Root Cultures of *Linum* Species Section *Syllinum* as Rich Sources of 6-Methoxypodophyllotoxin

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Dedicated to Dr. Abulghasem Soltani, formerly professor of Pharmacognosy, Tehran Faculty of Pharmacy, Tehran on the occasion of his birthday

Linum spp. from section *Syllinum* are promising for the production of aryltetralin lignans like podophyllotoxin (PTOX) and 6-methoxypodophyllotoxin (MPTOX). MPTOX is a PTOX congener that has cytotoxic activity comparable with PTOX. In this study root cultures of *Linum Bungei* from section *Dasyllinum*, *L. strictum* from section *Linastrum*, *L. album*, *L. mucronatum* ssp. *mucronatum* and *L. nodiflorum* from section *Syllinum* were established and their MPTOX levels were investigated in 1000 ml flasks. Root cultures of *L. mucronatum* ssp. *mucronatum* and *L. nodiflorum* were used to examine cell growth and production of MPTOX during a culture period of 36 days in 250 ml flasks. Considerable amounts of MPTOX in root cultures (1000 ml flasks) of *L. album* (6 mg/100 g DW), *L. mucronatum* ssp. *mucronatum* (770 mg/100 g DW) and *L. nodiflorum* (91 mg/100 g DW) were detected while it wasn't detected in root cultures of *L. Bungei* and *L. strictum*. In time course experiments, the maximum amount of MPTOX in *L. nodiflorum* root culture was at day 16 with 480 mg/100 g DW and the maximum amount of MPTOX in *L. mucronatum* ssp. *mucronatum* root culture was at day 12 with 130 mg/100 g DW. The results showed that root cultures of *Linum* species from section *Syllinum* are rich sources of MPTOX and since this lignan has remarkable cytotoxic activity, it can be used as a precursor for the production of antitumor agents.

Key words: Linum Section *Syllinum*, 6-Methoxypodophyllotoxin, Root Culture

Introduction

Lignans are a group of phytochemicals with 8,8′-coupled dimers of coniferyl or cinnamyl alcohol (Moss, 2000). Numerous biological activities of lignans, including antitumor, antiviral, hepatoprotective, platelet activating factor antagonism and other properties (Botta *et al.*, 2001; Lee and Xiao, 2003), make them unique and very useful in promoting health and combating various diseases.

Of all the known plant lignans with numbers in the hundreds, the best known is PTOX that is used as a precursor for the production of anticancer drugs (etopophos®, etoposide and teniposide). The aryltetralin lactone PTOX is obtained commercially from *Podophyllum* spp. (Moraes *et al.*, 2002), but because of the limited supply of *Podophyllum* rhizomes, due to their intensive collection in the wild, there is considerable interest to have an alternative source for the production of this lignan. The genus *Linum* (Linaceae) could be an alternative source of lignans including PTOX. *Linum* species belonging to the section *Syllinum* are promising for the production of aryltetralin lignans and there isn't any well documented report about the production of aryltetralin lignans in other sections of the *Linum* genus (Berlin *et al.*, 1986, 1988; Broomhead and Dewick, 1990; Konuk-

