Marques et al. 2013). Most of these genes catalize enzymatic steps, starting from phenylalanine to matairesinol. Considering the phenylpropanoid pathway, phenylalanine is deaminated by *PAL* to give cinnamic acid, hydroxylation by *C4H* leads to p-coumaric acid and after additional steps coniferyl alcohol is formed by *CAD*(Whetten and Sederoff 1995; Dixon and Srinivasa 2003). However, no information exists

on remaining 14 genes, starting from D-erythrose-4-phosphate to p-coumaric acid in lignan producing species.

The amount of *p*-coumaric acid biosynthesized is crucial in providing supply of further intermediates such as caffeic acid, ferulic acid, coniferyl aldehyde, coniferyl alcohol, and so on in the pathway that eventually lead to the formation of PTOX.

Two molecules of coniferyl alcohol are coupled to pinoresinol (Scheme 1). A so called dirigent protein leads to the exclusive formation of (+)-pinoresinol in *Forsythia intermedia*(Davin and Lewis 2000).The same metabolic step was found in *Linum flavum* and *Podophyllum peltatum* and the gene encoding the dirigent protein was cloned and the correspondent recombinant protein was obtained (Xia et al. 2000). In contrast, the both enantiomers of pinoresinol has been founded in cell cultures of *L. album*(Von Heimendahl et al. 2005). (+)-Pinoresinol is reduced via (+)-lariciresinol to (-)-secoisolariciresinol by pinoresinol–lariciresinol reductase and subsequently oxidized to (-)-matairesinol (Dinkova-Kostova et al. 1996 ; Xia et al. 2000; Okunishi et al. 2004, (Von Heimendahl et al, 2005; Youn et al. 2005; Moinuddin et al. 2006). In *P. hexandrum* as well as in *L. flavum* the enantiospecific reduction of pinoresinol in two steps leading to secoisolariciresinol due to the action of the enzyme NADPH-dependent pinoresinol/lariciresinol reductase (*PLR*) has been confirmed and the characterization and clonation of this gene in *P. hexandrum* (*PhPLR*) has been carried out by Wankhede et al. (2013).

Matairesinol is consequence of the action of the enzyme secoisolariciresinol dehydrogenase (*SD*), which has been found by Arneaud and Porter (2015) in *Phialocephala podophylli*, an endophytic fungus of *Podophyllum peltatum*. In fact, these authors recently sequenced and cloned the gene encoding for the secoisolariciresinol dehydrogenase in the strain PPE7 of the fungus *Phialocephala podophylli*. Simultaneously, the same gene was also characterized from *P. peltatum* and cloned by the same authors.
Currently, from matairesinol only is known the successive structural changes in the molecule that involved aryltetrahydronaphthalene ring formation, additional hydroxylation an O-methylation steps as well as the formation of the methylendioxy bridge. This last step that at present is known as metabolic step leading to pluviatolide, the authors informed that it was catalyzed by a NADPH-dependent cytochrome P450 enzyme.

In relation to the pluviatolide formation, in 2013 (Marques et al. 2013) working with *P hexandrum* and *P. peltatum* founded two genes encoding two cytochrome P450s enzymes (CYP719A23 and CYP719A24, respectively) after sequencing the transcriptome by means of the Illumina system and bioinformatics studies. The activity of these two enzymes was *in vitro* assayed. The results showed that they catalize the formation methylenedioxy bridge in (-) matairesinol yielding pluviatolide, in the mentioned plant species, indicating that these two enzymes can be considered as pluviatolide synthases. Later transcriptomic, bioinformatic and metabolomic studies carried out by the same research team have shown a new pathway leading to the alkaloid a morphine in

Podophyllum species with high similarity with the podophyllotoxin biosynthesis (Marques et al.2014).

Very little information exists about the metabolic steps that lead pluviatolide to the deoxypodophyllotoxin (dPTOX), the accepted precursor of PTOX, since in *Podophyllum*, *Linum* and *Forsitia* species these steps have not yet been well characterized and the genes/enzymes that control them are still unknown.
In *Linum album in vitro* cultures (both cell and hairy root cultures), the main lignan founded was 6-methoxy podophyllotoxin(MPTOX), and PTOX was found in very little concentration (Federolf et al. 2007). These authors showed, by means of feeding experiments that, dPTOX obtained from matairesinol, was the compound from which both the PTOX and the MPTOX are formed.

In order to clarify the steps leading to PTOX and MPTOX, feeding experiments were already conducted in 1984 in *Podophyllum hexandrum* plants. When these plants were fed with labeled phenylalanine, cinnamic acid or ferulic acid, labeled PTOX was isolated (Jackson and Dewick 1984). When dPTOX was added to cell cultures of *Linum flavum*, this compound was converted to MPTOX and its MPTOX-β-D-glucoside whereas PTOX was only converted to PTOX-β-D-glucoside indicating that dPTOX and not PTOX is the precursor for MPTOX (Van Uden et al.1995). Therefore, dPTOX might be the branching point to either PTOX or MPTOX biosynthesis. The hydroxylation at position 7 of dPTOX to PTOX (deoxypodophyllotoxin 7-hydroxylase, DOP7H) has still to be characterized. On the way to MPTOX the alternative hydroxylation

at position 6 of dPTOX by deoxypodophyllotoxin-6-hydroxylase (DOP6H) was proven to be catalyzed by a cytochrome P450 enzyme which was partially characterized in *L. flavum* and *L. nodiflorum*(Molog et al. 2001). This metabolic step leads to the formation of β-peltatin. This compound is converted to β-peltatin-A-methylether (PAM) by β-peltatin 6-O-methyltransferase (βP6OMT). This enzyme was characterized for the first time in *L. nodiflorum* in 2003 (Kranz & Petersen 2003). The enzyme for the last hydroxylation step to form MPTOX (β-peltatin-A-methylether 7-hydroxylase, PAM7H) is not yet known. In 2009, Li et al. corroborated the divergent route from dPTOX since in *Podophyllum hexandrum* tissue culture showed that the conditions that increased the activity of the DOP6H enzyme, resulted in more MPTOX accumulation whereas those conditions which reduced this activity caused more PTOX accumulation.

This fact indicates that PTOX only accumulates if DOP6H activity is missing. Further evidence for this hypothesis comes from feeding experiments. dPTOX administered to the culture medium leads to the formation of PTOX in cell cultures PTOX producer. The addition of dPTOX, however, to the cell line MPTOX producer gave only PTOX if the DOP6H was blocked by inhibitors for cytochrome P450 monooxygenases. Since PTOX formation by DOP7H is not blocked by these inhibitors this hydroxylase is probably not a cytochrome P450 monooxygenase. In the presence of DOP6H, PAM is formed and can serve as substrate for the 7-hydroxylation by PAM7H. In this case PTOX is almost not formed. If DOP6H is absent dPTOX could accumulate instead of PAM. In that case PAM7H can

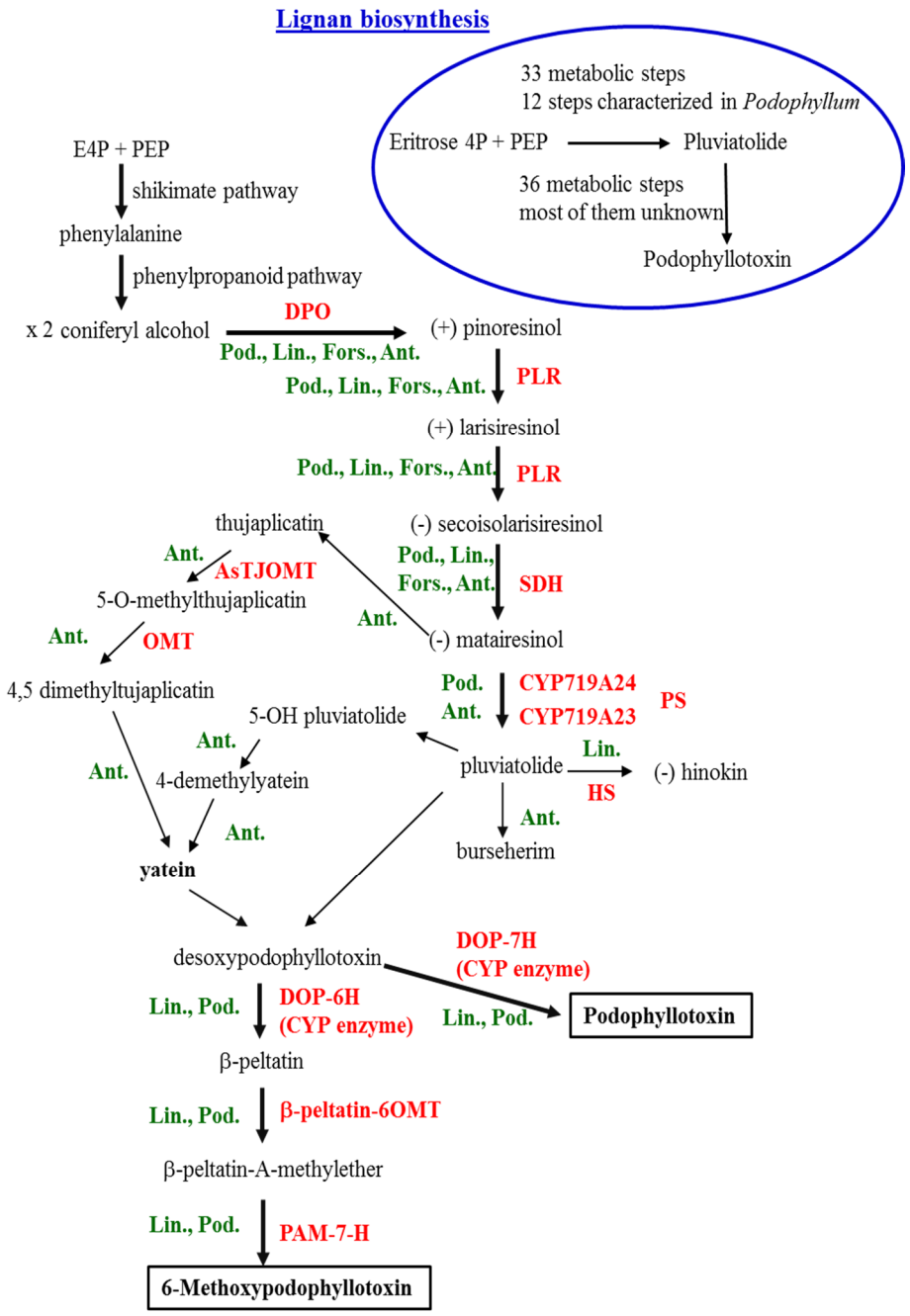
hydroxylate dPTOX (1) instead of PAM at position 7 to PTOX. This means that the hydroxylations at position 7 of PAM to MPTOX or DOP to PTOX might be catalyzed by the same enzyme, the PAM7H and not two different enzymes, PAM7H and DOP7H, respectively (Fuss 2003 and Federolf et al. 2007).

In *Anthriscus* spp., other metabolic steps leading to PTOX and MPTOX and other related compounds has been determined. The biosynthetic pathway leading from 2x coniferyl alcohol until (-) matairesinol seems to be the same as indicated for *Podophyllum*, and *Linum* species however, after the formation of this compound other metabolic steps are found in these plant species.

A new metabolic pathway that forms yatein, as a precursor of dPTOX, from matairesinol was proposed by Sakakibara et al. (2003) in *Anthriscus sylvestris* after feeding young shoots with labeled lignan precursors. In this species the authors indicated that from matairesinol, in 4 metabolic steps, through the formation of thujaplicatin, 5-methylthujaplicatin, 4,5 dimethyl thujaplicatin, the compound yatein is formed. Consequently in *Anthriscus sylvestris* yatein formation from matairesinol needs the action of two O-methyltransferase: the first transforms region-selectively thujaplicatin to 5-O-methylthujaplicatin and the second, also region-selectively, from this compound forms 4,5-O,O-dimethylthujaplication.

Ragamustari et al. (2013), isolated and characterized a cDNA encoding the first of these two O-methyltransferases from roots and

young shoots of *A. sylvestris*. The O-methyltransferase (OMT) was expressed as a recombinant protein using the pET expression system. From the substrates that were tested, the recombinant OMT exclusively catalyzed the regio-selective methylation of thujaplicatin to produce 5-O-methylthujaplicatin, and thus was designated as *A. sylvestris* thujaplicatin OMT (AsTJOMT). Kinetic analyses with this substrate (thujaplicatin) were carried out showing that AsTJOMT had a Km value of 3.8 μM and kcat value of 0.29 min^{-1}. Quantitative real-time polymerase chain reaction showed that AsTJOMT had the highest expression level in roots compared with other organs. This was in accordance with plant protein assays in which specific activity for thujaplicatin was significantly higher in roots compared with other organs. To the best of our knowledge, this is the first report on the isolation and characterization of a thujaplicatin-specific plant OMT. The formation of thujaplicatin has been also confirmed in *Tuja occidentalis*(Sakakibara et al 2003). This pathway, as indicated above, has not been confirmed in *Podophyllum* or other lignan producing species. In fact, (Wankhede et al 2013) informed that on the basis of the biosynthetic studies carried in different plant species, it is not clear in which extend the information obtained in one PTOX producing plant species can be applicable to other species producing the same lignans. In *Anthriscus sylvestris*, the same authors found that from matairesinol, the lignan burseherim could be obtained through the pluviatolide formation, although this last compound could not be a yatein or podophyllotoxin precursor in this species.



Scheme 1. Possible biosynthetic pathways for PTOX production currently described in different sources.

2.6.Biotechnological approaches for PTOX production

Biotechnological production using plant cell cultures may be considered as an alternative source for PTOX production to the cultivation of the plants in the field. Kadkade (1981)was the first researcher to report PTOX bioproduction in tissue cultures of *Podophyllum* species. Further studies performed by other authors confirmed the potential of the plant cell cultures of *P. hexandrum* and *P. Peltatum* as alternative sources for PTOX production(Kadkade 1982,Anbazhagan et al. 2008). However, the main problem of these cultures is that are quite recalcitrant, suffer browning and grow very slow (Fuss 2007). Due to these drawbacks, over forty species were studiedfor in vitro production of therapeutic lignans. Among them,in vitro cultures of *Linum* species accumulated the higher quantity of cytotoxic lignans.

For example, cell suspension cultures of *Linum nodiflorum* accumulate 0.18% PTOX and 0.6% MPTOX in dry weight (DW) basis (Konuklugil et al. 2007). The highest yields of MPTOX in plant *in vitro* cultures was found in a root-like suspension culture of *Linum flavum* with 0.7% DW (Van Uden et al.1990) and in cell suspension cultures of *Linum nodifloruma* quantity of 1.7% DW MPTOX was reached (Kuhlmann et al. 2002). Specially, in vitro cultures of Iranian flax, *Linum album*, have become an attractive source of PTOX (Ionkova 2008), with high productivities among other *Linum* species, which shown to be PTOX producer as *L.*

flavum, *L. usitatissimun*, *L. persicum*, *L. nodofilum* (Schmidt et al. 2010,2012 ;Malik et al. 2014).

The PTOX and some derivatives production are reported by several authors in callus, cell suspension or organ cultures of *L. album.* In regard to cell suspension cultures, the studies performed demonstrated their suitability as a PTOX production platform, but the bioproduction obtained differs, Smollny et al.(1998) reported values of 0,50 %DW of PTOX, while Van Fürden et al.(2005) reported of 0,77% DW, Federolf et al.(2007) 0,86 % DW, among other authors which indicated strong variability in the lignan bioproduction in *L. album*. Besides, other biotechnological platforms are presented by the transformed cultures, as the hairy root cultures. Baldi et al. (2008) and Farkya and Bisaria (2008) founded in hairy roots from *L. album* a 0,5 %DW of PTOX production, conversely Wink et al.(2005) found the MPTOX as main lignan in hairy roots, latter Chashmi et al. (2013) corroborate that the hairy roots are mainly MPTOX producer reaches a maximum of 48 mg/g DW. On the other hand, Baldi et al. (2008) evaluated other systems like as, the transformed cell cultures, which presented high grown and obtained PTOX quantities of 52,9 mg/L. from cell lines derived of *L. album* plants transformed with *A. rizhogenes.*

2.5.1.Empirical Factors

In plant biofactories, the physiological stage of the plant when is excised, as well as the explants type to establish*in vitro* cultures for the selection of cell lines and some empirical factors have been

become important aspects to evaluated the variability of biomass and target compounds production (Arroo et al. 2002; Farkya et al. 2004). For example, variations intra species of *L. album* from different regions have been reported, evaluated from the capacities to biosynthesize secondary metabolites view (Konuklugil et al. 2007). This was corroborated by Federolf et al.(2007), who distinguished the accumulation patterns differ in two cell lines, one was a PTOX producer line (2.6 mg/g DW) and the order MPTOX producer line (5.4 mg/g DW). In order to obtain a more stable or improved bioproduction some strategies has been developed focused mainly in the optimization of culture media, carbon sources and plant growth regulators(PGR) addition, as well as, the use of elicitors, feeding experiments and metabolic engineering.

In this scenario, the effects of PGR addition in *Linum* cultures have been reported reported by several authors.The use of indolacetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2-4 D) increased PTOX production in 1,86 fold, and some authors as Yousefzadi et al. (2010 b and 2012) ; Bahabadi et al.(2011,2012) evidence the use of naphtalenacetic acid (NAA) and kinetin (Kin)had positive effects on growth and PTOX production. Van Uden et al. (1990)confirmed that the use of NAA containing media accumulated 40-50 folds more of lignans in *L. favum.* In regard to the carbon source, the sucrose was found to yield higher biomass to maximum values to 0,66 g/g and PTOX contents of 52,9 mg/L than others, as glucose which present 27,8 g/L of PTOX content (Baldi et al. 2008;Van Uden et al 1990).

2.5.2.Elicitors

A special mention deserves the use of elicitors in empirical and rational approaches carried out for improving the biotechnological production of target compounds. The elicitation is the most widely and effective strategy to boost the production of secondary metabolites in hairy root and cell suspension cultures. Elicitors are agents that induce plant defense responses to pathogen infection and stress, and in their mode of action, elicitors induce transcriptional reprogramming of different types of target genes. The elicitor is usually referred to as an extracellular signal compound which can be classified as endogenous or exogenous (Kombrink 2012). Endogenous elicitors are derived from the plant host and interact with the aggressor. The most representatives in this category are cell wall oligosaccharides, intracellular signaling compounds such as salicylic acid (SA), jasmonic acid (JA) and its derivative methyl jasmomate (MeJA). Exogenous elicitors are compounds released by microbial pathogens or aggressors, and include certain peptides, polysaccharides and glycoproteins.

Thus, approaches in *L. album* cultures treated with different elicitors exhibit higher production of lignans. Van Fürden et al. (2005) reported that treatment of *Linum album* cell cultures with 100 uM MeJA increased the PTOX yield to 7.69 mg/g DW compared with 2.66 mg/g DW obtained in the non-induced control conditions, a similar trend was observed with MPTOX, which reached a concentration of 1,11 mg/g DW in MeJA treated cells and 0,53 mg/g DW in non induced conditions. Bahabadi et al. (2011; 2012)

reported PTOX levels of 140 ug/g DW and 120 ug/g DW when cell suspension cultures were elicited with *Fusarium. graminearium* and Sclerotinia *sclerotiorum*, respectively. Similar effects have been observed in other PTOX derivatives such as lariciresinol, which bioproduction is susceptible to be increased by elicitors from *Rhizopus. stolonifer.* Abiotic elicitors such as 1 mM Ag^{+} have been tested in cell suspension cultures with positive results for PTOX production (0,24 mg/g DW) (Shams-Ardakani et al. 2005).

Our group has recently determined that salicylic acid (SA) at 10 μM is a potent elicitor for *L. album* cell cultures, increasing PTOX production by more than 200% without any effect on growth (Yousefzadi et al 2010 a). In the same way, light has a potent elicitor effect not only in the synthesis of primary but also secondary metabolites. PTOX was markedly increased by red and blue light with 2.5 fold more than the control maintained in the dark (Yousefzadi et al 2012). Other studies with chitosan and chitin oligomers evidenced that cells treated with chitosan exhibited the highest yield of PTOX and lariciresinol, with three and two- fold increases than the control respectively (Bahabadi et al 2014). In regard to roots culture studies, stimulation of hairy roots with fungal elicitors *(Fusarium graminearum)* induced the highest increases of PTOX (two-fold) and lariciresinol (three-fold) compared with the untreated controls (Bahabadi et al 2014).

Many genes that encode biosynthetic enzymes involved in plant secondary metabolism have been found to modify their expression patterns in response to elicitors (Kombrink 2012). However, little is

known about the transcriptional responses of genes involved in lignan biosynthetic pathways upon elicitation. The works referred above demonstrate that elicitors increase the expression of genes in *L. album* cultures, with a significant effect on genes involved in the early steps of the phenylpropanoid pathway. It has also been observed a correlation between *PLR* expression and PTOX production. These investigations suggested that PLR is a key enzyme for the up-regulation of the lignans biosynthetic pathway, thus confirming previous studies (Bahabadi et al 2012;2014). Most genes related with the phenylpropanoid pathway are stimulated within 24 h after elicitation, based on the transcriptional profiling of *PAL* (Yousefzadi et al 2012).

Coronatine is a pathogenic toxin naturally produced by the bacteria *Pseudomonas syringae*, which acts as molecular mimic for the isoleucine conjugated form of jasmonic acid (Ja-Ile), thus activating the host's jasmonate signaling pathway. Overlapping effects of coronatine and jasmonates have been confirmed in many types of experiments since the first report by Greulichi et al. (1995) until now. The use of MeJA and coronatine as elicitors resulted in enhanced mRNA levels as well as enzyme activities of flavonoid biosynthetic enzymes PAL, and chalcone synthase(Dietrich et al 1992); while Gundlach et al. (1992) reported PAL activation in jasmonic treatments; similar to reported byQian et al. (2004).Other studies indicated that PAL and chalcone synthase were induced by yeast elicitor in *Medicago truncatula*(Suzuki et al 2005) and in *L. nodofilum*, DOP6H and POMT, two enzyme involved in lignan pathway increased up to 21.9-fold and 14.6-fold, respectively in the

treated cultures with yeast elicitor(Berim et al. 2005). Comparisons between concentration and yield have been established, in which coronatine has been found to be more active than MeJA(Fliegmann et al. 2003;Krumm et al. 1994). Biological assays in tomato revealed other biological functions of elicitors in the host such as tendril coiling, inhibition of root elongation, hypertrophy, chlorosis and ethylene emission among others (Farmer andRyan 1990; Tamogami and Kodama2000;Yao et al. 2002; Schüler et al. 2004).

Previous assays carried out by our group indicated the enhancement of taxanes production in Taxus cell cultures(Onrubia et al. 2013) and hazel cell cultures by coronatine (Gallego et al 2015). Assays carried out in *Linum nodofillum* with two coronatine analogs (coronalon and indanoyl-isoleucine) induced higher accumulation of MPTOX compared with the elicitation with MeJA, which showed a more moderate influence (Schüler et al. 2004) These results suggested that coronatine is a promising elicitor for improving the bioproduction in *L. album*cell cultures.

2.7.Metabolic engineering for the production of PTOX

Metabolic engineeringprovide insight into the metabolic pathway involved in complex regulatory mechanisms, transport and limiting steps. The analysis of DNA, mRNA, proteins and metabolites in the cell will aid in the directional exploration of plants as cell factories for the production of therapeutic compounds. In this context several strategies have been develop some of them included regulatory

genes, over-expression or suppression genes or the partial identification of key enzyme in a specific pathway.

A part from the already mentioned transcriptomics studies carried out in lignan producing species, which permitted the characterization of several genes involved in these biosynthetic pathways. The expression of these genes was in accordance with the PTOX accumulation. In *Podophyllum*species, two suppression substractive hybridation libraries were synthesized obtaining several expression sequence tags (EST) with high similarity with known genes involved in PTOX biosynthesis from other lignan producing species like *Linum* spp. or *Forsythia intermedia*. Among them the EST corresponding to pinoresinol/lariciresinol reductase was identified, sequenced and cloned. The expression level of *PhPLR* gene as well as of other genes such as *PhSDH* and *PhDPO* was determined and the results showed that the biosynthesis of lignans of interest was not only restricted to subterranean organs but also can take place in leaves, although these compounds can be quickly glycosylated (Vankhede et al., 2013). These transcriptomic studies together with metabolomics analyses probably will permit to get more insights and to deeply know the *Podophylumspp* lignan biosynthesis giving information about the target steps that can be activated by means of metabolic engineering technics.

Taking into account all of the above, it suggests that approachesaiming at increasing podophyllotoxin content require the

genetic engineering of multiple enzymatic steps thatfacilitate the metabolic flow to the final products. The enzymes involved in several secondary metabolite pathways are often assembled in multiprotein complexes that enable an efficient canalization and avoid the diffusion of intermediates. The identification of such complexes can be performed through yeast two hybrid approaches, in which direct protein-protein interactions are detected (Kiemer&Cesareni 2007). This approach has been used to identify enzymatic interactions in the flavonoid biosynthetic pathway (Burbulis and Winkel-Shirley). In the case of the PTOX pathway, the identification of interacting proteins with already known enzymes may identify other enzymes in the pathway that belong to a potential PTOX biosynthetic complex

2.6.1.Yeast two hybrid system

Previous statements in this field have led to techniques such as the yeast two hybrid system (Y2H) (Fields and Song 1989),which is widely used to identify proteins of interest and could be applied for the discovery of new enzymes involved in plant secondary pathways such as lignan biosynthesis. The Y2H allows access to almost the entire cellular proteome, including membrane proteins, transcriptionally active proteins and proteins localized in specific subcellular compartments. Currently, a majority of published proteins interactions have been detected using thissystem. The screen of target biomolecules in Y2H systems takes advantage of the properties of the galactose-gene activating transcription factor

(GAL4) of yeast (*Saccharomyces cerevisiae*) (Brückner et al. 2009; Suter et al. 2008).

The GAL4 protein has two separable domains; the N-terminal domain also named DNA binding domain (BD) and the C-terminal domain with the activating domain (AD).When the two domains recognize each other the reconstituted AD+BD protein binds specific sequences in the DNA and stimulates transcription. Any protein can be fused to each of these AD and BD separable domains.One will be the bait (an already know protein) in frame with the BD domain and interactors of this protein are known as a prey, which are fused proteins to the AD domain and are represented in cDNA libraries.In the bait-prey interaction, the GAL4 protein is reconstituted by co-transformation in yeast which activates the transcription of one or more reporter genes that generate a color reaction or enable growth of auxotrophic yeast on specific media(Chien et al. 1991).

The most common reporter is the GAL1-lacZ fusion gene, which codes for the beta-galactosidase and allowsthe identification of interacting proteins in yeast cells by colorimetric change. Other are auxotrophic markers such as LEU2, HIS3, ADE2, URA3 and LYS2 that enable growth on minimal yeast media (Durfee et al. 1993 ; Ito et al. 2001). Two or more reporters are used to increase the stringency of the Y2H screens and avoid false positive interactions. The Y2H technique allows detection of interacting proteins on a genome-wide scale by two screening approaches which can be

distinguished: the matrix (or array) and the library approach. Figure 8.

