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In vitro organogenesis from transformed root cultures of *Plantago lanceolata* and phytochemical analysis by HPLC and GC-MS

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Article info:
Received: 12 June 2021
Accepted: 06 December 2021

ABSTRACT

Plantago lanceolata (ribwort plantain) is a well-known medicinal plant that has been used for traditional purposes. *In vitro* shoot, organogenesis, and plant regeneration of *P. lanceolata* were established from transgenic root explants, and then the main medicinal compounds were detected by HPLC and GC-MS. The highest frequency of transformation, shoot organogenesis (100%) and the number of shoots per explant (177) was achieved using the *Agrobacterium rhizogenes* strain A4 in ½ salt B_5 medium without the addition of plant growth regulators (PGRs) for 14-20 days after infection by *A. rhizogenes*. Root induction occurred from the regenerated shoots sub-cultured on the ½ MS without the addition of any PGRs after 7-14 days. The results of HPLC analysis showed that apigenin and gallic acid content in transgenic plants are more than transgenic roots. The content of catalpol did not show a significant difference in both transgenic samples. The methanolic extracts of transformed roots showed remarkable compounds, including Cycloheptasiloxane, tetradecamethyl-(1.43-fold increase); Palmitic acid methyl ester-(4.15-fold increase); 9,12-Octadecadienoic acid, methyl ester-(1.72-fold increase); Methyl isostearate--(4.26-fold increase) in comparison to the methanolic extract of *P.lanceolata* root. *P. lanceolata* is a medicinal plant that showed worthy potential for *in vitro* culture. As the first report, that established a reliable protocol to optimize the produce hairy roots and HR-plants of *P. lanceolata*. hairy roots offer suitable prospects for the production of valuable compounds due to the overproduction of shoot and root.

Key words: *Agrobacterium rhizogenes*, Phytochemical analysis, *Plantago lanceolata*, Transformed plants, Transgenic roots

Introduction

Plantago is the largest genus from the *Plantaginaceae* family comprising about 275 species (Goncalves and Romano 2016). It was used throughout the world as an herbal medicine for the traditional treatment of many diseases and functional foods. Some of them have commercial value due to their uses as pharmaceuticals and nutraceuticals. It is also used for the treatment of the upper respiratory tract, mouth, throat, and skin diseases (Oloumi et al. 2011). Polar extracts (methanolic, 30% acetonitrile, 80% methanol, 80% ethanol, and hot water) of leaves, roots, flowers, fruits, and seeds of *P. lanceolata* have been studied for their bioactivities. These extracts showed strong cytotoxic (Gálvez et al. 2003), anti-inflammatory (Beara et al. 2012), anti-oxidant (Zhou et al. 2013), anti-obesity (Yoshida et al. 2013), wound healing (Kovač et al. 2015) and anti-microbial effects (Ferrazzano et al. 2015). *Plantago* species are rich in iridoids, iridoid glycosides, polyphenols, flavonoids, polysaccharides, and triterpene acids (Samuelsen et al. 1999; Ronsted et al. 2000; Velazquez-Fiz et al. 2000; Tarvainen et al. 2010; Zubair et al. 2011).

Previous studies have shown that apigenin, catalpol and gallic acid considered in this study showed medicinal properties. Most of the studies on apigenin focus on its therapy effects on different cancers including breast melanoma, lung cancer, osteosarcoma, liver, prostate (Yan et al. 2017) colorectal cancer cell lines SW-480 and DLD1(Dai et al. 2016) as well as induction apoptosis HCT-116 cells (Lee et al. 2014). Apigenin has also been used for anti-viral, anti-bacterial properties (Özçelik et al, 2011).

Catalpol is an allelopathic, anti-microbial (Joanne et al. 1996; Wang et al. 2010) and an anti-cancer agent that inhibited Human gastric cancer cells (MKN-45) (García et al. 2010), Athymic nude mice (García et al. 2010), lung cancer (NSCLC) cells- A549 cells (Liu et al. 2017), Human solid tumor cell lines (A-2780, HBL-100, HeLa, SW-1573, T-47D and WiDr) (Wang and Zhan-Sheng 2018) and Human non–small-cell, Human colorectal cancer cells (HCT116) (Wang et al. 2019).

Gallic acid has been reported to be effective against pathogenic bacteria (*Plesiomonas shigelloides* and *Salmonella* spp) (Rattanata et al. 2016). gallic acid also is an anti-cancer agent, which inhibited the proliferation of cholangiocarcinoma cell lines (M213, M214) (Rattanata et al. 2016) but it didn't show the cytotoxic effect on normal cells (Van der Heijden et al. 1986).

The current study contains four main sections including optimization of the condition of transformed root induction (Details of optimization of hairy root induction published (Rahamouz-Haghighi et al. 2020) and continuously, adventitious shoot formation on hairy roots and determining the content of valuable medicinal compounds by HPLC and GC-MS methods. Thus, this study, an efficient protocol for the *in vitro* shoot organogenesis and plant regeneration of *P. lanceolata* was established from transgenic root explants. We introduced a simple and reliable approach for achieving transgenic roots (hairy roots) and hairy root-derived plantlets (HR-plants) of *P. lanceolata.* Moreover, in this study, the main medicinal compounds exist in hairy roots by High-performance liquid chromatography (HPLC) and Gas chromatography–Mass spectrometry (GC-MS) methods were detected.