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lugil *et al.*, 1999, 2001; Mohagheghzadeh *et al.*, 2003; Smollny *et al.*, 1998). Besides, there are some PTOX congeners like deoxypodophyllotoxin, 4′-demethylepipodophyllotoxin, 4′-demethyl-6-methoxypodophyllotoxin, 6-methoxypodophyllotoxin (MPTOX) which have cytotoxic activity (Lee and Xiao, 2003; van Uden *et al.*, 1992; Vasilev *et al.*, 2005). MPTOX derivatives, 7-*O*-*β*-D-glucopyranoside-MPTOX and 7-*O*-acetyl-MPTOX, are mentioned as two derivatives of MPTOX with cytotoxic and antitumor activity (Buckingham, 2006). Occurrence of MPTOX was reported for the first time from root cultures of *L. flavum* by Berlin *et al.* in 1986. Two years later its pharmacological properties were reported (Berlin *et al.*, 1988). After that, the isolation of MPTOX from *Juniperus sabina* (Cupressaceae) (San Feliciano *et al.*, 1990) and *L. capitatum* (Broomhead and Dewick, 1990) was reported. *L. flavum* was subject of many studies investigating the production of MPTOX in different plant parts and cultures. Wichers *et al.* (1990) demonstrated that cultures derived from the green parts of *L. flavum* contain considerably less MPTOX than its root-derived cultures. Van Uden *et al.* (1991) showed the accumulation of MPTOX in hairy root cultures of *L. flavum*, and cytotoxic aspects of MPTOX on cancerous cell lines were investigated (van Uden *et al.*, 1992). In 1993 it was reported that root cultures and the usually faster growing hairy root cultures are the most attractive systems for the production of MPTOX (Oostdam *et al.*, 1993). Some studies on the biosynthetic pathway of MPTOX were carried out but until now it isn't completely understood (Kuhlmann *et al.*, 2002). The occurrence of MPTOX was studied in other *Linum* spp. like *L. mucronatum* ssp. *armenum* (Konuklugil *et al.*, 2001), *L. nodiflorum* (Konuklugil *et al.*, 1999) and *L. mucronatum* ssp. *mucronatum* (Mohagheghzadeh *et al.*, 2005).

The aim of this study is to establish root cultures from *Linum* species as well as to study the production of MPTOX in the cultures.

Experimental

Plant material

The collection of *L. album* Ky. ex Boiss. seeds, Katan-e-Sefid in Persian (Mozaffarian, 1998), was carried out as described earlier by Smollny *et al.* (1998). Seeds of *L. nodiflorum* L., Katan-e-Gol Gereii in Persian (Mozaffarian, 1998), were collected from Roudbar, Darestan forest, Guilan province, Iran, at an altitude of 900 m in June 1999. Seeds of *L. Bungei* Boiss., Katan-e-Gorgani in Persian (Mozaffarian, 1998), were collected from Chalous Road, Kandovan, Mazandaran province, Iran, at an altitude of 2700 m in June 2000. Seeds of *L. strictum* L., Katan-e-Garmsiry in Persian (Mozaffarian, 1998), and intact plants of *L. mucronatum* Bertol. ssp. *mucronatum*, Katan-e-Zard in Persian (Mozaffarian, 1998), were collected from Boustan, Cuhgiluyeh and Boyer Ahmad province, Iran, at an altitude of 900 m in June and April 1996, respectively.

In vitro cultures

Seeds of *L. album* germinated under sterile conditions as described by Mohagheghzadeh *et al.* (2006). Seeds of *L. Bungei* and *L. strictum* germinated under sterile conditions in continuous lightness on hormone-free MS (Murashige and Skoog, 1962) medium, and agar and coconut media, respectively. The resulting seedlings were transferred to $^1/_2$ MS medium and infected by *A. rhizogenes* (ATCC 15834). After 2 d the infected samples were washed with 500 mg l^{-1} Claforan® solution (Hoechst Marion Roussel, Frankfurt, Germany) to inhibit the growth of *Agrobacterium*. Samples were transferred to $^1/_2$ B_5 solid medium (Gamborg *et al.*, 1968) containing Claforan®. After 3 weeks samples produced hairy roots and were transferred to $^1/_2$ B_5 liquid medium. Root cultures were scaled up from 250 ml to 1000 ml flasks.

Seeds of *L. nodiflorum* were put on agar and coconut media under sterile conditions and incubated in continuous light. The resulting seedlings were transferred to $^1/_2$ MS medium. After culture establishment, normal roots were transferred and established in 250 ml and 1000 ml flasks containing $^1/_2$ B_5 liquid medium.

