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Effect of in vitro morphogenesis on the production of podophyllotoxin derivatives in callus cultures of *Linum album*

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Abstract:

The anticancer compound podophyllotoxin and other related lignans can be produced in *Linum album* *in vitro* cultures, although their biosynthesis varies according to the degree of differentiation of the plant material. In general, *L.album* cell cultures do not form the same lignans as roots or other culture systems. Our aim was to explore how the lignan-producing capacity of organogenic cell masses is affected by the conditions that promote their formation and growth. Thus, *L.album* biomass obtained from plantlets was cultured in darkness or light, with or without the addition of plant growth regulators, and the levels of podophyllotoxin, methoxypodophyllotoxin and other

related lignans were determined in each of these conditions. The organogenic capacity of the cell biomass grown in the different conditions was studied directly and also with light and scanning electronic microscopy, leading to the observation of several somatic embryos and well-formed shoots. The main lignan produced was methoxypodophyllotoxin, whose production was clearly linked to the organogenic capacity of the cell biomass, which to a lesser extent was also the case for podophyllotoxin.

Keywords: Organogenic callus cultures; deoxypodophyllotoxin; podophyllotoxin; 6-methoxy-podophyllotoxin

Introduction

Plant secondary metabolism constitutes a vast source of diverse compounds whose main function is to protect plants and facilitate their adaptation to the ecosystem where they grow. However, most of these secondary compounds also have biological activities, which have been used since ancient times to improve human health and welfare. Specific secondary metabolites, unlike primary compounds, are generally restricted to certain plant families, genera or even species, and may only be active in particular organs or tissues at a certain time or development stage of the plant (Verpoorte, 2000, Cusido et al., 2014).

Secondary metabolism has its own genes and consequently is controlled by specialized proteins that respond to diverse development and differentiation programs. For this reason, the biotechnological production of therapeutic secondary compounds generally

requires cell differentiation, which does not usually occur in cell cultures. Root, shoot, and embryo cultures have therefore been used to obtain secondary products, since such organ cultures produce similar patterns of secondary metabolites to the plant. For example, the tropane alkaloids hyoscyamine and scopolamine are produced in hairy roots of *Hyoscyamus* and *Datura* (Oksman-Caldentey et al., 2000), anthraquinone in *Morinda citrifolia* adventitious root cultures (Baque et al., 2012) and camptothecin in shoot cultures of *Nathapodytes nimmoniana* (Dandin and Murthy, 2012). Also, higher ginsenoside levels have been achieved in *Panax ginseng* hairy roots than in cell cultures (Palazon et al., 2003; Mallol et al. 2001). However, complete organogenesis is not always required and cell differentiation in an optimized two-stage culture can be enough to activate secondary compound production (Hosakatte et al., 2014). For example, *Taxus* sp. cell cultures require a metabolic induction but not a visible differentiation. The highest taxol production is obtained after cells finish growing and enter the stationary phase, when the genetic expression pattern changes (Cusido et al., 2014).

The aryltetralin lactone podophyllotoxin (PTOX) is one of the main precursors of the effective anticancer drugs etoposide, etopophos or teniposide, which present inhibitory action against the topoisomerase II enzyme. These semi-synthetic derivatives are being used to treat several cancer processes, including lung, testicular and stomach and also non-Hodgkin lymphoma (Lalaleo et al., 2016). PTOX has been traditionally obtained from rhizomes of *Podophyllum*, and the biotechnological production of PTOX and related products will provide a viable alternative source (Giri and Narasu, 2000; Bahabadi et al., 2014). According to a review by Yousefzadi et al. (2010a), more than 40 plant and fungus species have been tested for *in vitro* PTOX production, but only *in vitro* cultures derived from *Podophyllum* sp., *Callistris drummondii* and the Iranian

Linum species, *L.album* and *L.persicum*, yielded significant amounts. The remaining species mainly produced other lignans such as 6-methoxy-podophyllotoxin (MPTOX), justicidin B, matairesinol, and β -peltatin (Fuss 2003; Ionkova 2007; Ionkova 2008). More recently, Julian-Pawlicki et al. (2014) reported the production of aryl-naphthalene lignans as well as aryltetralin-type lignans such as 6-MPTOX and one of its diastereoisomers in *L. perenne* hairy root cultures.

