Podophyllotoxin and 6-methoxy podophyllotoxin Production in Hairy Root Cultures of *Liunm mucronatum* ssp. mucronatum

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ABSTRACT

Aim: Two bacterial strains of *Agrobacterium rhizogenes*, A13 and 9534 were evaluated for induction of transformed hairy roots in *Linum mucronatum* ssp. mucronatum, a high value medicinal plant. **Materials and Methods:** The hairy roots were successfully initiated, through infecting the hypocotyl and root explants and the A13 strain performed a high transformation frequency for hairy roots induction. Transgenic status of hairy roots was confi rmed by polymerase chain reaction (PCR) analysis of the *rol* genes. Growth kinetics of transgenic roots induced by two strains indicated a similar pattern of growth, with maximum growth occurring between 42 to 56 days. The lignan contents in hairy roots were analyzed using high-performance liquid chromatography (HPLC) method. **Results:** Transformed cultures showed signifi cant differences ($P < 0.05$) in lignan content. The highest amount of Podophyllotoxin (PTOX, 5.78 mg/g DW) and 6-methoxy podophyllotoxin (MPTOX, 49.19 mg/g DW) was found in transformed lines induced by strain A13, which was four times higher than those of non-transformed roots. The results showed that hairy root cultures of *L. mucronatum* are rich sources of MPTOX. **Conclusion:** hairy root cultures from *L. mucronatum* can be used as a useful system for scale-up producing MPTOX and precursors for the production of antitumor agents in substitution with PTOX by considering the appropriate optimizations in future studies.

Key words: *Agrobacterium rhizogenes*, hairy roots, *linum mucronatum*, podophyllotoxin, polymerase chain reaction, 6-methoxy podophyllotoxin

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INTRODUCTION

Roots of numerous plant families are the site for biosynthesis or accumulation of major secondary metabolites including alkaloids, polyacetylene, sesquiterpenes and lignans. Multiple uses of these plants species have potential to use in large scale as raw material in the pharmaceutical industry, leading to over exploitation and disappearing in the wild. Hence, alternative sources have to be sought to meet the demand. *In vitro* root culture has become an alternative method for the production of valuable secondary metabolites on commercial scale. Adventitious roots induced by *in vitro* methods showed high rate of proliferation and active secondary metabolism.[1] The development of genetically transformed plant tissue cultures and mainly of roots transformed by *A. rhizogenes* (hairy roots), is a key step in the use of *in vitro* cultures for the production of secondary metabolites.[2] Hairy roots are natural, grow vigorously in plant hormone free medium and have shown tremendous potential of accumulation of valuable secondary metabolites. They exhibit a comparable or even greater level of metabolites production within shorter time than is commonly expected from naturally grown plants.[3,4] The hairy roots offer a valuable source of root derived phytochemicals that are useful as pharmaceuticals, cosmetics and food additives. Hairy roots represent an organ culture system with stable genetic and biochemical properties since biosynthetic pathway are also controlled by tissue specific gene expression. Whereas many pathways are switched off in undifferentiated cell suspension cultures, these pathways are active in organized tissue cultures. There is only one report concerning the production of podophyllotoxin (POTX) and 6-mrthoxypodophyllotoxin (MPTX) in callus and suspension cultures of *L. mucronatum* ssp. armenum[5] and to

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our knowledge, there are no publications about the lignans in hairy root cultures of this species (ssp. mucronatum).

Successful induction of hairy root has been reported in some *Linum* spp. such as *L. flavum,*[6] *L. austriacum,*[7] *L. tauricum*[8] and main metabolites (lignans) from resulted hairy roots have been shown to have biological activity.

MATERIALS AND METHODS

Chemicals

PTOX standard was gifted by Prof. Randolph Arroo from Leicester School of Pharmacy De Montfort University, German; β-glucosidase enzyme was purchased from Sigma (USA); MS medium culture from Duchefa (The Netherlands) agar and solvents were from Merck.