Materials and Methods.

Preparation and Disinfection of Plant material

The *P. lanceolata* wild plant was collected from the University of Zanjan, Iran (geographical coordinates of the collection sites: 36°41'15.5"N 48°24'02.2"E) and then were identified at the Department of Botany, University of Zanjan. Seeds of *P. lanceolata* were obtained from the gene bank of Research Institute of Forests and Rangelands of Tehran, Iran. Seeds were sterilized with 70% ethanol for 30 sec and then 5% sodium hypochlorite solution containing one drop of Tween-20 for 10 min and rinsed three times in distilled water. The seeds were cultured on ⅛- strength Murashige and Skoog basal medium (⅛ × salt MS) (Murashige and Skoog 1962). The pH of medium was adjusted to 5.8 before adding %7 agar and sterilized at 121 °C for 15 min by an autoclave. The seeds were incubated at 25 °C under a 16h light photoperiod.

Culture of A. rhizogenes strains

A4, A13, ATCC15834, and MSU440 were provided from the bank of microbes at Zanjan Pharmaceutical Biotechnology Research Center. The strains were cultured in Luria-Bertani (LB) liquid medium containing 50 mg/L rifampicin, (pH 7.2; OD= 0.6) at 28°C, 120 rpm on a shaker incubator. The bacteria were pelleted by centrifugation for 12 min at 3500 rpm and resuspended to a cell density of OD_{600} 0.7 in liquid inoculation medium (half MS salts and vitamins along with 50 mg/L sucrose).

Plant Transformation

The leaf explants from *in vitro* 4-8 week old *P. lanceolata* were transferred to pre-culture medium (full-strength MS) containing 0.5 mg/L 6-Benzylaminopurine (BAP), pH: 5.8 for 24 h and then explants were infected with four strains of *A. rhizogenes* by immersion method (pH: 5.5) for 5 min with 150 µM acetosyringone concentration in inoculation medium. Then explants were incubated in dark on co-cultivation medium (½MS) supplemented with 50 g/L (w/v) sucrose and 7g/L agar for two days (pH:5.5). Control explants did not inoculate by *A. rhizogenes.* Finally, infected explants transferred to selective medium (½MS or ½-strength Gamborg′s basal medium (B_5)) (Gamborg et al. 1968) containing 400 mg/L Cefotaxime.

DNA extraction and PCR analysis

Genomic DNA was isolated from hairy root, HR-plants samples and control roots using the CTAB DNA extraction method. PCR analysis of the isolated DNA was performed with primers 5′ ATG GAT CCG AAA TTG CTA TTC CTT CCA CGA 3′ (forward) and 5′ TTA GGC TTCTTT CTT CAG CAG GTT TAC TGC AGC 3′ (reverse) to detect a 780-bp fragment of the *rolB* gene the *rolB* gene integrated to the plant genome. Plasmid DNA of A4 and genomic DNA isolated from non-transformed roots was applied as a positive control and a negative control. The amplified PCR product (780 bp) was excised and purification by a Qiagen gel extraction kit (Qiagen, Germany). The eluted product was sequenced (Genomin company, Tehran, Iran) for confirmation of the presence of the *rolB* gene in hairy roots and HR-plants. The sequence similarity of the PCR products was analyzed with the *rolB* gene sequence obtained from the NCBI database.

HPLC analysis

The HPLC system to include a pump (WATER, USA), UV detector (WATERS, Breeze, USA), at a wavelength of 204 nm by a software (Breeze, USA) on 150 mm*4.6mm, particle size 5µm; Perfectsill, C8 analytical column (MZ-Analysentechnik, Germany) equipped by a guard column of the same packing. The mobile phase composed of HPLC grade acetonitrile (ACN) and orthophosphoric acid (1:1 v/v) with a flow rate of 1 mL/min. The sample injection volume into the system (20 µL) was created by a loop injector (Rheodyne 7725i, Cotati, CA, USA).

Preparation of Stock Solutions and Working Solutions

Apigenin (98% purity), catalpol (96% purity) and gallic acid (96% purity) were used as standard. Catalpol and gallic acid were prepared in sterile purified water and apigenin was prepared in HPLC grade methanol and Dimethyl sulfoxide (DMSO) (95: 5 v/v). Working solutions by dilution method

were prepared at 0.15625 to 10 µg/mL for apigenin and gallic acid and 1.5625 to 100 µg/mL for catalpol.

Preparation of P. lanceolata extracts for HPLC

The powdered of aerial (exception spike) and root parts (30g) and of hairy roots and HR-plantlets (30 mg) were extracted by maceration methods with HPLC grade methanol for 72 h at room temperature and then 30 min in ultrasonic bath, extracts concentrated by rotary evaporator and dried in room temperature for 7days. Dried extracts were dissolved in HPLC grade methanol to obtain stock solutions and then each extract solution was filtered through sterile 0.22µm member filter.