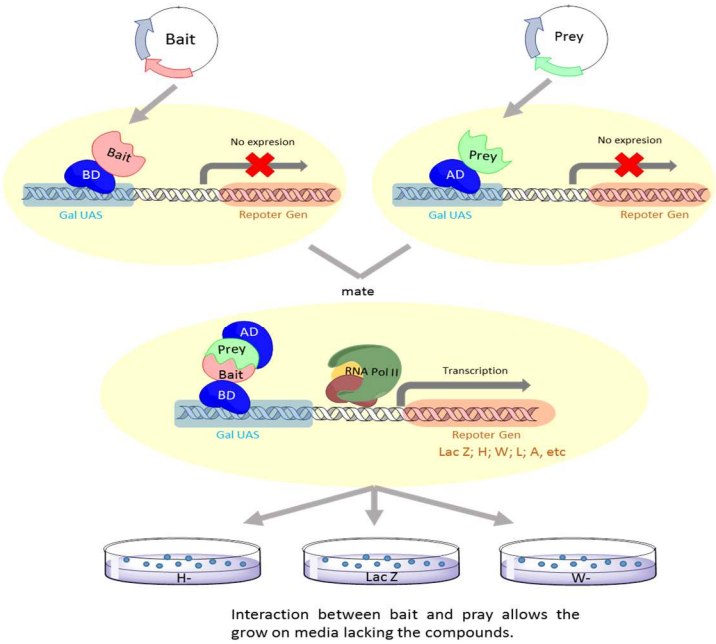


Figure 8. The classical yeast two hybrid system based in the activation of reporter genes

Evaluating several combinations between full-length open reading frames (ORFs) by mating a set of baits *versus* a set of preys is possible in a matrix screening but is conditioned to only few known proteins and the risk of false negatives is high. The second approach is not targeted and uses the entire plant proteome. In this approach, cDNA library preys are generated that may contain cDNA fragments in addition to full length ORFs, thus largely covering the transcriptome. In this type of screen, the number of false positives increases,so the interactions need to be further

confirmed by other methods (Fromont-Racine et al. 2000;Auerbach et al. 2002)

Efforts to minimize the drawbacks are focused in adjusting the stringency of the medium but concomitantly penalize detection of weak and transient interactions. Another possibility is to use 3-AT (3-aminotriazole), which has been demonstrated to be a competitive inhibitor of the HIS3 reporter gene product and it is usually required to reduce the level of background growth. Other limitations of the original Y2H approach have been reported and include differences in expression levels, which depend on the promoter used and may affect sensitivity and specificity of the screen, post-translational modifications, or that the system relies on proteins localizing in the nucleus, which may not correspond to the natural cellular environment (Brückner et al. 2009, Ehlert et al. 2006).

Alternative Y2H methodshave been developed for specific casesbut all of them share a similar principle. Some of them are based on the repression of transactivation or the use of alternative polymerase III transcription in cases in which the proteins can directly activate transcription. Such transactive baits would activate transcription in absence of any interaction with a prey, such as in the split-ubiquitin system (Dutta et al. 2014), RNA polymerase III based two-hybrid (Pol III) (Petrascheck et al. 2001), repressed transactivator system (RTA) (Hirst et al. 2001), Cytosolic split-ubiquitin system (cytoY2H) (Möckli et al. 2007), Ras recruitment system (RRS), SCINEX-P system(Broder et al. 1998).

To identify possible interactors localized in other cellular compartments than the nucleus, other methods can be used such as the SOS recruitment system (SRS)(Aronheim et al. 1994), the membrane split- ubiquitin system (MbY2H) (Stagljar et al 1998), G-protein fusion system,reverse Ras recruitment system (rRRS)(Hubsman et al. 2001), split-Trp system (Tafelmeyer et al. 2004).It has also been applied to screen for novel interactions from a variety of transcriptional activators.

3. MATERIALS AND METHODS

3.1.Establishment of in vitro plantlet cultures

Linum album seeds were kindly ceded by Dr. Yousefzadi (Bander Abbas University – Iran) (Chashmi et al. 2013). Theywere sterilized with a solution of 70% ethanol, washed with distilled water, and dipped in a solution of 0.1% $HgCl_2$ for 15 min in an ultrasonic bath before being placed in 3% $NaCl_2$ solution. Seeds were then treated with a solution of gibberellin [500 mg/L] for 1h with constant stirring, and transferred to Murashige and Skoog solid medium (MS) (Murashige and Skoog 1962) and maintained at 25°C with a photoperiod of 16 h light/8 h dark. After 30 days, small plantlets were developed and subcultured in Magenta jars (SIGMA) in a hormone-free MS medium that was changed every month.

3.2.Initiation and maintained of adventitious and hairy roots cultures of L. album

The adventitious roots were established from *L. album* plantlets. The roots were separated and cultured in MS medium at half concentration of salts supplemented with 0.5 mg/L indole-3-acetic acid (IAA) as a plant growth regulator (PGR) to promote root elongation, and were kept in the dark at 25°C for 30 days. After this period of time, adventitious roots were subcultured every 30 days in PGRs-free medium at half concentration of salts and maintained in the same conditions as described above (Figure 9).

For the induction of transformed roots, leaf discs of *in vitroL. album* plantlets were infected with the LBA9402 strain of the *Agrobacterium rhizogenes* in PGRs-free MS medium as described by Chashmi et al. (2013). Samples were kept in the dark at 25°C for 48 h to allow the infection of the bacteria. The leaves were then transferred to MS medium supplemented with 500 mg/L of claforan to eliminate the bacteria. After 30 days, roots appeared in most of the inoculated explants. Subsequently, roots were isolated from the leaf discs and the established root lines were maintained by subculturing every 20-30 days in fresh medium (Figure 10).Initiation and maintenance of *L. album* wild type and transformed callus cultures

To establish the callus cultures, sterilized *L. album* plantlets were cut into pieces and grown in MS medium, supplemented with 2 mg/L α-naphthaleneacetic acid (NAA) and 0.4 mg/L kinetin (KIN) as PGRs. They were grown in darkness for a period of 30 days at 25ºC, after this period, most of the explants had developed callus. Subsequently, callus mass was separated from the explant to establish callus lines, some of them showing organogenesis. Wild type (WT) calli were maintained by subculturing every 20-30 days in the same culture medium, in dark (Figure 10).

To establish transformed callus cultures, L. album hairy roots were dedifferentiated in MS medium supplemented with 2 mg/L of N-2-chloro-4-pyridyl-N-phenylurea (4-CPPU) and 0.1 mg/L indole 3-butyric acid (IBA) and cultured in the same conditions as the WT

callus, until to evidence of non-differentiated mass of cells (Figure 10).

3.3.Establishment of the cell suspension cultures

In order to obtain fine cell suspension lines, friable callus cultures, maintained as described above, were transferred to MS liquid medium added with the same PGR combinations (2 mg/L NAA + 0.4 mg/L KIN) for WT calli and for transformed callus (2 mg/L 4-CPPU + 0.1 mg/L IBA) and cultured in an orbital shaker at 25ºC of Tª and 110 as described by Yousefzadi et al. (2010 a). After several subcultures of 2 weeks, when the friable calli was completely disintegrated, cell biomass were filtered through Nylon filter of 150 µm diameter of porous in order to get the fine cell suspensions (Figure 10).

3.4.Genetic confirmation of the transformed cultures

The transformed nature of hairy roots and cells obtained from the dedifferentiation of hairy roots was checked by polymerase chain reaction (PCR). Total genomic DNA was performed with the method proposed by Dellaporta et al. (1983). Around 200 µg of initial material was used for the extraction. PCR analysis was performed using Dream Tag Polymerase with 800 ng of DNA for the hairy roots and 1300 ng for the cells. Gene-specific primers

designed by Exposito et al. (2010) were used (Table 1). The cycling conditions of gene sequences were: *ROLC* (94ºC 5 min; 35 cycles; 94ºC 1 min; 60ºC 30 seg; 72ºC 1 min and 72ºC 5 min); *AGS* (94ºC 5 min; 35 cycles; 94ºC per 1 min; 64ºC 30 seg; 72ºC during 45 seg and 72ºC 5 min); *MAS* (94ºC 5 min; 35 cycles; 94ºC 1 min; 62ºC 30 seg; 72ºC 45 seg and 72ºC 5 min), *AUX* (94ºC 5 min; 32 cycles; 94ºC 1 min; 56ºC 1 min; 72ºC 1:30 min and 72ºC 5 min). The amplicon size was analyzed in bromide agarose gel at 1%.

Table 1. Sequences of the primers used to amplify the genes of transformation confirmation

Gen	Primers	Amplicon Size	Tm
ROLC	Fw: 5'-TAACATGGCTGAAGACGACC-3' Rv: 5'-AAACTTGCACTCGCCATGCC-3'	534 pb	60ºC
AGS	Fw: 5'-GGCGTCAGCACCTCATATCCG-3' Rv: 5'-TTCGAAGCCTTTGCCTGCAAA-3'	347 pb	64ºC
MAS1	Fw: 5'-ACCTTGGTACTGCCCAGCCAC-3 Rv: 5'-CTTCAGTGGTCCATACCCACC-3'	343 pb	62ºC
AUX1	Fw: 5'-TTCGAAGGAAGCTTGTCAGAA-3' Rv: 5'-CTTAAATCCGTCTGACCATAG-3'	350 pb	56ºC

3.5.Study of the growth capacity

The growth capacity of the plant material was measured as dry weight (DW) by freeze-drying the fresh biomass (FW) and the cell viability was determined using fluorescein diacetate (FDA) and propidium iodide (IP), both at 0.01% (w/v) (Pollard, 1990).

Extraction and quantification of lignans by high performance liquid chromatography electro spray mass ionization (HPLC-ESI-MS)

The extraction of lignans was performed as described by Yousefzadi et al. (2010 c) with some modifications. A solution of hydrochloric acid [0.2 M] was added after the methanol extraction in order to hydrolyze the glycoside group of the lignans to obtain the aglycon. Lignans (PTOX, MPTOX, β-peltatin and dPTOX) separation and quantification was performed in a Brisa-LC2-C18 column (3µm 15 x 0.46 mm, Teknokroma) using an HPLC-ESI-MS platform (Varian IT 500MS).

Tweenty microliters of sample were injected into the column and eluted with acetonitrile (A) and water with formic acid at 0.1% (B) as mobile phase, with the following gradient (min : % A); (00 min : 40 %), (10 min : 67 %), (12 min : 100 %), (17 min : 100 %), (18 min : 40 %), (24 min : 40 %). The flow rate was 0.8 mL/min, and a 1/3b split was performed before detection by MS. The five standards were diluted in a matrix of absolute methanol. Every sample was assayed in triplicate. The lignan production was determined using a calibration curve and expressed as micrograms per gram dry weight.

3.6.Gene expression analysis

3.5.1.Total RNA extraction and cDNA preparation.

Samples frozen in liquid nitrogen were used for RNA extraction to evaluate the level of gene expression. Total RNA was isolated

using TRI Reagent® solution according to the specifications of Invitrogen. About 100 mg of homogenized material were used per sample. RNA integrity was determined in an ethidium bromide agarose gel (2%) and the quantity determined with a ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE,USA).

Two micrograms of RNA were treated with DNAse I (Invitrogen). First strand cDNA was synthesized using Superscript II (Invitrogen) and a mixture of oligo dT and random hexamers (1:1) according to manufacturer's instructions. cDNA was stored at -80º C.

3.5.2.Primer designing and qPCR analysis

Quantitative real-time PCR (qRT-PCR) was used to analyze the expression of CINNAMYL-COA REDUCTASE (*CCR*), CINNAMYL-ALCOHOL DESHIDROGENASE (*CAD*), PINORESINOL-LARICIRESINOL REDUCTASE (*PLR*) and ACTIN 1 (*ACT1*) as housekeeping gene. Primer sequences of genes under study were designed using Primer-BLAST and are listed in the Table 2..

Table 2. Sequences of the primers used to amplify in the gene expression analysis.

Oligo	Sequences	Amplicon size	Genbank accession Nº
CCR fw	5'-CTTGCTCTTCCTGCCTCCAA-3'	148 bp	AJ440712.1
CCR rv	5'-GGGTGGTTTGTTGTTCAGCC-3'		
CAD fw	5'-TGCTCGGGAGGAAGGTGATA-3'	64 bp	AJ811963.1
CAD rv	5'-AGCATCTCCTCCGTCTCCTT-3'		
PLR fw	5'-GAATTCCCGGTAACGTCAAGAGGT-3'	95 bp	AB525816.1
PLR rv	5'-GTCGTCGAATGTGATCCTTCCTG-3'		
ACT fw	5'-CTTCCCTCAGCACCTTCCAG-3'	86 bp	AY857865.1
ACT rv	5'-GAAGCACTTCCTGTGGAC-3'		

The qPCR was performed using the SYBR Green I dye method with 1:2 dilution of cDNA template on a Roche LightCycler 480 II detector system using the following conditions: 95°C 2 min, 40 cycles (95°C, 15 s; 60°C, 10 s; 68°C, 20 s) followed by a melting curve. The standard curves for each primer set were performed for quantification. The analyses were always performed on at least three biological replicates each with three technical replicates.

3.7.Histological Analysis

3.6.1.Fixation and dehydration of the sample

The sample preparation started with the fixing process, in which the samples were placed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and stored for 24 h at 10ºC. After fixing, the samples were washed for 10 times with 0.1M phosphate buffer (pH 7.4) and then kept in the same solution for another 24 h.

Subsequently, the samples were treated with 2% osmium tetroxide for 2 h at room temperature and then washed with water as in the previous step. Samples were divided to undergo analysis by either transmission electron microscopy (TEM) or scanning electron microscopy (SEM). The dehydration phase was performed with an ascending series of ethanol for TEM or acetone for SEM at different concentrations (30-100%; v/v). The analyses lasted 10 min and were performed three times.

3.6.2.Transmission electron microscopy

To polymerize the tissues, they were molded in pure silicon resin (EPON) and dried in a forced-air oven at 60°C for 48 h. Subsequently, the blocks were cut into semi-thin (600 nm) sections by using an ultra-microtome (Ultracut UC6 of Leica Vienne) with a diamond blade. The sections were mounted on cytological glass slides, and stained with 1% Methylene Blue dye dissolved in 0.5% borax water (4 min).

3.6.3.Scanning electron microscopy

The samples were dried in a critical point dryer CPD Emitech k850 using liquid CO_2 and then sputtered with 99.9% gold with Fine Coat Ion Sputter JFC 1100, prior to SEM analysis. Observations were made using an electron microscope (LEO Evo 040) operating between 10 and 20 kV.

3.8.Methodological design of a comparative study of lignan production in four biotechnological systems

In this experiment, we evaluated the growth capacity and lignan pattern and production, in four different biological systems, wild type and hairy root cultures and wild type and transformed cell suspension lines, the last ones, derived from hairy roots (Figure 10). The cultures were performed with 2 g of the root-inoculum in

20 ml of the corresponding culture media. The culture was performed in an orbital shaker at 25ºC of T, 110 rpm and in dark conditions. Samples were taken every 7 days for a culture period of 28 days, with three repetitions per sample.

Suspension cultures were established as described in section 3.4. Cells inoculum of 2g were transferred into 20 ml of the corresponding media. For WT cell lines, the MS liquid medium was supplemented with 2mg/L NAA and 0.4 mg/L KIN, and for the transformed ones with 2 mg/L 4-CPPU and 0.1 mg/L IBA, the flaks were cultured for a period of 14 days. The samples were taken every 3-5 days until the end of the experiment.

3.9.Methodological design for coronatine-elicitation studies

In order to evaluate the effect of elicitors in *L. album* cells and root lines, studies of coronatine (CORO) elicitation were carried out in all biotechnological systems described above, WT roots and transformed roots as well as, WT and transformed cells (Figure 9). In this study, 2 g of cells/roots were transferred to 20 mL of liquid medium MS (which composition is specific for each line) and supplemented with CORO 1 µM after 7 days of preculture in cell suspension cultures, or at day 23 (mid stationary phase), in root cultures. Cells samples were taken after 0 h, 6 h, 12 h and 1 – 7 days after elicitation. Root samples were taken each day after the addition of CORO during five days. All treatments were performed

with three replicates and a non-elicited control. The parameters analyzed in this study were growth capacity, lignin production as well as expression profile of the genes *CCR*, *CAD* and *PLR*.

3.10.Methodological design for studying in vitro morphogenesis and lignan production in L. album callus cultures

This study has been carried out with not-friable callus obtained from *L. album* plantlets as described in the section 3.3, with the following experimental design:

a) *Callus induction:* As previously mentioned, segment of *L. album* plantlets were inoculated in MS solid medium supplemented with 2 mg/L NAA and 0.4 mg/L KIN, in darkness for a period of 30 days at 25ºC. After this time the explants had developed calli, some of them was not friable and organogenic, which were separated from the explants and subcultured every 20-30 days in the same medium and conditions until to obtain enough biomass for this experiment.

b) *Increase of organogenic callus biomass and preliminary assay:* Part of the not friable calli was transferred to the same culture medium (MS added with the indicated PGRs) and maintained, the half in darkness (condition C1) and the other half in light conditions (C2) (photoperiod of 16 h light/8 h darkness), whereas the other part of not friable calli was transferred to a PGRs-free MS medium and cultured also in

darkness (C3) or under the same light conditions (C4). Calli grown in these four conditions, constituted the starting material for evaluating their growth, organogenesis and lignin production. Calli grown in MS supplemented with PGRs in the light (C4) did not growth and was discarded (Figure 9).

c) *Study of growth and organogenic capacity and lignan production of the callus cultures under the experimental conditions assayed:* In order to know the effects of light conditions and/or PGRs addition in not friable- organogenic calli, three kind of callus starting material from the already indicated conditions (C1, C2 and C3) were used in this experiment. With these materials, the assayed cultured conditions were: callus grown previously in condition 1 (darkness plus PGRs) was maintained in darkness with PGRs (C1.1) or transferred to light without PGRs (C1.2). Callus derived from condition 2 (darkness without PGRs) was maintained in darkness without PGRs (C2.1) or transferred to light also without PGRs (C2.2). Finally, callus derived from condition 3 (light without PGRs) was maintained in light without PGR (C3.1), or transferred to darkness without (C3.2) and with PGRs (C3.3) (Figure 9).

For all of the treatments, callus pieces of two grams of biomass were used as inoculum and placed in the different culture media and conditions for a culture period of 6 weeks. Samples were taken at weeks 1, 2, 4 and 6 for each treatment with 4 biological replicates.

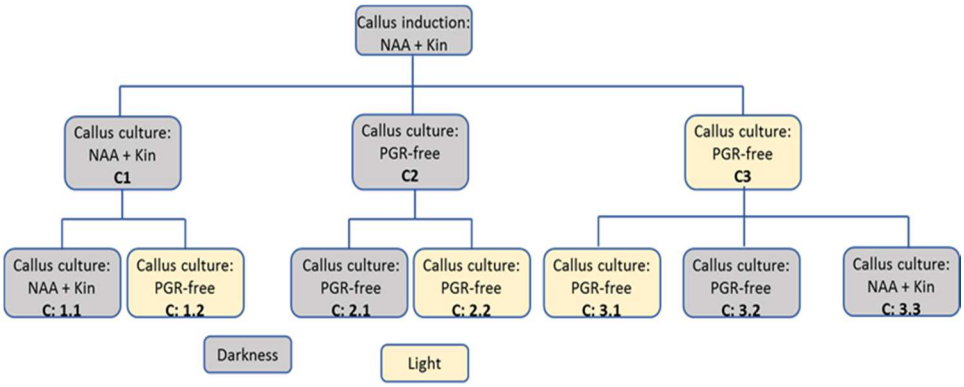


Figure 9. Description of the experimental conditions utilized for studying the effect of the light and PGRs addition in *L. album* callus cultures

The Figure 10 summarizes all the *in vitro*cultures established from *L. album* plantlets that has been utilized in the different studies carried out in this PhD thesis, as well as, the different culture conditions assayed for deepen in the knowledge about lignan production and its control.

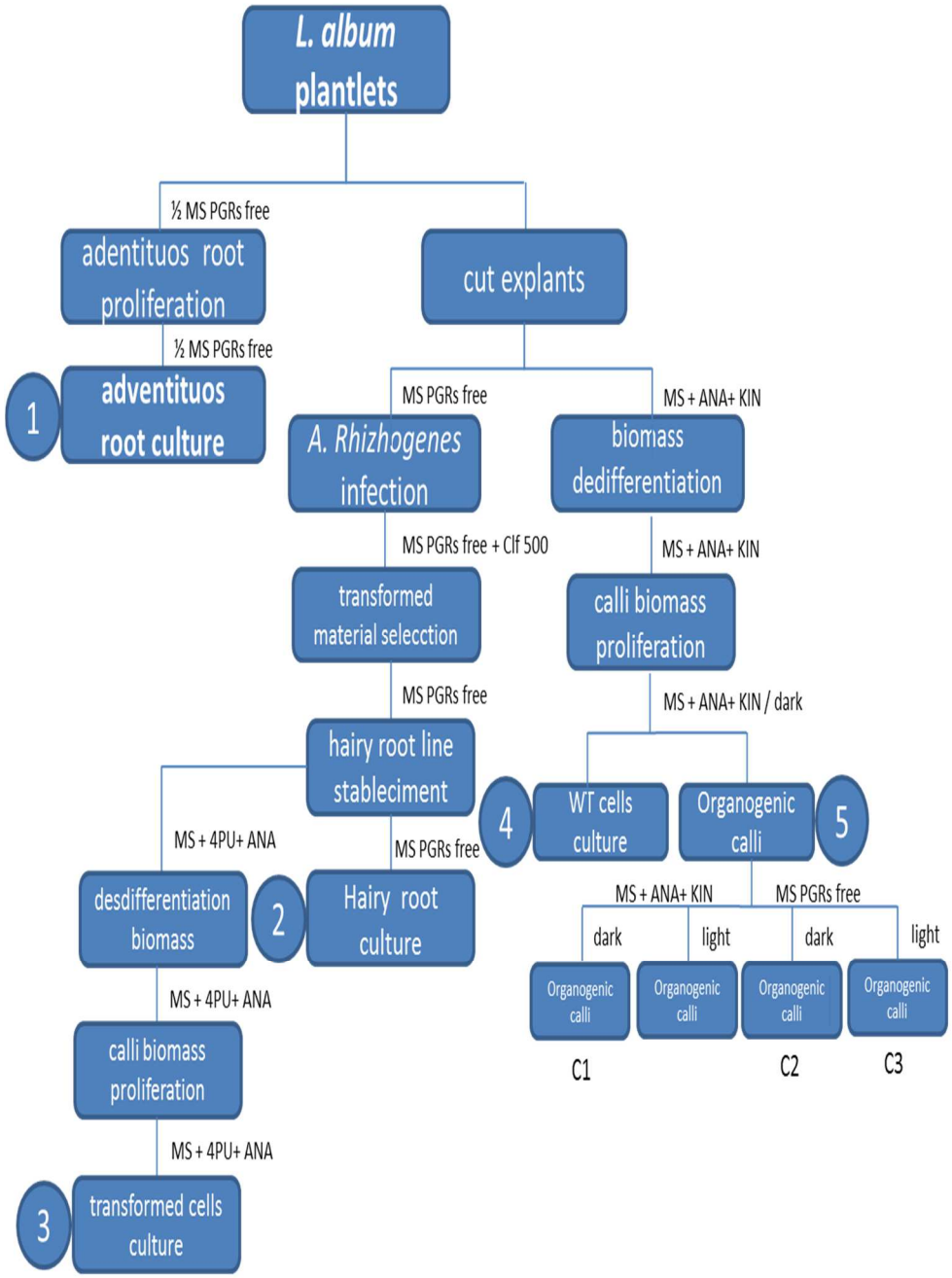


Figure 10.Flow chart summarizing the different plant material utilized in the study.

3.11.Yeast two hibrid system in Linum album

3.10.1.Yeast Media

Yeast potato dextro agar (YPDA) medium is a blend of peptone, yeast extract and dextrose in optimal proportion for growth of most strains of *Saccharomyces cerevisiae* (20g/L; 10g/L; 2% of glucose solution and 0,2% of adenine hemisulfate).

Minimal SD base and minimal SD agar base, are composed by yeast nitrogen without aminoacids, supplied with a dextrose source (2% glucose solution) and 10x Dropout (DO) solution. The combination of SD base and a DO supplement will produce a synthetic, defined minimal lacking one or more specific nutrients. The specific nutrients omitted depend on the selection medium desired. The DO is composed with the listed amino acids: L-adenina hemisulfate salt (200mg/L); L-arginine HCl (200 mg/L); L Histidine HCl monohydrate (200 mg/L); L- isoleucine (300 mg/L); L/K/M/F/T/W/T/U/V. The S/D/E makes the medium too acid and the yeast can synthesize these amino acids endogenously.

In the transformation of yeast the use of the following solutions were required: 1.1X TE/LiAC (10X of TE and 1M LiAC) and PEG/LiAc solution (50% PEG 3350; 10X TE Buffer and 1M LiAc).

3.10.2.Yeast strains/ media and manipulation

Two yeast strains were used throughout the experiments. The Y187 *Saccharomyces cereviciae* strain yeast (MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80 Δ, met-, URA3::GAL1UAS – GAL1TATA –LacZ, MEL1. and Y2HGold yeast strain : MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS – Gal1TATA –His3, GAL2UAS – Gal2TATA – Ade2, URA3::MEL1uas – Mel1TATA, AUR1-C, MEL1; provided by Matchmarker® Gold Yeast Two-Hybrid System Kit (Clontech). So two reporters genes (lacZ and MEL1) under the controlling of two promoters had the Y187 strain and four reporter genes AUR1-C, HIS3, ADE2 and MEL1 under tree promoters controlling are present un the Y2H gold strain. Yeast cells were grown on YPDA agar medium and minimal SD medium. Different minimal dropouts were prepared that omitted the amino acids used for selection by auxotrophic, accordingly with the characteristic described above and were grown at 30 ºC for 3-4 days in solid media.

3.10.3.Preparation of competent yeast cells

The competent yeast cells were generated using the Lithium Acetate (LiAc) method, following the instruction in the User manual of the YeastmakerTM Yeast transformation System 2 (Clontech). A yeast preculture was done in 5 ml of YPDA liquid medium at 30ºC with shaking at 170 rpm for 12 h, then a second culture with 100 µl of the first culture in 50 ml of YPDA fresh liquid medium was done.

When the culture reached 0.15 – 0.3 OD600 16-24 h, the cells were centrifuged at 700 g and resuspended again in YPDA liquid medium until reaching an OD600 0.4-0.5 (3-5 h). After, washes with deionized sterile H_2O and 1xTE/LiAc solution were performed and cells were finally resuspended in 600 µL of 1xTE/LiAc.