The biosynthetic pathway leading to PTOX has not been fully elucidated and may vary among the producing plants. PTOX and related compounds are derived from the shikimic acid/phenylpropanoid pathway (Jackson and Dewick 1984; Xia et al. 2000; Julian-Pawlicki et al (2014), and matairesinol is known to be the common intermediate. In *L.album* the steps between matairesinol and dPTOX have not been elucidated. The latter is the precursor of PTOX and MPTOX, following two divergent biosynthetic pathways (Federoff et al., 2007; Fuss, 2003; Yousefzadi et al., 2010a) (Fig. 1).

The development of embryogenic cultures as a micropropagation system constitutes a milestone achievement in plant biotechnology, permitting large-scale culture, and this may be a suitable approach for the production of the bioactive compound PTOX. Arumugam & Bhojwani (1990) found that in *P.hexamdrum* somatic embryo cultures, the interaction with auxin favored embryogenic development, contrary to the results obtained by Nadeem et al. (2000) in similar cultures, although both groups reported that establishing and maintaining these kind of cultures was difficult and time-consuming. A recent article to promote leaf somatic embryogenesis in *Q. alba*, has revealed that auxin accumulation is induced during embryogenesis initiation and differentially found in embryogenic cells (Corredoira et al. 2017).

The aim of this work was 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 with light and scanning electron microscopes, and the production level of the target compounds was determined. The results emphasize the importance of cell differentiation and organogenesis for obtaining a high yield of the lignans in a biotechnological production platform.

Materials and Methods

Plant material and experimental design

This study was carried out with cell masses (CM) obtained from *Linum album* plantlets after the following steps:

a) *Dedifferentiation and initial cell mass formation*: Sterilized *L.album* plantlets obtained from seeds were used as explants to establish CM. Pieces of these plantlets were inoculated in Murashige and Skoog solid medium (MS medium; Murashige and Skoog, 1962) supplemented with 2 mg/L 1-naphthaleneacetic acid (NAA) and 0.4 mg/L kinetin (KIN) as growth regulators (Yousefzadi et al., 2010b), and maintained in darkness for a period of 30 days at 25°C. After this time the explants had developed CM (Fig. 2), which were separated from the explants and subcultured every 20-30 days in the same medium and conditions until enough biomass was obtained. .

b) *Increase of cell biomass in the four initial experimental conditions*. Part of the CM (obtained in step a) presenting organogenesis were transferred to the same culture medium (MS with the aforementioned phytohormonal supplement) and divided: half were maintained in darkness (Conditions 1) and the other half in light (photoperiod of 16h light / 8h darkness) (Conditions 2). The remaining CM were transferred to a plant

growth regulator (PGR)-free MS medium and also cultured either in darkness (Conditions 3) or light (Conditions 4) (Fig. 3). CM grown in these four conditions constituted the starting material for the study: 24 callus pieces (2 ± 0.2 gFW) were cultured in each of the four conditions. CM grown in MS supplemented with PGRs in light (Conditions 2) did not show any growth and were finally discarded.

c) Establishment of CM cultures for studying growth, organogenesis and lignan production under different experimental conditions. In order to know if the growth, organogenic capacity and lignan production depended on the amount of light and/or PGR addition, the three aforementioned types of starting material were exposed to different treatments and conditions, as indicated in Fig. 3. Two pieces of 2 ± 0.2 gFW biomass (time 0) were used as the inoculum and placed in the different culture media for a culture period of 6 weeks. Samples were taken at weeks 1, 2, 4 and 6 for each treatment with 4 biological replicates. The entire experiment was performed with three replicates.

Fresh and dry weight determination

The growth of the organogenic CM was measured as fresh weight (FW) and dry weight (DW). FW was obtained as indicated in Yousefzadi et al., (2010b) and the cells were then lyophilized to obtain the DW.

Lignan extraction and HPLC MS-ESI analysis

Extraction was performed as described by Yousefzadi et al. (2010b) with some modifications. A 0.2 M solution of chlorohydric acid was added after the methanol extraction to hydrolyse the glucoside group of the lignans and obtain the aglycon. Quantification and separation of PTOX and its derivatives (MPOX, β -peltatin,

deoxypodophyllotoxin and yatein) was performed in a Brisa-LC2-C18 column (3 μ m 15 x 0.46 mm, Teknokroma) using an HPLC-ESI-MS platform (Varian IT 500MS).