Seed germination and explant preparation

L. mucronatum seeds were collected from mountain region around the city of Tabriz, West Azerbaijan Province, Iran, at an altitude of 1800m. The seeds were thoroughly washed under running tap water for 15 min and surface-sterilized by immersing in 70% (*v/v*) ethanol for 1 min and in 2% (*v/v*) solution of sodium hypochlorite (commercial bleach) for 10 min. Finally, sterilized seeds were immediately rinsed with sterile distilled water for 10 min to wash out the sterilization agents before placing onto glass vessels containing 7.5g/l water-agar for germination. The cultures were maintained in a growth chamber at 24 ± 2°C with a photoperiod of 16h light and 8h dark under light intensity of 40μmol/ms). The 4-week-old germinated seedlings were used as explants source. Different explants including cotyledon, hypocotyl (including cotyledonary node) and root were taken from the seedlings.

Bacterial strains and hairy root induction

Two wild-type strains 'A13' and '9534' of *A. rhizogenes*, were used in the transformation, which was kindly provided by the National Institute of Genetic Engineering and Biotechnology, Iran. For prepare of bacterial suspension for each strain, a single bacterial colony was cultured into liquid LB medium[9] supplemented with 50 mg/l rifampicin and maintained at 28°C for 48h on a rotary shaker at 200rpm speed. The overnight grown bacterial culture was centrifuged at 3,500rpm for 10 min and the bacterial pellet was re-suspended in liquid MS medium,[10] pH 5.5. Final Density of bacterial suspension was diluted with LB medium to 0.4-0.6 OD (optical density at 600nm) before using for the infection. The isolated explants were cut into small pieces of about 5-10 mm and then were submerged in the bacterial suspension for 5 min with occasional shaking. The explants were blotted on a sterile filter paper to remove the excess bacterial suspension. Inoculated explants were transferred to agar (7.5g/l) solidified hormone-free MS medium for co-cultivation under dark incubation at 24 ± 2°C for 2 days. As a control, a few explants were immersed in sterile distilled water and were cultured the same way. After 2 days, the explants were rinsed with sterile water to remove the bacteria from the surface and then transferred to MS medium supplemented with 500 mg/l of cefotaxime, 30g/l sucrose and 6g/l agar. The concentration of cefotaxime was gradually reduced in the subsequent sub-cultures and finally was completely omitted after 3rd sub-culture.

Establishment of hairy roots

Numerous hairy roots emerged from the wound sites of explants. The hairy roots were separated from the ex- plant tissue and sub-cultured on agar-solidified hormone-free MS medium and maintained in growth chamber at 24 ± 2°C in the dark. Rapidly growing hairy roots were transferred to 30 ml of MS liquid medium, containing 30g/l sucrose, in 100 ml Erlenmeyer flasks on a rotary shaker (110 rpm) at 24 ± 2°C in the dark and sub-culturing was carried out after every 14 days in the same medium for proliferation of hairy roots[8] [Figure 1].

Polymerase chain reaction analysis for hairy roots

Genomic DNA was extracted from both hairy roots and untransformed roots (control) by cetyltrimethylammonium bromide (CTAB) method.[11] For confirmation of the transgenic nature of hairy roots, the presence of the *rol* genes located on the T-DNA which are main determinants for the development of hairy roots were examined by polymerase chain reaction (PCR) analysis using corresponding gene-specific primer pairs. The Ri plasmid of *A. rhizogenes* strains 'A13 and 9534' was used as a positive control. The primer sequences to amplify a 1,794-bp portion of the *rol*A-B genes were 5'-CAGTTTCGCATCTTGACAG-3' and 5'- GTTCTCGCGAGAAGATGCA-3'. The primers 5'-ATGCCCGATCGAGCTCAAGT-3′ and 5′-CCTGACCCAAACATCTCGGCTGCCCA-3' were designed based on the *vir*D gene from the not-transferred virulence region of the *A. rhizogenes* Ri plasmid. The PCR reaction conditions were as follows: Initial denaturation for 5 min at 94°C, followed by 35 cycles consisted of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, extension for 1 min at 72°C and a further extension step for 10 min at 72°C. Amplified DNAs were analyzed by staining with ethidium bromide after electrophoresis in a 0.8% (*w/v*) agarose gel at 80 V for 90 min.

Growth measurements

Hairy root lines were transferred to a 250-ml Erlenmeyer flask containing 50 ml of hormone-free MS liquid medium for period of 10 weeks. Hairy root line growth was measured every 2 weeks.

