Plant Cell Reports (1993) 12:474–477

Plant Cell Reports

Establishment of hairy root cultures of *Linum flavum* producing the lignan 5-methoxypodophyllotoxin

André Oostdam [1], Jos N. M. Mol [2], and Linus H. W. van der Plas [3]

Departments of Physiology and Biochemistry of Plants [1] and Molecular Genetics [2], Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands
[3] Department of Plant Physiology, Agricultural University, Arboretumlaan 4 6703 BD, Wageningen, The Netherlands

Received January 21, 1993/Revised version received February 16, 1993 – Communicated by K. Hahlbrock

Abstract. Hairy root cultures were induced from leaf explants of *Linum flavum* by infection with *Agrobacterium rhizogenes*. The transformed nature of tissue was confirmed by the production of opines. The cultures produced 1.5 to 3.5% of the lignan 5-methoxypodophyllotoxin (5-MPT) on a dry weight basis, which was 2 to 5 times higher than the 5-MPT content in untransformed root cultures and 5 to 12 times higher than in *L. flavum* cell suspensions. The 5-MPT production as a function of time was up to four times higher than that in cell suspensions.

Abbreviations: NAA: 1-naphthaleneacetic acid; BAP: 6-benzylaminopurine; 5-MPT: 5-methoxypodophyllotoxin; DW: dry weight; GC-MS: gas-chromatography coupled electron impact mass spectrometry.

Introduction

Podophyllotoxin is a highly cytotoxic aryltetralin lignan that is formed by dimerisation of intermediates from the general phenylpropanoid pathway (Jackson and Dewick 1984). Its semisynthetic derivatives etoposide (VP-16-213) and teniposide (VM-26) are widely used in chemotherapy, having antineoplastic activity against testicular and small cell lung cancer among others (Van Maanen et al. 1988). Podophyllotoxin may accumulate up to 4% of the dry mass in rhizomes of *Podophyllum hexandrum* (Berberidaceae) (Broomhead and Dewick 1990). These plants which grow very slowly (Krishnamurthy et al. 1965), are collected from the wild and are thus becoming increasingly rare. This limits the supply of podophyllotoxin and necessitates the search for alternative ways of production. Chemical synthesis of the compound, however, is very lengthy and leaves much room for improvement (Ward 1990). *In vitro* culture of *Podophyllum* tissues was reported only recently (Van Uden et al. 1989; Heyenga et al. 1990) but production of podophyllotoxin in such cultures did not exceed 0.1% of the dry mass (Woerdenbag et al. 1990).

Another approach is to find derivatives of the podophyllotoxin family exhibiting cytostatic activity. *Linum flavum* (Linaceae) produces the compound 5-methoxypodophyllotoxin (5-MPT) (Berlin et al. 1986; Van Uden et al. 1990, Wichers et al. 1990). This lignan has an additional methoxy group at the 5 position that is absent in podophyllotoxin (Fig. 1).

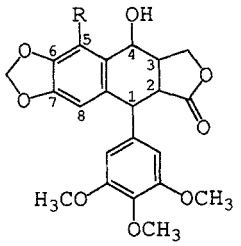

Fig. 1: Structural formula of the lignans podophyllotoxin (R=H) and 5-methoxypodophyllotoxin ($R=OCH_3$). The latter is produced by *L. flavum*.

5-MPT also has strong cytotoxic properties (Berlin et al. 1988; Van Uden et al. 1992) and thus may be an attractive alternative cytostatic lignan of the podophyllotoxin family. 5-MPT is mainly accumulated in the roots of *L. flavum* (Broomhead and Dewick 1990; Wichers et al. 1991), where it may accumulate up to 4% on a dry weight basis, either in the glycosidic form, or as the aglycon. It was shown (Berlin et al. 1988; Van Uden et al. 1991) that root cultures can be established from *L. flavum* and that 5-MPT may accumulate up to 0.7% on dry weight basis in these roots. In undifferentiated cell suspensions the 5-MPT content is lower, with a maximum of 0.3% (Wichers et al. 1990). In optimised production media, 5-MPT may accumulate to 0.7 % (Van Uden et al. 1991).