GC-MS analysis

The methanolic extracts of hairy root and untransformed root parts from *P. lanceolata* were used for GC-MS analysis. GC-MS analysis was performed using Agilent technologies 5975c. GC-MS analysis carried out by 1 µL of as subjected to analysis. The GC-MS system has been equipped with a capillary column (30m × 250µm × 0.25µm, Agilent). Helium was used at the flow rate of (1.0 mL/min). The injector and the interface temperature were maintained at 350°C. The column temperature has been programmed as follows: the initial temperature was 50°C (2 min) then increased at a rate of 4°C/min up to 230°C (2 min). The identification of the components was done by comparison of mass spectral fragmentation patterns stored in MS data libraries (NIST08.L) (Jamilah et al. 2012).

Preparation of P. lanceolata extracts for GC-MS

Approximately 20 grams of dried root part of *P.lanceolata* and 20mg of hairy roots were ground to a coarse powder and extracted by the reflux method using 200 mL and 20 mL of HPLC grade methanol for 8 h. Then obtained extracts were filtered and concentrated in a rotatory evaporator under reduced pressure at 35 to 45°C for 75 minutes. The extracts were stored at 4°C. The concentration for injection was 4 mg/mL in both extracts.

Statistical analysis

The experiments carried out with three replications and ten explants cultured in each replication. The means were compared using Duncan (P-value < 0.05 and 0.01) by the statistical SPSS version 21. The graphs were designed using Excel software. The results were reported as mean of replications ± standard deviation.

Results

Hairy root induction

A. rhizogenes-mediated transformation caused the formation of hairy root of *P. lanceolata* as well as hairy root induction appeared in A4, A13, ATCC15834 and MSU440 strains after 9 days of inoculation (Figure. 1).

Among the different strains evaluated for the production

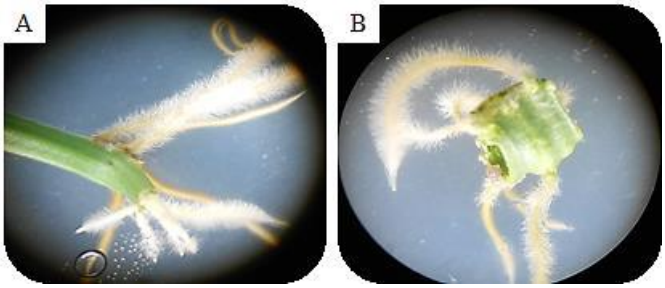

Figure 1. *Hairy root induction in leaf explants was developed through Agrobacterium rhizogenes mediated transformation. A4 (A), and MSU (B)*

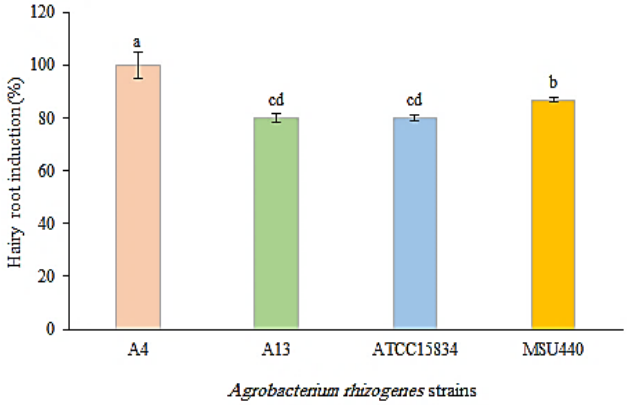

Figure 2. *Hairy roots induction by Agrobacterium rhizogenes strains in optimum condition. Values are mean ± SD, different letters show significant differences using Duncan test (P-value <0.05).*

of hairy root of *P. lanceolata*, the A4 and MSU440 strains showed the highest rates of hairy root induction. In the optimum conditions, A4, MSU440, A13, ATCC15834 and strains resulted in 100 %, 86.66 %, 80%, and 80 % transformation (Figure. 2). In the current research, it is observed that B_5 salt medium displayed more growth of hairy root lines in terms of fresh (FW) and dry weights (DW) compared to MS salt medium.

Comparison of two selective media for sub-culture

The transformed roots of *P. lanceolata* established from leaf explants were sub-cultured every 7 days. They were excised and put into ½ MS or ½ B_5 medium. After hairy roots transferred to ½ MS medium, calluses appear on the hairy roots (Figure. 3A). While the size of calluses increased, calluses regenerated without the addition of PGRs after 7-10 days. Several small buds were formed on the surface of hairy