3.10.4.Plasmid Vector Construction

Recombinant DNA constructs were obtained by the Gateway system. To screen for potential protein interaction patterns, the following genes were selected: *PINORESINOL-LAIRECINOL REDUCTASE 1* (PLR) and *THUJAPLICATIN O- METHYL TRANSFERASA* (AsTJOMT). Gene sequences are available at GenBank under the accession numbers: AB525816.1 and AB820130 respectively. The CDS (PLR and AsTJOMT) were supplied by GenScript (Piscataway,NJ USA). PCR-amplified CDS with the following primers:
PLR (Fw: 5'-GAATTCCGCSTGGGTTCCCTGGGGAAGTGAA-3'; Rev: 5'-GCGGCCGCCGACTGACTAGCTAGACGTAACGCTTT-3') TJOMT (Fw: 5'- GTCGACATGTCTAAACAAGATCAAGATGCCA-3' ;Rev: GCGGCCGCCTACACTTTCTTATGAAATTCTAG-3').They were cloned in pSPARKII plasmid using T4 DNA ligase (New England Biolabs) and transformed into *E. coli* DH10B by electroporation (Rusell 1999). Transformed clones were selected in LB medium supplied with ampicillin [100 µug/ml]. Plasmids from independent clones were purified using E.Z.N.A Plasmid DNA Mini Kit (OMEGA bio-tek) and following manufacturer's instructions. To confirm the identity of the clones, a digestion was performed with

XbaI/PstI restriction enzymes for PSPARK II-PLR and Hind III/PstI for PSPARK II-TJOMT. The digestion was checked by electrophoresis on 1.2% agarose/EthBr gels. Clones with the expected band patterns were then sequenced with flanking primers T7 and SP6. T7: 5'-TAATACGACTCACTATAGGG-3' and SP6: 5'-ATTTAGGTGACACTATAG-3'.

The coding sequence (CDS) were released from the pSPARK II vector by the digestion with restriction enzymes; EcoRI- NotI for PLR and SalI- NotI for the TJOMT and cloned into PENTRY plasmid. Each gene expression vector was confirmed by digestion with Hind III and by sequencing with T7 and SP6 primers. Finally, the CDS they were transferred to PGBKT7 and PGADT7 by LR clonase (Invitgrogen), which resulted in in-frame fusions of the proteins with BD and AD domains, respectively. The resulting clones (PGBKT7-PLR; PGADT7-PLR; PGBKT7-TJOMT; PGADT7-TJOMT) were sequenced again using GW primers:
(Fw:5'-GGGGACAAGTTTGTACAAAAAAGCAGGC-3');
(Rev:5'- GGGACCACTTTGTACAAGAAAGCTCGGT-3'.

3.10.5.Vector constructs for yeast expression

The transformation was performed using 200 µg of DNA plasmid (bait, prey and interactions). All the constructions are summarized in the Table 3. On the other hand, the prey plasmid construction in Y187 used for the screening process was 258.9 ng/µL of L. album cDNA. Denatured carrier DNA (10µg/µL) previous treated at 95ºC-5 min /cold and PEG/LiAc solution was required to transform the

yeast cell. It was incubated at 30ºC for 30 min. Followed DMSO was added and the reaction was placed in a water bath at 42ºC for 15 min. A centrifugation at 700 g for 5 min was necessary to recover the pellet yeast cells.

Table 3. Vector constructions with the bait proteins (PLR, TJOMT) and the possible interaction

Bait (GAL4 dna-bd)	**Prey (GAL4ad)**
PGBKT7-*PLR*	-
PGBKT7-*TJOMT*	-
-	PGADT7-*PLR*
-	PGADT7-*TJOMT*
PGBKT7-*PLR*	PGADT7Ø
PGBKT7-*TJOMT*	PGADT7 Ø
PGBKT7-*PLR*	PGADT7-*PLR*
PGBKT7-*PLR*	PGADT7-*TJOMT*
PGBKT7-*TJOMT*	PGADT7-*PLR*
PGBKT7-*TJOMT*	PGADT7-*TJOMT*

In order to promote the transformation efficiency, the yeast pellet was resuspended in 1 ml YPDA Plus medium (Clontech) and incubated for 90 min at 30 ºC with shaking. Finally, the pellet was recovered by centrifugation and resuspended in 15 ml of NaCl solution (0.9 % w/v). The yeast transformed cells were plated on the corresponding media, bait yeast strain (SD/-W), prey yeast strain (SD/-L) and the interaction in the (SD/-W/-L).

3.10.6.Determination of transformation efficiency

To determine the efficiency of transformation, 100 µl of serial dilutions (1/10; 1/100; 1/1000;1/1000) of transformed yeast cells were spread in SD medium/-L/-W and incubated at 30ºC until the colonies appeared (4-5 days). The transformation efficiency wascalculated by the relation of cfu obtained per µg of DNA

3.10.7.*L. album* cDNA library construction

For the construction of the cDNA library, mRNA was isolated as described in the section 3.8.1. NucleoSpin RNAII kits was used to purify total mRNA and the integrity was checked in an agarose gel (2%) and quantity determined in a ND-1000 Spectofotometer (NanoDrop Technologies, Rockland, DE,USA).

The cDNA was synthesized using the Make your Own “Mate &PlateTM” Library System kit from Clontech as briefly described below:
Around 1.5 µg of total RNA with 10 µM Oligo dT Primer (CDSIII Primer:5’ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30VN-3’), 5X first Strand Buffer , 100mM dNTPS mix and 100 MM aDTT were used for the first-strand cDNA synthesis according to the manufacturer. Following the second-strand synthesis, a Long Distance PCR (LD-PCR) was required. For the PCR reaction, the Advantage 2 PCR System (Clontech) was used. The PCR reaction was performed using the primers:

5'PCR primer:
5'-TCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3')
3'PCRPrimer:
(5' GTATCGATGCCCACCCTCTAGAGGCCGAGGCCGAGGCGGCCGACA-3').

The parameters for the LD-PCR were: 95ºC for 30 sec, 20 cycles at 95ºC for 10 sec, 68 ºC for 6 min and 68ºC during 5 min The optimal number of cycles was selected according to the amount of RNA used for the first strand cDNA synthesis as suggested by the manufacturer. A small quantity of PCR product was checked on 1.2% of agarose/EthBr gel to determine the quality and size of the cDNA generated. Finally, the double stranded cDNA (ds-cDNA) was purified with ACHROMA SPIN+TE-400 column previously equilibrated. The gel matrix was completely resuspended and purged by centrifugation at 700g for 5 min. Followed; the samples were passed through the column and collected. To precipitate the cDNA, 3 M sodium acetate and ice cold absolute ethanol was added and then placed at -20ºC overnight. The purified cDNA was centrifuged at 14,000 rpm for 20 min to discard the supernatant and resuspended in deionized water. The quantification was done in a ND-1000 Spectofotometer (NanoDrop Technologies, Rockland, DE,USA).

3.10.8.Creating mate and plate library

As explained before around 5 ug of cDNA was used to co-transform the yeast prey strain (Y187 strain [cDNA library]) with 3 ug of linearized pGADT7-Rec and transformed cells were spread in SD/-

L medium. After 4-5 days the pool of transformants was harvested with 5 ml of liquid medium per plate combining all the recovery in a flask. Then, the library was aliquoted and stored at -80ºC. The cell density was determined using a hemocytometer. The number of independent clones was calculated correlating the No of cfu/ ml of medium per resuspension volume (ml).

3.10.9.Self- activation bait test

To confirm that the bait does not automatically active the reporter gene in absence of a prey protein, the transformants in the Y2HGold yeast strain were assayed for the interactions previously reported in the Table 3**.** Different colonies were selected and tested in separate plates of minimal drop out SD media composition: SD/-W; SD/-W/-L; SD/-W/-L supplied with 0,1mM 3AT (3-amino-1,2,4-triazole; Sigma).

3.10.10.Two-Hybrid library screening using yeast mating

The bait strain (Y2HGold [pGBKT7+bait]) was inoculated in SD/-W liquid medium at 30ºC and 270 rpm until it reached an OD600 = 0.8. Then, cells were collected by centrifugation at 1000 g for 5 min and resuspended in the same media to obtain a density of >1x108 cell per ml.

Afterwards, 1 ml of the cDNA library was combined with the bait cell suspension in 2xYPDA media supplemented with 2% of glucose and 50 µg/ml of kanamycin. The culture was placed in an orbital

shaker at 30 rpm and, 30ºC for 20 h until to observe zygotes structures in the mating. The cells were rinsed several times with sterile water and resuspended in 10 ml 0.9% NaCl. Diploid cells formed were selected by the activation of HIS3 reporter gene in plates (SD/-W/-L/-H/+3AT). Serial dilutions as a controls were made and spread in different media plates (SD/-W; SD/-L; SD/-W/-L) to check the efficiency of the transformation. The number of screened clones was determined correlating the cfu/ml of diploids per resuspension volume (ml).

3.10.11.X-α-Gal assay for positive clones and sequencing

Positive colonies were restreak into selection media (SD/-W/-L/-H) supplemented with 20 µg/ml X-α-Gal (5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside) to confirm the activation of the reporter gene. The selected clones were subjected to colony PCR with primers flanking the cloning site of PGADT7 (PACT2-5' PCR primer:
5'-CTATTCGATGATGAAGATACCCCACCAAACCCA-3´;
PACT2-3' PCR primer:
5´-GGTGCACGATGCACAGTTGAAGTGAACTTGCG-3´;using standard PCR conditions. The products were visualized on 1 %agarose EthBr gels and bands extracted and purified using Qiagen Gel Extration Kit. The purified fragments were sequenced using the same PCR primer pairs.

3.10.12.Plasmid rescue

To recover the pGADT7-prey clones, the plasmid DNA from the positive yeast clones was recovered and transformed into *E. coli*. pairwise transformations were performed into Y2H Gold strain between the interactor as prey (pGADT7+interactor) and the proteins under study as bait (pGADT7- PLR/TJOMT or controls). The controls were used to identify real interactions by the presence of growth. pVA3-1 is a DNA-BD plasmid that provides a positive control when combined with the pTD1 activation domain construct (Li & Field, 1993; Iwabuchi 1993); and pLAM5´-1 plasmid, which encodes a GAL4 DNA –BD/human Lamin C hybrid provides a control for fortuitous interactions between an unrelated BD hybrid protein and own GAL4 AD/library plasmid. Both controls were provided by the MATCHMARKER Two-Hybrid System 2 system (Clontech).

3.10.13.*In silico* analysis

To determine which candidates should be selected o discarded, our approach was first to annotate the genes using similarity to orthologous sequences and homology with others genes in the same genome. To get information related to the enzyme function, computational alignments were determined using available sequence data from the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Phytozome 1.1 (https://phytozome.jgi.doe.gov/pz/portal.html). Additionally, the Plant Interactome database

(http://interactome.dfci.harvard.edu/A_thaliana/) provided information about possible interacting proteins in *A. thaliana*. Finally, a phylogenetic neighbor-joining (NJ) tree was constructed using Mega 7.0 (www.megasoftware.net/) software to evaluate the evolutionary relationship between the preys and homologous/orthologous sequences from other species.

The Figure 11 summarizes the experimental work in this section (3.13) to development of yeast two hibrid system in *Linum album.*

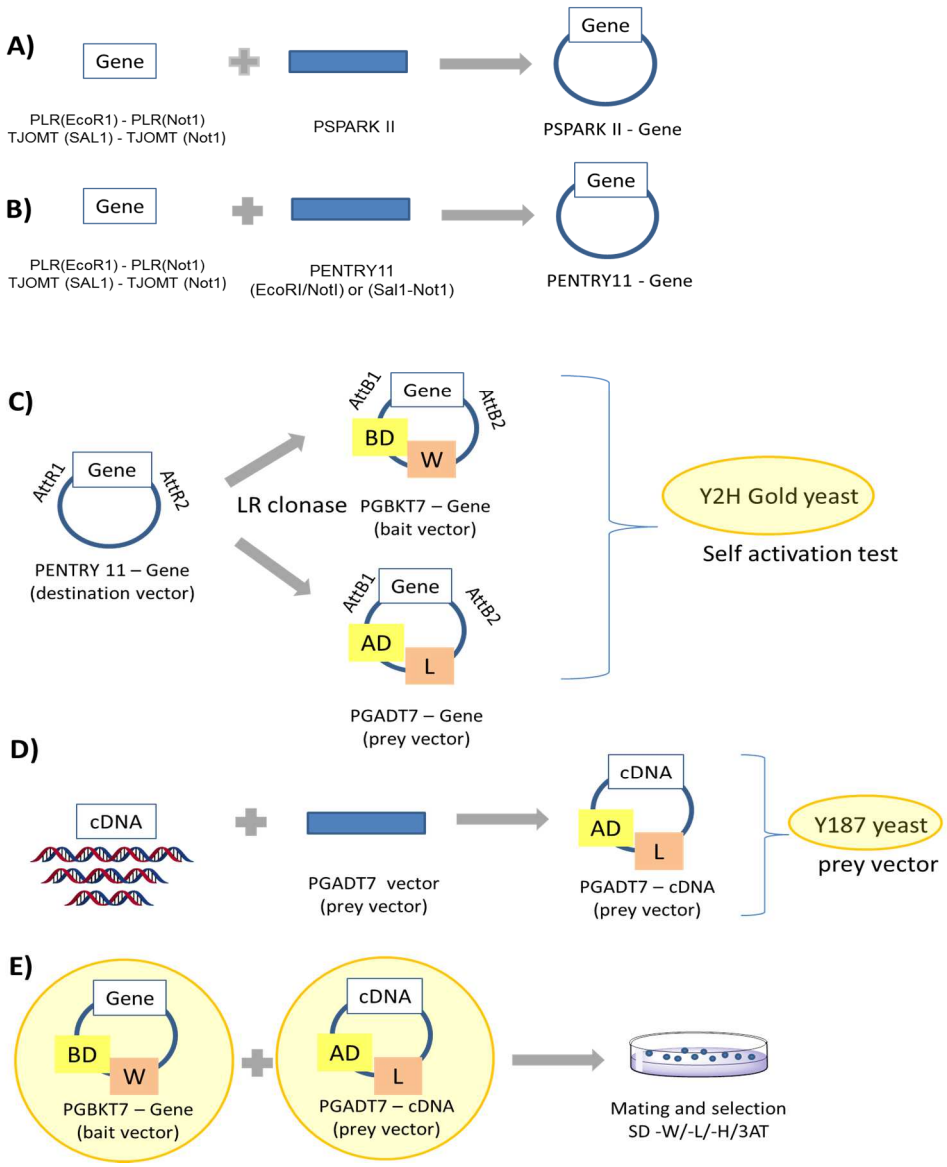


Figure 11. Ilustration of experimental work. **A)** Generate clones and amplify the genes of interest by PCR**. B)** Generate entry clones by topoisomerase based cloning. **C)** Generate the bait and prey plasmid by LR combination reactions and transformation in the Y2H Gold yeast. **D)** Generate the prey plasmid with the *L. album* cDNA and transform in the Y187 yeast. **E)** Mating and selection of possible interactors.

4. RESULTS AND DISCUSSION

4.1.Plant material obtention of *Linum album*

After sterilization of the *Linum album* seeds, preliminary studies were carried out to find high performance germination. The addition of gibberellin was tested in two ways: i) added to culture medium and ii) the seeds placed directly in a solution of gibberellin (1h, stirring) before transferring to MS medium (Figure 12). The best results were obtained by treating the seeds with gibberellin solution before transferring to the medium, resulting in 76.6% germination, in contrast with only 23.3% when added to the culture medium. Moreover, the shoots were larger when the seeds were treated with gibberellin before the transference to MS medium (data not shown). Ashrafi et al. (2013) and Samadi et al. (2012) provide further evidence that the germination in *Linum* species (including *L. album)* isimproved by the additionof higher concentrations of GA_3 (1600 ppm).*Linum* seeds have exogenous and endogenous dormancy and the use of gibberellin helps to disrupt, thus increasing the germination percentage.

Figure 12. *L. album* seeds germination

Four biotechnological platforms were established from leaves and young stems of *L. album*: two lines of cell suspensions (WT and transformed) and two root cultures (adventitious and hairy roots).

For the initiation of *L. album* hairy root cultures, once the first roots were obtained by infection with *A. rhizogenes* (LBA 9402 strain), they were kept in MS medium initially supplemented with growth regulators.Previous studies in our laboratory confirm that this medium generates a dense rooting. The adventitious root cultures were stablished from excised root from *L. albumin vitro* plantlets and maintained in hormone-free MS medium. However, after culturing, the roots did not proliferate and died. The effect of adding PGRs (auxins and cytokinins) to the media on root elongation and growth was previously evaluated. (Farkya and Bisaria 2008) reported that the addition of IAA to the basal medium increased the degree of branching in hairy roots. This approach had positives results in our adventitious root cultures. On the other hand, Anbazhagan et al. (2008) describes the using half-strength MS medium to induce adventitious roots in *Podophyllum peltatum.* We found this to be a successful approach with *L. album*, which resulted in good rooting and growth (Figure 13).

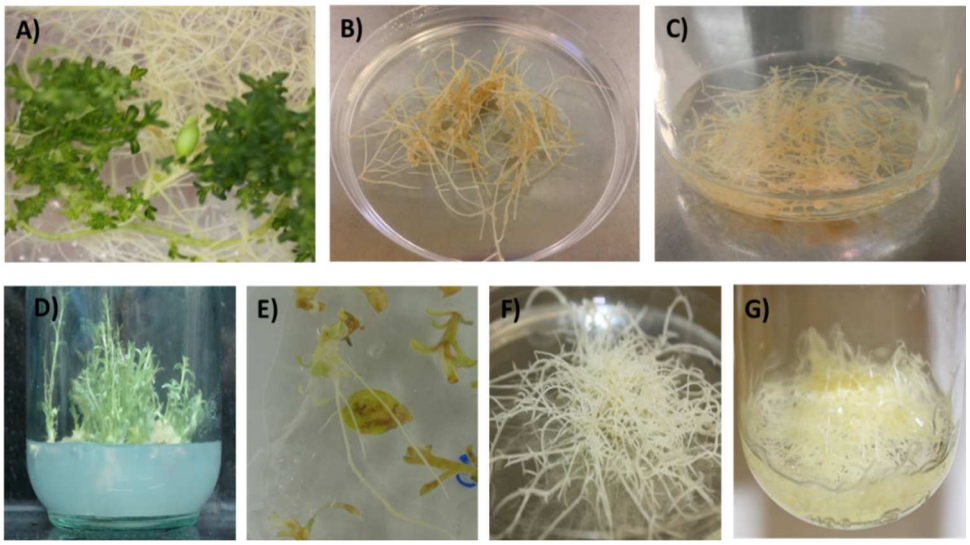


Figure 13*.** Establishment of hairy root and adventitious roots of *L. album*. **A)**. *L. album* plantlets. **B).** Adventitious roots excised directly from the plantlets. **C)** Adventitious roots cultures. **D)** L. album plantlets (leaves). **E)** Hairy roots from explants infected with *A. rhizogenes.F)** Establishment of hairy root line. **G)** Hairy roots cultures.

One of the greatest challenges of the study was to establish cell suspension cultures. For this purpose, different culture media with various combinations of growth regulators and concentrations were tested (Table 4). Several authors reported the establishment of stable *L. album* cell lines in MS supplemented with ANA and Kin as growth regulators. We also tested other combinations of plant growth regulators in case better results could be obtained, but no improvements in calli development were observed. On other hand, we had some difficulties in the differentiation and generation of biomass in the transformed callus became browning and died at the end. No studies of dedifferentiation from *L. album* hairy roots have been done before. Berlin et al. (1986) found that the concentration

of 2-4D in the medium influenced the growth of *L. flavum,* but in our case this did not yield good results. However, growth was observed when using MS supplemented with [0.1 mg/L] IBA and [2 mg/L] 4-CCPU as PGRs (Figure 14).

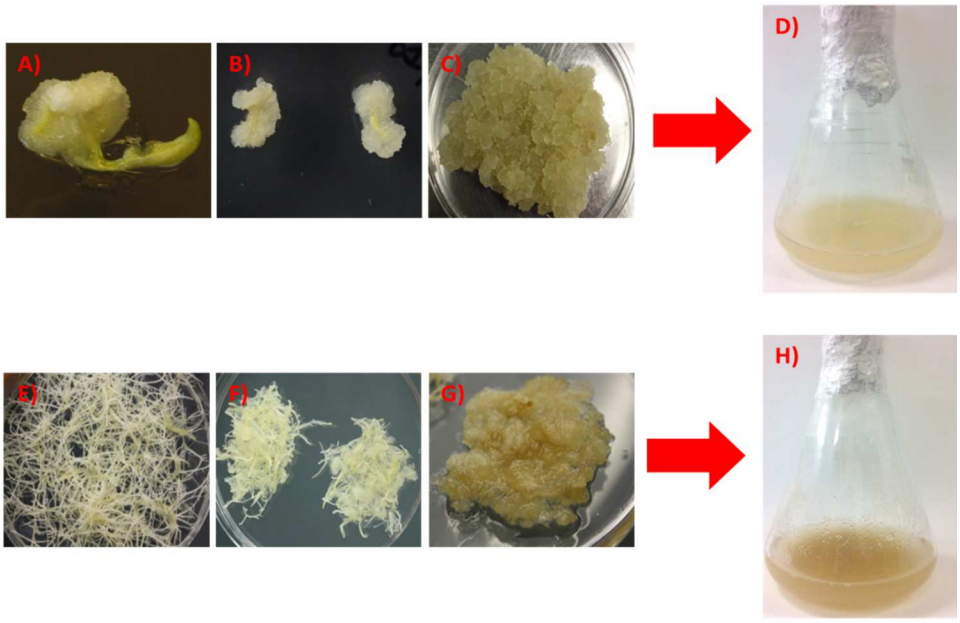


Figure 14. Establisment of wild type and transformed cell suspension. **A)** Dedifferentiation of *L. album* explants in PGRs MS media. **B)** Wild type biomass proliferation. **C)** Wild type calli formation. **D).** Wild type cell suspension. **E)** Hairy roots cultured in PGSs MS media. **F)** Dedifferentiation from hairy roots. **G)** Transformed calli formation. **H)** Transformed cell suspension.

Table 4.Summarize the different media used to establish cells lines and roots lines cultures

		Medium	Auxins		Citokinins		Observations
			Type	Concentration	Type	Concentration	
WT callus	**Dedifferentiation**	MS	ANA	2 mg/L	KIN	0,4 mg/L	friable callus
		MS	IBA	0,1 mg/L	4PU	2 mg/L	organogenic callus
	Callus culture	MS	ANA	2 mg/L	KIN	0,4 mg/L	friable callus
		MS	IBA	0,1 mg/L	4PU	2 mg/L	compact callus
		MS	2-4D	0,1 mg/L	4PU	2 mg/L	organogenic callus - low growth
		MS	2-4 D	0,1 mg/L	KIN	2 mg/L	friable callus - low growth
	Cell suspensión	MS	IBA	0,1 mg/L	4PU	2 mg/L	cluster formation
		MS	ANA	2 mg/L	KIN	0,4 mg/L	friable suspensión
Transfomed callus	**Dedifferentiation**	MS	ANA	2 mg/L	KIN	0,4 mg/L	Died
		MS	2-4D	1 mg/L	-	-	Died
		MS	IBA	0,1 mg/L	4PU	2 mg/L	friable callus
		MS	IAA	11,5 uM/L	KIN	1 uM/L	Died
	Callus culture	MS	IBA	0,1 mg/L	4PU	2 mg/L	friable callus
	Cell suspensión	MS	IBA	0,1 mg/L	4PU	2 mg/L	friable callus
Adventitious Root	**Elongation**	MS	ANA	2 mg/L	KIN	0,4 mg/L	Died
		MS	IAA	1 mg/L	-	-	Died
		MS	IAA	0,5 mg/L	-	-	Died
		MS	IBA	0,1 mg/L	4PU	2 mg/L	Callusing
		MS (1/2 sales) *	IBA	14,78 uM/L	-	-	Died
		MS (1/2 sales) *	-	-	-	-	Rooting
	Culture	MS (1/2 sales) *	-	-	-	-	Rooting
Hairy root	**Elongation**	MS	-	-	-	-	dense rooting
	Culture	MS	-	-	-	-	dense rooting

In other to assess the genetic status of the hairy roots and transformed cell lines, their genomic DNA was extracted and PCR analyses were performed for detection of rolC,AUX, MAS, AGS and virD genes. The rolC gene is diagnostic for the T_L-DNA integration into the host genome. In addition, the *ops* genes (mas -manopine synthase / ags -agropine synthase) and the AUX gene responsible for the auxin biosynthesis to proliferate the transformed cells are located in the T_R DNA regions of the pRi plasmid. With this battery of genes, the transfer of the T-DNA is initiated (Exposito et al. 2010).

In the case of the hairy root, which was also the starting material for the transformed callus development, as this is located outside the t-DNA. The PCR analysis confirmed the presence of the rolC (534 bp), AGS (347 bp), MAS1 (343), and AUX1 (350 pb) genes from the Ri plasmid in both lines (Figure 15).

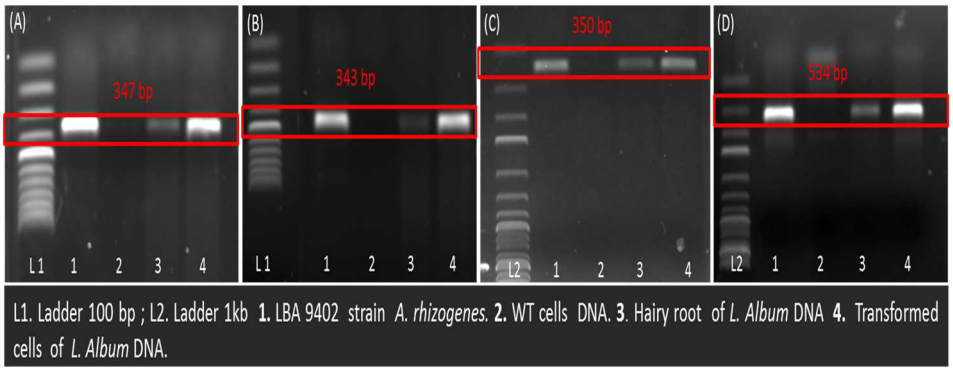


Figure 15. Presence of T-DNA genes of *A. rhizogenes*, analyzed by polymerase chain reaction (PCR). **(A).** AGS, **(B)**. MAS1, **(C)**. AUX 1 and **(D)** rolC.