Twenty microliters of sample were injected into the column and eluted with acetonitrile (A) and water with formic acid at 0.1% (B) as the 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. Lignan data were expressed as micrograms per gram DW.

Sample preparation for microscopic analysis

Fixation and Dehydration

The sample preparation started with the fixation 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 10°C. After fixing, the samples were washed at 10 min intervals 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 2h at room temperature and then washed with water as in the previous step. The samples were divided to be analyzed by either light microscopy (LM) or scanning electron microscopy (SEM). The dehydration phase was performed with an ascending series of ethanol for LM or acetone for SEM at different concentrations (30-100%; v/v). Each dehydration phase lasted 10 minutes and was done in triplicate.

Light Microscopy (LM)

Samples were finally embedded in Epon resin, which was polymerized 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 placed on glass slides, stained with 1% methylene blue dye dissolved in 0.5% borax water (4 min), and mounted with coverslips. Observations were made in a bright field light microscope (LEICA DM 2000 LD)

Scanning Electron Microscopy (SEM)

The samples, fixed and dehydrated as indicated previously, were dried in a critical point dryer CPD Emitech k850 using liquid CO₂ and then sputtered with 99.9% gold with a Fine Coat Ion Sputter JFC 1100, prior to SEM analysis. Observations were made using a scanning electron microscope (LEO Evo 040) operating between 10 and 20 kV.

Statistics

The multi-factorial ANOVA analysis followed by the Tukey multiple comparison tests were used for statistical comparisons. A $P \leq 0.05$ was considered for significant differences.

Results and Discussion

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 (Fig. 2). The obtained cell masses (CM), growing in light or darkness, and with or without the addition of

PGRs, were studied to determine their lignan production and the relationship between the lignin profile and organogenic capacity in the assayed conditions.

CM formed from the explants were excised and grown in darkness, supplemented with the aforementioned PGRs, for a period of 7-8 months, with routine subcultures every 2-3 weeks in the same conditions (Fig. 2).

After several subcultures, the biomass obtained was divided among the four different culture conditions described in Material and Methods (conditions 1: with PGRs in darkness (C1); conditions 2: with PGRs in light (C2); conditions 3: without PGRs in darkness (C3), and conditions 4: without PGRs in light: (C4)). In this way, the effect of PGRs and light on the growth and organogenic capacity of *L.album* cell cultures was elucidated. The CM maintained in these four conditions grew well for 3 more months, except for those transferred to light with the addition of PGRs (C2). The latter showed a decreased growth rate, turned yellow and then brownish, and were finally discarded. All the CM were routinely subcultured every 2 weeks in the same conditions until enough biomass was obtained to start the planned experiment. The cultures, except those corresponding to C2, are depicted in Figure 3.

Once enough biomass was obtained in the aforementioned starting conditions it was inoculated in fresh medium. The aim was to determine how the growth, organogenic capacity and lignan production in these three culture conditions would be affected by substituting the presence of PGRs and light by PGR-free medium and darkness, or vice versa. The biomass distribution was as depicted in Figure 4.

CM were harvested at 1, 2, 4 and 6 weeks after the new conditions were established, in order to study the growth, organogenenic/morphogenenic capacity, lignan production and the relationship between these parameters.

Growth

The growth course of the CM expressed as DW (g) presented a lag phase until the second week, except in those grown in the light without PGRs (conditions C4.1), which showed the highest capacity for biomass formation. The FW (g) of the latter increased constantly until the end of the experiment and at six weeks was almost 9 times higher than that of the inoculum (Fig. 4).

CM growing in darkness without the addition of PGRs (C3.1 and C4.2), derived from cultures also maintained in PGR-free conditions, either in darkness (C3) or light (C4), also showed good growth after 6 weeks, both achieving approximately 7 times the weight of the inoculum. An intermediate growth capacity was observed for CM kept in light without PGRs (C1.2). In this case, the slightly lower biomass formation (the final biomass was 6 times the inoculum) compared to the other CM cultured without PGRs, either in light or darkness, is probably due to their origin, since they were grown with PGRs before starting the experiment.

A lower growth capacity was observed in CM growing in darkness with PGRs (C1.1 and C4.3), regardless of their origin. In both cases the final biomass was only twice the inoculum (Fig. 4).