Figure 1: Hairy roots induced on different sites of hypocotyl and root explants in *L. mucronatum* one week after inoculation by A. rhizogenes strain 'A13'. (a) The emergence of hairy root from hypocotyl explant. (b) induction of the thin hairy roots from root explants (c-e) Variation in the number and the site of emergence of the hairy roots. (f) Close view of a hairy root. (g and h) Well development of hairy roots after 2nd sub-culture into the same medium, red arrows indicate sites of induction of hairy roots

Extraction and isolation of lignans

Lignans were extracted from powdered plant cell material (200 mg) with MeOH (2 ml). The mixture was homogenized in an ultrasonic bath (2 × 30 s) with intermediate cooling on ice. Distilled water (6 ml) was added and the pH was adjusted to 5.0 with 5% phosphoric acid. After adding β-glucosidase (1 mg) the sample was incubated at 35°C for 1h in a water bath. MeOH (12 ml) was added and the mixture was incubated for another 10 min at 70°C in an ultrasonic bath. After centrifugation for 7 min at 4500rpm, the volume of supernatant was determined. One milliliter of the supernatant was taken and centrifuged at 13,000rpm for 5 min at 25°C. This final solution was used for high performance liquid chromatography (HPLC) analysis.[8]

HPLC analysis

HPLC determination was performed on a Thermo Quest (Agident, USA) equipped with a Spectra SYSTEM UV6000LP detector. The separation column was a GROM-SIL 120 ODS-5 ST (250 × 4.6 mm, particle size 3.5μm). The gradient system was water with 0.01% phosphoric acid (85%0 (A) and acetonitrile (B) as follows: From 0 to 17 min from 40% to 67% B, from 17 to 18 min back to 40% B. The flow rate was 0.8 ml/min between 0 and 17 min, 1 ml/min between 17 and 18 min, and again 0.8 ml/min after 24 min; detection was performed at 290nm against reference standard. The lignans were identified by comparison of the retention time with authentic Standards. The retention time for PTOX and 6MPOX is about 6.76 and 9.18 min respectively. The detector wavelength was 290nm.

Statistical analysis

Data for root weight were set up in a completely randomized design (CRD) with three replicates per treatment. Data were subjected to the analysis of variance using SAS computer package[12] and means differing significantly were compared using Fisher's least significant difference[13] test at a 5% probability level.

RESULTS AND DISCUSSION

Establishment of hairy root cultures

Hairy root cultures of *L. mucronatum* were initiated by inoculation of root and hypocotyl explants with two strains, A13 and 9534, of *A. rhizogenes*. These roots exhibited characteristics typical of transformed roots, that is rapid growth, extensive lateral branch, root covered with a mass

of tiny root hairs [Figure 1] and the lack of geotropism.[14] The hairy roots had a suitable growth and prolific root development was evident after 8 weeks [Figure 1]. Whereas no adventitious roots formed from the control explants. Also, Agrobacterium-inoculated cotyledon explants did not show induction of roots (data not shown). Hypocotyl explants were highly susceptible to infection by strain 'A13' of *A. rhizogenes*, as shown by the percentage (60%) of them from which hairy roots emerged whereas, root explants exhibited thin hairy roots and the lowest infection frequency (20%) [Figure 1]. Also there was a variation in the site of emergence and growth of hairy roots [Figure 1] and in the number of hairy roots in per explants [Figure 1].

Detection of relevant transgenes in the selected hairy root lines

PCR method can be used simply for detecting T-DNA sequences in putative transformants.[15] In this study, the presence of the *rolA and rolB* genes in the hairy root lines was confirmed by PCR analysis [Figure 2] using, primers specific for core sequence located between the *rol*A and *rol*B genes. Genomic DNA was extracted and purified from both transformed and un-transformed roots grown in Murashige and Skoog (MS) media. In the selected transgenic hairy root lines, a sharp band of 1794-bp was amplified, but no such amplicon was observed in the untransformed root (negative control) sample [Figure 2]. The *vir*D gene, located outside the T-DNA, is diagnostic for the presence of any remaining agro-bacteria in the root tissue The negative results of PCR amplification for the *vir*D gene demonstrated that no bacterial DNA was involved in *rol*B amplification leading to false positives [Figure 2] This result indicated that *L. mucronatum* is susceptible for transformation with *A. rhizogenes* strain 'A13' and '9534' the roots and hypocotyls explants respond quite efficiently to transformation by *A. rhizogenes*.