The quantification of lignans by ESI/MS was done in positive ion mode. The main ions obtained from the standards were as follows in Table 5:

Table 5. ESI/MS fragmentation of lignans

Lignan	MW	m/z	Molecular Ion	RT
Podophyllotoxin	414	397.1	$[-H_2O]$	6.0
β-Peltatin	414	421.1	$[M+H]^+$	6.40
6-Methoxypodophyllotoxin	444.43	427.2	$[M+H]^+$	7.5
Deoxypodophyllotoxin	398.4	395	$[H^+]^+$	10
Yatein	400.42	401.1	$[M+H]^+$	10.3

4.2.CHAPTER I. Comparing patterns of aryltetralin lignan accumulation in four biotechnological systems of *Linum album*.

4.1.1.Adventitious and hairy roots cultures.

4.2.1.1 Growth studies

The growth profiles of *L. album* roots are depicted in Figure 16. A time course study (28 days with 7 day intervals) was performed to evaluate the kinetic growth. The adventitious roots showed an increase in growth up to day 21, continuing to grow thereafter at a lower rate until day 28, and reaching a maximum of 0.34 L, which represents a 2.1-fold increase in comparison with the initial inoculum. Figure 16A shows the different phases of the kinetic growth: the lag phase (first and second week), when the specific growth rate is essentially slow and the exponential growth phase, which began in the third week and continued until the fourth. There is no evidence of a constant growth rate or stationary phase.

The highest biomass in hairy roots (0.32 g) (Figure 16B) was obtained at day 23 (2.8-fold higher than the initial inoculum), declining thereafter. In the first week growth was comparatively slow (lag phase), and an increase in biomass was observed at day 7, when the exponential growth began. The specific growth rate was constant at day 21, which was the beginning of the stationary phase. These growth results are comparable with those of Chashmi et al. (2011;2013), who describe a similar kinetic pattern

with the highest biomass at the end of the experiment with hairy roots of *L. album* as well.

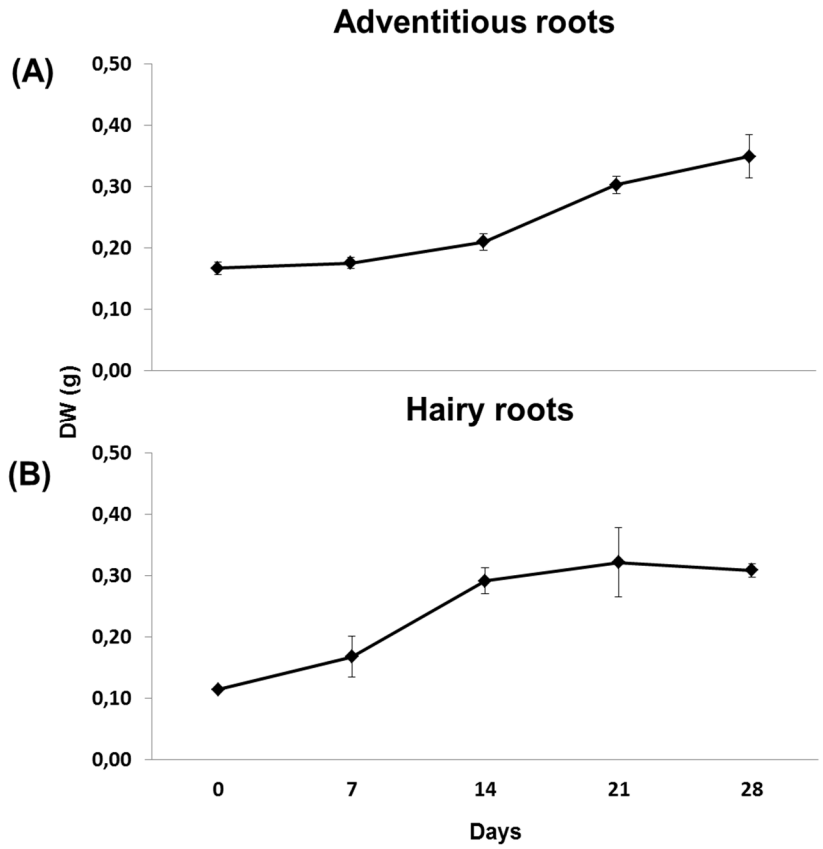


Figure 16.Time courses of biomass production (expressed as dry weight) of the adventitious root (A) and hairy root (B), cultured for 28 days. Data represent average values from three separates experiments ± SD.

Thus, the two roots lines of *L. album* achieved different levels of biomass, with adventitious showing better growth than hairy roots. While the biomass of airy root increased at day 7, and continued to grow until day 14, adventitious root showed a continuous growth

until the end of the experiment, suggesting that the lag phase went beyond the 28 days of the study.

Several studies suggest that most secondary metabolites are produced in the lag phase, when growth is continuous. It was interesting to know the kinetics of growth of the cell lines to establish bioproduction studies of the target metabolites.

4.2.1.2 Bioproduction analysis

The production of PTOX and derivatives in roots cultures was evaluated (Figure 17), with a considerable difference observed between the two lines of study. The results are expressed in µg/g DW. The most abundant compound in both cases was MPTOX (17B-E), although the highest value was recorded in adventitious root 15,355.0 µg/g DW compared to 9,074.322 µg/g DW in hairy roots. In addition, the production pattern also differed. While MPTOX amounts increased significantly in WT roots in the last days, in hairy roots there was a declining tendency after 21 days.

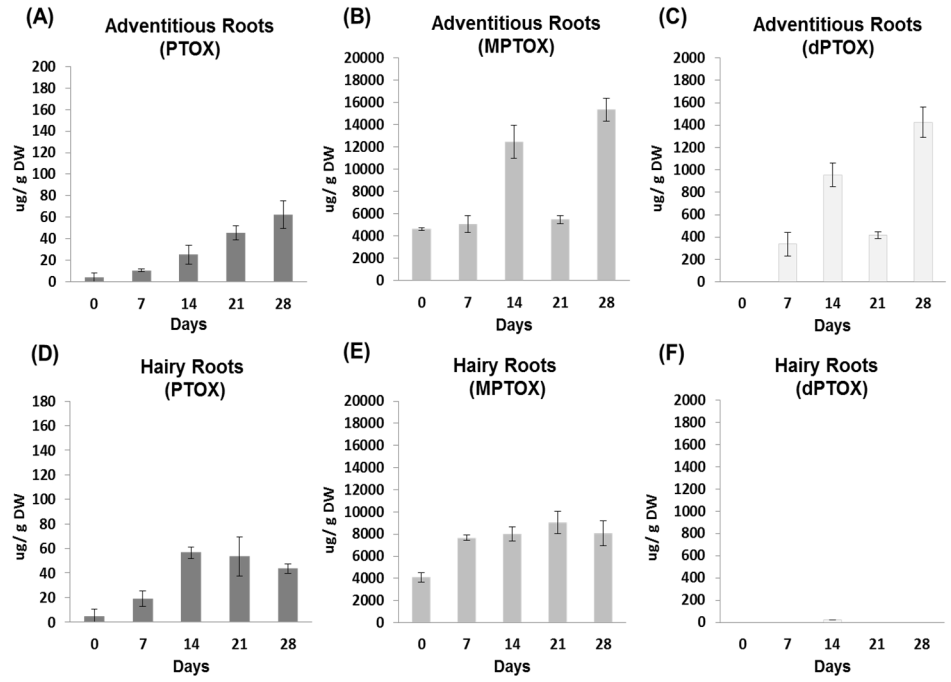


Figure 17.Time curse of lignans concentration (PTOX,MPTOX,dPTOX) expressed in µg/gDW in adventitious root (A-C) and hairy root (D-F) cultured for 28 days. Data represent average values from three separates experiments ± SD.

Most reports about *Linum* root cultures establish MPTOX as the major lignan produced, not PTOX, despite variations in quantities (Kranz and Petersen 2003; Federolf et al. 2007). In concordance with the bioproduction profile reported by Chashmi et al. (2011), the hairy roots derived from the LBA9402 strain in *L. album* produced more MPTOX, with 48 mg/g DW recorded, in comparison with 2.5 mg/g DW of Farkya and Bisaria (2008) and 0.06 mg/gDW of Mohagheghzadeh et al. (2007).

Additionally, a high amount of β-peltatin (17C-F) was only detected in adventitious root, reaching a maximum production of 1,425.419 µg/g DW, with the same pattern as observed for MPTOX. In contrast, in hairy roots only traces of β-Peltatin were identified. β-peltatin is a precursor for MPTOX production in *Linum* cultures, where it is methylated at position 6 to β-peltatin-A methylether 5 and finally hydroxylated at position 7 to 6-methoxy-podophyllotoxin. The transformation of deoxypodophyllotoxin into β-peltatin and PTOX has been demonstrated by feeding experiment (Widad et al, 1986). These results raise questions about the immediate bioconversion of β-peltatin in the two systems, as well as the nonexistence of dPTOX. Probably they are related to the biological system and productivity of the line.

The highest PTOX content (62.32 ug/gDW) was found in adventitious roots at day 14, with a slight tendency to decrease in the following days. In hairy roots, the pattern was the same as for the other lignans reported above, with the highest quantity of PTOX (56.67 µg/gDW) recorded in the middle of the experiment (day 14) during the exponential phase, after which the bioproduction decreased. Similar fluctuations in PTOX yield in hairy root lines have been reported by different authors. Chashmi et al (2013) reported a maximum concentration of PTOX of 105 ug/gDW, which is higher than our results. There are no reports of PTOX production in *L. album* adventitious roots to make comparisons, although the same authors reported a PTOX concentration of 14.11 µg/gDW in the roots of mother plants collected from their natural habitat. And

either deoxypodophyllotoxin no yatein were detected in the roots cultures.

In general, the root cultures, especially the hairy roots, grew rapidly and were stable, in agreement with other studies. The bioproduction of total lignans was highest in the adventitious roots and took place at the end of the experiment, while in the hairy roots bioproduction peaked at day 14at the start of the stationary phase. However, these results are in contrast with those of Chasmi et al (2013), who report that the insertion sites of Ri-T-DNA affected the secondary metabolite biosynthetic pathway of lignans in hairy root cultures and increased their bioproduction (Mishra et al. 2011; Chandra et al. 2007).

4.1.2.Wild Type and transformed cell cultures.

4.2.2.1 Growth study

The growth kinetics of the cell suspensions along 14 days were investigated at intervals of 3.5 days (Figure 18). The wild type (WT) cell suspension had a maximum dry cell weight (0.34 g) at day 11, with 70% of cell viability, which decreased thereafter. This value was 2.45-fold higher than that of the initial inoculum. The WT cell suspension showed a linear growth phase up to day 11, which declined thereafter, in correlation with a decreasing cell viability, as reported by Yousefzadi et al. (2012). Similar results have also been described previously; (Seidel et al 2002; Federolf et al.2007), reporting a maximum dry weight at day 8.

The highest accumulation of biomass was 0.26 g at the last day of the experiment in the transformed cell suspension. This cell line was the slowest growing in the first 7 days, when it reached an exponential phase that continued until the end of the experiment. It is possible that growth would reach more after the days of experiments until to evidence lag phase. The cell viability was in correlation with the growth, declining in the first days until 67%, and then showing a slight increase on the last day of experiment to over 70%.

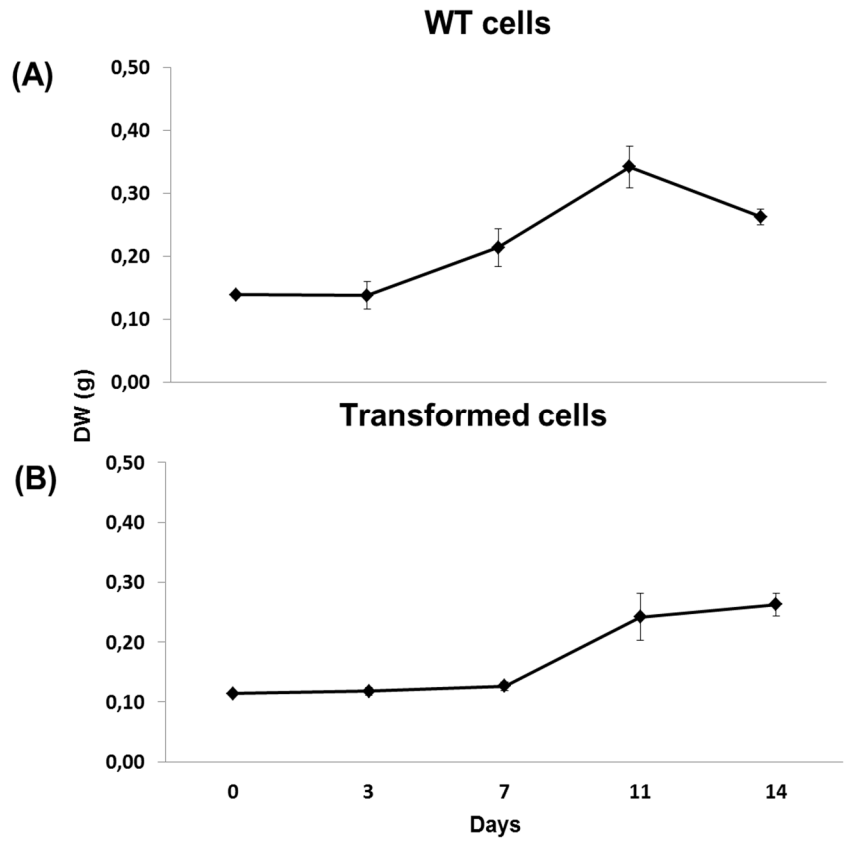


Figure 18.Time courses of biomass production (expressed as dry weight g) of the WT cells and transformed cells cultured for 14 days. Data represent average values from three separates experiments ± SD.

4.2.2.2 Bioproduction analysis

Regarding the lignan bioproduction in the *L. album* cell suspensions, we can infer they are primarily PTOX-producing cell lines. The results are represented in the Figure 19 in µg/gDW units. The highest production of PTOX in both WT cells and transformed cells was achieved in the first 3 days of the experiment, declining thereafter. However, the highest amount was recorded in WT cells:

44.07 ug/g DW in comparison with 37.54 µg/g DW in transformed cells. Empt et al. (2000) reported in a *L. album* cell line a production of 28 mg/L of PTOX within 11 days. In other cell lines, a production of 2.6 mg/g DW was obtained by Federolf et al (2007), 0.4 mg/g DW by Yousefzadi et al. (2010a,b) and in previous studies in our laboratory 100 µg/g DW. In studies on transformed calli of *P. hexandrum* with *A. rizhogenes* in a culture medium supplied with phytohormones, the PTOX content increased 3-fold compared to the non-transformed material. Farkya and Bisaria (2008), achiving a production of 10.50 mg/g in *L. album* calli and Baldi et al. (2008) 52 mg/L in calli line derived from transformed with A. rizhogenes.

The bioproduction of deoxypodophyllotoxin was constant throughout the experiment, with values of around 40 µg/g DW in both WT cells and transformed cells. The first studies using isotope-labeled dPTOX in feeding experiments confirm it to be a direct precursor of PTOX (Kamil and Dewick 1986). An alternative biosynthetic route for dPTOX was subsequently found, as it is involved in MPTOX formation via β-peltatin (Malik et al. 2014). The results in these cell lines suggest that MPTOX is not strongly influenced by dPTOX formation, due to the traces of MPTOX found.

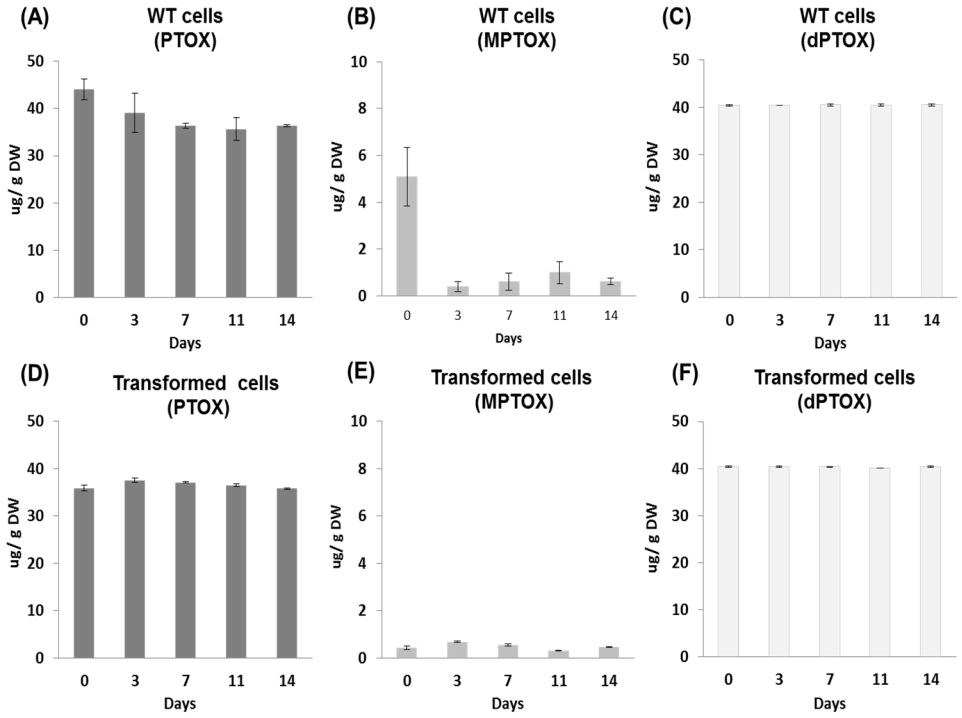


Figure 19.Time curse of lignans concentration (PTOX, MPTOX, dPTOX) expressed in µg/g DW in WT cell (A-C) and transformed cells (D-F) cultured for 28 days. Data represent average values from three separates experiments ± SD.

Comparing our results with previous by other authors, noticed the variability in the PTOX production in the same system and between system under study. However the pattern of bioproduction to each system are according with other reports, being the cellular systems majority producers of PTOX and the roots cultures of MPTOX. Surprisingly the transformed cells, which derive from the dedifferentiation of a system mainly producer of MPTOX changed its pattern of production by PTOX.

4.3.CHAPTER II. Lignan bioproduction in the four biotechnological systems non- elicited and elicited with coronatine

4.2.1.Adventitious and hairy roots cultures

4.3.1.1 Growth studies

Growth curves in adventitious and hairy roots, both elicited and non-elicited (control) were determined (Figure 20). The transition to the stationary phase was observed on day 24 in adventitious roots and one day earlier in hairy roots (see section 4.2.1.1.). In order to evaluate the effect of coronatine on growth, the elicitor was added at the beginning of the stationary phase. Previous studies report that the most common effect in elicited cultures is decreased the biomass, thus the addition of elicitors in the stationary phase is more beneficial to avoid the arrest of the biomass growth in early development stages, and allow being in sufficient contact time to produce an effect (Wang & Wu 2013; Ramirez et al. 2016).

Adventious roots system exhibited higher sensitivity to coronatine in terms of growth inhibition upon elicitation, at least during the first two days of treatment. Thereafter, growth slightly recovered and slights increases in biomass were observed. The maximum growth achieved was 0.39 g DW in the control (day 1) and 0.31 g DW in the elicited culture (day 5, after elictitation). Conversely, growth of hairy roots was not affected by the addition of the elicitor. The control and elicited hairy roots growth curves were similar and tended to decrease in the same way.

Our assays indicate that adventitious roots have a slightly higher growth rate than hairy roots (Figure 20A and B). Regarding to the effect of the elicitor in the systems, coronatine did not promote the growth of adventitious or hairy roots. Comparison of these growth profiles with others studies is not possible because the use of coronatine in *L. album* cultures has not been reported before. On other hand, growth of elicited hairy roots at the end of the cultivation period was very similar to the control, this tendency is comparable to reported by Bahabadi et al. (2014) with *L. album* hairy roots treated with fungal elicitors, where no differences between the non-treated and the elicited with respect to the growth.

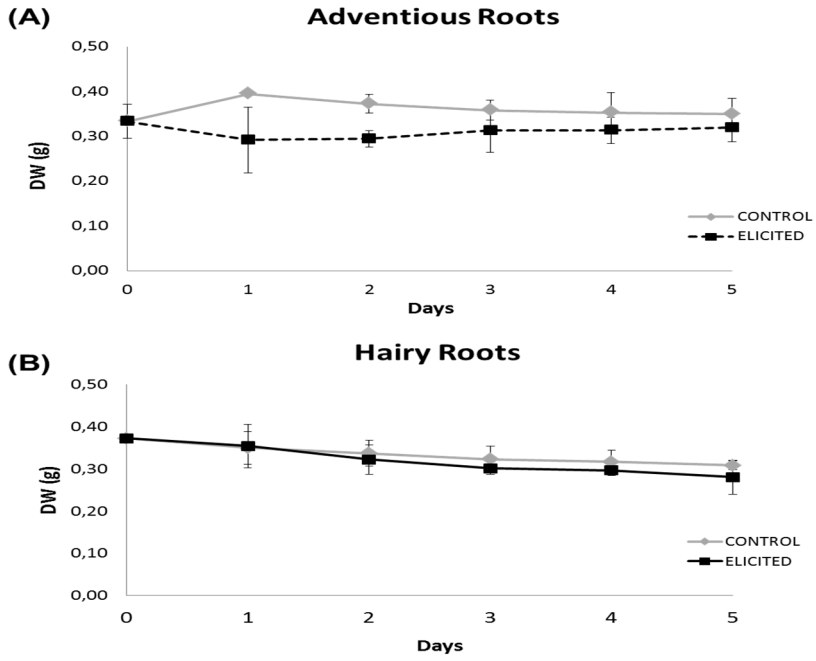


Figure 20.Time courses of biomass production (expressed as dry weight g) of the adventitious root and hairy root cultured for 28 days in non-treated (control) and treated with coronatine at 1mM conditions. Data represent average values from three separates experiments ± SD**.**

4.3.1.2 Bioproduction analysis

The analysis of lignan patterns is showed in Figure 21. The results indicate that MPTOX is the major lignan produced in all conditions assayed. Adventitious roots are more productive than hairy roots (with maximum values of 15,000 µg/gDW and 9,500 µg/gDW, respectively). No comparative data are available for *L. album* adventitious roots in *in vitro* cultures, since they have not been studied before.

However, Chashmi et al. (2013) compared the bioproduction of hairy roots with roots of *L. album* mother plants. These were collected from its natural habitats and presented lower concentrations of MPTOX (1.13 mg/g DW) than hairy roots, for which the productivity is 3.2-fold higher than the one reported here.

Wink et al. (2005) and Oostdam et al. (1993) reported that MPTOX production was 2 to 5-fold higher in hairy roots of *L. flavum* than in untransformed roots. These results are in contrast with our results, in which the adventitious roots seem the most productive system. Elicitation with coronatine increased MPTOX production in relation with the controls in both adventitious and hairy roots, reaching peak values of 17,000 µg/gDW and 10,000 µg/gDW, respectively (Figure 21B-E).

All the roots systems assayed (adventitious and hairy roots, control and elicited) showed a lower production of PTOX than MPTOX (approximately 100-fold less) (Figure 21A-D). In the control of

adventitious roots, the PTOX production was higher than in the control hairy roots, peaking at day 2 (135 µg/gDW), this value increased to 15000 µg/g DW after elicitation with coronatine (Figure 21A-C). In control hairy roots, the PTOX production was less than 60 ug/g DW, about half the yield of the control adventitious roots and did not increase with elicitation (Figure 21A-C). Chasmi et al. (2013) reported root from *L. album* mother plants are mainly PTOX productor, reaches (11,4 mg/g DW) and studies by Anbazhagan et al. (2008) in *P. peltatum* adventitious root cultures corroborate the same trend, roots with higher PTOX contend, suggesting that root *in vitro* cultures could be adopted to produce these lignan.

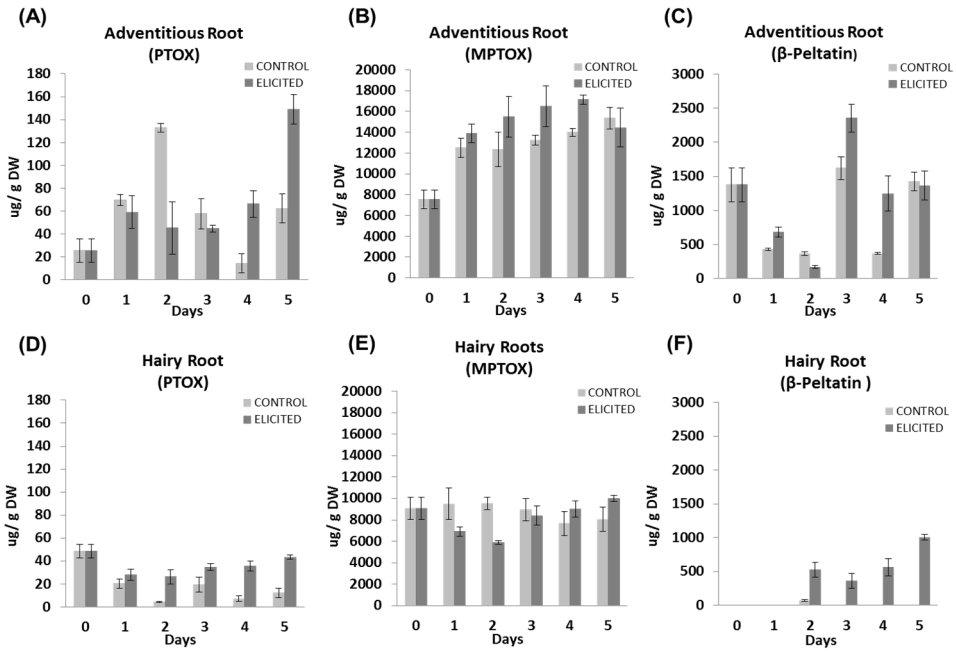


Figure 21. Time curse of lignans concentration (PTOX, MPTOX, dPTOX) expressed in µg/g DW in adventitious root (A-C) and hairy root (D-F) cultured for 28 days in non-treated (control) and treated with coronatine at 1mM conditions. Data represent average values from three separates experiments ± SD.

Collectively, these studies represent examples of the variability in the lignans content among organ cultures of the same species which previously reported by Schmidt et al. (2012). Notably, the more elicited route in each system was the less productive one. Thus, in the roots, the PTOX pathway was more elicited than that of MPTOX (Figure 21A-C). Conversely to one report with biotic elicitor in hairy roots in which the more elicited route was that from the main lignan productor (Bahabadi et al 2014).

The first reports of β-peltatin are referred to roots derived from the dedifferentiation of *L. flavum* callus (9 mg/g DW) by Berlin et al. (1986). In our analyses, high values of β-peltatin were found in both control and elicited adventitious roots, above all in the latter, peaking on day 3 (1,600 ug/g DW and 2,350 ug/g DW, respectively). In elicited hairy roots, the maximum β-peltatin production was 1,000 ug/g DW on day 5. dPTOX was not detected in any root system. A ratio of 100/10/1 could be assigned for 6-MPTOX/ β-peltatin/PTOX levels.