According to these results, an absence of PGRs in the medium conditioned the best biomass growth, independently of the available light. Under these conditions (C3.1 and especially C4.1 and C4.2), the CM also showed a high organogenic capacity. However, the lowest growth capacity was observed in CM grown for 6 weeks in light without PGRs (C3.2), the biomass scarcely increasing during the experiment (Fig. 4). Although the original conditions of this biomass were possibly the best for growth,

i.e., darkness without PGRs, when they were abruptly exposed to light, growth was dramatically reduced.

From these results, we can infer that although the dedifferentiation of *L.album* plants and the initial CM formation was completely dependent on the addition of NAA and KIN, the best conditions to increase the *L.album* biomass were a PGR-free medium in darkness. When CM were cultured without PGRs in light (conditions C3), their growth capacity and chlorophyll production were also high, as was their organogenic capacity, which was not lost after transfer to darkness. In *Podophyllum hexandrum* Royle the presence of auxins is described as essential for embryogenic callus proliferation, but it is usually inhibitory for further development and growth of somatic embryos (Rajesh et al., 2014).

CM grown under conditions 1 (darkness with PGRs), when maintained for 6 weeks in the same medium (C1.1), were friable and white, without any visible organogenesis. In contrast, when CM were subcultured and maintained for 6 weeks in light without PGRs (C1.2), they appeared greenish after the second week, and had turned green by week 6. Thus, the light promoted the formation of chlorophyll, and increased organogenesis after 4-6 weeks of culture.

In CM originating from conditions 3 (PGR-free in darkness), when grown for 6 weeks in light also without PGRs (C3.2), no chlorophyll or organogenesis was visible. These extremely slow-growing CM were less compact and healthy, and their color changed from white to yellow as growth decreased (Fig. 4). In contrast, the CM maintained in the original conditions (C3 to C3.1) presented a good growth capacity, with a white and friable appearance.

CM maintained in conditions 4 (PGR-free in light) were green and showed a very good constant growth (Fig. 4). When subcultured and maintained for 6 weeks in the same conditions (C4.1), their appearance remained the same: friable green biomass with high growth and initially without any visible organogenic capacity. After 4 weeks of culture, granular organogenic-like zones were detected and at the end of the experiment organogenesis features were observed with the naked eye.

The CM derived from conditions 4, when subcultured and maintained in darkness without or with PGRs (C4.2 and C4.3, respectively), turned brownish-white. After 4 weeks of growth in darkness, clear differences were observed: those without PGRs (C4.2) became organogenic, developing somatic embryos, whereas those grown with PGRs (C4.3) had turned dark yellow by the end of the experiment, their growth stopping almost completely.

To gain more insight into the organogenic capacity of the CM grown in the experimental conditions for 6 weeks, they were studied with light and scanning microscopes.

Light microscopy study

CM grown in conditions 1 and 3 (darkness with and without PGRs, respectively) presented small cells with thin cell walls, together with large cells with thick walls, and prominent intercellular spaces. No organization of CM was visible.

During the first 4 weeks of culture, few differences were observed between CM derived from conditions 1 maintained in the same conditions after subculture, i.e., with PGRs and in darkness (Fig.5a,c,d), and those subcultured without PGRs in light (Fig.

5g,h). As shown in the figures, clusters of small cells were surrounded by larger cells with intercellular spaces. However, mainly after 6 weeks of culture, CM subcultured in light without PGRs showed small organized areas that were probably meristematic and linked with their greenish aspect and larger size (C1.2, Fig. 5i,j). In contrast, those grown without light (C1.1, Fig. 5e,f) did not show any cell organization.

The same lack of organogenesis was observed in C3-derived CM when subcultured without PGRs in light (C3.2) (Fig.5b,o,p). At the beginning of the experiment (weeks 1 and 2), the cells exposed to light were smaller than those in darkness (C3.1) (Fig. 5 k,l), although no organization was observed and there were prominent intercellular spaces. After 4 and 6 weeks of culture, the CM were mainly composed of large cells. At the end of the experiment, small, probably meristematic cell areas were observed in CM maintained in light (Fig 5r), which in this case did not have a green appearance.