Leaves of *L. mucronatum* ssp. *mucronatum* were put on MS medium supplemented with 1 mg l^{-1} NAA (*α*-naphthalene acetic acid), 0.5 mg l^{-1} Kn (kinetin), 0.5 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) and 15% coconut milk. After callus production and culture establishment, calli were transferred to liquid MS media supplemented with 1 mg l^{-1} NAA, 0.5 mg l^{-1} Kn, 0.5 mg l^{-1} 2,4-D and 15% coconut milk to produce aggregate cell suspension cultures under lightness. *L. mucronatum* roots regenerated from 27-day-old arrogated suspension cells. The regenerated root-like structures were transferred to $^1/_2$ B_5 liquid me-

dium for producing root cultures. Root cultures were established in 250 ml and 1000 ml flasks containing $^1/_2$ B_5 liquid medium. All root cultures were maintained under darkness at (25 ± 2) °C on a rotary shaker (80 rpm) and refreshed by new media every two weeks. All 1000 ml flask root cultures were maintained on a 130 rpm rotary shaker.

Root cultures of *L. nodiflorum* and *L. mucronatum* were used to examine the cell growth and production of lignans during a culture period of 36 d. 1 g fresh weight (FW) [*ca* 0.083 mg dry weight (DW)] of *L. nodiflorum* root culture and 2 g FW (*ca* 0.15 mg DW) of *L. mucronatum* root culture were inoculated in 50 ml medium in 250 ml flasks, separately. For determination of FW, roots were removed every 4 d from the medium and weighed immediately. DW was determined after lyophilization. pH value, conductivity and sugar content (determined by refractive index) were measured in the medium with the appropriate electrodes and a refractrometer, respectively.

General experimental procedures

UV spectra were measured on-line using a Thermo Quest (Egelsbach, Germany) HPLC system equipped with a Spectra System KO 6000 LP photodiode array detector. The column and the solvent system were as described below. NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) on a Bruker DRX500 spectrometer at room temperature in $CDCl_3$. The data are referenced to the solvent signals (7.270 ppm C*H*Cl_3, 77.20 ppm *C*DCl_3).

Extraction, isolation, and determination of MPTOX

Separation of MPTOX from *L. mucronatum* roots was performed using a Nucleosil 100-C18 column (8.0 mm i. d., 40 + 250 mm length, particle size 5 μm) and a gradient system with water (A) and acetonitrile (B) as eluents as follows: 0 to 17 min from 45 to 67% B, from 17 to 18 min to 50% B, and then 18 to 25 min back to 45% B. The flow rate was 3.0 ml/min between 0 and 9.0 min, 0.2 ml/min between 9.0 and 20.0 min, and again 3.0 ml/min between 20.0 and 25.0 min. MPTOX was collected at Rt 20.22 min.

For quantification of the lignans, fresh plant material was frozen and then lyophilized by freeze-drying. Extraction and determination of lignans were performed as described by Empt *et al.* (2000). HPLC analysis was performed using a similar C18 column (4.6 mm i. d.) and gradient system (flow rate 0.8 ml/min). The detector wavelength was 290 nm. Accuracy of each quantified lignan was proven by retention time, co-chromatography and measuring on-line UV spectra using a Thermo Quest HPLC system equipped with a Spectra System KO 6000 LP photodiode array detector. Isolated MPTOX was used for quantification.

Results and Discussion

Hairy root cultures of *L. Bungei*, *L. strictum* and *L. album* and root cultures of *L. mucronatum* ssp. *mucronatum* and *L. nodiflorum* were established in 1000 ml flasks. Chromatographic separation of MPTOX (Fig. 1) was performed by preparative HPLC from root extracts of *L. mucronatum*. All NMR spectroscopic data for MPTOX were in full agreement with reports (Broomhead and Dewick, 1990; van Uden *et al.*, 1992). Following 3 subcultures, MPTOX levels of *Linum* root cultures were investigated by HPLC (Table I). *L. Bungei* and *L. strictum* hairy root cultures failed to produce any levels of MPTOX.

