

Hairy Root Induction in *Linum mucronatum* ssp. *mucronatum*, an Anti-Tumor Lignans Producing Plant

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Abstract

Transgenic hairy root system is a promising source of secondary metabolites in medicinal plants with high pharmaceutical value. For the first time, hairy roots were established in different explants of *Linum mucronatum*, an anti-cancer agent producing plant, via a mikimopine type strain of *Agrobacterium rhizogenes*, 'A13'. The percentage of hairy root induction varied from 0 to 60% depended on the explants and hypocotyl (including cotyledonary node) explants were found to be highly susceptible to *A. rhizogenes* infection with the highest (60%) rate of hairy root induction. four different Murashige and Skoog (MS)-based liquid culture media were used for well establishment of hairy roots. Hairy root growth medium D (HRGM-D) containing hormone-free MS basal medium with an extra one day pre-incubation period at 35°C was found to be more efficient for profuse growth (fresh weight; 8500 mg per 25 ml culture medium) of hairy roots. Hairy root system presented in this study may offer a suitable platform for optimization and production of satisfactory level of aryltetralin lignans like podophyllotoxin and its derivatives from *L. mucronatum*.

Keywords: *Agrobacterium rhizogenes*, explant, growth medium, medicinal plants, secondary metabolites

Introduction

Linum spp. from section *Syllinum* are promising for the production of aryltetralin lignans like podophyllotoxin (PTOX) and 6-methoxypodophyllotoxin (6-MPTOX) (Mohagheghzadeh *et al.*, 2007). PTOX is the main lignan in the cell cultures of *Linum album* and 6-MPTOX is predominantly accumulated in cell lines of *L. flavum*, *L. nodiflorum*, *L. mucronatum*, and *L. tauricum*. This lignan and its derivatives possess anti-cancer properties (Ionkova, 2007). The semi-synthetic derivatives of this compound like etoposide, etophos, and teniposide are used clinically as chemotherapeutic agents for a variety of tumors, including small cell lung carcinoma, testicular cancer, and malignant lymphoma (Ionkova *et al.*, 2010). *Agrobacterium rhizogenes* mediated transformation system was found to be very useful for hairy root induction and production of phytochemicals (Choi *et al.*, 2000; Veena and Taylor 2007). Transformed roots of many plant species have been widely studied for the *in vitro* production of secondary metabolites (Christensen and Muller, 2009; Mukundan *et al.*, 1998). Hairy roots are genetically stable and not repressed during the growth phase of its culture (Bourgaud *et al.*, 1999). The greatest advantage of hairy roots is that their cultures often exhibit approximately the same or greater biosynthetic capacity for secondary metabolite

production as compared to their mother plants (Kim *et al.*, 2002). They can be a promising source for the continuous and standardized production of secondary metabolites under controlled conditions without losing genetic or biosynthetic stability (Giri and Narasu, 2000). They would be the best choice for metabolic engineering of the secondary metabolite pathways to enhance the accumulation and secretion of high value metabolites (Arroo *et al.*, 2002). Even in cases where secondary metabolites accumulate only in the aerial part of an intact plant, hairy root cultures have been shown to accumulate the metabolites (Bakkali *et al.*, 1997; Wheathers *et al.*, 2005). Successful induction of hairy root has been reported in some *Linum* spp. such as *L. flavum* (Lin *et al.*, 2003; Oostdam *et al.*, 1993), *L. austriacum* (Mohagheghzadeh *et al.*, 2002), *L. leonii* (Vasilev *et al.*, 2006) and *L. tauricum* (Ionkova and Fuss, 2009) and main metabolites (Lignans) from resulted hairy roots have been shown to have biological activity. Based on the scientific literature currently available, there is no publication on the hairy root induction in *L. mucronatum*. Therefore, in this study, successful hairy root production of this species was achieved by *Agrobacterium rhizogenes* mediated transformation. In addition, the effects of culture media were also evaluated for the growth enhancement of hairy roots.

Materials and methods

Seed germination and explant preparation

L. mucronatum seeds were collected from mountain region around the city of Tabriz, West Azerbaijan Province, Iran. 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 7.5 g/l water-agar for germination. The cultures were maintained in a growth chamber at 24±2°C with a photoperiod of 16 h light and 8 h dark under light intensity of 40 µmol/ms). The 4-week-old germinated seedlings were used as explant source. Different explants including cotyledon, hypocotyl (including cotyledonary node) and root were taken from the seedlings.

