Pharmacognosy Magazine Vol 4, Issue 17, Jan-Mar, 2009 Page 14-18
(An Official Publication of Pharmacognosy Network Worldwide)
Received: 14/05/2008
Modified: 6/11/2008
Accepted: 6/11/2008

PHCOG MAG.: Research Article

Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and lignan production in *Linum tauricum ssp. tauricum*

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ABSTRACT
Hairy root cultures were induced from leaf explants of *Linum tauricum* ssp. *tauricum* by infection with *Agrobacterium rhizogenes*. Different bacterial strains of *Agrobacterium rhizogenes* - TR 105 and ATCC 15834 were evaluated for induction of transformed hairy roots in *Linum tauricum* ssp. *tauricum*. These different strains varied in their virulence for induction of hairy roots in this species. Acetosyringon in cultivation medium was used to increase of frequency of hairy root induction. Growth kinetics of transgenic roots indicated a similar pattern of growth, with maximum growth occurring between 17 and 20 days. The transformed nature of tissue was confirmed by the production of opines. The lignan production of different clones was found to be growth-related. The cultures produced to 2.6% of the lignan 4'-demethyl-6-methoxypodophylotoxin (4'-DM-MPTOX) and to 3.5% of the lignan 6-methoxypodophyllotoxin (6MPTOX) on a dry weight basis, which was 10 to 12 times higher than in *Linum tauricum* ssp. *tauricum* cell suspensions. Transformed cultures showed significant differences in lignan content. The highest amount of 4'-DM-MPTOX and MPTOX was found in transformed line induced by strain ATCC 15834. Rapidly growing root lines were selected to increase the efficiency of he production of lignans.

KEY WORDS: *Linum tauricum* ssp. *tauricum,* hairy roots, cytotoxic activity, 4'-demethyl-6-methoxypodophylotoxin, 6-methoxypodophyllotoxin

INTRODUCTION

The hairy roots offer a valuable source of root derived phytochemicals that are useful as pharmaceuticals, cosmetics and food additives. Transformed roots of many plant species have been widely studied for the in vitro production of secondary metabolites (1).

Linum tauricum ssp. tauricum (Willd.) Petrova, endemic to the Balkan region, belongs to the Section Syllinum of the genus *Linum* (Linaceae). After recent taxonomic revision (2) of the four subspecies of *Linum tauricum* (ssp. *tauricum*, ssp. *bulgaricum*, ssp. *linearifolium* и ssp. *serbicum)* this subspecies is now know as distinct taxa at species level as a *Linum tauricum*. To our knowledge, there are no publications about the lignans in hairy roots of this species.

The podophyllotoxin derivatives 4'-demethyl-6-methoxypodophylotoxin (4'-DM-6MPTOX) and 6-methoxypodophyllotoxin (6MPTOX) have been isolated and identified by NMR and UV as the two main lignans in the aerial parts of *Linum tauricum* ssp. *tauricum* (3). Here we report the identification of the both derivatives as the main lignans in hairy root cultures of *Linum tauricum*. The compound 4'DM-6MPTOX was for the first time isolated from wild medicinal plant from us and is interesting with its pharmacological activity. The *in vitro* investigation of its cytostatic properties has demonstrated 2 to 3.5 times higher activity than that of the referent antineoplastic drug etoposide (4). This is the first report on the induction of hairy roots

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of *Linum tauricum* and production of ariltetralin lignans in these transformed root clones.

MATERIALS AND METHODS

Plant material

The plant material from *Linum tauricum* ssp. *tauricum* was collected near Varna (Bulgaria) in July 2004. A plant specimen was deposited in the Herbarium of the Faculty of Pharmacy, Medical University of Sofia, Bulgaria (No FAF 0001, coll. by N. Vasilev). The seeds were germinated on modified MS medium, which consisted of half strength MS macro element (5).

Bacterial strains.

Agrobacterium rhizogenes strains: ATCC 15834 and TR 105 were grown on liquid YMB medium (6), containing yeast extract (1 g/l).

Culture method.

Leaf segments from sterile grown plants, were wounded with a sterile needle and the bacteria from the media were spread onto the leafs. After four days, the explants were transferred to MS medium without phytochormones, containing 500 $mg.l^{-1}$ sodium cefotaxim (Claforan, Hoechst AG, Frankfurt) to remove the excess of bacteria. The cultures were kept in dark, at 25°C. Roots developed after 3-4 weeks of incubation. After completely removing free-living Agrobacteria, the hairy roots are cultivated in the usual manner. Single roots (ca 20-30 mm long) were transferred to liquid MS medium without phytochormones. For the time course of growth and lignan production, 2-3 cm (0.5g fresh weight) root tips were inoculated in 25 ml medium in 100 ml flasks or 50 ml in 300 ml flasks and cultivated on a gyratory shaker at 120 rpm in darkness at 25± 2 °C and subcultured at a four-week interval. The growth and development of hairy roots were studied during a 4-week period. Hairy roots were cultured initially on full, half and one-fourth strength of MS media to evaluate the optimal medium. Excised roots of in vitro germinated seedlings were cultured similarly and served as controls.

