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Kinetics of glucosylated and non-glucosylated aryltetralin lignans in *Linum* hairy root cultures

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ABSTRACT

Due to their pronounced cytotoxic activity, a number of aryltetralin lignans (ATLs), such as podophyllotoxin (PTOX), are used as antitumor compounds. The production of such molecules from entire plants or plant cell-tissue-organ cultures is thus of interest to the pharmaceutical industry. Hairy root cultures constitute a good tool not only for phytochemical production but also for investigating plant secondary metabolism. This work reports on the growth and ATL biosynthesis in two hairy root cultures of *Linum album* Kotschy ex Boiss. and *Linum flavum*. The kinetics of accumulation of the intermediates of MPTOX biosynthesis and of their glucosylated forms are described over a 21-day period of growth. An accumulation of non-glucosylated forms of the ATLs during the exponential phase of the cultures is followed by an accumulation of the glucosylated forms during the stationary phase. Our results show a strong coordination of the biosynthetic paths derived from deoxypodophyllotoxin via deoxypodophyllotoxin 6-hydroxylase and deoxypodophyllotoxin 7-hydroxylase, and a coordinated glucosylation of podophyllotoxin, methoxypodophyllotoxin, and 5′-demethoxymethoxypodophyllotoxin. Furthermore, our results suggest an important role of β-peltatin-6-glucoside formation in the control of ATL accumulation in *Linum* hairy root cultures.

1. Introduction

Lignans are secondary metabolites belonging to a complex family originating from the shikimic acid pathway. Some of them present a carbocycle between two phenylpropane units bound *via* their side chains in the 8–8′ positions (Arroo et al., 2002). Within this group of molecules, podophyllotoxin (PTOX), an aryltetralin lignan (ATL), and its derivatives are highly cytotoxic compounds. This cytotoxicity is best known in humans, due to its therapeutic applications. Podophyllotoxin inhibits tubulin polymerization and its semi-synthetic glucoside, known as etoposide, displays binding activity to the enzyme topoisomerase II during the late S and early G2 stage of mitosis (Gordaliza et al., 2004). ATLs have been successfully tested for their antitumor activity, and have shown good clinical effects against several types of

Abbreviations: ATL(s), aryltetralin lignan(s); β-*P6OMT*, β-peltatin 6-0-methyltransferase; DMeDOP, demethyl-deoxypodophyllotoxin; DMeDOPG, demethyl-deoxypodophyllotoxin glucoside; DMeMPTOX, 4′-demethyl-methoxypodophyllotoxin; DMeMPTOXG, 4′-demethyl-methoxypodophyllotoxin glucoside; dMPTOX, 5′-demethoxy-methoxypodophyllotoxin; dMPTOXG, 5′-demethoxy-methoxypodophyllotoxin-7-glucoside; DOP, deoxypodophyllotoxin; *DOP6H*, deoxypodophyllotoxin 6-hydroxylase; *DOP7H*, deoxypodophyllotoxin 7-hydroxylase; DT, doubling time; DW, dry weight; FW, fresh weight; HRC(s), hairy root culture(s); M&S, Murashige and Skoog; µM, micromole; MPTOX, methoxypodophyllotoxin; MPTOXG, methoxypodophyllotoxin-6-glucoside; (*M*)*PTOX7G*, (methoxy)podophyllotoxin 7-O-glucosyltransferase; *PAM7H*, β-peltatin-A-methylether-7-hydrolase; PCR, polymerase chain reaction; PELT, β-peltatin; PELTG, β-peltatin-6-glucoside; PELT ME, β-peltatin-A-methylether; *PEL6G*, β-peltatin 6-O-glucosyltransferase; PTOX, podophyllotoxin; PTOXG, podophyllotoxin-7-glucoside; *SDH*, secoisolariciresinoldeshydrogenase; µ, specific growth rate per day.

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cancer (Canel et al., 2000). PTOX, and other related lignans, are extracted from the rhizomes of the *Berberidaceae* species *Podophyllum peltatum* L. and *Podophyllum hexandrum* Royle (syn. *Podophyllum emodi*) (Broomhead and Dewick, 1990). The supply of *P. hexandrum* rhizomes, which contain ca. 4% of PTOX on a dry weight basis, has become increasingly limited due to both intensive collection and lack of cultivation (Koulman et al., 2003). In fact, the species *P. hexandrum* is listed in appendix II of CITES (Convention for International Trade in Endangered Species). ATL structures have also been described in other genera and families such as *Linaceae*. These lignans show many structural similarities to the podophyllotoxin-like compounds from *Podophyllum* spp., *Linum album* Kotschy and *Linum flavum* L. (*Linaceae*, section *Syllinum*). They accumulate mainly 6-methoxypodophyllotoxin (MPTOX), its 7-O-glucoside derivative (MPTOXG) and also trace amounts of PTOX.

The biosynthesis of PTOX and MPTOX starts with the general phenylpropanoid pathway and deoxypodophyllotoxin (DOP) is the precursor for these two compounds (Federolf et al., 2007). Hydroxylation at position 7 of DOP gives PTOX while the alternative oxidation at position 6 gives β-peltatin (PELT). PELT is then converted to β-peltatin-A-methylether (PELT ME) and the hydroxylation step forms MPTOX (Broomhead and Dewick, 1990).