Bacterial strain and plant transformation

A mikimopine producing wild-type strain 'A13' of *Agrobacterium rhizogenes*, was used in the transformation, which was kindly provided by the National Institute of Genetic Engineering and Biotechnology, Iran. A single bacterial colony was cultured into liquid LB medium (Bertani, 1952) supplemented with 50 mg/l rifampicin and maintained at 28°C for 48 h on a rotary shaker at 200 rpm speed. Transformation procedure was done as described previously (Jafari et al., 2009). The overnight grown bacterial culture was centrifuged at 3,500 rpm for 10 min and the bacterial pellet was re-suspended in liquid MS medium (Murashige and Skoog, 1962), pH 5.5. Final Density of bacterial suspension was diluted with LB medium to 0.4-0.6 OD (optical density at 600 nm) 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 (7.5 g/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, 30 g/l sucrose and 6 g/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 30 g/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 (Mohagheghzadeh et al., 2002) (Fig. 1).

Polymerase chain reaction analysis for hairy roots

Genomic DNA was extracted from both hairy roots and untransformed roots (control) by CTAB method (Khan et al., 2007). 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* strain 'A13' was used as a positive control. The primer sequences to amplify a 1,794-bp portion of the *rolA-B* genes were 5'-CAGTTTCGCATCTTGACAG-3' and 5'-GT-TCTCGCGAGAAGATGCA-3'. 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.

Treatment of hairy roots growth in liquid media

Two to three weeks after the emergence of hairy roots, some of them were transferred to a fresh liquid MS basal medium including 3% sucrose namely Hairy Root Growth Medium A (HRGM-A) as well as to three other media including HRGM-B, HRGM-C and HRGM-D containing MS basal medium supplemented with 0.2 mg/l α -naphthalene acetic acid (NAA), hormone free MS medium with 2% sucrose, and the same as HRGM-A with pre-incubation at 35°C for 24 h, respectively. All cultures were incubated in 100 ml Erlenmeyer flasks on a rotary shaker (110 rpm) at 24±2°C in the dark and after 2 weeks of incubation, the extent of development of hairy roots in terms of fresh weight (FW, mg/25 ml culture medium) was assessed.

Statistical analysis

Data for root weight were collected from four growth media treatments, each of them were set up in a completely randomized design (CRD) with three replicates per treatment. Data were subjected to the analysis of variance (ANOVA) using SAS computer package (SAS Institute Inc., 2004) and means differing significantly were compared using Fisher's least significant difference (Fisher, 1954) test at a 5% probability level.

Results and discussion

Induction of hairy root

Transgenic hairy root system is of great importance, particularly in several economically important plants, like *L. mucronatum*, where transgenic plants are difficult to achieve. This investigation was able to establish hairy roots in this medicinal plant using *A. rhizogenes* strain 'A13'. Hairy root cultures were initiated from different inoculation sites (Fig. 1A-D) of used explants isolated from young seedlings of *L. mucronatum* after 2-3 weeks infection. The hairy roots had a suitable growth and prolific root development was evident after 8 weeks (Fig. 1E), 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 the lowest infection frequency (20%). We did not find any hairy roots from cotyledonary leaves. The results indicated that wild type strain of *A. rhizogenes* used in this study were able to im-

pressively induce hairy roots on hypocotyl explants that can be used for mass production of hairy roots in *L. mucronatum*. In general the variation in hairy root induction could possibly be attributed to the variation in virulence of different *Agrobacterium* strains as well as to plant species and type of explant used in transformation (Porter, 1991). In *L. flavum*, hairy roots were initiated from leaf discs with a success rate of approximately 50% using *A. rhizogenes* strains, 'LBA9402' and 'TR105'. In contrast, very low hairy root induction rates were obtained with strains '15834' and 'A4' (Lin et al., 2003). Evidently, the selection of an effective *Agrobacterium* strain for the production of transformed root cultures is highly dependent on the plant species, and must be determined empirically (Lee et al., 2010). Therewith, the results of the present study with consistent to previous research showed definite role of explant type in efficient transformation rate and prolific hairy root induction.