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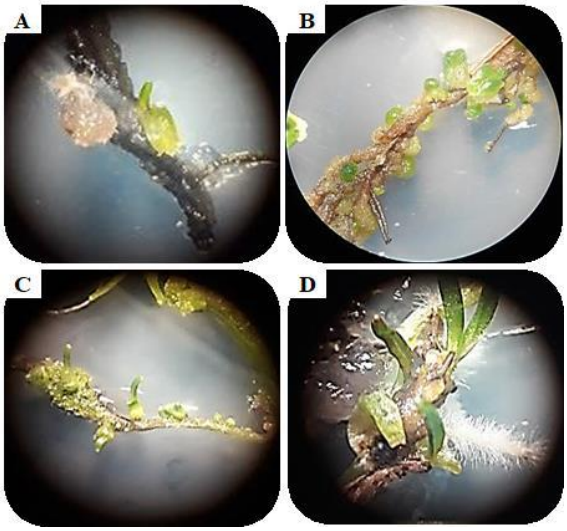

Figure 3. *Callus formation on the hairy root surface of Plantago lanceolata and produce indirectly regeneration.*

roots calli (Figure. 3B, C). The number of adventitious shoots increased with time (Figure. 3D).

The obtained hairy roots showed adventitious shoot formation, when transferred to ½ B_5 medium without the addition of PGRs and direct regeneration appeared on hairy roots (Figure. 4). The roots became narrower and less developed. The shoots (approximately 1 cm long) were

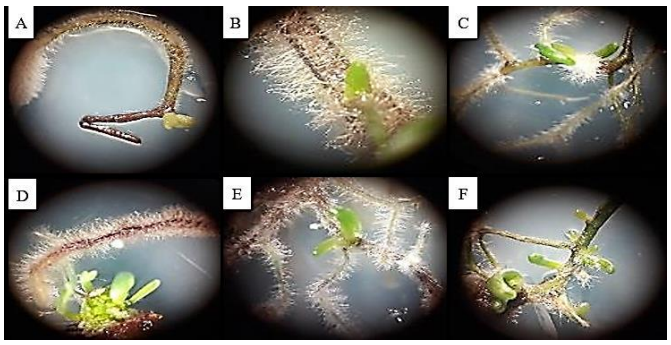

Figure 4. *Direct shoot organogenesis on hairy roots surface of P. lanceolata produced brown roots (A-F).*

excised from hairy roots and transferred on shoot multiplication medium (full MS or B_5 medium). There was no difference between the two media for the growth of HR-plants. In the present study, shoot production (indirectly and directly organogenesis) was observed in the PGR-free medium. The highest number of shoots per explants (28) observed by A4 strain. The shoots were sub-cultured for 4 weeks. The average fresh weight (FW) and dry weight (DW) of hairy roots were 12.36 g ± 0.32 and 4.2 g ± 0.26, respectively.

Four-week-old HR-plants were rooted in ½ MS medium containing 0.5 mg/L IBA or neither. The rooting frequency (100 % after two weeks), number of roots per shoot (average

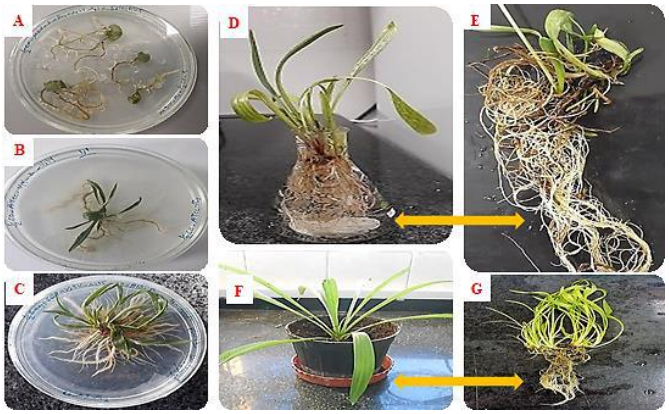

Figure 5. *Hairy root produces by Agrobacterium rhizogenes (A), selective the best explant and line (line4) (B) shoot organogenesis formation on the hairy root (C), morphological characterizes of regenerated plantlets of hairy roots (D, E). Acclimatized regenerated plantlets of hairy root (F) and characteristic of shoot and root of regenerated plantlets of hairy root after 4 weeks (G).*

42) and length of roots (64 cm) were greatest in the presence of auxin. The plantlets were acclimatized in the growth chamber for four weeks and the survival rate was 100 %. The survival rate of the HR-plants of *P. lanceolata* after the acclimatization period in the pots was 100 % similar to

Figure 6. *Spontaneous regenerations of the roots of Hairy root-plants. Hairy root produce by Agrobacterium rhizogenes rhizogenes A4. Shoot organogenesis was obtained from transgenic root cultures of P. lanceolata and then root of Hairy root-plantlets regenerated spontaneously.*

Figure 7. *The characteristics of hairy root-derived plantlets by Agrobacterium rhizogenes strains*

untransformed plants (Figure. 5).

The presence of T-DNA in the genome of hairy roots and HR-plants of *P. lanceolata* confirmed by PCR analysis. The

Table 1. *Comparison of morphology and biomass yield of transformed and untransformed plants of P. lanceolata after one month of growth in a growth chamber*.