4.3.1.3 Gene expression analyses

The effect of coronatine on the transcriptional profile of *CCR*, *CAD* and *PLR* genesin root systemsis shown in Figure 22.The expression levels of *CCR* and *PLR* were similar in adventitious and hairy root under control conditions. Elicitation always enhanced the expression level of the genes in both root systems, in general, but in hairy roots coronatine greatly increased the expression level of *CAD* on day 3 after elicitation (Figure 22B-E). Previous studies

published by Bahabadi et al. (2014) corroborate the increase ingene expression under elicited conditions in *L. album* hairy roots, especially in PLR expression (2.5- fold more than the control).

In adventitious and hairy roots, CCR and PLR mRNA levels were similar, peaking two or three days after elicitation. It is worth to mention that after elicitation of adventitious and hairy roots, *PLR* transcripts increased 18- and 60-fold, respectively, compared with no elicited (control), although the total mRNA levels were low. Similarly, *CCR* gene expression in hairy roots after elicitation increased around 45-fold in comparison with the control, but the total transcript levels were low.

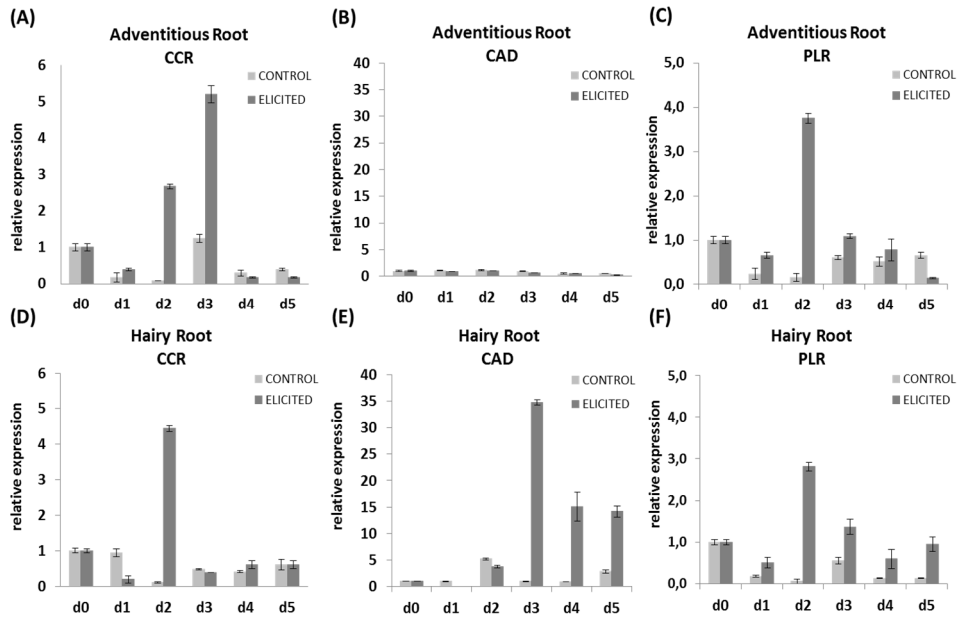


Figure 22.Relative expressionof *CCR, CAD, PLR* genes in adventitious root (A-C) and hairy root (D-F) cultured during 5 days after the elicitation in non-treated (control) and treated with coronatine at 1 µM conditions. Data represent average values from three separates experiments ± SD.

However, the production of MPTOX (10 mg/g DW) and β-peltatin (1,000 µg /g DW) was lower than in the adventitious root (MPTOX (1,700 µg/g DW) and β-peltatin (2,350 µg/g DW)). This suggests that the expression of *CCR* and *CAD* is not necessarily directly correlated with the lignans patterns in study, and coronatine affects more the expression of early genes in the pathway (phenylpropanoid-related genes) than those of the PTOX pathway in the root system. Unfortunately, many latter steps of the pathway remain to be characterized. The identification of such genes would be required to evaluate the overall impact of elicitor treatment on the transcriptional profiling of the entire lignan biosynthetic pathway. Nevertheless, through the analysis of *PLR* gene can provide a good insight into the PTOX route.

4.2.2.Wild type and transformed cell cultures.

4.3.3.1 Growth studies

The growth of wild-type (WT) and transformed cells, was determined during 14 days (section 4.2.2.1,Figure 18). Based on the these results, we decided to elicit the cultures after 7 days of the previous culture, when both systems under study are nearly to the late lag phase phase (Figure 18).

In control WT cells (Figure 24A), the maximum growth achieved corresponded to a biomass of 0.37 g (day 2 after elicitation time). Thereafter, a slight decrease on growth was observed. Conversely, elicitation of WT cells with coronatine, imposed a transient

decrease on growth which slightly recovered on day 3 after elicitation (Figure 23A), reaching a maximum growth of 0.23 g DW. It seems to be more sensitive to the coronatine presence at the first days than the transformed line. Cell viability in WT cells was high under control conditions, reaching values of 90% the first day after elicitation decreasing to 78% (day 7). Consistent with the observed growth effects, coronatine treatment reduced cell viability to 80% and 70% after 3 and 7 days of elicitation, respectively.

A gradual and extended increase in growth was observed in control transformed cells (Figure 23B), reaching a maximum production of 0.30 g DW on day 6 after elicitation, near the growth achieved in control WT the same day (Figure 23A). However, elicited transformed cells exhibited a slight increase in growth until the day 3, similarly to elicited WT cells, and thereafter growth declined. Viability was somewhat lower in transformed than WT cells, with values of 80 % (day 0) and 70% (day 7) and 70% after 7 days of the elicitation.

Our results indicate that growth is markedly higher in WT than transformed cells, and decreases with elicitation in both systems, more noticeably in transformed cells. Several authors have reported that biomass is significantly reduced by the addition of the elicitor. MeJA in *L.album* cells, leading to a reduction in DW as soon as 24 h after inoculation (Van Fürden et al. 2005). Similar results have been reported using chitosan and chitin oligomers (Bahabadi et al 2014), salicylic acid (Yousefzadi et al 2014) and

other biotic elicitors such as *Fusarium graminearum* or *Rhizopus stolonifer* fungal extracts (Bahabadi et al 2011).

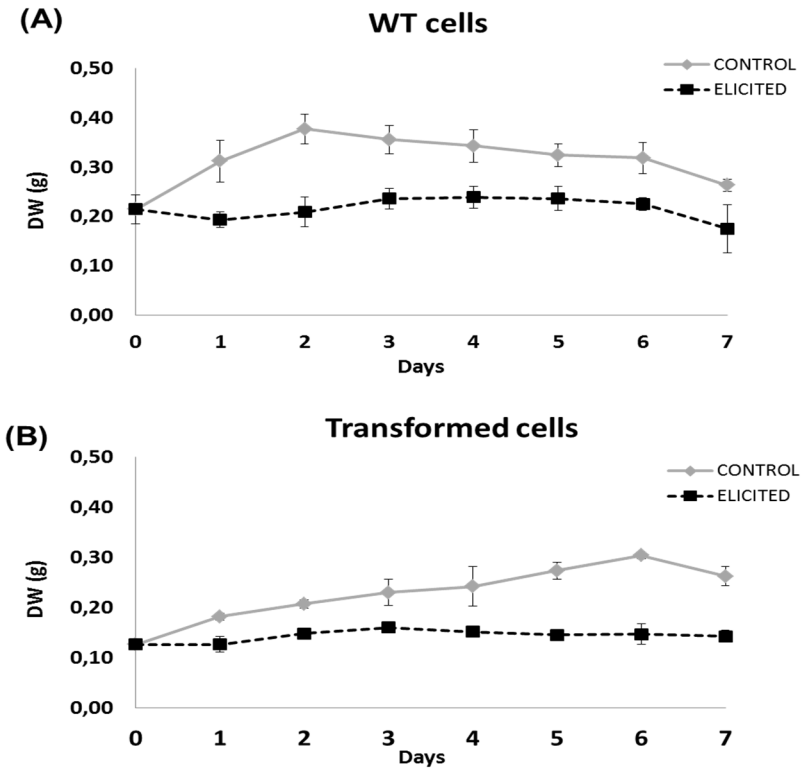


Figure 23.Time courses of biomass production (expressed as dry weight g) of wild type (WT) and the transformed cells cultured for 7 days after coronatine elicitation treatment compared with non-treated (control) conditions. Data represent average values from three separates experiments ± SD.

To date, only one work has reported the use *L. album* transformed cells (Baldi et al 2010), in which the main objective was to evaluate the changes in PTOX and MPTOX levels by treatment with fungal extracts, as elicitors. Remarkably, the transformed cells were not derived from hairy roots but obtained by co-culture of explants with

A. rhizogenes (Baldi et al. 2008). Therefore, the current and previous studies are not fully comparable. Nevertheless, the results from this work are consistent with a suppressive effect on growth of *L. album* cells included by elicitation.

4.3.3.2 Bioproduction analysis

The pattern of lignan production in wild type and transformed suspension cells, in control and elicited conditions, are shown in Figure 25. PTOX was the main aryltetralin lignan produced in all the conditions studied. In previous studies in our laboratory, callus lines obtained from predominantly PTOX-producer *L. albumin vitro* plants were also PTOX producers (Yousefzadi 2010,2012) and corroborated by other authors (Smollny et al. 1998 and Mohagheghzadeh et al. 2006). The maximum PTOX production in control WT cells was observed on day 2 (47 µg/g DW) (Figure 25A).In the elicited WT cells, the maximum PTOX production was achieved after 4 days of elicitation (42 µg/g DW), but the yield was slightly lower than in the control cells. Although PTOX production was increased by the elicitation at day 4, it remained below the maximum levels of the control cells. Several studies have been done using elicitors for the lignan bioproduction in *L. album* cells. Van Fürden et al. (2005) reported that MeJA increases PTOX production two fold and other mimic elicitors such as coronalon or indanoyl-isoleucine also increased the lignan production in *Linum*species (Berim et al 2005).

In transformed cells, PTOX production (Figure 24A-C) was similar than in control and elicited cells, and its levels were kept constant at an average of 36 µg/g DW. It is important to notethat the chiefly PTOX-producing transformed cells were mainly derived from MPTOX-producing hairy roots, and thus the lignan profile changed after desdifferentiation. In this experiment, the transformed cells seem to be less susceptible to coronatine treatment, which agrees with the growth curves since there is not a markedly depletion in biomass.

Thus, WT cells enable the maximum production of PTOX on day 2, whereas transformed cells provide a more stable and constant system for PTOX production independently of the growth curves. Interestingly, a temporary shift was observed in coronatine-treated WT cells, which exhibited the maximum production of PTOX, 4 days later than control.All biotechnological systems used (WT and transformed cells, elicited or not) exhibited a lower production of MPTOX than PTOX (Figure 24B-E). In control WT cells the highest 6-MPTOX production was 9.5 µg/g DW on day 3, compared with a maximum peak of 5.8 µg/g DW in elicited WT cells on day 4.

Coronatine, maintained PTOX production constant instead of decreasing its levels, as it would be expected when the cell viability is compromised (Figure 24A-D). However, the elicitation effect was more apparent in MPTOX (which is the less productive route) than PTOX, although it did not lead to significant increases in MPTOX production (Figure 24B-E). In transformed cells, the levels of

MPTOX were very low, regardless of the elicitation (approx. 2 µg/g DW) (Figure 24E). This pattern was also observed in root systems.

The levels of dPTOX, precursor for both PTOX and MPTOX, remained constant at 40 µg/g DW in the different biotechnological systems and conditions used. This indicates that a pool of this metabolite is maintained, and thus, it does not represent a limiting substrate for PTOX and MPTOX production (Figure 24C-F). β-peltatin, precursor of MPTOX, could not be detected in any of the systems analyzed, before or after elicitation.Thus, we can infer that not being MPTOX producers systems, the production of β- peltatin will also be very low as well.

Our results indicate that the levels of MPTOX/dPTOX/ PTOX are maintained at a ratio of 1:10:10 in both types of cell suspension cultures studied.

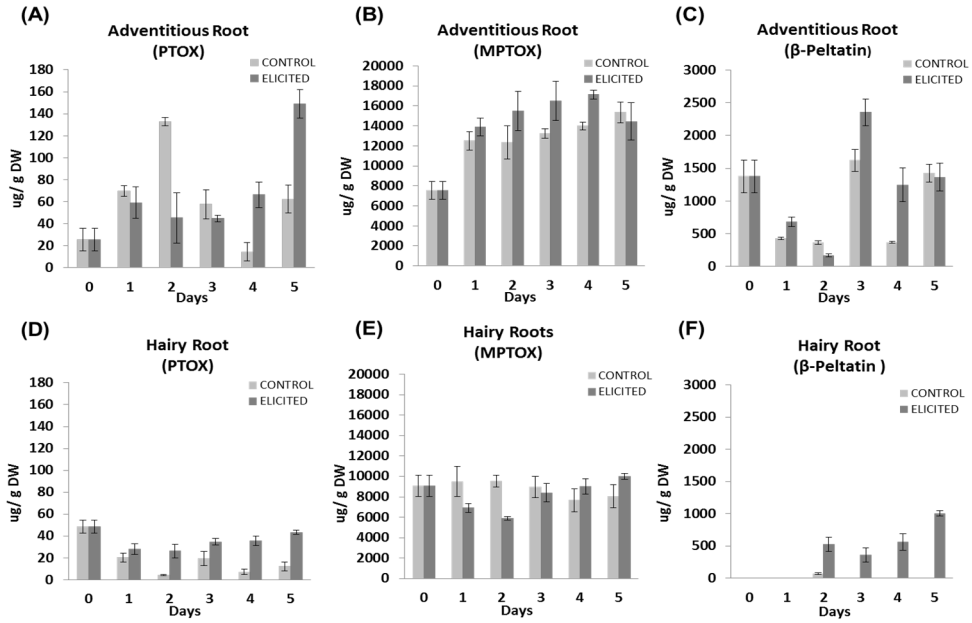


Figure 24.Time curse of lignans concentration (PTOX,MPTOX,dPTOX) expressed in µg/gDW in wild type (WT) (A-C) and transformed (D-F) cultured for 14 days in non-treated (control) and treated with coronatine at 1mM conditions. Data represent average values from three separates experiments ± SD.

4.3.3.3 Gene expression analysis

Elicitation produced an increase in the expression of the genes analyzed in both cell systems but its effects were higher in transformed than WT cell cultures (Figure 25), and remarkably in the case of the PLR gene.

In WT cells, the expression level of PLR was lower, peaking after 12 h of elicitation. The maximum transcript accumulation was observed for *CCR* and *CAD* 6 h after of elicitation. However, PTOX and dPTOX production did not increase and MPTOX production

increased on day 4 but remained low. Van Fürden et al. (2005) first reported an increase in the expression of genes in the PTOX pathway, mainly in *CAD* and *CCR* by fungal elicitors. Yousefzadi et al. (2010, 2012) and Bahabadi et al. (2011, 2014) corroborated these expression patterns. However, Bahabadi et al. (2014) also reported that the expression of *PLR* was more affected by chitosan than any of the other gene expression analyzed. Apart from this, a similarly tendency has been observed in transformed cells for the tree genes, but coronatine dramatically increased the transcript levels of *PLR* three days after elicitation. In this study the transformed cells seem to be more sensible to coronatine elicitation.

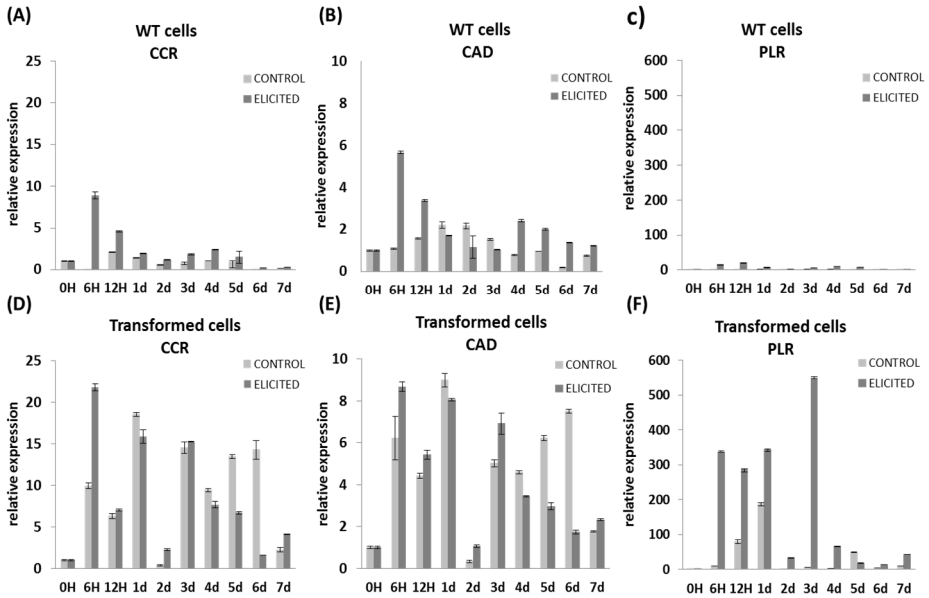


Figure 25. Relative expression of *CCR, CAD, PLR* in wild type (A-C) and transformed cells (D-F) after the 6 hours and then each day during 7 days after the elicitation in non-treated (control) and treated with coronatine at 1mM conditions. Data represent average values from three separates experiments ± SD.

Overall, we conclude that *CCR*, *CAD* and *PLR* expression in both systems exhibits two maximum peaks of expression, one after coronatine elicitation, which is coincident with the beginning of the growth phase, the system patterns previous studied in the section 4.2.2.1 and the second peak of expression during the stationary phase (Figure 18). This agrees with the patterns observed for PTOX and MPTOX production, which also exhibited two maximum peaks, although at very low levels.

4.4.CHAPTER III. Effect of the in vitro morphogenesis in the production of podophyllotoxin derivatives in callus cultures of *Linum album*

L. album plantlets grown *in vitro* were dedifferentiated in darkness in an MS culture medium supplemented with 2 mg/L NAA and 0.4 mg/L KIN in order to study the lignan production of the obtained cell biomasses when growing in light or darkness, and with or without the addition of PGRs (Figure 26), as well as the relationship between their lignan pattern and organogenic capacity in the assayed conditions. The experimental design included the following steps:

a) Dedifferentiation. Cell masses formed from the explants were excised and grown in dark conditions with the addition of the aforementioned PGRs, for a period of 7-8 months with routinely subcultures every 2-3 weeks in the same conditions. After this time, the compact and white biomasses obtained showed, most of them, organogenic capacity (Figure 26).

b) Increase of biomass in different experimental conditions. After several subcultures, the biomass obtained was divided in four different conditions in order to elucidate the effect of PGRs and light on growth, and organogenic capacity in cell cultures of *L. album*. For this reason, the cell biomass was distributed in: MS-PGRs free or MS with PGRs and each of them in the light or darkness conditions. The cell biomasses maintained in these four conditions

showed good growth capacity for 3 more months, except those transferred to the light with the addition of PGRs, which showed a decreased growth, turning yellow and then brown, and finally were discarded. All the calli were routinely subcultured every 2 weeks in the same conditions until enough biomass was obtained to start the planned experiment. The aspect of these cell cultures are shown in Figure 26.

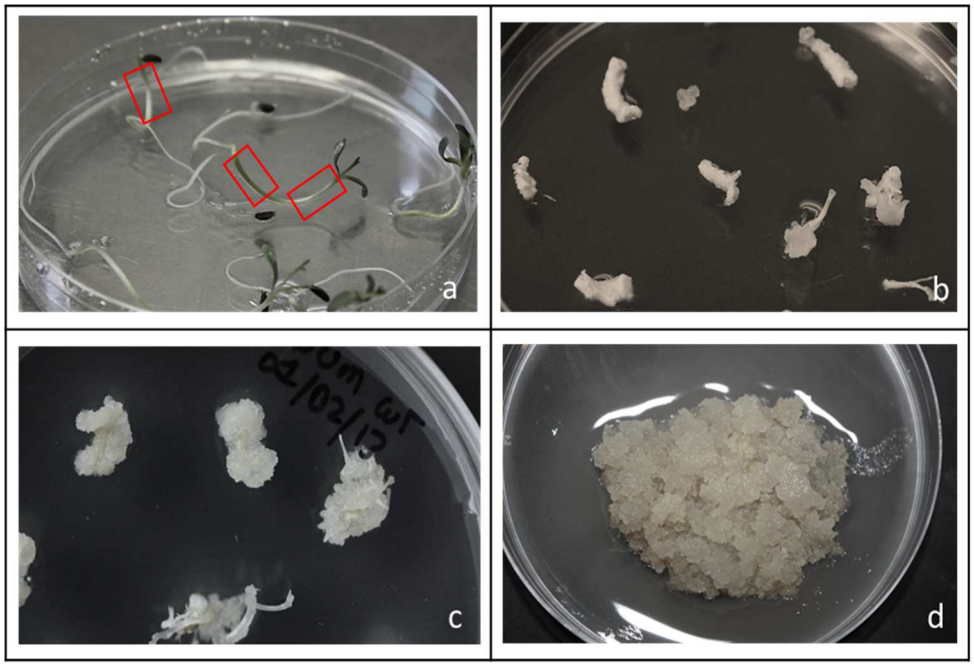


Figure 26. Dedifferentiation of the explants and cell masses induction. **a)** Plantlet segments used as explants; **b-c)** cell mass formation; **d)** isolated cell mass culture.

c) *Establishment of CM cultures in the experimental conditions assayed*. Once enough biomass was obtained in the aforementioned starting conditions (darkness with or without PGRs

and light without PGRs: conditions 1, 2 and 3, respectively) and in order to determine how the three culture conditions or the change from these conditions to others where the presence of PGRs or light was substituted by PGR-free or darkness or viceversa, affected to growth, organogenic capacity and lignan production of the cultures, the obtained biomass was inoculated in fresh medium with the following distribution (Figure 27):

- Conditions 1.1 and 1.2: biomass (derived from conditions 1 - darkness plus PGRs) was grown in darkness with PGRs (1.1), and in light without PGRs (1.2), respectively.

- Conditions 2.1 and 2.2: biomass (derived from conditions 2 - darkness without PGRs) was grown in darkness without PGRs (2.1), and in light also without PGRs (2.2), respectively.

- Conditions 3.1, 3.2 and 3.3: biomass (derived from conditions 3 - light without PGRs) was grown in light without PGR (3.1), and in darkness without (3.2) and with PGRs (3.3), respectively.

Callus sultures were harvested at one, two, four and six weeks after the establishment of the new conditions, with the aim of studying the growth, organogenesis/morphogenesis capacity, lignan production and the interrelation among all these parameters.

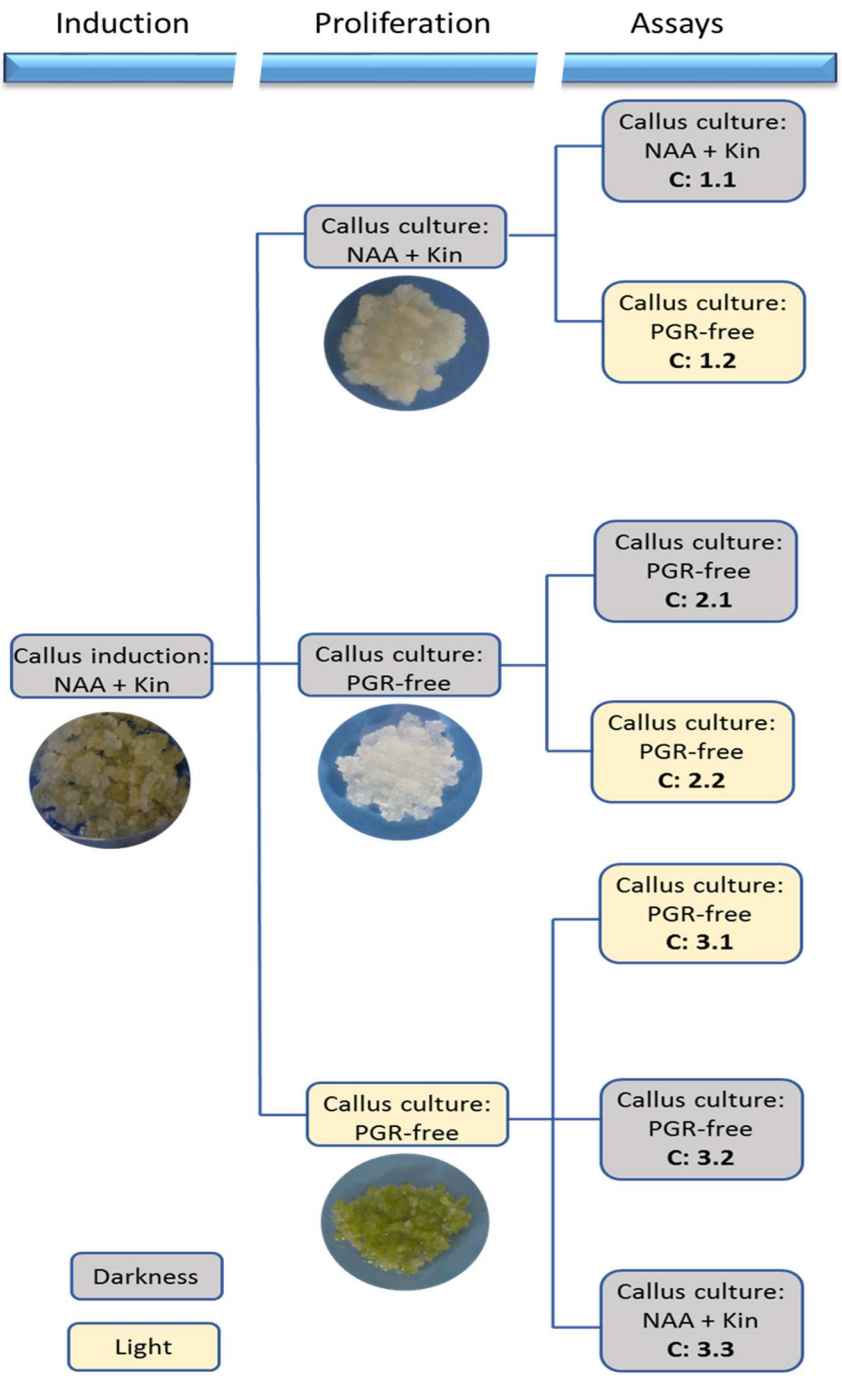


Figure 27.Schematic drawing of the methodological design employed in the study of the organogenesis effects.

4.3.1.Study of the growth capacity

The growth course of the calli expressed as FW (g) presented a lag phase until the second week, except in the case of calli grown in the light without PGRs (conditions 3.1), which increased the FW constantly until the end of the experiment, showing the highest capacity for biomass formation. After six weeks, the fresh weight obtained in this case was almost 9 times higher than that of the inoculum (Figure 28A).