It is well known that infection with the soil bacterium

Correspondence to: A. Oostdam

Agrobacterium rhizogenes leads to hairy root cultures (Tempé and Casse-Delbart 1989). These cultures often grow vigorously and produce high levels of secondary metabolites (Hamill et al. 1987; Flores et al. 1987). We have examined the possibility of generating hairy root cultures of *L. flavum* with the objective of increasing the 5-MPT production. In this paper we describe the transformation of *L. flavum* leaf explants by *A. rhizogenes* and show that the production of 5-MPT in the resulting hairy root cultures is increased 2 to 5 fold relative to untransformed root cultures. These cultures are so far the most attractive system for the production of 5-MPT.

Materials and methods

Plant material and culture conditions. Linum flavum L. plantlets were grown axenically on MS medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg et al. 1968), sucrose (20 g/l) and agar (8 g/l). Plantlets were subcultured at least monthly and were grown at 25°C in continuous light. The hairy root cultures were grown in 50 ml of the same medium without agar at 25°C in the dark and subcultured monthly. *A. rhizogenes* strains LBA 9402, 8490 and 9365 (NCPPB 1855 *rif*^{r}, 2659 and 8196 resp.) were grown on YMB medium (Hooykaas et al. 1977) at 28°C.

Induction of hairy root cultures. For the infection of *L. flavum* leaf explants with *A. rhizogenes*, a modification of the protocol described by Koornneef et al. (1986) was followed. Young leaves of *L. flavum* that had been carefully abscised and cut transversally in half, were placed on feederlayers. These consisted of 2 ml of a *Petunia hybrida* cv Commanche-albino cell suspension on MS medium supplemented with sucrose (20 g/l), NAA (2 mg/l), BAP (1 mg/l) and agar (10 g/l), covered with a filter paper disc and prepared a day in advance. The leaf explants were infected by dipping them into an inoculum that consisted of 100 ml of an overnight culture of *A. rhizogenes* which was pelleted and resuspended in 25 ml of fresh medium. Following infection, the explants were blotted with filter paper to remove excess bacteria. After two days of co-cultivation, the explants were blotted dty abd transferred to MS medium containing zeatin (2 mg/l) and carbenicillin (500 mg/l). Control explants were given the same treatment but were dipped in sterile YMB medium.

When approximately 1 gram of hairy roots had formed on a leaf explant, they were transferred to a separate shake flask with liquid medium containing carbenicillin (500 mg/l). Axenic hairy root cultures were established after five successive subcultures in the presence of carbenicillin. Growth rates were determined by monitoring the fresh weight. Opine assays using paper electrophoresis were performed according to Peerbolte et al. (1985).

Extraction and detection of 5-MPT. 5-MPT was extracted from 100 mg of oven-dried material (24 h 60°C) according to Van Uden et al. (1990). HPLC equipment consisted of a Gilson model 303 peristaltic pump, a Rheodyne injection valve fitted with a 20 µl loop and a Perkin Elmer LC 235 diode array detector. Detection was at 280 nm. A chromatographic procedure was adapted from Lim and Ayres (1983) using a LiChrosorb SI-60-7 column (i.d. 250x4.6 mm), and a heptane, dichloromethane, methanol (90:10:4 v/v/v) mobile phase. The flow rate was 3 ml/min and the retention time of 5-MPT was 7.6 min.

Gas chromatography and electron impact mass spectrometry (GC-MS) were performed as described (Wichers et al. 1990). The 5-MPT content of the examined hairy root lines was determined from peak areas of HPLC chromatograms, using purified 5-MPT as a reference. In combination with the data on growth rate, the 5-MPT production per gram dry weight per week was calculated.

Results and discussion

Establishment of hairy root cultures

During initial experiments, leaf explants of *L. flavum* were infected separately with the *A. rhizogenes* strains LBA 9402, 8490 and 9365. Only strain 9402, which is known to be one of the more virulent strains (Hamill et al. 1987), showed activity with *L. flavum*, so further experiments were performed with only this strain. Infection of *in vitro* grown plantlets with *A. rhizogenes* using a hypodermic