Most of the cells observed in the CM grown under conditions 4 (light without PGRs) were small and isodiametric, leaving large intercellular areas (Fig. 6a). After one week of culture, the CM subcultured under conditions C4.1, C4.2 and C4.3 showed few differences, displaying large cells with some clusters of small cells and without any clearly visible organization. After two weeks of growth, the CM grown in the light (C4.1) presented highly organized meristematic cell areas (Fig. 6c), and those grown in darkness (C4.2) showed embryogenesis (Fig. 6g), with very well organized somatic embryos at different developmental stages. Under darkness with PGRs (C4.3), there was less organization, although some meristematic areas were visible. After 4-6 weeks of culture, the meristematic areas observed at the beginning of the experiment persisted, and cell organization was evident, mainly in C4.1 and C4.2. In these conditions, after 4 weeks of culture several vascular bundles and somatic embryos were observed (Figs. 6d and h, respectively). After 6 weeks in conditions C4.1, several

somatic embryos at different stages and leaf primordia were observed (Fig. 6e), and in C4.2, very well developed meristems and shoots were visible (Fig. 6i). In C4.3, the morphogenesis was more limited, although several meristemoids and organized zones were observed (Fig. 6m).

All these results, obtained after observation of the CM grown in the assayed conditions, confirmed the aforementioned morphological aspects. The experimental conditions that most inductive of somatic embryo formation, structured meristematic cell areas and shoots were those derived from conditions 4, especially C4.1 and C4.2.

At different stages the CM organogenic development usually presented grouped cells with few intercellular spaces (Fig. 7). It was only after two weeks of culture that most of the organogenic CM developed tracheids near the meristemoid zones. Early somatic embryos and meristems with leaf primordia are visible in Figs. 7b and 7c, which depict CM grown in the aforementioned conditions, especially those that most clearly induced organogenesis. A detailed shoot meristem with leaf primordia is shown in Fig. 7d.

Scanning electronic microscope study

Scanning electronic microscopy of the CM grown for 6 weeks in the experimental conditions confirmed the organogenic capacity observed with light microscopy. In general, their external surface presented large, oblique and elongated cells and clusters of small isodiametric cells with several intercellular spaces (Fig. 8a).

In C4-derived CM, especially those maintained in conditions C4.1 and C4.2, organogenesis was clearly visualized. Fig.8b shows an early stage of organogenic

biomass, and leaf primordia and shoots can be observed in Figs. 8c and 8d, respectively. The developmental stage of these organogenic CM was advanced, since several stomata were visible in the leaf primordia (Fig. 8e) and they had also formed several trichomes with a stalk and 6-8 branches, like the one shown in Fig. 8f. Shoots were mainly found in CM maintained in conditions C4.1 and C4.2, indicating that the shoots had begun to differentiate when the original CM were maintained in light, even though morphogenesis does not need light. Additionally, the organogenic CM showed several vascular bundles, which are essential for transport between tissues and distant organs, ensuring biomass growth and maintenance. Thus, the differentiation of xylem and phloem is critical for the formation of organogenic structures.

Lignan production

The lignans studied were deoxypodophyllotoxin (dPTOX), podophyllotoxin (PTOX), β -peltatin (PTT), yatein (YT) and 6-methoxy-podophyllotoxin (MPTOX). PTT was found only in traces, and YT was not detected. PTT is a compound formed from dPTOX by the action of a CYTP450 enzyme, whereas YT seems to be involved in an alternative pathway leading to PTOX via matairesinol (Fig. 1).

The production and/or accumulation of dPTOX in the CM remained in the range of 40-43 $\mu\text{g/g}$ DW under all conditions studied. The production was similar in the initial calli that formed the basis of the experiment, although no dPTOX was found in the *in vitro* plants. Also, no relationship between CM organogenesis and dPTOX levels was observed (Fig. 9A). This indicates that the route of this common precursor toward the formation of PTOX or MPTOX depends on the activity of the enzymes that control both biosynthetic pathways.

In contrast, the production of PTOX and particularly MPTOX was clearly affected by the CM growth conditions. In a study with *L. album* cell suspensions the precursor of both PTOX and MPTOX was reported to be dPTOX, but the variable accumulation of these lignans depended mainly on the original plant and the established cell line (Federolf et al. 2007). Recently, Javadian et al., (2017) showed that polyploidy affects PTOX production in *L. album* plants, since the expression of several genes and the activity of some enzymes involved in PTOX biosynthesis were clearly higher in tetraploid plants.