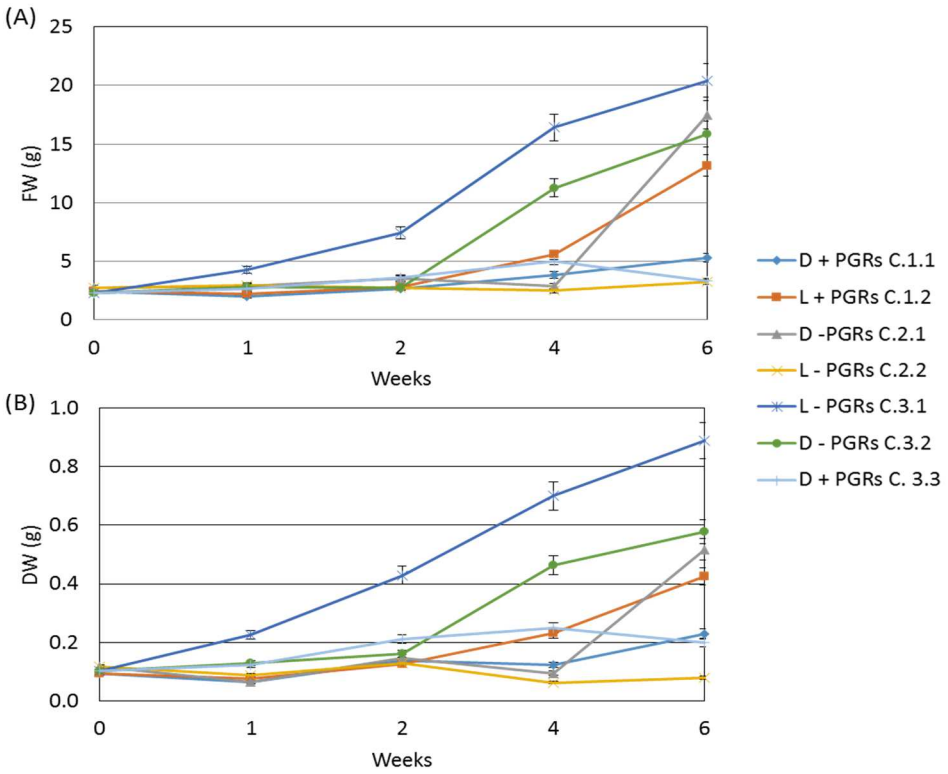


Figure 28. Study of the growth course measured as FW (A) and DW (B) during a period of 6 weeks. Measures are the average of 3 biological replicates ± SD.

Calli growing in darkness without the addition of PGRs (conditions 2.1 and 3.2), derived from cultures also free of plant growth regulators, either in darkness (conditions 2) or light (conditions 3), also achieved good growth after 6 weeks of culture (both achieving approximately 7 times the weight of the inoculum). Intermediate growth capacity was observed for calli kept in light without the addition of PGRs (conditions 1.2). In this case, the slight reduction of biomass formation (the final biomass was 6 times the inoculum) compared to the other calli growing without PGRs, either in light or darkness, is probably due to their origin, since they were grown with the addition of PGRs before starting the experiment.

Lower growth capacity was achieved by calli growing in darkness but with the addition of PGRs (conditions 1.1 and 3.3) regardless of their origin. In these two cases the final biomass was only twice the inoculum (Figure 28A).

From these results, we can infer that a lack of PGRs in the medium permitted the best growth in the calli studied, independently if they were grown in light or darkness. These conditions (2.1 and especially 3.1, and 3.2) also induced high organogenic capacity of the callus (Table 6).

However, the calli grown for 6 weeks in the light without phytohormones (condition 2.2), but derived from calli maintained in darkness, also without PGRs, showed the lowest growth capacity, the biomass increasing only 1.2 times in relation to the inoculum (Figure 28A). The origin of these calli was darkness without PGRs,

possibly the best conditions for growth, yet when these conditions were abruptly changed to light, the growth was dramatically reduced.

The time course of the DW during the 6-week experiment corroborates the growth capacity of the studied calli indicated by the FW (Figure 28B).

Table 6. Changes in the growth and morphology of the calli during 6 weeks of culture in the assayed conditions. (PGRs: 2 mg/L NAA and 0.4 mg/L KIN).

CALLI	TREATMENT	Proliferation Rate				Callus characteristics (week 6)
		week 1	week 2	week 4	week 6	
Darkness with PGRs (1)	Darkness with PGRs 1.1	-	-	-	-	White, friable, non embryogenic
	Light without PGRs 1.2	-	-	+	++	Lush green, compact, Embryogenic
Darkness without PGRs (2)	Darkness without PGRs 2.1	-	-	+	++	Brownish, very small
	Light without PGRs 2.2	-	-	-	-	White, granular, embryogenic
Light without PGRs (3)	Light without PGRs 3.1	++	++	+++	++++	Lush green, granular, Embryogenic
	Darkness without PGRs 3.2	+	+	+++	++++	Brownish white,compact, embriogenic
	Darkness with PGRs 3.3	+	+	++	+	Brownish, very small

From these results, we can infer that although the dedifferentiation of *L. album* plants and the initial callus formation was completely dependent on the addition of NAA and KIN, the best conditions to increase the biomass of the *L. album* calli were maintenance in darkness without the addition of PGRs. This was the initial treatment of calli submitted to conditions 2, while conditions 1 and 3 were darkness with PGRs, and light without PGRs, respectively. When calli were cultured in light without PGRs (condition 3), their capacity to form biomass, as well as chlorophyll, was also high (Table 6).They also showed notable organogenic capacity, which was not lost after transfer to darkness. It has been shown (Rajesh et al., 2014) that the presence of auxins is essential for embryogenic callus proliferation, but it is usually inhibitory for further development and growth of somatic embryos in Podophyllum hexandrum Royle.

Calli grown under conditions 1 (darkness with PGRs), when maintained for 6 weeks in the same medium (condition 1.1), were friable and white, without any visible organogenesis. In contrast, when calli were subcultured and maintained for 6 weeks in light without PGRs (condition 1.2), they appeared greenish after the second week, and had turned green by week 6. Thus, the light promoted the formation of chlorophyll, and increased organogenic capacity after 4-6 weeks of culture (Tables 6, 7). Table 7 presents the morphological changes of the calli according to the culture conditions, together with their capacity to produce biomass.

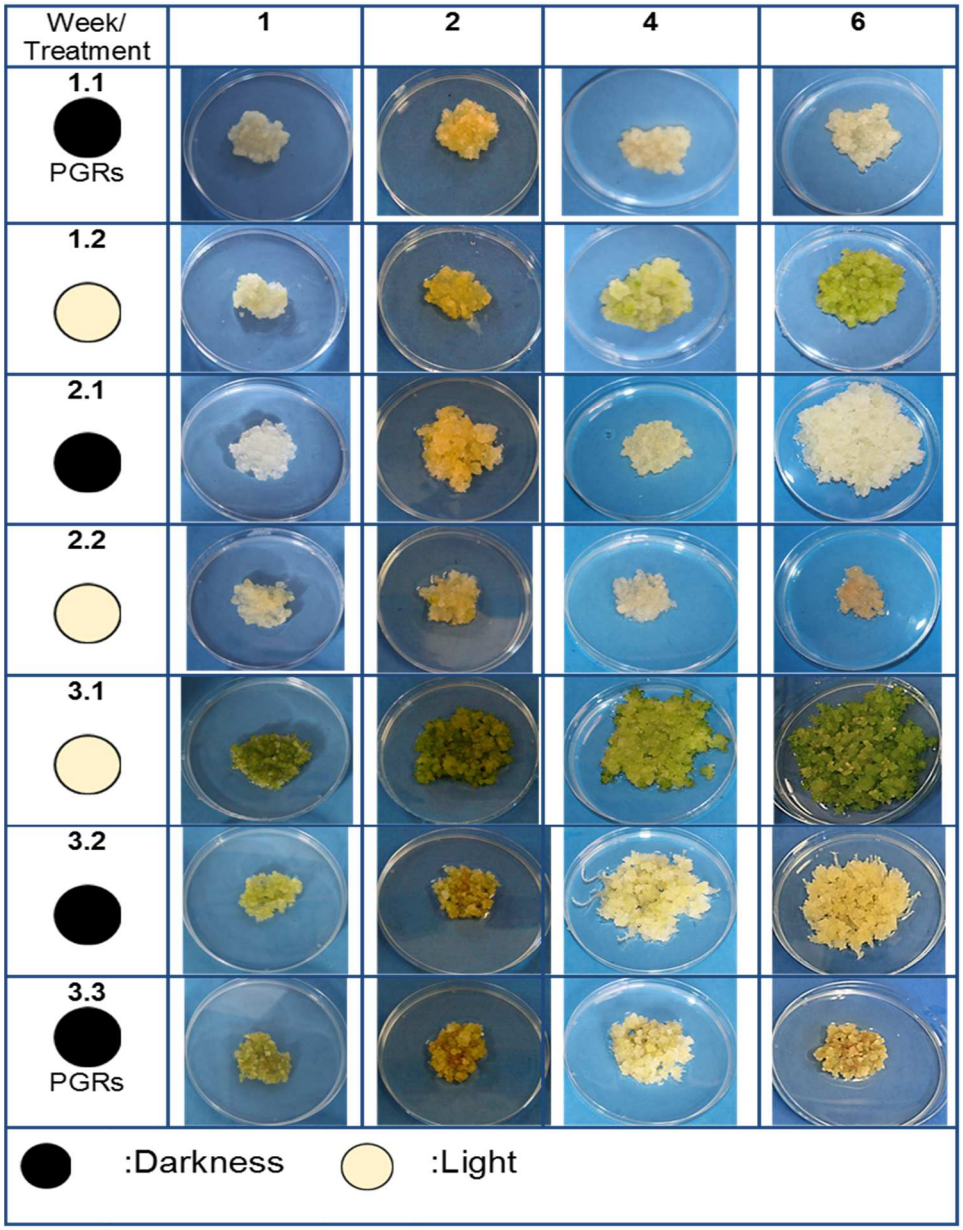
The appearance of calli originating from conditions 2 growing for 6 weeks in darkness or in light without PGRs is showed in Table 7 In light (condition 2.2) no chlorophyll or organogenesis was visible, and the appearance of the calli corroborates the low growth capacity of the cultures under this conditions. The growth of these calli was very slow and they were less compact and healthy. Their color changed from white to yellow as growth decreased. In contrast, the calli maintained in the original conditions presented highest growth capacity, with a white and friable appearance (Table 7).

Calli maintained in the light without the addition of phytohormones (condition 3) were green and showed a very good and constant growth (Table 7). When subcultured and maintained for 6 weeks in the same conditions (conditions 3.1), their appearance was the same: friable green calli with high growth but no visible organogenic capacity at the beginning, but after 4 weeks of culture showing granular organogenic-like zones (Table 7), although this could not be confirmed without a microscopic study.

The calli growing in conditions 3, when subcultured and maintained in darkness, without or with the addition of plant growth regulators (conditions 3.2 and 3.3, respectively), turned brownish white with time. After 4 weeks of growth, clear differences were observed in calli maintained in darkness: those without PGRs became organogenic, developing somatic embryos, whereas those grown with PGRs had turned dark yellow by the end of the experiment,

Their growth stopping almost completely. In the latter calli, very little organogenesis could be observed with the naked eye (Table 7).

Table 7.Changes in the appearance of the callus cultures during a growth period of 6 weeks under the studied conditions.



To gain more insight into the organogenic capacity of the calli grown in the experimental conditions for 6 weeks, a light and scanning microscope study was carried out.

4.3.2.Light microscopy study

Calli grown in conditions 1.2 and 2.2 (light without PGRs, coming from dark and with or without PGRs, respectively) presented small cells with thin cell walls together with large cells with thick walls, and intercellular spaces were prominent. There was no organization visible (Figure 29).

During the first 4 weeks of culture, few differences were observed among calli derived from condition 1 when subcultured in the same medium and maintained in the dark or subcultured without phytohormones in light (Figure 29). As shown in the figure, clusters of small cells were surrounded by larger cells with intercellular spaces and there was an absence of any significant cell organization. However, mainly after 6 weeks of culture, calli subcultured in light without phytohormones showed small organized areas that were probably meristematic and linked with their greenish aspect and larger size. In contrast, the calli grown without light (condition 1.1) did not show any organization (data not shown).

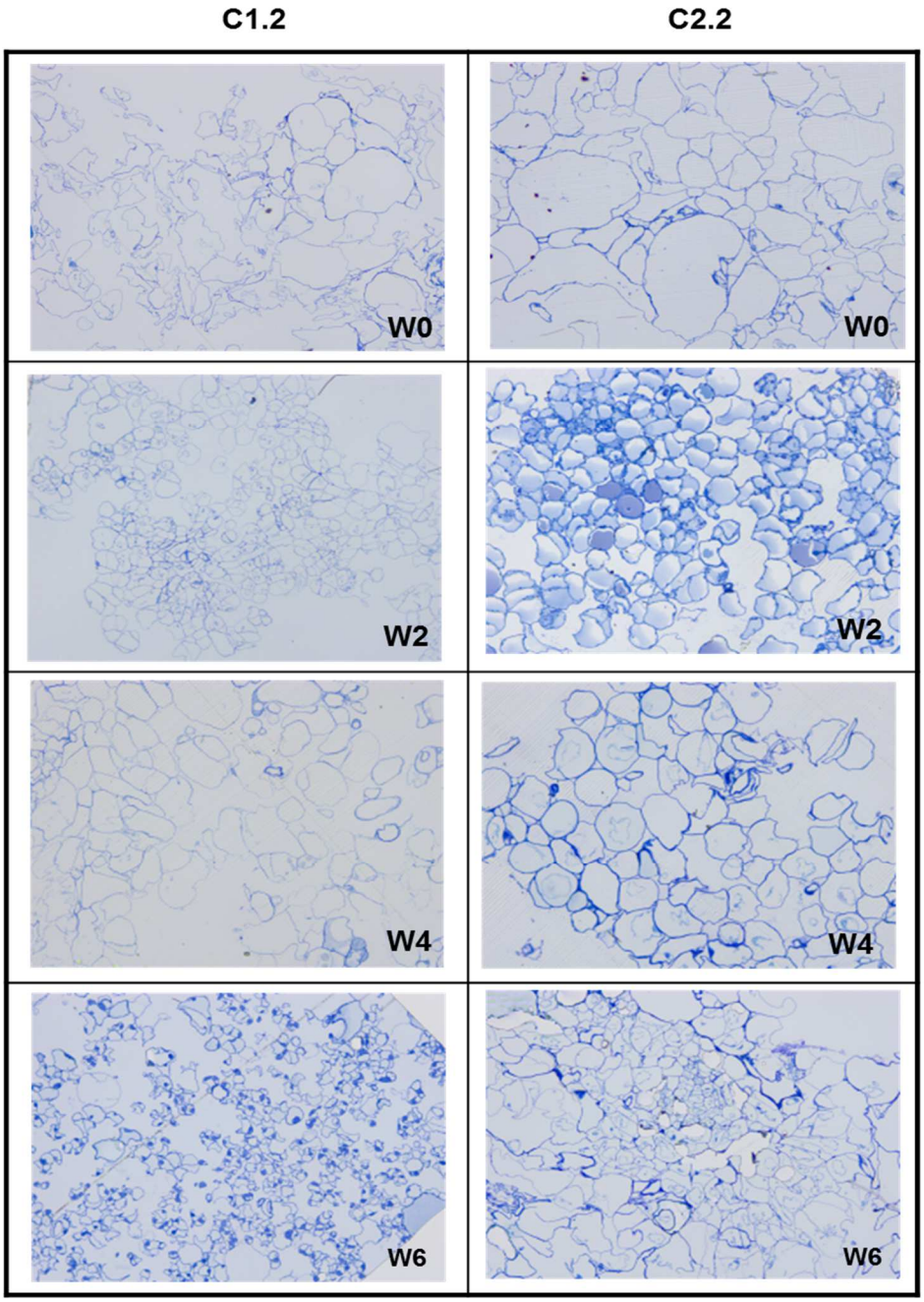


Figure 29.Cellular organization of the callus cultures during a period of 6 weeks under conditionsC1.2 and C2.2. W: week.

Calli subcultured without phytohormones in light (conditions 2.1 coming from darkness and PGRs) are shown in Figure 29. At the beginning of the experiment, the cells exposed to light were smaller than those in darkness, although no organization was observed and there were predominant intercellular spaces. After 4 and 6 weeks of culture, the calli were mainly composed of large cells, with fewer spaces among them. At the end of the experiment, small meristematic areas were observed in calli in light, which in this case did not have a green appearance (Table 7).

Most of the cells observed in the calli grown under conditions 3 (light without PGRs) were small and isodiametric, leaving large intercellular areas (Figure 30). At the beginning of the experiment, calli subcultured from these conditions to conditions 3.1, 3.2 and 3.3 showed few differences, displaying large cells with some clusters of small cells (probably meristematic areas) and without visible organization (data not shown). After two weeks of growth, the calli grown in the light (conditions 3.1) presented very well organized meristematic areas, and those grown in darkness showed embriogenesis, with very well organized somatic embryos at different developmental stages (Figure 30). Under darkness with phytohormones (conditions 3.3), there was less organization, although some meristematic areas were found.

After 4-6 weeks of culture, the meristematic areas observed at the beginning of the experiment persisted, and cell organization was visible, mainly in conditions 3.1 and 3.2. In these conditions, after 4 weeks of culture several vascular bundles and somatic embryos

were observed (Figure 30). After 6 weeks in conditions 3.1, several somatic embryos at different stages were observed, and in conditions 3.2, very well developed meristems and shoots were evident (Figure 30). In conditions 3.3, the morphogenesis was more limited, although several meristemoids and organized zones were observed (data not shown).

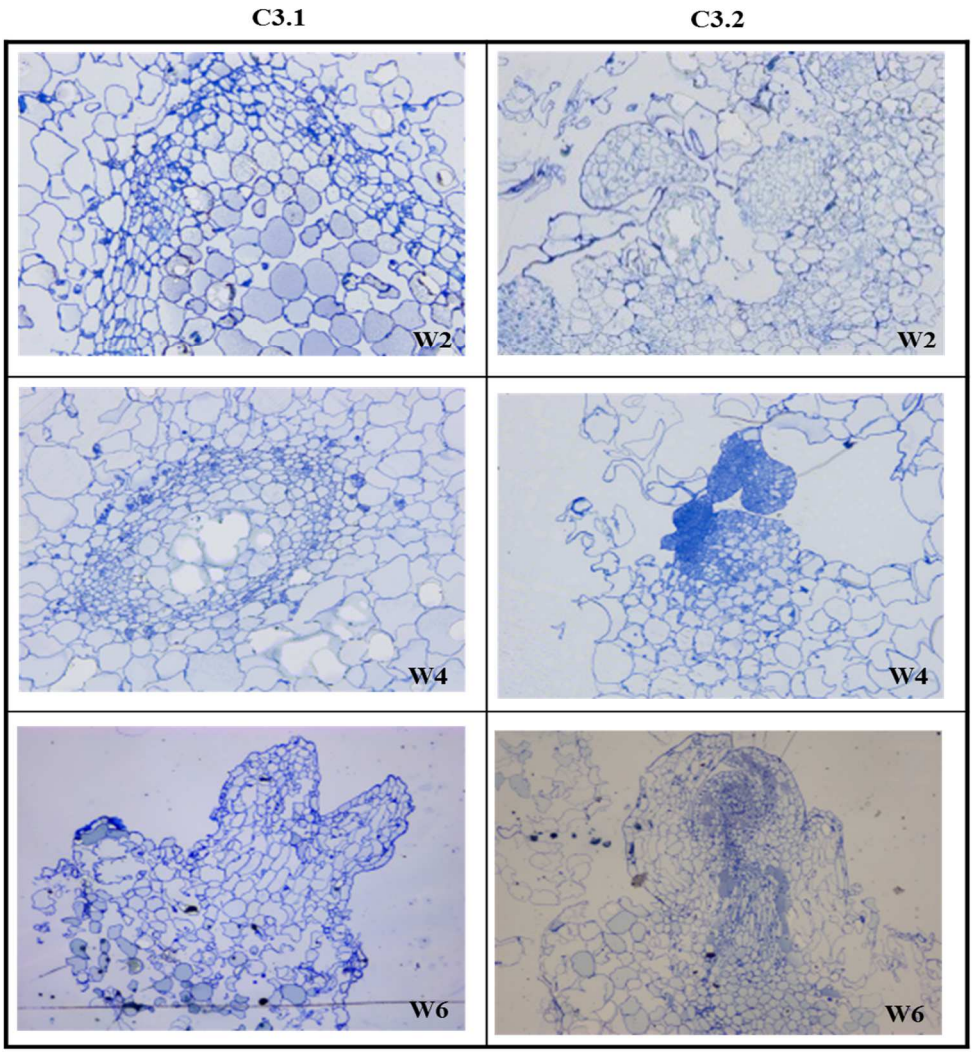


Figure 30. Cellular organization of the callus cultures during a period of 6 weeks under conditions C3.1 and C3.2. W: week.

All these results, obtained after observation of the calli grown throughout the experiment in the assayed conditions, confirm the morphological aspects indicated above. The experimental conditions that induced more somatic embryo formation, more structured meristematic areas and shoots were those derived from conditions 3, especially 3.1 and 3.2. In Figure 30 it is also possible to see the different stages during the shoot development. The organogenic calli usually presented grouped cells with few intercellular spaces (C3.&, W&). It was only after two weeks of culture that most of the organogenic calli developed tracheids near the meristemoid zones (C3.&, W&).In Figure 31 it is showing somatic embryos at different development stages, and meristems with leaf primordia are visible in (Figure 31c-d). These stages correspond to the calli grown in the aforementioned conditions, especially those that induced clearer organogenesis

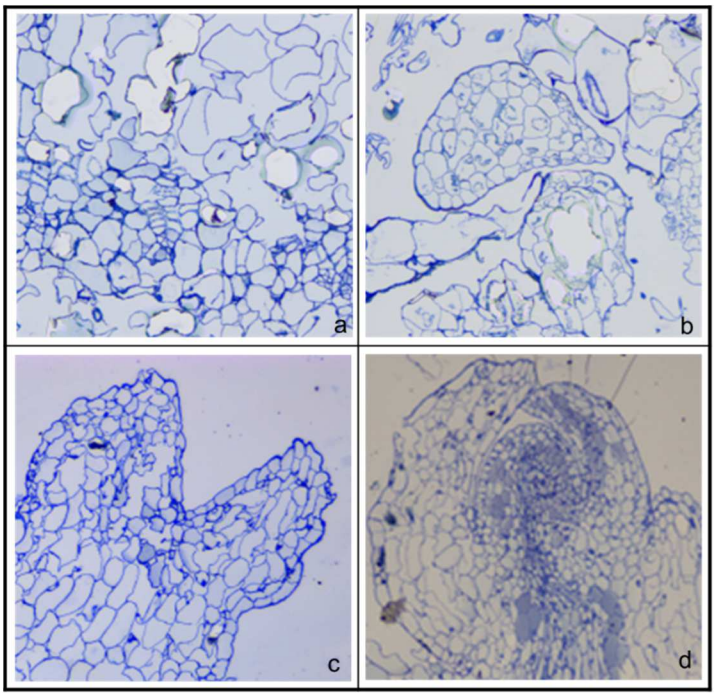


Figure 31. Details of cell differentiation and organogenesis at cellular level. a) Typical callus cells and proliferative areas (small cells). B) Organogenic mass isolated from the rest of the callus. c) Initiation of cell differentiation to epidermis. d) Formation of leave primordium and apical meristem.

4.3.3.Scanning microscope study

The observation of the calli grown for 6 weeks in the experimental conditions with the scanning microscope corroborated the organogenic capacity of some of them, already observed at light microscopy. In general, the external surface of calli presented large, oblique and elongated cells and clusters of small isodiametric cells with several intercellular spaces (Figure 32).

In some calli, mainly grown in the dark and without phytohormones, the formation of 5-7-celled filamentous trichomes initiated from

external (possibly epidermal) cells was clearly observed (Figure 32f).

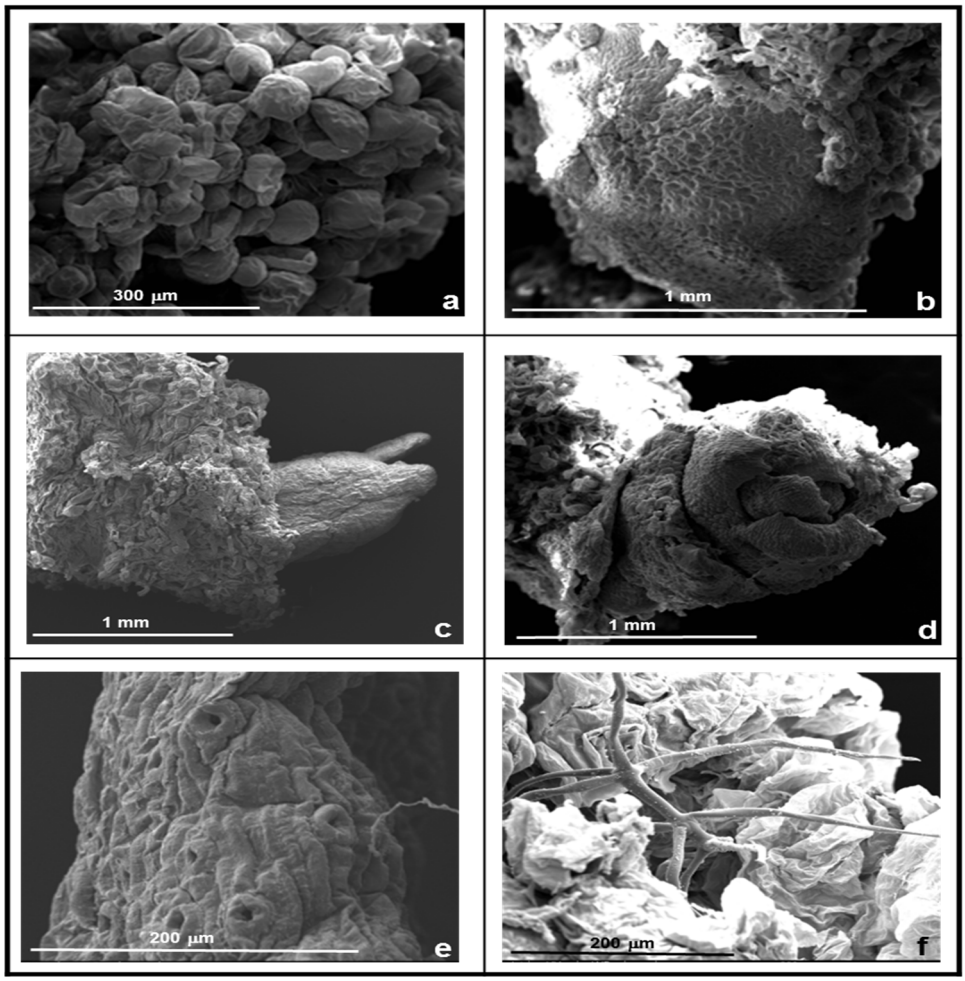


Figure 32.Scaning electron microscopy. **a)** callus mass composed by undifferentiated big cells. **b)** Initiation of the organogenic process showing organogenic masses composed by small cells. **c)** Organogenic growth developing leaf primordia. **c)** Shoot formation. Last steps of the organogenesis, **e)** Stomas. **f)** Trichrome.