Confirmation of transgenic nature of hairy roots

Opine assay. Opines in the hairy roots were extracted as described in (6). The opines were detected on TLC with the reagent of Travelyan. Agropine and mannopine (Sigma) were used as standard.

Extraction and isolation of lignan aglycons.

A fine powder (0.2 g) of the lyophilised plant material was extracted with methanol (2 ml) in an ultrasonic bath (two times for 30 s). Distilled water (6 ml) was added, and the pH was adjusted to 5.0 by *o*-phosphoric acid. After adding of β-glucosidase (1 mg), the sample was incubated at 35 °C for 1 h. Methanol (12 ml) was added and the mixture incubated for another 10 min at 70 °C in an ultrasonic bath. After centrifugation, the supernatant was used directly or storage at –18 °C.

HPLC, NMR spectroscopy

HPLC with a Thermo Quest HPLC system (Egelsbach, Germany) equipped with a photodiode array detector was used. Separation was performed using a GROM-SIL 120 ODS-5ST column with guard column (250 mm long, 4.6 mm i.d. and 40 mm long, 4.6 mm i.d., respectively; Grom Company, Herrenberg, Germany) and a gradient program with water (A) and acetonitrile (B) as eluents as follows: 0 to 17 min from 40 to 67% B, from 17 to 18 min to 40% B, and until 24 min back to 40% B. The flow rate increased from 0.8 mL/min at 0 min to 1.0 mL/min at 17.0 min and decreased again to 0.8 mL/min between 18 and 24 min.

A Waters/Millipore HPLC system equipped with a photodiode array detector and a fraction collector was used for the collection of the compounds. After the lignan extraction (mentioned above) the methanolic part of the supernatant was evaporated. The aqueous remnant was extracted twice with equal volumes of ethylacetate p.a. After the ethylacetate phases were combined and evaporated, the remains were dissolved in methanol p.a. Collection of the two main lignans was carried out by using a semi-preparative GROM-SIL 120 ODS-5ST column with guard column (250 mm long, 8 mm i.d. and 50 mm long, 8 mm i.d., respectively; 5 μM particle; Grom Company, Herrenberg, Germany) and the same gradient system as mentioned above, but the flow rate was doubled.

1H-NMR spectra were recorded on a Bruker DRX 200 spectrometer at 200.13 MHz in $CDCl_3$. Conditions and data respectively, were as those reported early (3).

RESULTS AND DISCUSSION

Effect of bacterial strains.

The main objective of this investigation was to establish hairy roots in an important wild medicinal plant *Linum tauricum* using *Agrobacterium rhizogenes*. Different strains of *Agrobacterium rhizogenes* were evaluated for induction of transformed hairy roots in *Linum tauricum* using leaf explants. Various bacterial strains exhibit different levels of virulence to this plant species. In the present study, induction of hairy roots occurred at a 32 % frequency for ATCC 15834 and a success rate of approximately 55% using *A. rhizogenes* strains TR 105. Wild type *Agrobacterium rhizogenes* ATCC 15834 (harbouring pRi 15834) shows more resistance for hairy induction in *Linum tauricum,* but production of ariltetralin lignans in this hairy root

clones was highly, compared with hairy root clones, transformed with TR 105 (Tabl.2).

Effect of acetosyringon.

In order to improve the frequency of transformation, two methods were attempted. It has been demonstrated that *Agrobacterium* are attracted to host across chemical gradients of phenolic compounds released by injured plant cells. One specific highly active compound in this respect has been identified as acetosyringone. Acetosyringone have been reported to increase *Agrobacterium* - mediated transformation frequencies in a number of plants (1). In some of the more recalcitrant species, successful transformation was achieved by including 10 ¤M acetylsyringon in the medium in which the bacteria are suspended. The addition to the cocultivation medium of acetylsyringone changed the frequency of transformation and resulted in a substantial increase in the number of transformation events. This compound, produced during the wounding response of plants, activates the *vir* genes of *Agrobacterium*, aiding plasmid T-DNA transfer (1). The number of individual roots formed has been used as a measure of virulence. The results show that supplementation of acetosyringone in bacterial culture and co-cultivation medium increased the frequency of hairy root induction in *Linum tauricum* and can be used to increase *Agrobacterium*-mediated transformation frequencies in *Linum* species as well.