The PTOX levels (Fig. 9B) in C1-derived CM that remained in darkness with PGRs increased slightly (1.2-fold) from the beginning of the culture period to week 4. A similar increase (1.3-fold) was observed in the C1-derived CM when transferred to the light and cultured without PGRs. In both cases the PTOX levels had decreased at 6 weeks. When CM grown in darkness without PGRs (C3) were subcultured in the same medium, either in light or darkness, PTOX levels clearly peaked after one week of culture (being 1.4 and 1.8-fold higher than at time zero) with significant differences ($P \leq 0.05$), decreasing thereafter (Fig. 9B). It is worth mentioning that the PTOX content in the initial calli was 48 $\mu\text{g/g}$ and in the *in vitro* plants 509 $\mu\text{g/g}$.

From the results obtained with CM derived from C1 and C3 (darkness with and without PGRs, respectively), we can infer that PTOX accumulation is more positively affected by the absence of PGRs than the presence or absence of light. This was corroborated by the fact that the highest PTOX levels in the C3-derived cultures (without PGRs) were achieved both in darkness and light. In contrast, in the CM grown in conditions C1.1 and C1.2 PTOX levels were lowest at the beginning of the experiment (Fig. 9B).

In CM grown in light without PGRs (C4), once again the absence of PGRs resulted in the highest PTOX accumulation (90 $\mu\text{g/g}$ at week 6 with significant differences ($P \leq 0.05$)). When switched to darkness, PTOX levels peaked at 2 (55 $\mu\text{g/g}$) and 6 weeks of culture without PGRs, and at 1 (60 $\mu\text{g/g}$) and 4 weeks with PGRs (Fig. 9B). Overall, while PGR-free medium was most conducive to PTOX production, light also had a positive effect.

The highest PTOX levels were also associated with the organogenic capacity of the CM, albeit with some exceptions (e.g. the CM cultured in conditions C4.2). Conditions C4.1 (light without PGRs), which induced the highest PTOX production, also had a high organogenic capacity, as described below.

MPTOX is a lignan formed from dPTOX after a hydroxylation at C6 leading to peltatin, and a methylation followed by another hydroxylation at C7 (Fig. 1). As mentioned, the dPTOX levels in the CM were approximately the same under all the conditions studied. Strikingly, the MPTOX levels in CM derived from those grown in the light without PGRs (C4) were very high (approx. 300 $\mu\text{g/g DW}$), regardless of whether they continued to grow in the light or were transferred to darkness, in both cases remaining in a PGR-free medium. The highest production levels were obtained after 4-6 weeks of culture (Fig. 9C) and differed significantly ($P \leq 0.05$) from the highest values in CM grown with PGR (200 $\mu\text{g/g DW}$).

Thus, the MPTOX production in CM grown for 6 weeks in darkness or light, with or without PGRs, clearly depended on the origin of the CM. When they were derived from biomass cultured in darkness (C1 and C3), the highest MPTOX yield was approximately 25-30 $\mu\text{g/g DW}$. When the original CM were cultured in light (C4), MPTOX levels increased up to 280 and 300 $\mu\text{g/g DW}$ after subculturing without PGRs

in C4.1 and C4.2, respectively. High MPTOX levels were also obtained when PGRs were added (C4.3), reaching a maximum of 200 $\mu\text{g/gDW}$. The MPTOX production in organogenic CM was higher than in the initial calli (approx. 10 $\mu\text{g/gDW}$) but lower than in the *in vitro* plant (approx. 100 $\mu\text{g/gDW}$).

These results show that MPTOX biosynthesis and accumulation was activated by light, since the highest average levels throughout the 6 weeks studied (Fig 9C) were found in CM derived from those maintained in the light, particularly those from PGR-free medium. Additionally, MPTOX production was promoted by organogenesis. There are many reports of morphogenesis increasing secondary metabolite production. For instance, Kumar et al. (2014), studying the regeneration of *Svertiachirayita* plants from calli, showed that the production of swertiamarin (a secoiridoid glucoside), polyphenols such as amarogentin, and the xanthone mangiferin was far higher in regenerated *in vitro* plantlets than in biomass without organogenesis.