Glucosylated ATLs were described by Lin (1996) in *P. peltatum*. Konuklugil et al. (2007) reported that methoxypodophyllotoxin-6-glucoside (MPTOXG), β-peltatin-6-glucoside (PELTG) and podophyllotoxin glucoside (PTOXG) are found in cell cultures of *L. album* and *L. flavum*. For Gordaliza et al. (2004), the glucosylated forms have greater water solubility, which enables vacuolar storage; they believe that this conversion from the aglycone form to the glucosylated form might well occur as part of a detoxification process. Berim et al. (2008) showed that the glucosylation involves (i) a (methoxy)podophyllotoxin 7-O-glucosyltransferase (*(M)PTOX7G*) for podophyllotoxin, methoxypodophyllotoxin and demethoxymethoxypodophyllotoxin, and (ii) a β-peltatin 6-O-glucosyltransferase (*PEL6G*) for β-peltatin. They also believe that the return to the aglycone form is possible.

Biotechnology is an opportunity to diversify sources of secondary metabolites (Ono and Tian, 2010; Sasheva et al., 2012). Hairy root cultures (HRCs) have been established from a wide variety of plant species, including flax-related species (Chashmi et al., 2013).

HRCs are a tool of choice for investigating plant secondary metabolism and phytochemical production. They generally accumulate phytochemicals to levels comparable to those of intact plants and are usually stable in their biosynthetic capacity (Lanoue et al., 2002; Ono and Tian, 2010; Jousse et al., 2010; Al Balkhi et al., 2012).

Cell cultures and/or HRCs of *L. album* and/or *L. flavum* have been used to produce ATLs. Baldi et al. (2008) increased PTOX in cell cultures of *L. album* through the action of different culture media and carbon sources during growth. Oostdam et al. (1993) established HRCs of *L. flavum* producing MPTOX. Lin et al. (2003a,b) studied the production of PTOX using cross-species co-culture of *L. flavum* hairy roots and *P. hexandrum* cell suspensions. Farkya and Bisaria (2008) showed that exogenous hormones affected the morphology and biosynthetic potential of HRCs of *L. album*. Kumar et al. (2012) showed that a culture filtrate of the root endophytic fungus *Piriformospora indica* promoted the growth and lignan production of *L. album* hairy root cultures. The authors provided kinetics for one ATL (PTOX) or two ATLs (PTOX and MPTOX), but they did not study the relation between the aglycone and glucosylated forms.

In this report, we study the kinetics of ATL accumulation in HRCs of *L. album* and *L. flavum* during the 21 days of total hairy root growth. Particular attention is paid to the balance between ATL aglycones and their ATL glucosides.

2. Results and discussion

2.1. Hairy root induction, selection and characterization of growth

In the *L. flavum* species, no hairy root induction was observed using cotyledon disks as the starting explants. With hypocotyl pieces, root induction was obtained at a level of 16% and 18% using *Agrobacterium rhizogenes* strains ATCC 15834 and LBA 9402, respectively; these levels were equal to or higher than those obtained by Lin et al. (2003a,b) or Oostdam et al. (1993).

After 4 weeks of cultivation, individual root lines were isolated and cultivated on hormone-free medium containing antibiotics. With the ATCC 15834 strain, a true hairy root phenotype associated with vigorous growth was most frequently observed (see details in SI1).

For *L. album*, transformation could be obtained with *A. rhizogenes* LBA 9402 (6% transformation efficiency) and ATCC 15834 (4% transformation efficiency).

For the two *Linum* species, the complete and stable transformation status of the isolated HRCs was confirmed by both (i) PCR on their genomic DNA for the detection of the presence of the pro-oncogenes *ROL-B* and *ROL-C* and the absence of VIR-D2, and (ii) sqRT PCR analysis for the expression of these two pro-oncogenes. At the end of this selection phase, six *L. flavum* and five *L. album* HRC lines were chosen.

In a preliminary experiment (completed by phytochemical analysis described below in Section 2.2.1), the growth of the different HRCs obtained were compared. *L. flavum* HRLF15.3 and *L. album* HRLA94.3 lines were selected for further studies because of their faster growth.

The growth kinetics of these two lines were established as previously described in Mairet et al. (2010). The lag phase, exponential growth phase and stationary phase were characterized, enabling the calculation of the doubling time (DT; in days) and specific growth rate per day ($\mu = \ln 2/\mathrm{DT}$; in d^{-1}). The growth kinetics of *L. album* and *L. flavum* HRCs in B5 media are presented in Fig. 1 and the characteristics of the growth in Table 1. For *L. flavum*, these data are similar to values given by Lin et al. (2003a,b). For *L. album*, Kumar et al. (2012) obtained the best mass of hairy roots (7.5 g of fresh weight (FW)) over 16 days while, more recently, Chashmi et al. (2013) obtained 12.45 g FW over 4 weeks from an initial explant of 2.4 g FW.

2.2. ATL profiling by LC–MS and LC–MS/MS analysis

2.2.1. Preliminary ATL extraction and identification

As a preliminary analysis, the six *L. flavum* and five *L. album* HRC lines were screened for their ATL production. HPLC analysis indicated the same pattern of accumulation for each HRC analyzed but significant quantitative variations were observed (Table SI.1.1.). The two main components in hairy roots of *L. flavum* and *L. album* were proposed by LC–MS as MPTOX and its glucoside MPTOXG. The MS fragmentation of the isolated compounds showed an ion at *m*/*z* 467.13 for MPTOX and at 629.18 for MPTOXG as well as MS/MS fragmentation ions at *m*/*z* 427, 343, 312, 277, 259 and 215 for MPTOX and for MPTOXG (Table 2). Thus, as already described by Berim et al. (2008) in a suspension culture of *Linum nodiflorum*, we have shown that MPTOX and MPTOXG are the main ATLs accumulated in *L. album* and *L. flavum* HRCs.

In order to quantify these two main compounds in our HRCs, and because no commercial standards of these compounds were available, we isolated them from the dry matter of our HR using the protocol described by van Uden et al. (1992). These isolates were analyzed by means of LC–MS.

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