Growth kinetics of transgenic hairy roots

To determine the growth rate of the transformed cultures, root tips 2-3 cm long were excised from 10-day-old cultures and an inoculum size of 50 mg fresh weight was inoculated into 50 ml of half strength MS liquid medium supplemented with 2% sucrose in 300 ml flask in triplicates. Growth rate was determined by checking the fresh weight of hairy roots every second day up to 28 days. The growth curve of hairy roots is characterized by a short lag-phase followed by exponential increase of biomass. Untransformed control roots obtained from shoot tip explants on MS basal medium with hormones were also grown to serve as a control. Doubling time after inoculation of various hairy root cultures was 24-90 h. The growth rate of *Linum tauricum* hairy roots depends on two parameters - the linear extension of the root tip and exponential formation of lateral roots. These cultures showed maximal increase in biomass about 20-fold at the end of 4 weeks compared with inoculum. Growth kinetics of transgenic hairy roots induced by different strains indicated a similar pattern of growth, with maximum growth occurring between 17 and 20 days. The effect of exogenous hormones on the growth has been investigated. Although hairy roots in general are hormone-autotrophic, supplementary auxin (NAA-naphthalene acetic acid) has proved beneficial for root growth in the first two passages.

Opine production

The occurrence of N-(carboxyalkyl)-amino acids (opines) is a characteristic feature of Agrobacterium-mediated transformation of plant tissue (1).To prove the genetic transformation, the opines manopine/agropine were identified in several HR clones of *Linum tauricum*. Preparation of extracts from plant material and HR clone, opine analysis by TLC, and detection with alkaline silver nitrate reagent were carried out as described earlier (6). HR of transformed root lines were tested positive for opines, showing that the HR contained the appropriate enzymes from the plasmids responsible for opine synthesis. The roots of parent plant species did not synthesize opines.

Production of lignans in hairy roots of Linum tauricum

The two main lignans, which were present in about equal amounts, were isolated from the above ground parts of this species (3). 6-Methoxypodophylotoxin (MPTOX) was readily identified by comparison of its HPLC retention time and UV spectrum with that of an authentic sample, as well as by its ^{1}H NMR spectral data which were identical with literature data. The second isolated lignan differed from MPTOX in the absence of the 4'-O-methyl group. Thus, besides a compound with a trimethoxylated (6-methoxypodophyllotoxin) pendant ring also a 4'-demethyl analogue occurs. The ^{1}H-NMR spectral data of both compounds are given in Table 1. The 4'-demethyl-6-methoxypodophyllotoxin was isolated previously only in traces from cell cultures *in vitro* of *Linum flavum* L. (also section Syllinum), but is there not a main lignan (7). The detection of both podophyllotoxin derivatives: 6-methoxypodophyllotoxin and of 4'-demethyl-6-methoxy podophyllotoxin in *Linum tauricum* confirms the hypothesis of Broomhead and Dewick (8), that the presence of aryltetralin lignans is typical for the section Syllinum of the genus *Linum*.

The biosynthetic capacity was different between root lines induced with strains ATCC15834 and TR 105. Transformed hairy root cultures showed significant differences in lignan content. A hairy root line induced by strain ATCC 15835 was found to contain highest amount of both lignans (Table 2). A 28 days old hairy root cultures of *Linum tauricum* produced 2.6% of the

Table 1. ^{1}***H-NMR spectral data of 4'-demethyl-6-methoxypodophylotoxin (4'-DM-MPTOX) and 6-methoxypodophyllotoxin (6MPTOX) on Brüker DRX 200 (200.13 MHz) in*** $CDCl_3$

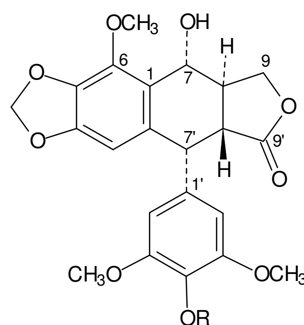
H-	4'-DM-MPTOX			6MPTOX		
	δ (ppm)	mult	*J* (Hz)	δ (ppm)	Mult	*J* (Hz)
3	6.34	*s*		6.34	*s*	
7	5.06	*d*	8.8	5.07	*d*	8.8
8	2.88	*dddd*		2.89	*dddd*	
9a	4.66	*dd*	7.0, 8.4	4.68	*dd*	6.9, 8.4
9b	4.06	*dd*	9.9, 8.7	4.10	*dd*	8.4, 9.8
2' + 6'	6.46	*s*	(2H)	6.48	*s*	(2H)
7'	4.57	*d*	4.3	4.58	*d*	4.3
8'	2.76	*dd*	4.3, 14.5	2.78	*dd*	4.3, 14.6
OCH_2O	5.99	*s*	(2H)	5.99	*s*	(2H)
OCH_3 at 6	4.20	*s*	(3H)	4.20	*s*	(3H)
OCH_3 at 3' + 5'	3.84	*s*	(6H)	3.81	*s*	(6H)
OCH_3 at 4'	n.a.			3.85	*s*	(3H)
OH at 7 *	4.08	*d*	1.5	4.07	*d*	1.5
OH at 4′ *	5.48	*br s*		n.a.		