As mentioned, the highest levels of MPTOX were achieved by CM grown without PGRs, either in light or darkness. Lower quantities (about 20-39 $\mu\text{g/gDW}$) were found in all the studied conditions only after 4 weeks, except in those most detrimental for growth (C3.2). In contrast, the PTOX levels remained steady throughout the experiment and under the different conditions studied (Fig. 9B).

Studies have shown that the main lignan found in cell cultures depends primarily on the original plant and cell/root line, irrespective of experimental conditions. Federolf et al. (2007) established a *L. album* cell line that mainly produced MPTOX and another, obtained from the same plant, whose main lignan was PTOX at the peak of production (8-16 days of culture). Smollny et al. (1998) found several lignans, predominantly PTOX, in *L. album* cell cultures. Another example of cells, calli or root cultures

forming different lignans has been reported by Wawrosch et al. (2014), who found the pharmacologically active lignans leoligin and 5-methoxy-leoligin in Edelweiss hairy roots, mainly after elicitation.

The trace levels of β -peltatin found in the CM may indicate that after being produced by the hydroxylation of dPTOX, this precursor was metabolized very quickly to MPTOX (Fig.1). The absence of the lignan yatein in the CM in all the culture conditions suggests that the pathway leading from matairesinol to PTOX via yatein was not active. More detailed genetic and metabolic studies on the presence of this biosynthetic route in *L. album* are required.

Based on all the results obtained, we can conclude that among the experimental conditions assayed, the best for growth and organogenesis of *L. album* cell masses were those without plant growth regulators (C4), either in light (C4.1) or darkness (C4.2). The main lignan accumulated in these conditions was MPTOX, in levels approximately 4-fold higher than PTOX. Previous studies with *L. album* have reported PTOX as the main lignan in cell cultures (Yousefzadi et al., 2010b) and MPTOX in transformed roots (Chasmi et al., 2011). The results of our study suggest that although the biomass did not form any root structures, the organogenesis observed also led to MPTOX production.

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Figure legends:

Fig. 1. PTOX biosynthetic pathway. PS, pinoresinol synthase; PLR, pinoresinol-lariciresinol reductase; DOP7H, deoxypodophyllotoxin 7-hydroxylase; DOP6H, deoxypodophyllotoxin 6-hydroxylase.

Figure 1

Fig. 2. Steps for CM induction and culture. (A) *In vitro* *Linum album* plantlet culture. (B) First stage of CM induction. (C) 2 weeks later. (D) Isolated CM culture.

Figure 2

Fig. 3. Schematic drawing of the CM culture treatments.

Fig. 4. Time course of the growth expressed as DW of the CM cultured for 6 weeks in the assayed conditions. Each value is the average of 3 biological replicates $\pm$ SD.

Figure 4

Fig. 5. Light microscopy study. Semi-thin sections observed by optical microscope of CM grown under conditions 1.1, 1.2, 3.1 and 3.2 at 0, 2, 4 and 6 weeks of culture.

Figure 5

Fig. 6. Light microscopy study. Semi-thin sections observed by optical microscope of the CM grown in conditions 4.1, 4.2 and 4.3 at 0, 2, 4 and 6 weeks of culture. mz: meristematic zone; se: somatic embryo; vb: vascular bundle; am: apical meristem; lf: leaf primordia.

Figure 6

Fig. 7. Details of the evolution of the organogenesis process at the cellular level.

a- Initial organogenesis stage: small proliferative cells.

b- Organogenic mass separated from the calli. **se**: somatic embryo.

c- More advanced cell differentiation. **ec**: epidermal cells.

d- Formation of leaf primordia and apical meristems. **am**: apical meristems; **lp**: leaf primordia.

Figure 7

Fig. 8. Scanning electron microscope of organogenic *Linum album* biomass.

a- Large undifferentiated cells. **b-** Early stage of organogenic biomass with small cells. **c-** Organogenic development: leaf primordia. **d-** Shoot formation. **e** and **f-** Details showing more advanced stages in the leave organogenesis: stomata (st) and trichomes (tch).

Figure 8

(Fig. 9. Lignan production expressed as $\mu\text{g/gDW}$ in *L. album* calli grown for 6 weeks in the conditions established in this work. Deoxypodophyllotoxin (A), podophyllotoxin (B) and 6-methoxy-podophyllotoxin (C). Data are shown as means $\pm$ SD from three independent biological replicates. Different letters indicate significantly differences ($P < 0.05$) among values.