In calli obtained from conditions 3, especially those maintained in conditions 3.1 and 3.2, organogenesis was clearly observed by the scanning microscope. Figure 32 shows in detail the steps from undifferentiated callus mass (Figure 32a) to the development of typical leave structures such as, stomas (Figure 32e) or trychomes (Figure 32f). Intermediate steps conducting to leave primordia and the formation of the shoots are also visible in Figure 32c-d. Shoots formation were mainly found in calli derived from conditions 3 and maintained in conditions 3.1 and 3.2. Consequently, the shoots observed in these conditions could start their differentiation when the original calli were in the light, although morphogenesis is a process that in this case does not need light. Finally, the development of vascular bundles, that are essential to guarantee the transport between tissues and distant organs, assuring the growth and maintenance of the callus was also observed (data not shown). In this sense, the differentiation of xylem and phloem is critical for the formation of organogenic structures.

4.3.4.Lignan production

The lignans studied were deoxypodophylotoxin (dPTOX), podophylotoxin (PTOX), peltatin (β-Peltatin), yatein (YT) and 6-methoxy-podophyllotoxin (MPTOX). β-Peltatin was found only in traces, and YT was not present in the calli at all. β-Peltatin is a compound formed from dPTOX by the action of a CYP enzyme, whereas YT seems to be involved in an alternative pathway leading to PTOX via matairesinol, without requiring the precursor pluviatolide (Scheme 1).

The production and/or accumulation of dPTOX in the calli did not change significantly under the different conditions studied. The levels achieved were in the range of 40-43 µg/g DW, independently of the presence of light or the addition of phytohormones in the cultures. Also, no relationship between callus organogensis and dPTOX levels was observed (Figure 33). This indicates that this common precursor of PTOX and MPTOX can be directed to the formation of these two compounds depending on the activity of the enzymes that control both biosynthetic pathways.

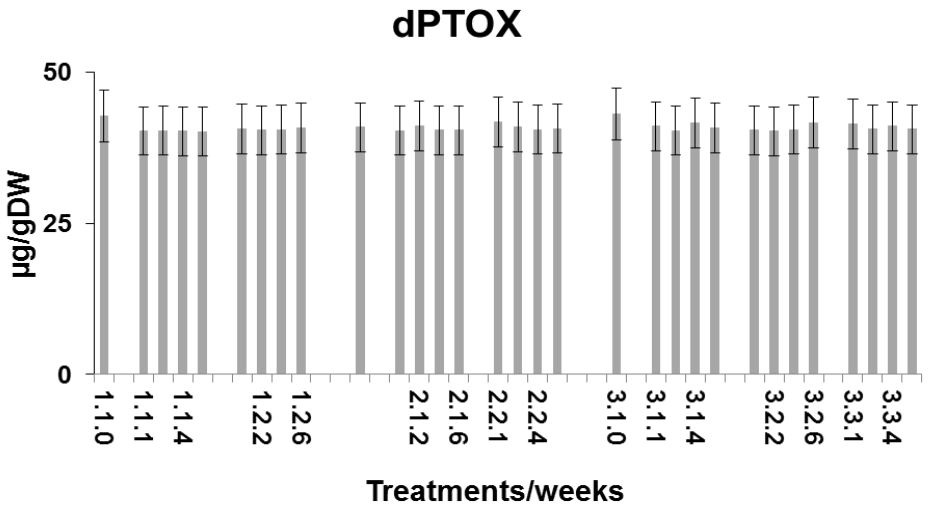


Figure 33. Time course of DOPTOX production in the callus cultures of L. album growing under the different condition asayed. Each value is the average of 3 biological replicates ± SD.

In contrast, the production of PTOX and particularly MPTOX was clearly affected by the calli growth conditions. As indicated

previously (Federolf et al. 2007), in cell suspensions of *L. album*, dPTOX was the precursor of both PTOX and MPTOX, however the different accumulation of these compounds was depending basically on the original plant and the cell line established.

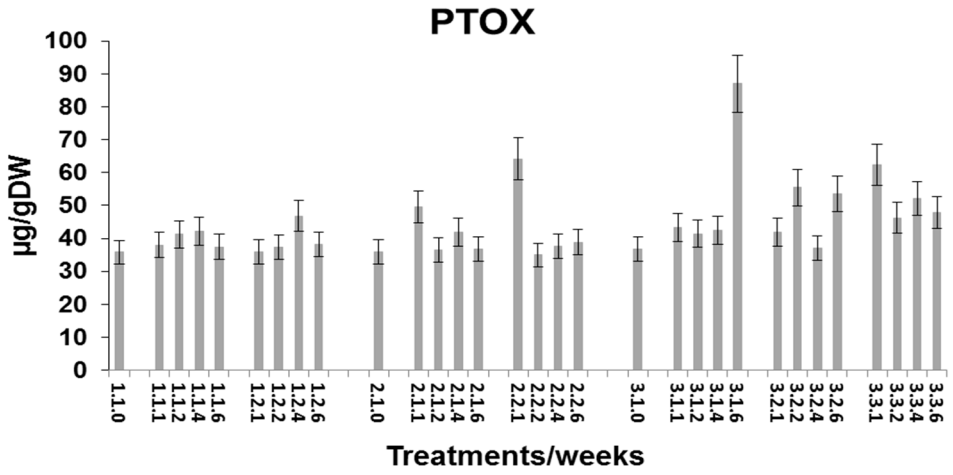


Figure 34. Time course of PTOX production in the callus cultures of *L. album* growing under the different condition assayed. Each value is the average of 3 biological replicates ± SD.

The PTOX levels (Figure 34) in calli derived from condition 1 and grown in darkness with the addition of PGRs (C1.1) increased slightly (1.2-fold) from the beginning of the culture period to week 4. A similar increase (1.3-fold) was observed when the calli also grown in condition 1 were transferred to the light and without PGRs (C1.2). After 6 weeks of culture, the PTOX levels decreased in both cases.

When calli grown in darkness without the addition of PGRs (condition 2) were transferred to the same medium, either in darkness (C2.1) or light (C2.2), PTOX levels clearly peaked after one week of culture (being 1.4 and 1.8-fold higher than at time zero), decreasing thereafter (Figure 34).

From the results obtained under the two initial conditions (conditions 1 and 2: darkness with and without PGRs), we could infer that the accumulation of PTOX is more positively affected by the absence of phytohormones than the presence or absence of light. This was corroborated by the fact that the highest levels of this lignan in the cultures derived from condition 2 (absence of PGRs) were achieved the first week of culture both in darkness and light conditions, unlike the calli grown in conditions 1.1 and 1.2 where the lower levels were obtained at the beginning of the experiment (Figure 34).

When calli were grown under light without PGRs supplements (third condition), once again the absence of plant growth regulators resulted in the highest PTOX accumulation (2.3-fold higher at week 6 than at time 0). When the calli were grown in darkness, PTOX levels peaked after 2 and 6 weeks of culture without PGRs and after 1 and 4 weeks with the supplement (Figure 34).

Taken all together, these results suggest that the PTOX production was generally more dependent on the addition of PGRs than on conditions of light or darkness, since the highest levels were achieved when the culture medium was not supplemented with

phytohormones. Nevertheless, light had also a positive effect on the PTOX yield.

When considering the morphogenetic capacity of calli, it can be inferred that the highest PTOX levels corresponded to organogenic calli, although not all the organogenic calli presented the highest levels of this lignan, as it happened, for instance, with calli cultured in the 3.2 condition. However conditions 3.1, calli grown in the light without phytohormones, was the one that induced the highest PTOX production and also high organogenic capacity, as it will be indicated later.

MPTOX is a lignan formed from dPTOX after a hydroxylation at C6 giving peltatin, and a methylation followed by another hydroxylation at C7 (Scheme 1). As mentioned above, the levels of dPTOX in the calli were approximately the same under all the different conditions studied. However, the MPTOX levels in calli derived from calli grown in the light without PGRs (condition 3) were very high (approx. 300 μg/ g DW), regardless of whether they continued to grow in the light or were moved to darkness, but in both cases still without plant growth regulators. The highest values were obtained after 4-6 weeks of culture (Figure 35).

Notably, the MPTOX production in calli grown for 6 weeks in darkness or light with or without phytohormones clearly depended on the origin of the calli. When calli were derived from biomass cultured in darkness (condition 1 and 2), the highest MPTOX obtained was approximately 25-30 μg/g DW, but when culture was

originated in the light (condition 3), the level of this compound increased up to 280 and 300 μg/g DW, when subcultured without PGRs, in 3.1 and 3.2 condition, respectively. High levels were also obtained when phytohormones were added (3.3 condition), however the MPTOX level arrived only to 200 μg/g DW (Figure 35).

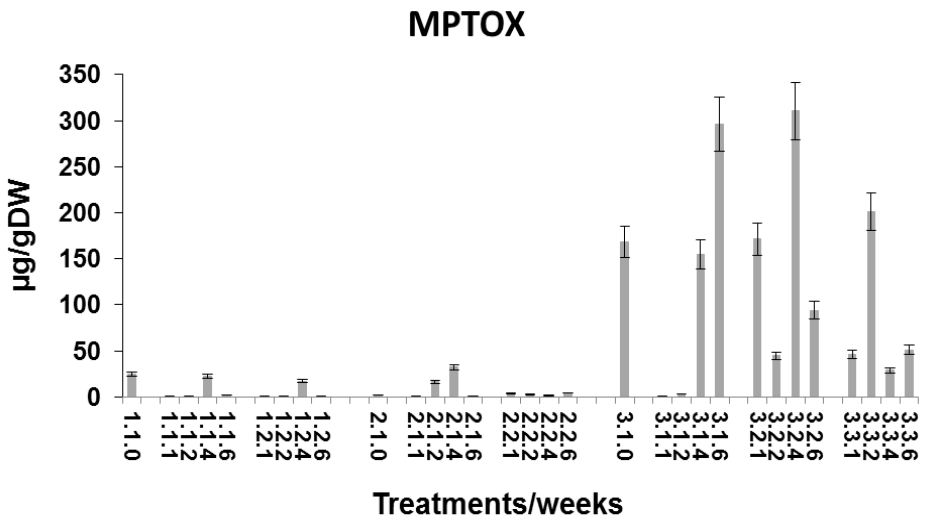


Figure 35. Time course of MPTOX production in the callus cultures of *L. album* growing under the different condition assayed. Each value is the average of 3 biological replicates ± SD.

These results showed that the MPTOX biosynthesis and accumulation is activated by the light since the highest average levels, throughout the 6 weeks studied (Figure 35), were found in calli originated from those maintained in the light and especially without the addition of PGRs. At the same time, organogenesis potentiates very positively the production of this compound. It has

been frequently reported that morphogenesis increased the secondary metabolite production. For instance, Kumar et al. (2014) studying the regeneration of *Svertia chirayita* plants from calli, showed that the production of swertiamarin (one secoiridoid glucoside), poliphenols such as amarogentin, and the xanthone mangiferin was much more higher in regenerated in vitro plantlets than the calli without organogenesis.

Considering the PTOX and MPTOX production, the results obtained, as indicated above, showed that the highest levels of MPTOX were achieved by calli grown without the addition of phytohormones both in light and in darkness. Lower quantities (about 20-39 µg/g DW) were found only after 4 weeks in all the studied conditions, except in the worse condition for growth that is 2.2 condition (Figure 34). Contrary, the production of PTOX presented a more steady behavior throughout the time of the experiment and the different conditions studied (Figure 34).

It has been shown that the main lignan found in cell cultures, a part from the experimental conditions established, was clearly depending on the original plant and on the cell/root line studied. Federolf et al. (2007) established a *L. album* cell line which mainly produced MPTOX and another, obtained from the same plant, whose main lignan was PTOX when the production was at its highest (8-16 days of culture). Smollny et al. (1998) described in cell cultures of *L. album* found several lignans and also PTOX being the main compounds. More other examples of cell, calli or

root cultures forming different lignans have been reported by Fuss (2003).

The levels of peltatin were very low in the calli studied and only traces were found. This fact indicates that the hydroxylation of dPTOX for producing peltatin is a metabolic step that really takes places, but the transformation of peltatin into MPTOX involves two teps that are more efficient and active than the first step leading to MPTOX from dPTOX (Scheme1).

In the calli studied, independently from the conditions of growth, the lignan yatein was not found, indicating that the pathway that leads matairesinol to PTOX by this via could not be operative or, may be, that the quantities of yatein formed were very quickly converted to dPTOX, as it was indicated in the case of the transformation of β-petatin to PTOX. More detailed studies at genetic and metabolic level would be necessary to assure the presence of this biosynthetic route in *Linum album*.

4.5.CHAPTER IV. Yeast two hybrid screen of PLR and TJOMT in Linum album

4.4.1.Generation of bait and prey constructs

The *PLR* and *TJOMT* CDS were inserted into the pSPARK II vector using the T4 ligase, resulting in two different bait vectors. The inserts were confirmed by enzymatic digestion and sequencing confirmed that the inserts were free of mutations. The same process was performed once the insert was cloned into the PENTRY1 destination vector. Secondly, the expression products in *E.ColiDH10B* derived from the LR reaction between the entry clones (PENTRY1A Gm-*PLR*; PENTRY1AGm *TJOMT*) and destinations vectors (PGBKT7 and PGADT7) were tested by digestion with Hind III. All the transformants appeared to be consistent with the expected band sizes. The results were later confirmed by sequencing with GW primers in which the analysis indicated that all the four transformants contained the corresponding gene without mutations.

4.4.2.Self activation test of PLR and TJMOT. Test for direct interactions.

The analyses for self protein- activation and direct protein interactions were evaluated by yeast growth on stringent selective media. For self-activation assays, the PLR or TJOMT baits were co-transformed with the pGADT7 (AD) empty vector. To test for direct interactions between PLR and TJOMT, both proteins were

co-expressed in yeast by fusion to AD and BD domains, respectively. In addition, the possibility of self-dimerization was tested by co-transformation of PLR fused to AD and BD, and TJOMT to AD and BD. Three colonies were takenfrom each of the co-transformed yeasts and grown on SD W- L- and SD W-L-H- supplemented or not with 3-AT.

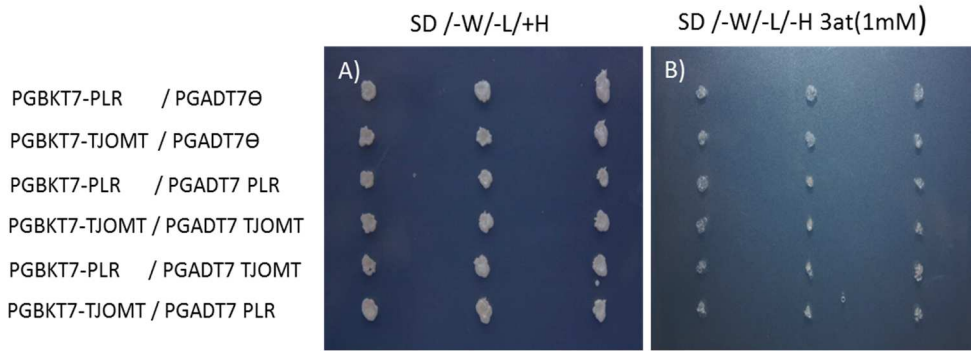


Figure 36. Self-activation test for PLR and TJOMT proteins in stringent media. **A)** Transformation control. **B)** HIS3 reporter gene activation test**.**

The results indicated the absence of self-activation in PLR and TJOMT, as well as absence of direct interactions between PLR-TJOMT. In addition, there was no evidence of self-dimerization for PLR or TJOMT.

Absence of self-activation enables the use of PLR and TJOMT baits in Y2H screen with cDNA libraries, otherwise the number of false positives would be high and the need of a more stringent media or truncated proteins required.

Absence of PLR/TJOMT interaction was also expected. Even though these proteins are involved in the PTOX biosynthesis pathway (Von Heimendahl et al 2005, Dinkova-Kostova et al 1996,Fujita et al 1999) data summarized in the Scheme 1, they participate in different steps in the route which are not close. Another possibility is that *Linum album* does not have a *TJOMT* protein, notwithstanding it was confirmed to take part in the PTOX pathway in *Antriscus silvestris* (Ragamustari et al 2013,Ibrahim et al.1998*;* Joshi and Chiang 1998*;* Umezawa et al. 2013*;* Schöder et al 2002;Zubieta et al 2001) or that this inteaction exists,but is not direct between both proteins.

From these data we conclude that PLR and TJOMT proteins are suitable for the yeast two hybrid system and the results are summarized in the Table 8.

Table 8.Summarized results of self-activation test of PLR and TJMOT with the correspond interactions

Bait (GAL4 dna-bd)	**Prey (GAL4 dna- ad)**	**Observations**
PGBKT7-*PLR*	PGADT7Ɵ	Non interaction
PGBKT7-*TJOMT*	PGADT7Ɵ	Non interaction
PGBKT7-*PLR*	PGADT7-*PLR*	Non dimerization
PGBKT7-*PLR*	PGADT7-*TJOMT*	Non interaction
PGBKT7-*TJOMT*	PGADT7-*PLR*	Non dimerization
PGBKT7-*TJOMT*	PGADT7-*TJOMT*	Non interaction

4.4.3.cDNA library and generation of Linum album cDNA yeast strain as a prey.

The *L. album* cDNA library was generated using RNA isolated from WT callus previously treated with 1 mM coronatine for 6 hours. Under the premise that elicitors induce the expression of genes involved in the secondary metabolism, in this case PTOX pathway previously discussed in the sections 4.3.1.3 and 4.3.2.3, this approach attempted to obtain en enrichment of cDNAs representing the PTOX pathway in the library. The purified cDNA was checked on 1 % agarose Eth/Br gel. This revealed a homogeneous distribution of cDNA sizes that ranged from 100 bp to 1.5 kb (Figure 37).

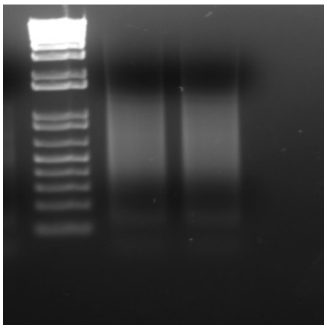


Figure 37. 2% Et/Br agarose gel of LD-PCR amplification product of *Linum album* cDNA . Two samples are represented in this gel.

Transformed yeast cells with the library (pGADT7-cDNA) were selected in SD/-L media. Serial dilutions of the library determined a total of approximately $125x10^7$ independent clones, which is an indicator of the library complexity.

4.4.4.Screen of L. album cDNA library fragments with bait proteins

Yeast mating is a convenient method of introducing two different plasmids into the same host cell. The formation of diploid cell (zygotes) enable a high-throughput protein–protein interaction screen and result in the mating of cDNA library as prey with *PLR* or *TJOMT* are represented in the Figure 39.

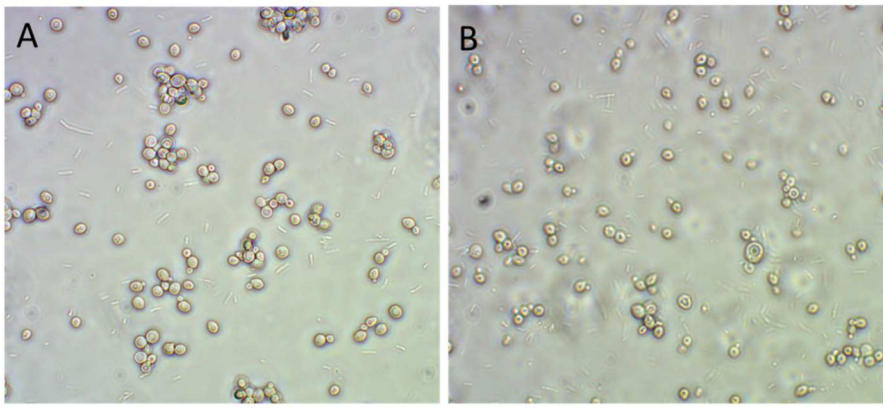


Figure 38. Zygote yeast formation in two hibrid library screening using yeast mating. **A)** screening with PLR bait protein. **B)** screening with AsTJOMT as a bait protein.

4.5.4.1 Screen with PLR bait protein

In the screen with PLR as bait (pGBKT7-PLR), 450,000 clones were tested for potential interactions. Evidence of yeast growth on highly stringent dropout medium (SD/-L/-W/-H/+A/+3AT) by the activation of reporter genes, was detected.

Four colonies resulted from the assays as possible interactors. Later the strength of interactions was confirmed by X-α-Gal assays, resulting three as genuine protein interactors with the PLR, which turned into blue colonies as consequence of GAL4 reported gene activation (Figure 40).Prey plasmid from positive clones were amplified by colony PCR, isolated and sequenced. Basic Local Alignment Search Tool (BLAST) analyses identified the three PLR-interacting proteins: i)Kunitz-type protease inhibitor B mRNA, ii)cytochrome b5 isoform E protein, iii)P-loop containing nucleoside triphosphate hydrolases superfamily protein.

Kunitz –type protease inhibitor proteins play a role in plant defense against pathogens and insects.Herbers et al (1994) reported that exogenous application of abscisic acid (ABA) induced such proteinase inhibitors similarly to the effect of mechanical wounding (Heibges et al 2003) and methyl jasmonate (MeJA), thus providing a link between JA and ABA pathways. This was also observed by Peña et al (1988) and Hildmann et al (1992) in *Solanum tuberosum*. Even though the kunitz –type protease inhibitor proteins play a role in plant defense against pathogens and insects triggered by elicitors, there is no evidence of kunitz-type protease inhibitors involved in the regulation lignan biosynthetic pathway.

The sequence analysis of the P-loop containing nucleoside triphosphate hydrolases superfamily interacting protein identified some homologs in *Arabidopsis* which are transcription factors that regulate the CBF pathway, involved in cold-stress signaling (reference). Due to the nature of the interactor and limited

connection with the lignan biosythetic pathway, we argued that this could be a self-activator prey.

Finally, the cytochrome b5 (CTB5) protein interactor was analyzed. CTB5 is a small, cylindrical acidic membrane protein with 6 helices and 5 β-strands. CTB5 is folded into two domains: i) the larger domain is the amino- terminal cytosolic heme-containing, hydrophilic region ii) the smaller domain is the C-terminus hydrophobic, membrane-binding (14–18 residues) joined by proline to a globular domain (Mathews, 1985). Due to this protein containing anionic residues in the heme edge, it exhibits charge-pairing interactions with other electron transfer proteins, as natural partners.

The cytochrome b5 is known to be an electron transfer component in a number of oxidative reactions involving steroids and fatty acids anabolism, xenobiotic and compounds of endogenous metabolism catalyzing this process, in different biological tissues. Interestingly, CTB5 also interacts with NADPH-cytochrome P450 reductase or NADH-cytochrome b5 reductase proteins, and appear to function as obligate electron donors in the reaction. Several authors indicate that a number of cytochrome P450 isoforms require cytochrome b5 for their function (Schenkman & Jansson 2003 ; Hlavica and Lewis 2001).

Based on this, some mechanisms of actions are suggested to describe cytochrome b5-imposed positive modifier action of the cytochrome P450 monooxygenase reaction: i) donor electron into

the monooxygenase cycle, ii) acting as positive modifier of the monooxygenase by decreasing the extent of uncoupling the monooxygenase reaction, iii) making a hemoprotein complex with cytochrome P450 hemoprotein complex, allows the acceptance of two electrons from NADPH-cytochrome P450 reductase iv) serving as an effector without a reduction-oxidation role in the monooxygenation reaction (Schenkman and Jackson 2003). In all the statements before, the CTB5 facilitates the flow of electrons through into a system.

Considering that the biosynthesis of lignans involves NADPH-dependent reductases such as *PLR* and *C4H* argue that *CTB5* might be involved in the regulation of the lignan pathway, perhaps forming a multiproteomic complex, which are increasingly recognized as molecular regulators of cellular fluxes of molecules, signals and energy.

To further confirm a genuine interaction between this putative CTB5 with the PLR protein, the positive yeast clone was grown in selective media (SD/+W/-L/+H/+A) and the plasmid prey rescued, and re-tested for bait dependency by carrying out pairwise bait-prey interaction with some control vectors (pVA3-1; pLAM5'-1). These were confirmed by assessing expression from the LacZ reporter gene using α-Gal assays (Figure 39).Only the interaction CT5-PLR grew on the selective dropout medium, which confirmed that this represents a true interaction in yeast.

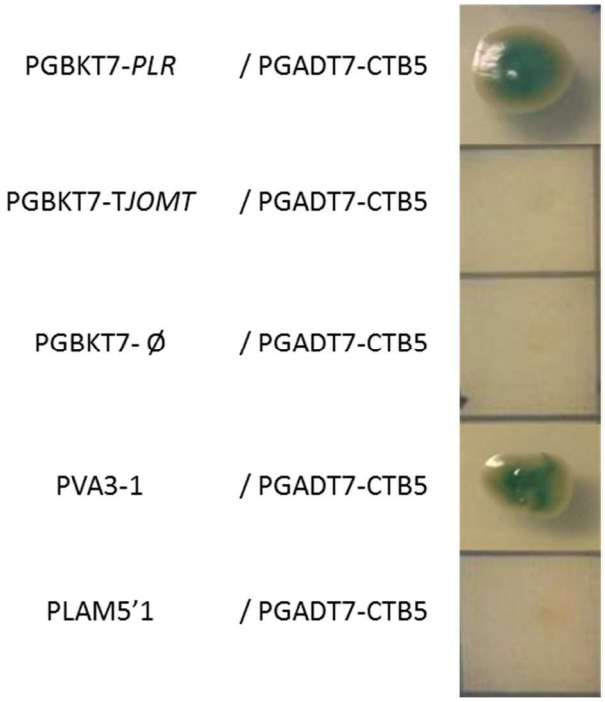


Figure 39.α Gal assay between CTB5 as prey [PGADt7/CTB5] and *PLR* as bait [PGBKT7/*PLR*];PVA3-1 (positive control), PLAM5`1 and PGBKT7Θ (negative control).

4.5.4.2 *In silico*phylogenetic analysis of interactor CTB5

The data described above provided information about a putative protein involved in the PTOX pathway. To further study the nature of the gene, an *in silico* analysis was performed. The genome of *Linum album* is not sequenced yet, but the complete genome sequence of a close relative (*Linum usitatissimum*) is available (Wang, Z. Hobson, N. Galindo, L. Zhu, S. Shi, D. Mcdill et al. 2012). A BLAST search was conducted with data from *Linum* usitatissimum to find orthologous CTB5 sequences.