Results of *L. nodiflorum* and *L. mucronatum* ssp. *mucronatum* root cultures are shown in Figs. 2 and 3, respectively. Fig. 2 shows that in root cultures of *L. nodiflorum*, within a culture period of 36 d, maximum formation of biomass (6.143 mg FW or 0.263 mg DW) was achieved after day 24 (Fig. 2A), and then declined until the end of the observation period. The highest MPTOX level was at day 16 with *ca* 480 mg/100 g DW (Fig. 2A). This correlated with the maximal uptake of sugar and ions observed until day 16 (Figs. 2B and C). The increase in conductivity, which indicated cell lyses at the end of the period, caused an increase in the pH value throughout the root cultivation period (Fig. 2D).

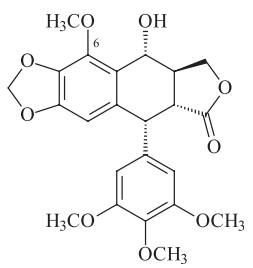

Fig. 1. Structure of 6-methoxypodophyllotoxin.

Table I. MPTOX levels of root cultures of *Linum* species.

Linum species	Culture[a]	Flask volume[b] [ml]	MPTOX level [mg/100 g DW]
L. album	HRC	1000	6
L. Bungei	HRC	1000	–
L. mucronatum ssp. *mucronatum*	RC	250	130
		1000	770
L. nodiflorum	RC	250	480
		1000	91
L. strictum	HRC	1000	–

[a] HRC, hairy root culture; RC, root culture.
[b] $^1/_2$ B_5 media.

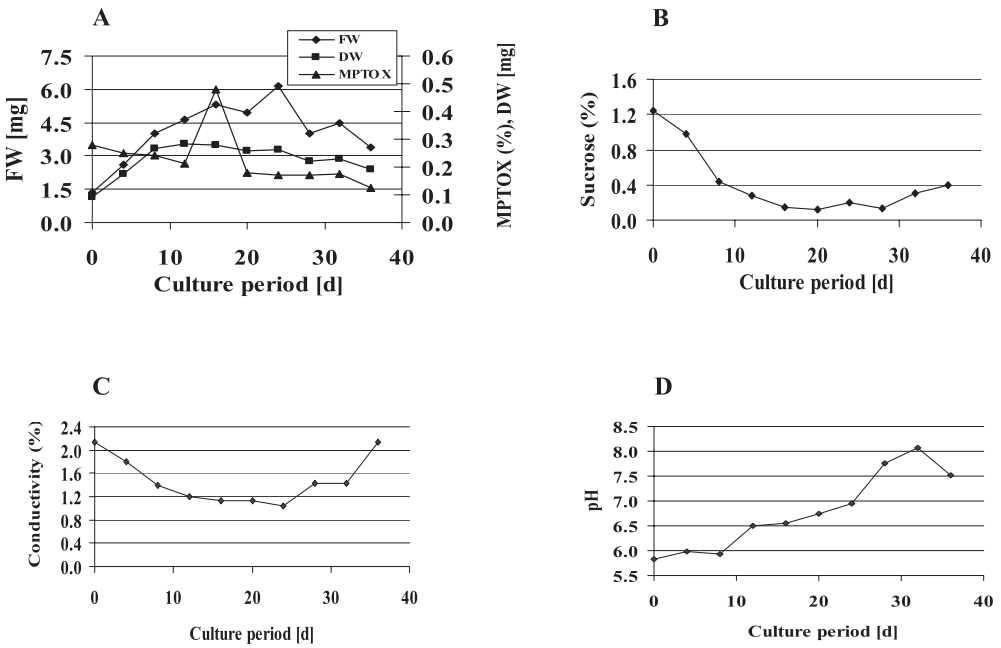

Fig. 2. Characterization of *L. nodiflorum* root culture; (A) MPTOX level (mg/100 g DW), fresh weight (FW) (mg), dry weight (DW) (mg); (B) sucrose (%); (C) conductivity (%); (D) pH value.