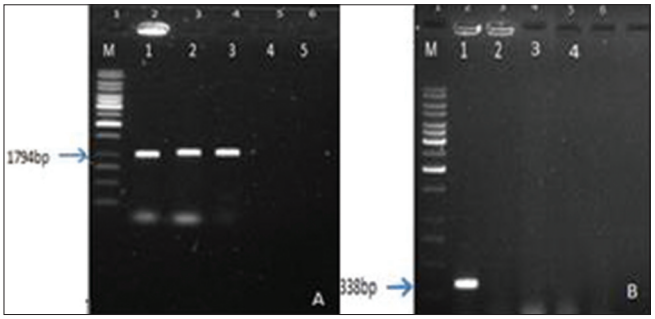

Figure 2: PCR analysis for hairy roots lines of *L. mucronatum*. (a) PCR analysis using the *rolA* and *rolB* genes specific primers. M: 1 Kb DNA Ladder (Fermantas), 1: Ri plasmid from *A. rhizogenes* strain 'A13' as a positive control, 2 to 3: Transgenic hairy roots induced on hypocotyl and root ex-plants infected by Agrobacterium, 4: Wild plant root as first negative control, 5: Non-DNA template PCR reaction as second negative control. (b) PCR analysis using virD genes specific primers. M: 1 Kb DNA Ladder (Fermantas), 1: Ri plasmid from *A. rhizogenes* strain 'A13' as a positive control, 2, 3: Transgenic hairy roots induced on hypocotyl and root explants infected by Agrobacterium, 4: Wild plant root as first negative control, 5: Non-DNA template PCR reaction as second negative control

Effects of bacterial strains

Two different *A. rhizogenes* strains: A13 and 9534 were investigated to evaluate their ability of transformation and lignan production of *L. mucronatum* ssp. mucronatum, regarding the differential efficiency of various *A. rhizogenes* strains in promoting, induction, growth and secondary metabolite production of hairy roots.[16] In *L. flavum*, hairy roots were initiated from leaf discs with a success rate of approximately 50% using *A. rhizogenes* strains 'LBA9402'and 'TR105'.[6] Evidently, the selection of an effective bacterial strain for the production of transformed root cultures is highly dependent on the plant species, and must be determined empirically.[17] The bacterial strains used in this study exhibit different levels of virulence to this plant species and the induction of hairy root occurred at different time depended on strains. Also the percentage of responsive explants and numbers of hairy root per explants was varied in each inoculation. The results of this study showed that the highest number of hairy root per explants was 3 and 15 roots induced by 9534 and A13 strains, respectively. Although, the ability of hairy root induction and frequency of transformation varied with respect to the type of explants. A morphological variation between the hairy roots emerged from the explants was also seen, as the hairy roots induced on root explants were thin and little branched [Figure 1]. This could be due to slight differences in wound-induced phytohormone production or to differences in *rol* genes expression.[18] In this study, explants infected by A13 strain, formed callus tissue and shoot regeneration shoot regeneration was also observed [Figure 3].

The time period required for hairy root induction and frequency of transformation was varied with respect to the age and type of explants and also the strain of bacteria. Hypocotyl and root explants 4 to 5 weeks old *in vitro* grown seedlings were only the responsive explants for hairy root induction and exhibited 80% and 50% transformation frequency after 15 and 22 days of bacterial infection by A13 strain and transformation rate of 25% and 9% after 23 and 27 days by 9534 strain of *A. rhizogenes*. Young explants showed higher transformation frequency (80 and 25%)

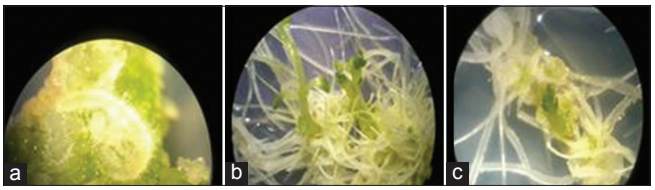

Figure 3: (a) Callus formation in explants inoculated with bacteria. (b-c) Seedling growth from explants inoculated

which decreased with an increase in explants age. Also wild type *A. rhizogenes*, 9534, shows more resistance for hairy induction in *L. mucronatum*, and production of aryltetralin lignans in this hairy root clones was lowly, compared with hairy root clones, induced by A13 [Table 1].