Until now, we only referred to the putative interactor as CTB5 , but the data previously provided by BLAST analysis mentioned as CTB5, isoform E. Previous alignment in *Linum usitatisimun* found a high similarity with other isoform sequences mainly CTB5A (Lus10032612; Lus10008838; Lus10022357; Lus10005992; Lus10030219; Lus10012615; Lus10030219; Lus10012615; Lus10010111 and related CTB5A Lus1000651) and two CTB5B (Lus10022794; Lus10011858). Such annotations are predictions made by bioinformatic analyses. In few species the CTB5 genes have been biologically characterized, among them in *Arabidopsis*. In order to have a detailed analysis of the isoforms and asign the correct isoform to *Linum album*, the CTB5 members of Arabidopsis were included in the analysis: AtCb5-A (At1g26340); AtCb5-B (At2g32720); AtCb5-C (At2g46650); AtCb5-D (At5g48810); AtCb5-E (At5g53560). The homology between CTB5 Isofoms from the different species is shown in multiple sequence alignment (Figure 40).

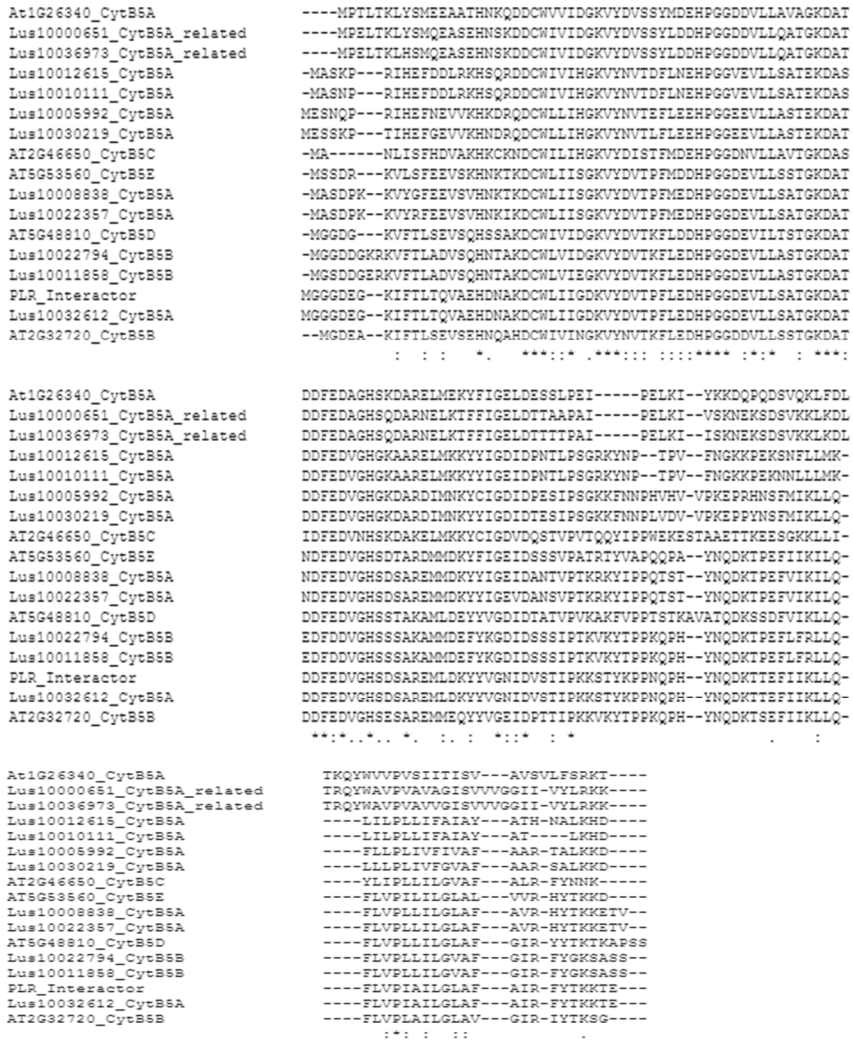


Figure 40. Aligment of the putative CTB5 interactor and related CTB5 isosform in *Linum usitassimum* and *Arabidopsis thaliana.*

There is poor information about CTB5 isoforms, but one fact is that the occurrence of multiple Cb5 isoforms is related with the cellular localization. This appears to be an evolutionary characteristic found in most eukaryotic organisms. Plants have at least two differential

localized forms of the enzyme, whereas yeast and other lower eukaryotes, only ER-localized have been reported (Schenkman and Jansson, 2003). It is suggested that mitocondrial CB5 is characteristic of higher eukaryotes from their ER- localized counterparts, and in lower eukaryotes other proteins-like are doing this function. Therefore Cyts b5 have been considered mostly as ER proteins in plants (Smith, M.A. Stobart & Shewry, P. Napier 1994). However, Zhao et al. (2003); Hwang et al. (2004) and Kumar et al (2012) indicate in their studies the presence of isoforms targeted to other compartments.They concluded that three of the putative CTB5 are located in the ER (cyt A, B, and C) and one (cyt D) in mitochondria in studies performed in *A. thaliana*. Based on the amino acid sequences of this protein family, they often have a neutral or net positive charge in the C-terminal polar domain in plants, however the charge of residues in the C-.terminal with basic amino acids relocate the protein from the ER to another membrane in plants and other organism.

With this premise, the phylogenetic neighbor joining tree is shown in the Figure 41,establishing the relationship between the different CTB5's in *Linum usitatisimun* and *A. thaliana*. Our putative CBT5 homologus PLR interactor is close to Lus10032612 CTB5A (*L. usitatissimum*) and followed by At2G32720 CTB5B (*A thaliana*), which is not distant and grouped in the same cluster. This evidences the presence of this CTB5 family among species, participating in different metabolic reactions as the modulation fatty acid desaturation or cell death suppressor in *A. thaliana.* widely

studied by Kumar et al (2012); Nagano et al. (2009) in which evaluated the participation of the CTB5 isoforms.

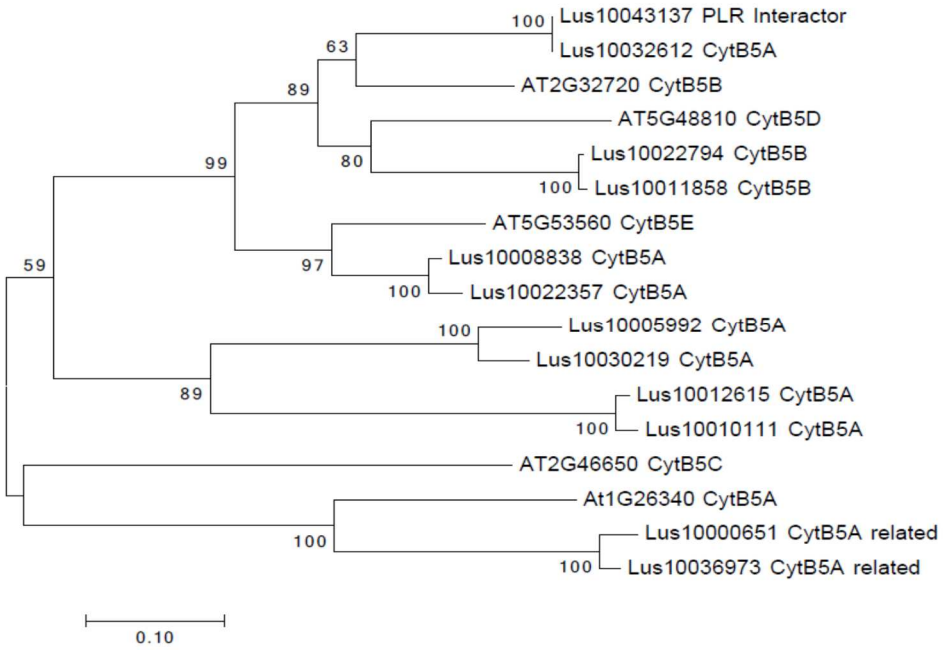


Figure 41. NJ-tree of PLR interactor with Linum usitatissimum and Arabidopsis thaliana homologs. The evolutionary history was inferred using the Neighbor-Joining method [1]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [3] and are in the units of the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 120 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

To more profoundly reveal its function in the lignan catalysis, we search for possible interactors taking the high sequences similarity found in *A. thaliana* (AT2G32720). Keeping the criteria of search

genes involved in stress/defense response in mind, we found many CPY450s proteins involved in the phenylpropanoid metabolism which have been identified, cloned and characterized. Interestingly among them, AT4836220 gene is found as interactor of CTB5. It encodes for a CYP84A1 protein, which acts as feluric acid 5 deshydrogenase in the phenylpropanoid pathway (Anderson et al 2015;Guo et al 2015; Maruta et al 2014).Thus increasing the probability to be more close to a genuine interactor in the lignan pathway.

4.5.4.3 Y2H screen with TJOMT bait protein

In order to get a molecular insight into potential complexes involving the TJOMT a Y2H screen was performed using TJOMT as bait. This protein is an OMT protein (O-Methyl transferase) involved in the PTOX pathway by methylation from yatein. The Y2H screen did not detect any positive interactor, even though the number of the screened clones resulted 2.5 $x10^6$. The lack of capacity to identify positive interactors among prey proteins produced by this *L. album* cDNA library could be attributed to some factors as: i) this route from yatein to PTOX is not present in *L.album*, ii) this protein does not need to interact with others, forming any protein complex iii) own drawbacks of yeast two hybrid system.

Several authors provide strong evidence of defects in vivo compatibility taking the point that the interaction involves others events in plants that aren't present in yeast cells, which can derived

in the localization of the prey and the bait protein. Some modification in the receptor binding site when is expressing in yeast cell is other factor to considerate. Also, the HIS3 reported activation assays based in increased the stringency to identify the real interaction, could to reduce the number of interactions and the addition of 3AT can affect in high concentration as well.Taking into account all factors above mentioned and the fact that elucidation of PTOX biosynthetic pathway has been poorly explored in plants as the precise localization and timing of the synthesis, all the statement derived from this assays are still hypothetical.

4.5.4.4 *In silico* phylogenetic analysis of TJOMT in *Linum ustatisumun*

We attempted to expand the understanding of the lignan pathway in *Linum* species by a phylogenic analysis using available data from *Linum ussitatisimun*, which could result in highlights to our study in *Linum album*. AsTJOMT which derived from *Antriscus silvestris* was found to be grouped in the same cluster with two OMT in *L.* usistatisum (Lus100115576, Lus 10032929) (Figure 42).

The results suggest a possible presence of this enzyme and consequently an alternative route to produce PTOX until now described in *Linum* species. Conversely, we need to accept the fact, no all information obtained from one plant species can be applied to podophyllotoxin producing- plants by the genetical and production variability. So the possibility of OMT which performs

some step in the route from the mataresinol to yatein in *L. album* exits and its interesting subject to study.

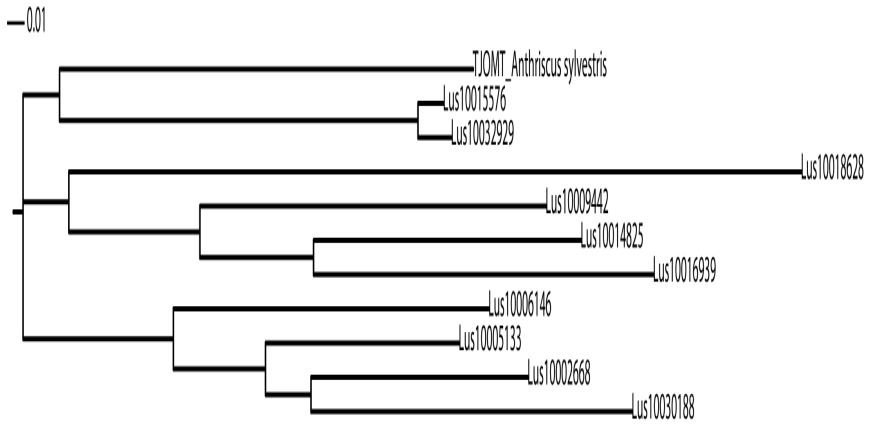


***Figure 42*.** NJ-tree of AsTJOMT with *Linum usitatissimum* homologs. The evolutionary history was inferred using the Neighbor-Joining method [1]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [3] and are in the units of the number of amino acid substitutions per site. The analysis involved 11 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 120 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

Summarizing the overall result, the screening between cDNA library with the PLR as bait allow to find one interactor, which has present strong homology with a CTb5 b. Literature reported that homologous gene in A thaliana interacts with a CPY450. It participates in the phenylpropanoid pathway. Taken into count that extensive studies support the interaction between CTB5 and CTP450 family proteins in oxide reduction reactions, some enzymes in the lignan pathway belong to this family. Interesting the

deoxypodophyllotoxin 6-hydroxylase which catalyze the deoxypodophyllotoxin (DOP) to PTOX in enzyme preparations from *L. album* cells (downstream steps) is presumably carried out by a cytochrome P450-dependent monooxygenase. So there is a highlight that CTB5 could interact with the PLR and with some enzyme in upstream steps or downstream in the route. Figure 43 show a hypothetical pathway where the CTB5 participated.

A computational alignment suggests that even if we could not be able to identify any interaction with TJOMT in *Linum album*, it might be present in *Linum* species (*Linum ussitatisumun*). A hypothetical pathway in *Linum album* could be proposed based on our previous results and the literature. However, further assays are needed to confirm the participation of a putative CTB5 in the lignan pathway and the possible interactions.

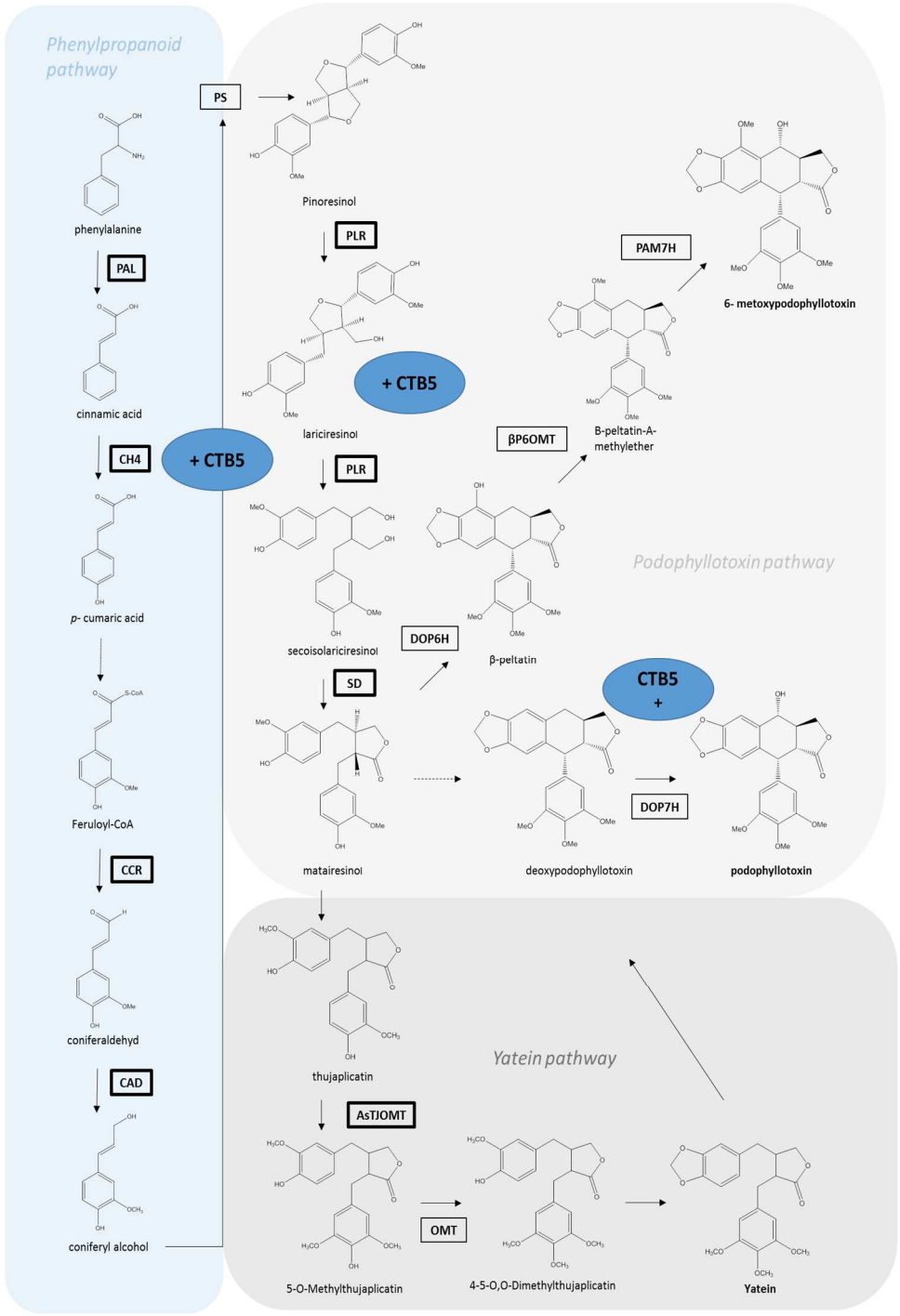


Figure 43. Hypotetical pathway where the CTB5 participated

5. CONCLUSIONS

5.1. The four biotechnological platforms studied (wild type cells, transformed cells, adventitious roots and hairy roots) showed different patterns of growth and aryltetralin lignan bioproduction.

5.2. According to our results, the growth and aryltetralin lignan bioproduction of the wild type systems (cell suspensions and adventitious roots) were greater than that of the transformed systems (transformed cells and hairy roots).

5.3. The cell systems (wild type and transformed cell suspensions) were more PTOX producers than MPTOX and both root systems (adventitious and hairy roots) were more MPTOX producers than MPTOX.

5.4. Comparing both cell systems, PTOX bioproduction was slightly higher in the wild type cells, which also grew more. Wild type cells produced more MPTOX and similar levels of β-peltatin than the transformed cells. To our knowledge, this is the first time that lignan content has been studied in transformed cells.

5.5. Comparing both roots systems, MPTOX bioproduction was higher in the adventitious roots, which also grew more. Adventitious roots also produced more PTOX and β-peltatin than the hairy roots.

5.6. Also we can infer that the production of these secondary metabolites are related with the cell differentiation and not with the transformation, since the transformed cells (PTOX producers) came from the dedifferentiation of the hairy roots (MPTOX producers) and the transformed cells show a different lignan pattern than the hairy roots.

5.7. This is the first report in which the lignan profile is studied in *L.album* systems treated with the elicitor coronatine. The growth decreased after elicitation in all four biotechnological systems. However it did not induce a significant depletion in both root systems.

5.8. Coronatine did not changed the aryltetralin lignan profile in any of the systems and increased the lignan production mainly in the roots, specially the adventitious ones.

5.9. It is interesting to point out that after the treatement with coronatine, in each system the minority lignan was the most elicited, that is, PTOX in the roots, and 6-MPTOX in the cells, although in the latter the production levels remained negligible.

5.10. The transcriptomic profiling analysis of CCR, CAD and PLR genes, showed that CCR and PLR mRNA levels were higher in cells than in roots systems, with the exception of CAD, which levels were higher in hairy roots.

5.11. In all systems, coronatine changed the transcriptome with no changes on lignan profile. Notably, elicitation enhanced the expression level of the PLR gene in all four systems, above all in transformed cells, where the expression values were very high.

5.12. In all systems analyzed, the transformed material was more susceptible to the coronatine treatment, thus being reflected in overall higher increases of gene expression levels.

5.13. The results obtained in the morphogenesis development in the production of podophyllotoxin derivatives in callus cultures of Linum album show that, among the experimental conditions assayed, the best for growth and organogenesis of L. album calli were their culture in the condition 3, without plant growth regulators, both in the light (condition 3.1) and in the darkness (condition 3.2).

5.14. The conditions 3.1 and 3.2 also were the main lignan MPTOX producers, which was found in quantity approximately 4 times higher than the levels of PTOX. Unlike in L. album cell cultures where the main lignan was PTOX (Yousefzadi et al 2010) in transformed roots of the same species it was MPTOX (Chasmi et al. 2011).

5.15. In the present study, although the calli did not form any root structure, probably the biochemical differentiation needed to induce morphogenesis which led to the root formation, also

led to the MPTOX production. However, further enzymatic and genetic studies would be necessary to clarify this fact.

5.16. The use of Y2H system led us to find three potential interactors by means of the X-α-Gal assay, using PLR as bait protein. However, only one interactor, the CTB5 isoform E constitutes a strong interactor related to the lignan pathway. Considering that the biosynthesis of lignans involves NADPH-dependent reductases such as PLR and C4H,we reason that CTB5 might be involved in the regulation of the lignan pathway, perhaps forming a multiproteomic complex, which are increasingly recognized as molecular regulators of cellular fluxes of molecules, signals and energy.

5.17. The *in silico* analysis conducted tofind orthologous CTB5 sequences with genomic data from *Linum usitatissimum*, predominantly found isoforms annotated as A and B. In order to have a detailed analysis of the isoforms and to assign the correct isoform to *Linum album*, the CTB5 members of *Arabidopsis* were included in the analysis. Our putative CBT5 homologus PLR interactor is close to Lus10032612 CTB5A (*L. usitatissimum*) and the *Arabidopsis* At2G32720 CTB5B. This evidences the conservation of this CTB5 family in different species, including *Linum album*.

5.18. More deep knowledge concerning the participation of CTB5 in the lignan catalysis was studied, we searched for

potentialinteractors taking the high similar sequence found in A. thaliana (AT2G32720). Interestingly AT4G36220 protein is found as interactor of CTB5. It codes for a CYP84A1 protein, which acts as feluric acid 5 dehydrogenase in the phenylpropanoid pathway. Thus, increasing the probability to be more close to a genuine interactor in the lignan pathway.

5.19. The Y2H screen did not detect any positive interactor with the TJOMT as bait protein maybe because this route from yatein to PTOX is not present in *L.album*, or this protein does not need to interact with others for its function.

5.20. Resultsfrom the *in silico* analysissuggest the presence of TJOMT enzyme in *Linum ussitatissimum*and consequently,it cannot be excluded theoccurrence of an alternative route to produce PTOX in *Linum album*.

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7. ANNEX

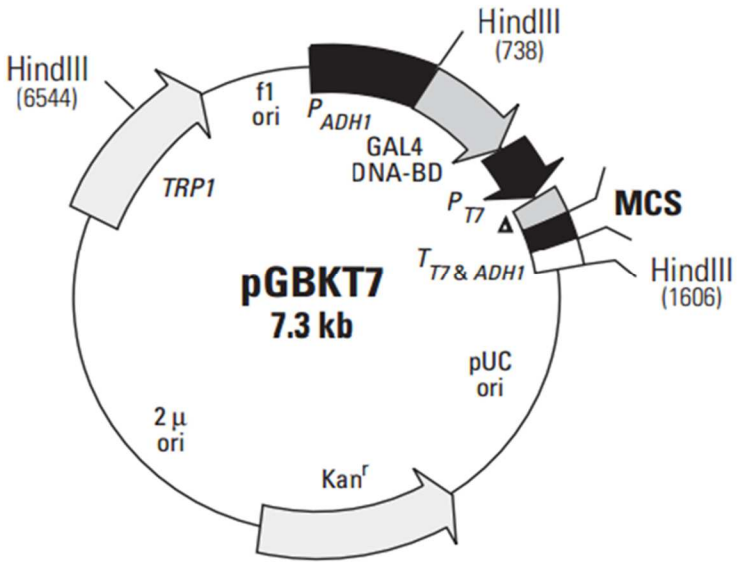

***Figure 44*.. Information bait vector (pGBKT7)**

The pGBKT7 vector expresses proteins fused to amino acids 1–147 of the GAL4 DNA binding domain (DNA-BD). In yeast, fusion proteins are expressed at high levels from the constitutive ADH1 promoter (PADH1); transcription is terminated by the T7 and ADH1 transcription termination signals (TT7 & ADH1). pGBKT7 also contains the T7 promoter, a c-Myc epitope tag, and a MCS. pGBKT7 replicates autonomously in both E. coli and S. cerevisiae from the pUC and 2 µ ori, respectively. The vector carries the Kanr for selection in E. coli and the TRP1 nutritional marker for selection in yeast. Yeast strains containing pGBKT7 exhibit a higher transformation efficiency than strains carrying other DNA-BD domain vectors. pGBKT7 is the DNA-BD Vector included with Clontech's Matchmaker™ Systems. The MCS of pGBKT7 contains unique restriction sites in frame with the 3' end of the GAL4 DNA-BD for constructing fusion proteins with a bait protein. The bait protein is also expressed as a fusion to a c-Myc epitope tag. c-Myc tagged proteins can be identified with antibodies raised to this common epitope, eliminating the need to generate specific antibodies to new proteins. The T7 promoter is used for in vitro transcription and translation of the epitope tagged fusion protein..

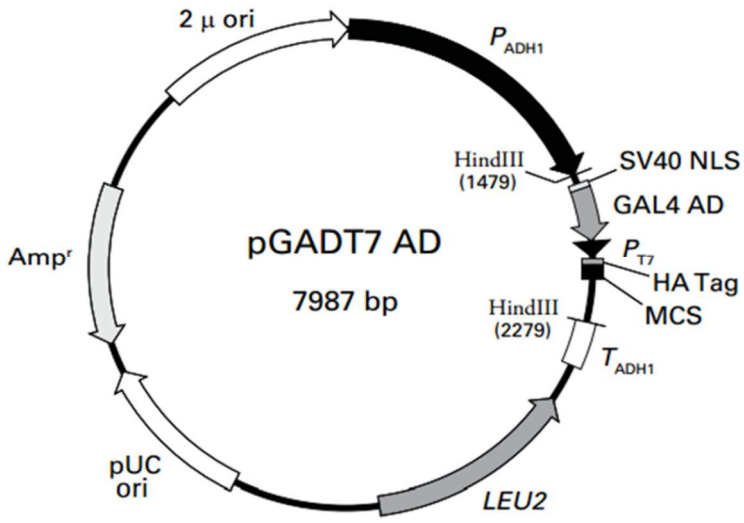

Figure 45..Information prey vector (pGADT7)

pGADT7 AD Vector Information PT3249-5 Cat. Nos. 630442 630489 630491 (010312) pGADT7 AD Vector Map and Multiple Cloning Site (MCS). Description pGADT7 AD is a yeast expression vector that is designed to express a protein of interest fused to a GAL4 activation domain (AD; amino acids 768–881). Transcription of the GAL4 AD fusion is driven by the constitutively active ADH1 promoter (PADH1), and is terminated at the ADH1 transcription termination signal (TADH1). The GAL4 AD fusion contains an N-terminal SV40 nuclear localization signal (SV40 NLS; 1) that targets the protein to the yeast nucleus, and a hemagglutinin epitope tag (HA Tag), located between the GAL4 AD and the protein of interest, that allows the protein to be easily detected with HA-tag antibodies. The T7 promoter (PT7), located just upstream of the HA tag sequence, allows in vitro transcription and translation of the HA-tagged protein of interest (without the GAL4 AD and the SV40 NLS). pGADT7 AD replicates autonomously in both E. coli and S. cerevisiae from the pUC and 2 μ ori, respectively. The vector also contains an ampicillin resistance gene (Ampr) for selection in E. coli and a LEU2 nutritional marker for selection in yeast