Successful production of hairy roots via wild type strain 'A13' of *A. rhizogenes* has been also reported in several medicinal plant species by other workers. For instance, the epicotyl explants derived from mature embryo axis of groundnut were infected with strain 'A13' of *A. rhizogenes*

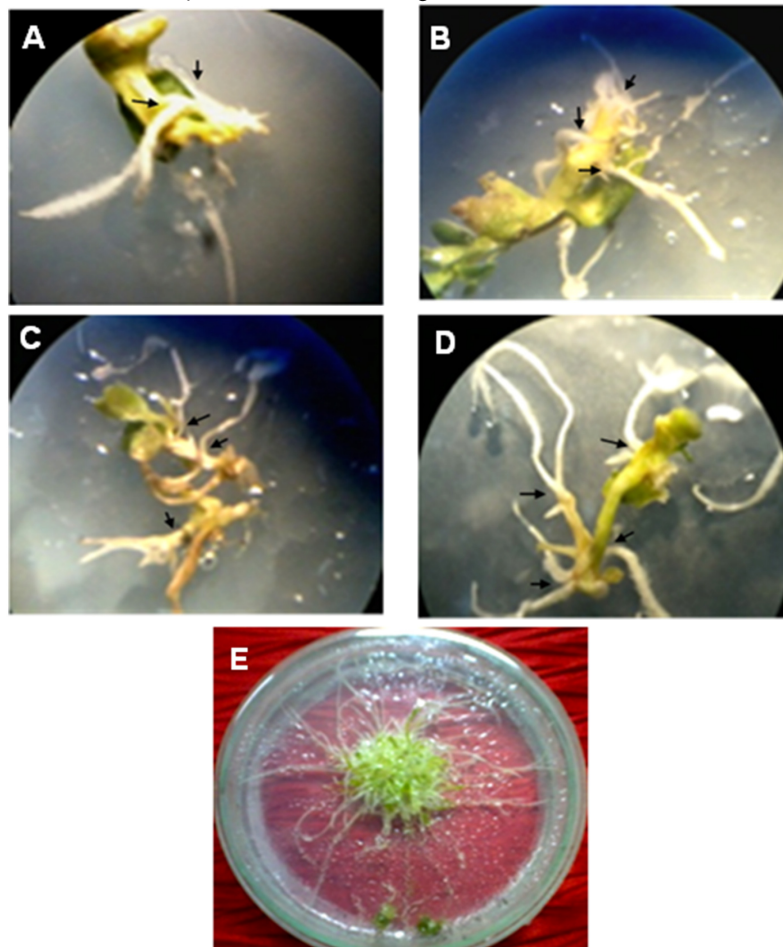


Fig. 1. Hairy roots induced on different sites of hypocotyl explants in *L. mucronatum* one week after inoculation by *A. rhizogenes* strain 'A13'. A-D: initiation and growth of hairy roots in phytohormone-free MS medium, Black arrows indicate sites of induction of hairy root, E: well development of hairy roots after 2nd sub-culture into the same medium

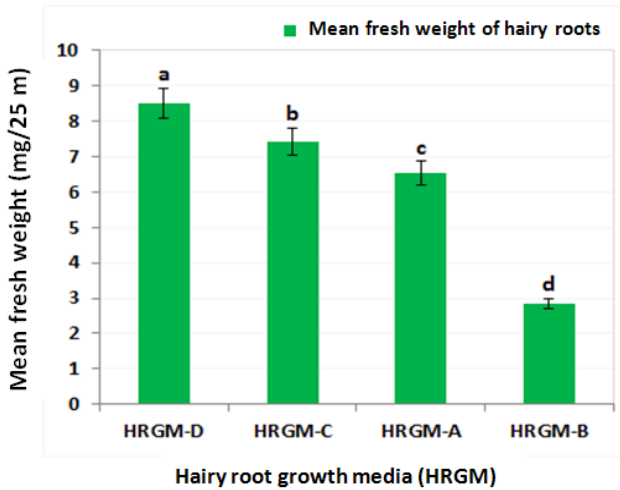


Fig. 2. Effects of various culture media on the growth of transgenic hairy root lines produced by *A. rhizogenes* strain 'A13' in *L. mucronatum*. The bars represent means \pm SE. Bars followed by different letters are significantly different ($p < 0.05$) according to FLSD

produces hairy roots (Akasaka *et al.*, 1998). Ohara *et al.* (2000) reported hairy roots induction from leaf segments of *Crotalaria juncea*, by infection with a mikimopine type wild strain 'A13' of *A. rhizogenes*. Regeneration of plants from hairy roots induced by strain 'A13' of *A. rhizogenes* is well established and has been reported in different medicinal plants as well (Akutsu *et al.*, 2004; Fukuda *et al.*, 2007; Godo *et al.*, 1997; Handa, 1992; Ishizaki *et al.*, 2002; Koike *et al.*, 2003, Ohara *et al.*, 2000).