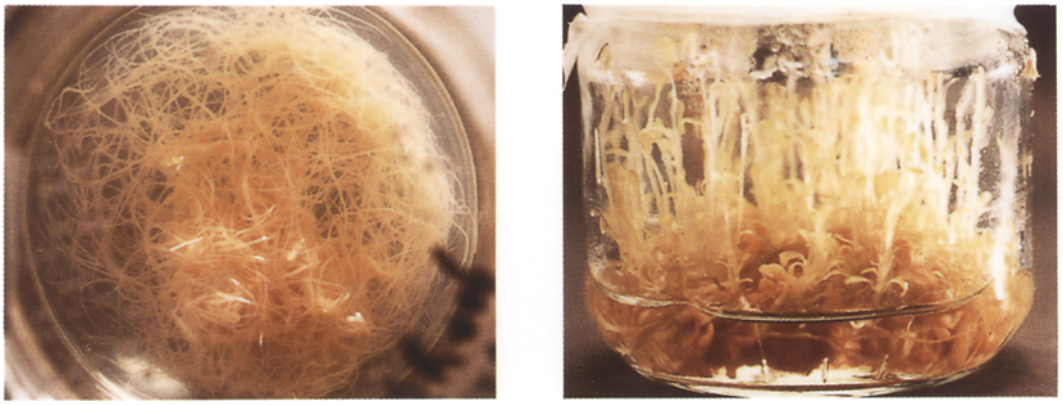

Fig. 2: Two different cultures exhibiting exclusively root formation (left) and shoot formation with very few roots (right). Both cultures synthesised opines and grew on medium lacking phytohormones. Both morphologies remained stable during prolonged subculture.

needle did not have the desired results. Attempts to infect roots of axenically-grown *P. hexandrum* seedlings were not successful either.

About 500 leaf-explants of *L. flavum* were infected in two separate experiments. Roots emerged from micro-calli after three to five weeks at the cut edges of 16% of the explants. Non-infected controls did not exhibit any growth and died after approximately 6 weeks. After several subcultures, large differences in the morphology of the transformed lines became apparent, ranging from undifferentiated callus to only root and even only shoot formation (Fig 2). Root hairs generally were sparse and the degree of lateral branching was low. Only four lines, (coded A-D in the following) displaying distinct root morphology were used for further analysis of 5-MPT production.

Detection of opines

A. rhizogenes strain LBA 9402 is of the agropine-mannopine type. Both opines were detected by paper electrophoresis in extracts from transformed tissues but not in untransformed control tissue taken from a cellsuspension (Fig. 3). The production of opines, along with the sustained growth on hormone-free medium, was taken as proof of transformation.

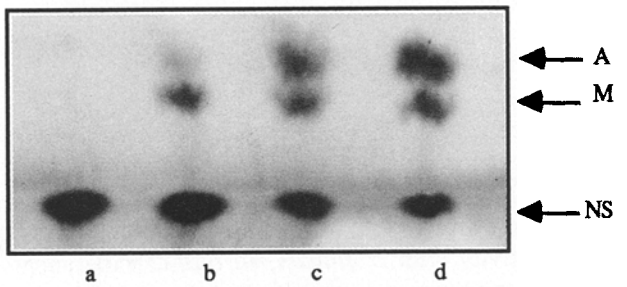

Fig. 3: Detection of agropine (A) and mannopine (M) by paper electrophoresis in extracts of two hairy root cultures (lanes b and c). Lane a: non-transformed tissue, lane d: authentic agropine and mannopine. NS: neutral sugars.

Identification of 5-MPT in transformed root cultures

HPLC analysis of extracts showed peaks with retention time and UV spectrum identical to those of authentic 5-MPT (Fig. 4). The compound was present in all extracts. GC-MS analysis showed the characteristic 5-MPT fragmentation pattern (Fig. 5) as described (Wichers et al. 1990), thus the presence of 5-MPT was well established. Apart from 5-MPT, trace amounts of the podophyllotoxins α-peltatin and β-peltatin were found. These could however not be quantified by HPLC because reference compounds were not available. The β-glucosidic form of 5-MPT, described by Berlin et al. (1988) was not detected. This may have been due to a strong β-glucosidase in *L. flavum* which is active even in the presence of high concentrations of organic solvents (Berlin et al. 1988).