Morphology	HR-plants (A4)	HR-plants (A13)	HR-plants (ATCC15834)	HR-plants (MSU440)	*In vitro*-plants (control)
Number of leaves/plants	28.00$_{a}$±1.7	14.00$_{b}$±2.02	16.61$_{b}$±0.35	17.27$_{b}$±1.19	10.00$_{b}$±2.02
Length of leaf (cm)	0.98$_{a}$±0.1	0.675$_{a}$±0.04	0.68$_{a}$±0.06	0.73$_{a}$±0.07	1.10$_{a}$±0.17
Width of leaf (cm)	13.12$_{a}$±0.64	11.95$_{ab}$±1.12	9.10$_{b}$±0.63	11.27$_{ab}$±0.44	10.58$_{ab}$±0.33
Number of lateral branches	4.00$_{a}$±.28	2.25$_{b}$±.14	2.81$_{ab}$±.46	3.66$_{a}$±.25	3.67$_{a}$±.098
Root biomass (g/plant)					
FW	12.36$_{a}$±0.32	5.80$_{b}$±.46	7.00$_{b}$±.57	10.33$_{a}$±.26	6.67$_{b}$±.67
DW	4.20$_{a}$±.23	2.90$_{b}$±.40	0.13$_{c}$±.017	3.60$_{ab}$±0.34	3.20$_{ab}$±0.11
Shoot biomass (g/plant)					
FW	9.1425$_{a}$±.08	3.67$_{c}$±0.38	4.77$_{ab}$±0.44	5.056$_{b}$±.034	5.38$_{b}$±0.21
DW	2.66$_{a}$±0.38	0.06$_{c}$±0.011	0.275$_{c}$±0.04	0.311$_{c}$±0.023	1.69$_{b}$±0.27

The characteristics of transformed plantlets

The direct shoot regeneration of *P. lanceolata*, is a desirable trait when the aim is obtaining of genetically stable and commercially viable plants. The mean total biomass of HR-plants calculated as FW of shoots was two times higher than that observed in untransformed plants of the same age (9.14 ± 0.8g and 5.38 ± 0.21 g). The phenotypical changes in HR-plants after a 4 weeks period of growth in the growth chamber were investigated (Figure. 5 and Table 1).

Leaves of HR-plants of *P. lanceolata* are similar to *in vitro* plants. The number of leaves increased but there was no change in leaf shape and size. These changes resulted in a significant increase in the total biomass of shoots. The superior properties of root of HR-plants were observed such as early emergence of roots, more number and root weight, high growth rate and frequent lateral branching unlike the natural roots of the wild plant which may be the result of the insertion of *rolB* genes in HR-plants. These changes were mainly associated with an increase in root biomass; however, the morphology of root of HR-plants was similar to hairy roots. However, root of HR-plants regenerated spontaneously (Figure. 6). However, no major alteration in leaf morphology was observed in any of the HR-regenerates of *P. lanceolata*. A considerable increase in the root biomass of the HR-regenerates of *P. lanceolata* was observed (Figure. 7).

Confirmation of transformation

amplification of the *rolB* gene by PCR showed that the *rolB* gene appeared at 780 bp which was similar to positive control. No bands not observed in the PCR product of non-transformed roots (Figure. 8). The eluted product has also confirmed the presence of the *rolB* gene in hairy roots and HR-plants.

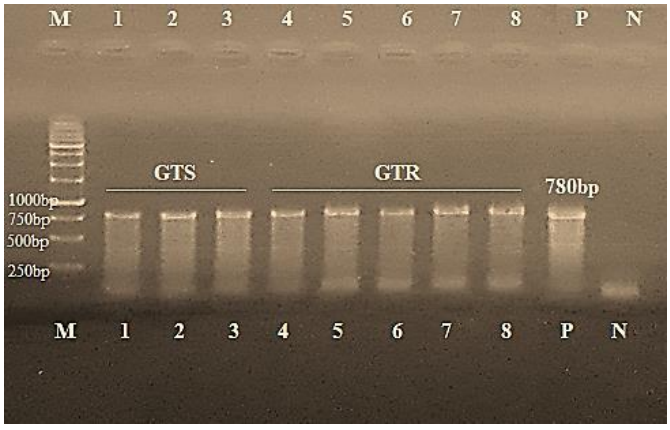

Figure 8. *PCR analysis of rolB gene in of P.lanceolata hairy roots . The figure shows the confirmation of selected hairy root clones (1 to 8) for production gene rolB (780 bp). (M) Molecular size marker (ladder Mix Fermentas Co.), (P) positive control (DNA plasmid of A4 strain of A.rhizogenes) and (N), an untransformed root as a negative control. Lines 1-3 is related to genetically transformed shoot (GTS) and genetically transformed root lines (GTR) were showed from 4-10 no.*

Apigenin, Catalpol and Gallic acid content of P. lanceolata hairy root and HR plants

The production of apigenin, catalpol and gallic acid was investigated in hairy roots and HR-plants by HPLC analysis (Figure. 9). The content of apigenin, catalpol and gallic acid