Effect of culture media in hairy root growth

The secondary metabolite synthesis in hairy roots is influenced by nutritional and environmental factors. Exogenous growth phytohormone, the sucrose level, the nature of nitrogen source and their relative amounts, light, temperature and the presence of chemicals can all affect the growth and total biomass yield and secondary metabolite production. Optimization of these components would enhance the production of desired secondary me-

tabolites. There are several reports indicating that sucrose and/or auxin supplementation could stimulate higher levels of hairy root production (Nilsson and Olsson, 1997; Sato *et al.*, 1991; Yoshikawa and Furuya, 1987). In this study, rapidly growing hairy roots were cultured in four different liquid media for 21 days. The rate of proliferation showed significant ($p < 0.05$) difference between the media in terms of hairy root fresh weight (Fig. 2), since the fresh weight of the hairy root cultures increased from low level of the original inoculum to 2.8-8.5 g/25 ml varied between HRGM media in a 3 weeks culture period. The results showed that HRGM-D was the best medium for well improvement of hairy root growth (8500 mg/25 ml fresh weight). The superior effect of a pre-incubation at 35°C on hairy root growth is shown in Fig. 3. In contrast, the hairy roots cultured on auxin (0.2 g/l NAA)-supplemented medium (HRGM-B) did not grow well and had the lowest (2800 mg/25 ml) level of fresh weight compared with the other media. Susumu *et al.* (1996) also reported the little effect of auxins on hairy root growth. However, it was demonstrated more recently that when testing systematically the effect of different types of phytohormones upon root growth and secondary metabolite production, some of them could enhance either growth or metabolites production. In the case of *Artemisia annua* hairy roots (Wheathers *et al.*, 2005), the response of cultures to five types of hormones: auxins, cytokinins, ethylene, gibberellins (GA) and abscisic acid (ABA) was evaluated. The highest biomass was obtained when 1-5 mg/l ABA was supplied in the medium, while 0.5-1 mg/l 2-isopentenyladenine (ipt) inhibited root growth but stimulated the production of artemisinin more than 2-fold.

Detection of relevant transgenes in the selected hairy root lines

Hairy root induction is due to the integration and subsequent expression of a portion of Transferred DNA (T-DNA) from the bacterial Ri (Root inducing) plasmid in the plant genome (Christensen and Muller, 2009). Four loci involved in root formation have been identified in the

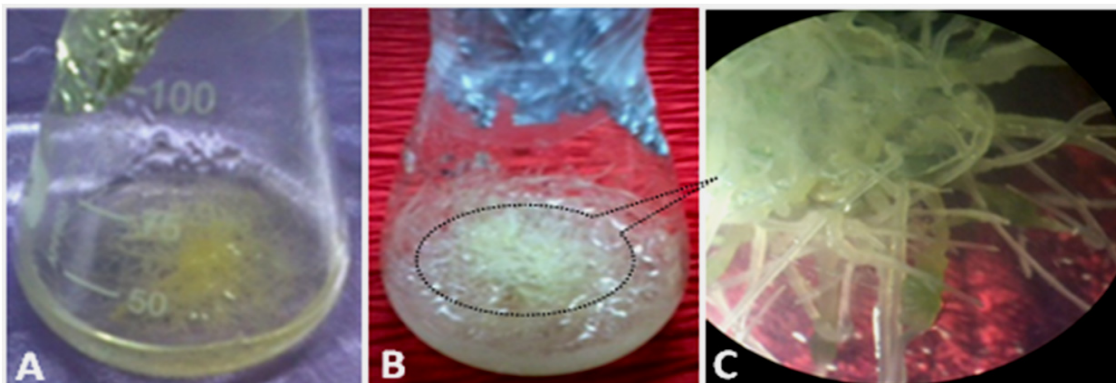


Fig. 3. Effect of high incubation temperature (35°C) on hairy roots growth. A: One pair of hairy roots at the beginning of treatment, B: Appearance of hairy roots 2 weeks after treatment, C: A close-up picture from plentiful growth of hairy roots