We also studied correlation between the time required for inoculation and the age of explants. The results showed that the time required for inoculation was increased with a decrease in explants age. In the other hand, increase the age of plants reduced their resistance to the bacteria. The type and age of explants have a great influence on hairy root induction since age of explants is a major factor that alters the physiological properties of the cell.[19] Besides, explants age is also an important factor that effects on further hairy root growth and even in production of transgenic production.[20]

Growth measurement

A time course experiment (10 weeks) was performed to monitor root growth and biomass production in 250 ml flasks. The hairy root growth rate is generally high, but great variation exists from one line to another especially among the hairy roots of different strains. Mean doubling time after inoculation was recorded 3 and 5 days for A13 and 9534 strains, respectively. A mean doubling time ranges from 1-4 days in *Linum tauricum* hairy root has been reported previously,[21] however a longer doubling time was also reported in other plant species. Doubling time of 6 and 15 days have been reported in *Cinchona* hairy roots[22] and *Galphimia glauca,* respectively.[23] In the present study, the highest hairy root growth was recorded after third subculture [Figure 4]. This means that the linear extension roots tips and exponential growth of hairy roots to form lateral roots and maximal increasing in biomass in *L. mucronatum* hairy roots nearly was taken two month [Figure 5]. In contrast, untransformed roots did not branch and they had slow growth. Also Growth kinetics of hairy roots induced by 2 strains, showed a similar pattern of growth, with maximum growth occurring between 42 to 56 days [Figure 4]. However, a short period of time (17 to 20 days) has been reported to maximum growth rate of hairy roots in *Linum tauricum*.[8]

Table 1: Content of Podophyllotoxin and 6-methoxypodophyllotoxin in the hairy root lines of *L. mucronatum*

No	The strains of the *A. rhizogene*	Podophyllotoxin (mg/gr DW[a] ± SD[b])	6-methoxy Podophyllotoxin (mg/gr DW ± SD)
1	A13	5.55 ± 0.76	41.38 ± 1.74
2	9534	4.96 ± 1.18	37.13 ± 1.15
3	Control Plants	1.06 ± 0.53	6.74 ± 0.79

[a] mg/gr DW Miligram/gram dry weight [b] SD standard deviation

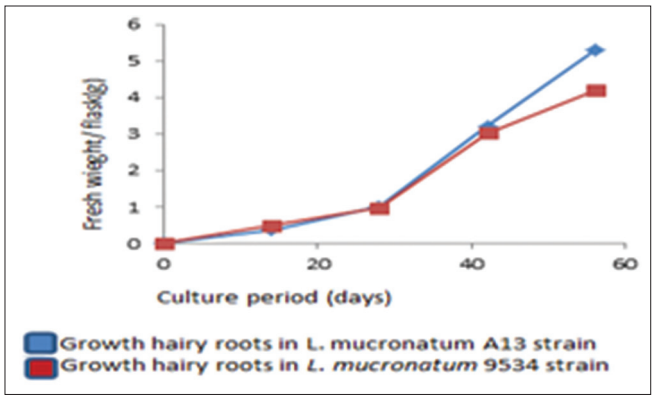

Figure 4: Growth of hairy roots culture of *L. mucronatum* over a cultivation period of 56 days in MS liquid medium. Each value is the mean of 3 replications ± SD