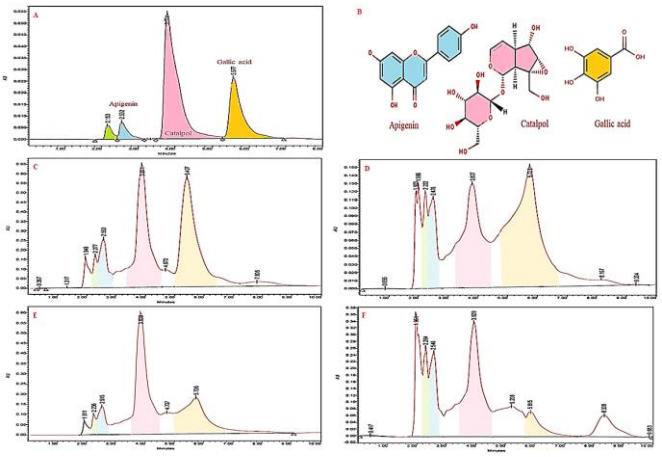

Figure 9. *HPLC chromatograms of (A) apigenin, catalpol and gallic acid standards (2.5, 100 and 5 µg.mL-1); (B) structure of apigenin, catalpol and gallic acid; (C) aerial part of wild P. lanceolata; (D) Root part of wild P. lanceolata; (E) hairy root-derived plantlets; (F) Hairy root of P. lanceolata. R2 values were yApigenin = 296784x + 13686; yCatalpol = 11865x + 4919.9 and yGallic acid = 355517x + 24027. The amount of apigenin, catalpol and gallic acid was also evaluated in aerial and root parts of wild P. lanceolata.*

**To compare, pictures C and D were repeated from Rahamouz-Haghighi et al., 2020*

were also measured in aerial and root parts of wild *P. lanceolata*.

The results showed that the aerial parts of this plant have more catalpol and gallic acid content than the root part (Figure. 10). Based on this result the content of apigenin and gallic acid in HR-plants is more than hairy roots but the content of catalpol did not display a significant difference in both transgenic samples.

Generally, the aerial part indicated 1.2, 1.6 and 2.3 -fold increase than the root part and also HR-plants 1.6, 1.09 and 3.2 -fold increase than hairy roots in the content of apigenin, catalpol and gallic acid, respectively.

Determination of the composition of methanolic extracts from P. lanceolata by GC-MS

The composition of methanolic extracts of root and hairy root of *P. lanceolata* was analyzed by GC-MS and its components were identified according to the NIST08.L library. The extracts of *P.lanceolata* showed the presence of fatty acids, siloxanes and esters derivatives. The presence of the volatile components was carried out using GC-MS which detected 1,2- Benzenedicarboxylic acid, mono(2-ethylhexyl) ester(47.12%), Cycloheptasiloxane, tetradecamethyl- (17.88%) and Cyclohexasiloxane, dodecamethyl- (16.36%) in methanolic extract of *P.lanceolata* root part, Cycloheptasiloxane, tetradecamethyl- (25.70%), Silane, dimethoxymethyl- (24.77%) and Hexadecanoic acid, methyl ester (16.99%) in methanolic extract of hairy roots of *P. lanceolata* as the dominant constituents (Table 2).

In the present study, the common compounds in the both of hairy root and root parts of *P. lanceolata* methanolic extracts were Cyclohexasiloxane, dodecamethyl-; Cycloheptasiloxane, tetradecamethyl-; Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-; Hexadecanoic acid, methyl ester; 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester and 9,12- Octadecadienoic acid, methyl ester.

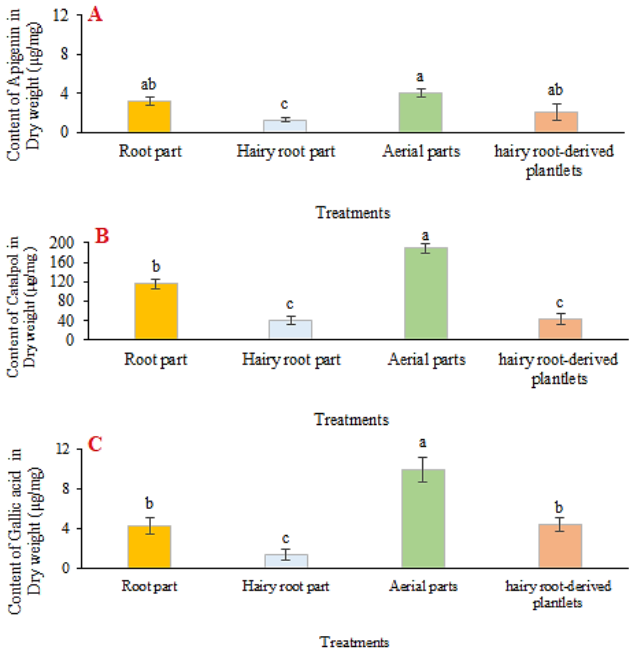

Figure 20. *Determination of Apigenin, Catalpol and Gallic acid contents in different parts and hairy roots of P. lanceolata.; the content of apigenin (A) catalpol (B) gallic acid (C) in Dry weight of samples (µg.mg-1). Values are mean ± SD and different letters show significant differences using Duncan's test (P-value < 0.05)*