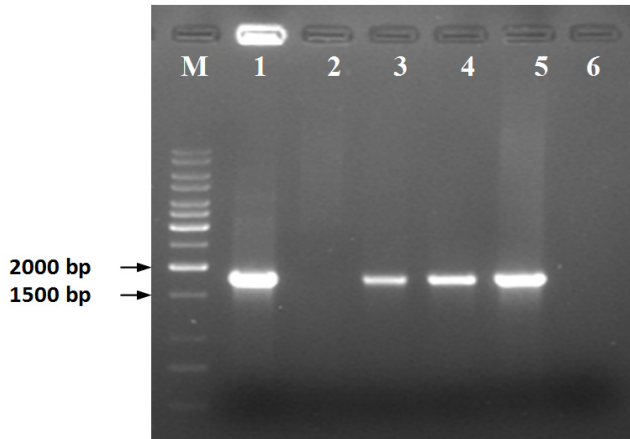


Fig. 4. PCR analysis for hairy roots lines of *L. mucronatum* using the *rolA* and *rolB* genes specific primers. M: 1 Kb DNA Ladder (Fermantas), 1: Ri plasmid from *A. rhizogenes* strain 'A13' as a positive control, 2: Wild plant root as first negative control, 3 to 5: Transgenic hairy roots induced on hypocotyl and root explants infected by *Agrobacterium*, 6: Non-DNA template PCR reaction as second negative control

T-DNA of the Ri plasmids and designated root loci (*rol*) A, B, C and D (Ayala-Silva *et al.*, 2007). The *rolB* gene is absolutely essential for the induction of hairy roots. Even when expressed alone, the *rolB* gene can induce significant hairy roots production (Nilsson and Olsson, 1997). Successful genetic transformation can be demonstrated in direct or indirect ways by detecting opines or T-DNA sequences, respectively. The direct method is preferred as in some cases. Nonetheless, opines production is not stable and may even cease (Sevon and Oksman-Caldentey, 2002). PCR method can be used simply for detecting T-DNA sequences in putative transformants (Palazon *et al.*, 2003). In this study, the presence of the *rolA* and *rolB* genes in the hairy root lines was confirmed by PCR analysis (Fig. 4) using primers specific for core sequence located between the *rolA* and *rolB* genes. 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. This result indicated that *L. mucronatum* is susceptible for transformation with *Agrobacterium rhizogenes* strain 'A13' and the roots and hypocotyls explants respond quite efficiently to transformation by *Agrobacterium*.

Root morphology

Transformed and untransformed roots were strikingly different in morphology and growth. The hairy root lines were characterized by vigorous growth and abundant lateral branching (Fig. 1E). They also formed a callus in phytohormone-free medium (Data are not shown). These are a typical characteristic for hairy roots induced by wild type strain 'A13' of *A. rhizogenes* mediated transformation that have also been reported by some researchers (Akutsu *et al.*, 2004; Fukuda *et al.*, 2007; Ishizaki *et al.*, 2002; Ohara *et al.*, 2000). In addition, they had fast growth rates; the

ratios of their final fresh weights to their initial fresh weights were greater than each generation of subculture. By contrast, untransformed roots did not branch and they had slow growth. It is also worth a note that transformed roots grew well in a hormone-free medium, while untransformed roots hardly grew in the same conditions (Fu *et al.*, 2005), consistently in this study observed their plentiful growth on hormone free HRGM media. It must be noted that hairy root morphology of the primary cultures also can be variable. This could be due to slight differences in wound-induced phytohormone production or to differences in *rol* genes expression (Palazon *et al.*, 1998).

Conclusions

The present study reports the successful production of transgenic hairy root lines in *L. mucronatum*. This report is the first, demonstrating the induction of hairy roots in this *Linum* species. The 'A13' strain of *A. rhizogenes* was capable of transforming hypocotyl (including cotyledonary node) and to some extent root explants, however, the relative efficiencies varied considerably between the explants. The results suggested that hypocotyl segments with high transformation rate (60%) were as responsive explants for production of transgenic hairy roots in *L. mucronatum* and the use of a phytohormone-free MS basal medium with a one-day pre-incubation at 35°C increased prominently hairy root growth. Hairy root induction protocol presented in this research could be used for further optimization with regard to various operational factors influencing the enhanced production of pharmaceutical metabolites like podophyllotoxin in this valuable wild *Linum* species. Additionally, this transformation method could also be applied as an alternative to *A. tumefaciens* system for genetic engineering of this plant using heterologous genes for biotechnological uses and enhanced production of bioactive compounds.

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