Production of PTOX and MPTOX in hairy roots

The natural lignan podophyllotoxin, a dimerized product of two phenylpropanoid moieties which occurs in a few plant species, is a pharmacologically important compound for its anti-cancer activities. It is used as a precursor for the chemical synthesis of the anticancer drugs etoposide, teniposide and etopophose. These compounds have been used for the treatment of lung and testicular cancers as well as certain leukemias [Figure 6].[24] In the present study, hairy root cultures induced of *L. mucronatum* were used for determining the accumulation of PTOX and MPTOX. Since, they are the 2 main lignan constituents of species belonging to *Linum* section *Syllinum* (Linaceae). The maximum concentrations of PTOX (5.78 mg/g DW), and MPTOX (43.19 mg/g DW) were recorded in hairy roots induced by strain A13 [Table 1]. While in untransformed roots, concentrations of PTOX and were 1.06 mg/g DW and 6.74 mg/g DW, respectively. In general, the amount of cytotoxic lignans (PTOX and MPTOX) in intact plants is considerably less than the hairy root lines.

The accumulation of PTOX and its congener lignans in fresh plant organs of *L. mucronatum* ssp. mucronatum (Linaceae) have been reported by other workers.[25] And the highest amounts of PTOX (0.595 ± 0.060%g/g DW) and

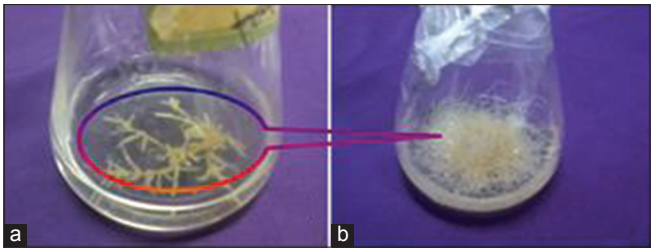

Figure 5: (a) Hairy roots before growth measuring. (b) Growth of the hairy roots after fifth subculture

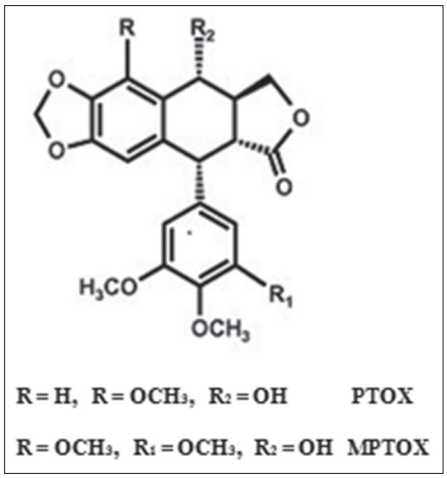

Figure 6: Chemical structure of isolated ariltetralin lignans

MPTOX (1.491 ± 0.125%g/g DW) were found in the plant sexual organs. Also previous studies, lignan contents of various organs of *L. mucronatum* ssp. mucronatum and hairy roots of the *L. flavum* species from the section *syllinum* were determined.[25] The results of these studies showed the accumulation of MPTOX as the main lignan in hairy root and root cultures of *Linum* spp.[26] *L. mucronatum* and *L. flavum* are yellow-flower *Linum* spp. of this section, while 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*. The root cultures of *Linum* spp. section *Syllinum* has been introduced as the main accumulation site for MPTOX.[25] Since MPTOX has a cytotoxic potency comparable with PTOX, it may be used as a precursor for the production of anticancer agents, in substitution with PTOX. In the present study, MPTOX was predominant metabolite accumulated in hairy root cultures (as mentioned above). So, hairy root lines obtained from this study can be the excellence organs for biosynthesis/accumulation of this metabolite.

CONCLUSION

The present study emphasizes the feasible production of high value medicinal compounds *i.e.* (PTOX and MPTOX) through hairy root cultures of *L. mucronatum* ssp. mucronatum. The results of present study demonstrated that hypocotyl and root explants isolated from 4 to 5-week-old seedlings were suitable explants to induce *L. mucronatum* hairy roots, and strain 'A13' of *A. rhizogenes* has a potentiality to be an effective means of inducing hairy root formation and especially for higher level of PTOX and MPTOX production in this plant species. The availability of this protocol for the production of PTOX and its related lignans provides a powerful system to study various aspects of the metabolic and molecular regulation of lignan biosynthesis in *L. mucronatum*. Finally, It is necessary to evaluate and screen the effects of various elicitors with different mechanisms on the production and accumulation of PTOX for possible the production on a commercial scale in the future investigations.

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