However, most of the compounds reported in the extracts have anti-bacterial, anti-fungal, anti-cancer, anti-oxidant and anti-inflammatory properties and are fatty acid. Fatty acids are one of the most important sources of medicinal properties, including anti-microbial and antifungal activities (Krishnaveni et al. 2014). Two compounds, Silane, dimethoxymethyl- and Silane, [[4-[1,2-

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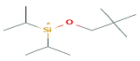
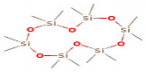
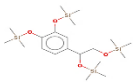
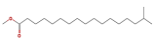
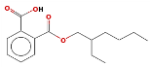
Table 2. *Identified compounds in untransformed and genetically transformed root parts of Plantago lanceolata methanolic extract by GC-MS analysis.*

RT	Name of compounds	UTR	GTR	Formula	M.W (g/mol)	Nature of compound	Biological activity	Structure of compound
3.23	Silane, dimethoxymeth	0	24.7766	$C_3H_{10}O_2Si$	105.19	Not determined	No activity found	
3.51	2,2-Dimethyl-1-diisopropylsilyloxypropane	3.3054	0	$C_{11}H_{25}OSi$	201.4	Not determined	No activity found	
20.84	Cyclohexasiloxane, dodecamethyl	16.3671	9.0667	$C_{12}H_{36}O_6Si_6$	444.92	Cyclic methyl siloxane	Antifungal, Emollient, Antimicrobial	
26.22	Cycloheptasiloxane, tetradecamethyl-	17.8895	25.7043	$C_{14}H_{42}O_7Si_7$	519.07	Cyclic methyl siloxane	Antibacterial, Antifungal, Antiseptic, Imunomodulatory, Antitumor	
31.08	Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)] bis[trimethyl-	3.8461	8.4157	$C_{20}H_{42}O_4Si_4$	458.89	Not determined	No activity found	
37.71	Hexadecanoic acid, methyl ester /Methyl palmitate/ Palmitic acid methyl ester	4.0863	16.9925	$C_{17}H_{34}O_2$	270.5	Fatty acid methyl esters	Antioxidant, Antibacterial, Antifungal, Anti-inflammatory, Anticancer, Hepatoprotective, Anticoronary	
39.32	Hexadecanoic acid, ethyl ester/Ethyl palmitate	3.4153	0	$C_{18}H_{36}O_2$	284.5	Fatty acid ethyl esters	Antioxidant, Antiandrogenic flavor, Hemolytic, Alphareductase inhibitor	
41.64	9,12-Octadecadienoic acid, methyl ester	2.0687	3.5624	$C_{19}H_{34}O_2$	294.4721	Fatty acid methyl ester	Hepatoprotective, Anti-histaminic, Antieczemic, Antioxidant, Antimicrobial	
42.37	Heptadecanoic acid, 16-methyl-, methyl ester/Methyl isostearate	0	4.2667	$C_{19}H_{38}O_2$	298.5	Fatty acid methyl ester	Antimicrobial	
43.13	9,12-Octadecadienoic acid, ethyl ester	1.8918	0	$C_{20}H_{36}O_2$	308.5	Fatty acid ethyl ester	Hepatoprotective, Antihistaminic, Anticoronary Antieczemic and Antiacne	
52.26	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester/Mono(2-ethylhexyl) phthalate	47.1298	7.2151	$C_{16}H_{22}O_4$	278.3435	Aromatic dicarboxylic ester	Antimicrobial, Cytotoxicity, Antioxidant, Anti-inflammatory, Antiviral	

*These compounds have been obtained by NIST08.L Library. With using of their CAS numbers, the popular names were determined

bis[(trimethylsilyl)oxy]ethyl]-1,2 phenylene]bis(oxy)]bis[trimethyl- were presented only in hairy roots. These characteristics comparisons of two methanolic extracts are summarized in Table 2.

Only one available protocol for hairy root induction by *A. rhizogenes* strain LBA9402 strain in *P. lanceolata*, was reported by Fons,1999. In their study the hairy roots produced more biomass than untransformed roots, but hairy roots quickly degenerated (Fons et al. 1999).

Nguyen et al. 1992 reported that at 100 mM concentration of nitrate, root growth was strongly inhibited. At very low nitrate concentrations (5 mM), growth was reduced without modification of the morphology. Root morphology did not change at phosphate concentrations below 1.1 mM but growth was strongly prevented. The present results suggest that the standard B_5 medium was close to optimal for the growth of the hairy roots (Nguyen et al. 1992). Generally, the hairy roots culture in ½ B5 was more competent than ½ MS due to inhibition of degeneration of hairy roots and callus formation on the surface of hairy roots.

The depletion of nutrient contents during continuous growth and changes in levels of sensitivity to phytohormones in transformed tissues can be considered as factors responsible for the spontaneous regeneration of shoots (Mehrotra et al. 2013).