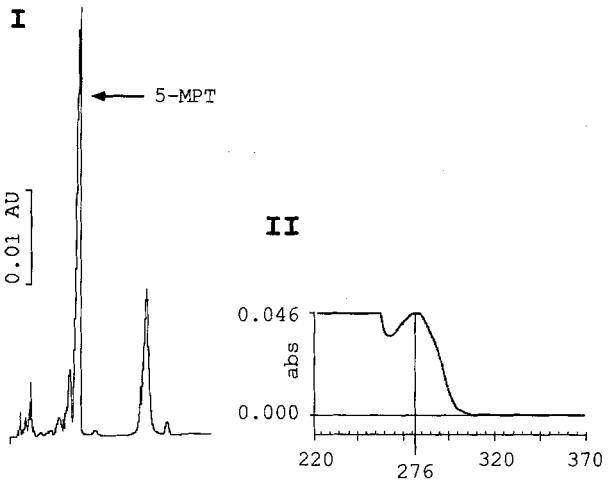

Fig. 4: HPLC chromatogram (I) of hairy root line B (see Table 1), with 5-MPT peak at 7.6 minutes and (II): UV spectrum of the 5-MPT peak with a maximum at 276 nm. AU: absorption units.

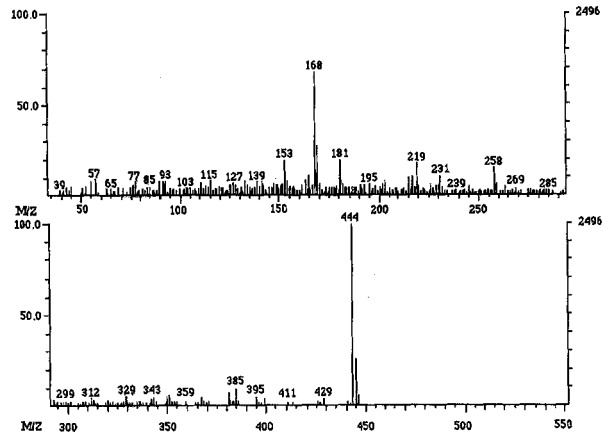

Fig. 5: Electron impact mass spectrum of 5-MPT in extract of hairy root line B (see Table 1), demonstrating the characteristic fragmentation pattern of 5-MPT, with m/e values of 168, 181, 219, 231 and 444.

Production of 5-MPT

The 5-MPT content ranged from 1.4 to 3.5 % DW (Table 1) in the four hairy root lines used for further analysis. The maximum amount of 5-MPT (line B) was remarkably similar to the 4.0 % found in roots of field grown plants (Broomhead and Dewick 1990; Wichers et al. 1991). This might indicate the maximum level of 5-MPT accumulation in *L. flavum* root tissues.

The doubling time of the hairy root lines varied between 11 and 29 days. This was longer than expected, on the basis of the doubling times reported in the literature for a variety of hairy root cultures (Christen et al. 1989; Nguyen et al. 1992). This may be caused by the low degree of lateral branching observed in our tissues. Since roots grow at their apical meristems, low branching frequency implies a high amount of mature, non-growing tissue.

Table 1: 5-MPT content, growth rate and 5-MPT production of four different hairy root lines (A-D) and of an untransformed cell suspension. Values of 5-MPT content are averages of at least three separate determinations.

Hairy root line	5-MPT content (%DW)	Doubling time (days)	5-MPT production (mg/gDW day^{-1})
A	1.7	9	0.57
B	3.5	28	1.23
C	1.4	19	0.73
D	1.5	11	1.40
cell suspension	0.3	8	0.37

Many reports describe effects of the medium composition on the growth rate of hairy roots (Sauerwein et al. 1991; Ho and Shanks 1992) so it seems likely that the growth rate of our tissues may be improved by medium optimization. Unfortunately, no data are available on the growth rate of field grown *L. flavum* roots. *L. flavum* cell suspensions have a doubling time of approximately 8 days. The growth rate of hairy root line D was comparable with this value. The 5-MPT content was however 5 fold lower in the cultured cells.

Despite the somewhat low growth rate of the hairy roots, the production of 5-MPT per day was in all cases higher than that observed in cell suspensions and almost four times higher in the best case (line D).

From our results it is clear that hairy root cultures provide a good system for the production of 5-MPT. Further optimization of the medium composition and selection of fast growing and high yielding lines may further increase the 5-MPT production.

Acknowledgements. This study was performed in co-operation with the Netherlands Organisation for Applied Scientific Research (MT-TNO) in Zeist and the University of Groningen Laboratory of Pharmacognosy. Purified 5-MPT was a generous gift from Dr. H. J. Wichers in Zeist, who also provided the GC-MS data. Authentic opines were a kind gift from Dr. A. Petit, C.N.R.S. France. T.J.A. Kneppers and Dr. J. Hille gave helpful advice concerning the transformation procedure.

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