L. mucronatum ssp. *mucronatum* root culture was investigated for its growth characteristics and MPTOX accumulation over a cultivation period of 36 d. Maximal FW and DW were achieved after day 12 (Fig. 3A). Until this time most of sucrose as well as mineral ions were consumed (Figs. 3B and C). The increase in conductivity after day 32 caused an increase in the pH value (Fig. 3D). The maximum level of MPTOX was at day 12 with about 130 mg/100 g on DW basis. When growing, root cultures alter the culture medium by taking up essential nutrients and releasing substances into the medium. The change of medium was monitored by determination of the pH value and conductivity as the measure of all ionic components of the medium. Since the dark-grown root cultures can not produce their own carbohydrate by photosynthesis, the roots are essentially dependent on sugar added to the culture medium; in this case sucrose was at an initial level of 1.5% before autoclaving. The initial level of sucrose (Figs. 2B and 3B) is 1.2% which indicates that sucrose cleavage may be due to autoclave conditions. The sugar content of the medium was determined refractometrically. Therefore it doesn't indicate solely the sucrose content of medium, but the overall content of sugar plus other substances interfering with the refractometrically determination (Empt *et al.*, 2000).

The main lignan which is produced in roots, root cultures and hairy root cultures of *L. flavum* is MPTOX (Berlin *et al.*, 1986, 1988; Broomhead and

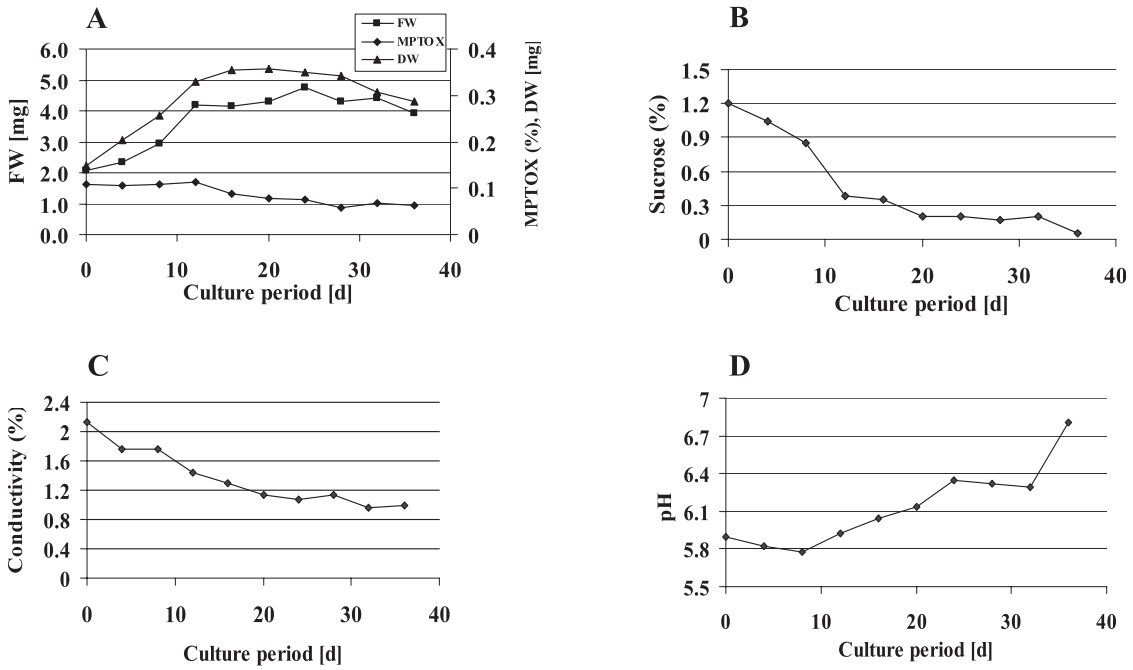

Fig. 3. Characterization of *L. mucronatum* ssp. *mucronatum* root culture; (A) MPTOX level (mg/100 g DW), fresh weight (FW) (mg), dry weight (DW) (mg); (B) sucrose (%); (C) conductivity (%); (D) pH value.