The effect of *rolB* integration into plant genome has been extensively studied. It is implicated in widespread rooting of them also the HR-plants show profuse rooting in the absence of an external auxin source (Geier et al. 2008). Some of the reports described that *rolB* can improve the shoot organogenesis (Altamura 2004).

Mano and Matsuhashi (1995) reported that horseradish plants can easily induce adventitious shoots on hairy roots in PGR-free medium. Spontaneous regeneration of hairy roots of *Rauwolfia serpentine* (Mehrotra et al. 2013), *Centaurium erythraea* (Subotić et al. 2009), *Plumbago indica* (Gangopadhyay et al. 2010), *Pogostemon cablin* (He-Ping et al. 2011), and *Ophiorrhiza rugosa* (Kamble et al. 2011) were reported.

Direct shoot organogenesis was observed in *Tylophora indicaas* a highly desirable feature in its tissue culture programs (Chaudhuri et al. 2006; Giri and ML 2000). It should be noted that the time required for spontaneous plant production from hairy roots was about 7 weeks for *P. indica*, whereas it was 2 months in *Atropa belladonna* (Jaziri et al. 1994) and as long as 6 months in Cephaelis ipecacuanha (Yoshimatsu et al. 2003) but the time required for produce HR-plant of *P.lanceolata* transgenic root was about 14-20 days since infected by different strains of *A. rhizogenes*.

Hwang (2005) and Zhou et al. (2007) have reported the regeneration of transformed root of *R. glutinosa* by ATCC15834 *via* the callus information in the presence of cytokinin. Also, regenerated shoots from the callus of hairy roots were formed in *Malus baccata* (Wu et al. 2012).

Piatczak et al. (2015) reported that the survival rate of the HR-plants of *R. glutinosa* was 100 % and higher than the untransformed plants of *R. glutinosa* (86–93 %) (Piątczak et al. 2015).

Normal leaves without a wrinkle in transformed *Alhagi pseudoalhagi* plants observed (Mei et al. 2001). Leaf wrinkling is a common trait in HR-plants and has been reported in several species as a sign formed after insertion of the *A. rhizogenes* Ri T-DNA (Nguyen et al. 1992; Zhou et al. 2007).

A considerable increase in the root biomass of the HR-regenerates is the most common properties observed in HR-plants of *R.glutinosa* (Piątczak et al. 2015), *Catharanthus roseus* (Cho et al. 2004), *Aralia elata* (Kang et al. 2006) and *P.indica* (Gangopadhyay et al. 2010). It has been reported that the expression of *rol* genes in HR-plants can affect not only the phenotype of the plants but also the accumulation of secondary metabolites (Grishchenko et al. 2013). The increase in root biomass of the HR-plants is due to the influence of the introduced rol genes on hormonal synthesis and sensitivity (Piątczak et al. 2015). The researchers reported that better rooting ability observed in HR-plants of *Dianthus caryophyllus* can be due to the *rolC* gene which exhibits both cytokinin-like and auxin-like activity (Casanova et al. 2003). In the present study, HR-plants of *P. lanceolata* displayed a better-developed root system with longer and higher numbers of branches.

Conclusions

P. lanceolata is a medicinal plant that showed worthy potential for *in vitro* culture. In the present study, hairy roots were produced under optimum conditions and sub-cultured every week. After 14-20 days, HR-plants appeared from hairy roots, without additional PGRs indicating a high potential of endogenous hormones. The superiority of transformed plants over untransformed plants in terms of shoot and root biomass suggests that the produce of HR-plants may be a valuable strategy to achieve the hemogenic transform plants as well as the higher yield of bioactive compounds.

In conclusion, this is the first report that established a reliable protocol to optimize the produce hairy roots and HR-plants of *P. lanceolata*. hairy roots offer suitable prospects for the production of valuable compounds due to the overproduction of shoot and root.

The results of the detection of compounds by HPLC showed that the aerial parts of *P. lanceolata* have more catalpol and gallic acid content than the root part and apigenin and gallic acid content in HR-plants is more than

hairy roots. The content of catalpol did not show a significant difference in both of transgenic samples.

Moreover, GC-MS was performed to investigate the components of the extracts of hairy root in comparison to the root parts of *P. lanceolata*. The methanolic extracts of hairy roots showed remarkable compounds, indicating the presence of Cycloheptasiloxane, tetradecamethyl-(1.43- fold increase); Palmitic acid methyl ester-(4.15-fold increase); 9,12-Octadecadienoic acid, methyl ester-(1.72-fold increase); Methyl isostearate-(4.26-fold increase) in comparison to the methanolic extract of *P.lanceolata* root that these compounds displayed medicinal values such as anti-microbial, anti-cancer, anti-oxidant, anti-inflammatory, anti-viral. Therefore, we might conclude that the extracts of *P. lanceolata* hairy roots can useable for further research and discover their biological activity, as well as to produce valuable metabolites that are also economically profitable.

Acknowledgement

The study was supported by University of Zanjan, Zanjan, Iran. The authors also would like to thank the authority of the School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran.

Conflict of interest

The authors declare that they have no conflict of interest.

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