* The signal disappear after adding of D_2O ; n.a.: not applicable

Table 2. Lignans in different in vitro cultures of L. tauricum

Cultures	Medium	4'-DM-MPTOX (mg/g dry weight)	6-MPTOX (mg/g dry weight)
Shoot cultures*	HP-9	1.21±0.09	1.41±0.006
Callus*	G-48	1.54±0.11	3.99±0.08
Suspension*	NAA	2.16±0.12	2.93±0.07
Hairy roots (ATCC 15835)	MS	25.92±0.31	35.16±0.23
Hairy roots (TR 105)	MS	17.30±0.33	22.62±0.21

*The data presented in (12)



1. R=H (4'-demethyl-6-methoxypodophylotoxin)
2. R= CH_3 (6-methoxypodophyllotoxin)

Fig. 1. Chemical structures of isolated ariltetralin lignans

lignan 4'-demethyl-6-methoxypodophylotoxin (4'-DM-MPTOX) and 3.5% of the lignan 6-methoxypodophyllotoxin (6MPTOX) on a dry weight basis, which was 10 to 12 times higher than in *Linum tauricum* cell suspensions. Lignan content of different hairy root lines was also found to be growth-related. The variation in hairy root induction could possibly be attributed to the variation in virulence of different *Agrobacterium rhizogenes* strains used. For example in *Linum flavum* hairy roots were initiated from leaf discs with a success rate of approximately 50% using *A. rhizogenes* strains LBA 9402 and TR 105. In contrast, very low root induction rates were obtained with strains ATCC 15834 and A4 (9). A fourteen days old hairy root culture of *Linum album* Kotschy. (Linaceae) was reported to produce about 2,5% of the dry mass basis 6MPTOX (10). Hairy root cultures of *Linum leonii*, obtained by genetic transformation using the agropine-

type strain *Agrobacterium rhizogenes* ATCC 15834, accumulate high amount of justicidin B (11).

Genetically modified hairy roots of *Linum tauricum* produced 10-12 fold higher yields of lignans (Table 2) - 25.92 mg/g dw and 35.16 mg/g dw for 4'-DM-6MPTOX and 6MPTOX, respectively, compared to suspensions (12) and show that differentiated roots produce higher amount of secondary compound. This suggests that this technique may be used to enhance the accumulation of both lignans. We can suggest that the hairy roots are the excellence organ for ariltetralin lignans biosynthesis/accumulation.

The soil-borne plant pathogen *Agrobacterium rhizogenes* responsible for adventitious (hairy) root formation at the site of infection also causes certain biochemical changes in the plant metabolism. Hairy root cultures have several properties that have promoted their use for plant biotechnological applications. Their fast growth and genetic and bio-synthetic stability offer an additional advantage for their use as an alternative to plant cell suspension cultures, for production of secondary metabolites of interest. Only a few studies have been carried out for the induction of hairy roots in *Linum* plants, because of its sometime highly resistance to infection by *A. rhizogenes*.

A systematic study using different strains of *A. rhizogenes* for the evaluation of transformation frequency, growth and ariltetralin lignan production from *Linum tauricum* hairy roots has not been carried out till date. In the present study, we have examined the possibility of generating high lignan-yielding hairy root cultures of *Linum tauricum* using different strains of *A. rhizogenes*. We have also studied the influence of acetosyringone on frequency of transformation and hairy root induction. In the present study, the best strains of *A. rhizogenes* for hairy root induction and media for their rapid growth has been optimized. The hairy root cultures of *Linum tauricum* established in liquid MS medium showed high growth rates as compared to non-transformed roots and could prove to be a useful system for production of secondary metabolites present in this important for production of lignans medicinal species.

ACKNOWLEDGMENT

Financial support from Ministry of Education and Science, Sofia, Bulgaria (grant TK-L-1607/06 I. Ionkova) is acknowledged. The authors are grateful to Dr. H. E. Flores (Pennsylvania State Univ.) and Dr. H.Huizing (Wageningen) for the supply of different *Agrobacterium* strains.

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