Dewick, 1990; van Uden *et al.*, 1991; Wichers *et al.* 1990). Recently, lignan contents of various organs of *L. persicum* and *L. mucronatum* ssp. *mucronatum* fresh plant materials were determined. The results of these two studies showed MPTOX accumulation in underground parts of both species (Mohagheghzadeh *et al.*, 2003, 2005). In this study, MPTOX profiles of *L. nodiflorum* and *L. mucronatum* ssp. *mucronatum* root cultures and *L. album* hairy root culture from section *Syllinum*, *L. strictum* from section *Linastrum* and *L. Bungei* hairy root cultures belonging to section *Dasyllinum* were determined (Table I). The results showed the accumulation of MPTOX as the main lignan in root cultures of *Linum* spp. belonging to the section *Syllinum*. The analysis of aerial parts of *L. album* and *L. persicum* showed that PTOX is major in the aerial parts of these two species (Mohagheghzadeh *et al.*, 2003), while MPTOX was major in the aerial parts of *L. mucronatum* and *L. flavum* (Mohagheghzadeh *et al.*, 2005; Wichers *et al.,* 1990). *L. album* and *L. persicum* are two examples of white-flower *Linum* species of the section *Syllinum* while *L. mucronatum* and *L. flavum* are yellow-flower *Linum* spp. of this section (Mohagheghzadeh *et al.*, 2003). Although their lignan contents in aerial parts are different, all *Linum* from this section accumulate MPTOX in their roots and root cultures as main lignan, whereas, *Linum* spp. belonging to other sections accumulate different types of lignans; *e.g. L. narbonense*, *L. austriacum* and *L. leonii* from the section *Linum* produce arylnaphthalene type lignans, including justicidin B and isojusticidin B (Mohagheghzadeh *et al.*, 2002; Vasilev and Ionkova, 2005). Therefore any aryltetralin lignans including MPTOX were not detected in root cultures of *L. strictum* from the section *Linastrum* and *L. Bungei* belonging to the section *Dasyllinum*. These results introduce root cultures of *Linum* spp. section *Syllinum* as the main accumulation site for MPTOX. The optimization of the medium composition and growth conditions will guide us to conditions to increase the production of MPTOX in these cultures.

L. nodiflorum normal root cultures scaling up from 250 ml to 1000 ml shaking flasks resulted in a considerable decrease in the accumulation of MPTOX from 480 to 91 mg/100 g DW (Table I). The higher level of MPTOX in root cultures of 1000 ml flasks of *L. mucronatum* (770 mg/100 g DW) compared to 250 ml flask samples may be

partially due to the formation of thick and well organized root-like organs accumulating MPTOX in the bigger flasks. Such organs wouldn't be established readily during a 36 days period of time in 250 ml flasks (130 mg/100 g DW). With the production of hairy root cultures of *Linum* spp. section *Syllinum* in large scale bioreactors (Wink *et al.*, 2005) we may get large amounts of MPTOX. On the other hand, production of PTOX by plant cell cultures is still insufficient and expensive (Fuss, 2003). Since MPTOX has a cytotoxic potency comparable with PTOX (van Uden *et al.*, 1992) it may be used as a precursor for the production of anticancer agents, and therefore it can be used as an alternative agent for PTOX. Above all, root cultures, normal as well as transformed, provide us with a good experimental system which can be used to elucidate enzymatic pathways, identify key intermediates and other aspects of the biosynthesis of MPTOX in *Linum* spp.

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