Effects of explant age and strain of *Agrobacterium rhizogenes* on hairy root induction in Fenugreek (*Trigonella foenum– graecum* L.)

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Abstract

Induced hairy roots by *Agrobacterium rhizogenes* are suitable organs for the production of secondary metabolites, due to the stability and high production of roots without phyhormones in a short time. Fenugreek (*Trigonella foenum– graecum* L.) is one of the most important medicinal plants with valuable medicinal compounds. For optimizing the hairy root induction of *T. foenum-graecum* L., six strains of *A. rhizogenes* ATCC15834, ATCC11325, K599, A7, A4 and A13 (MAFF-02-10266) and three explant ages (7, 14 and 21 day old seedlings) were tested. The explants were cultured in B5 in a completely randomized design with 4 replications. Hairy roots appeared after 3 to 4 weeks. Transgenic status of the roots was confirmed by PCR using *rolB* specific primers. The results revealed that all strains were able to induce hairy roots after 3-4 weeks. The maximum percentage of hairy root induction (87.5%) was obtained from A7 and ATCC11325 strains and 7-day-old explants. Among the strains, ATCC11325 strain showed the highest hairy root number (4.25) and root length (2.1 cm) using 14-day-old explant. Also, the highest fresh (0.203 g) and dry weights (0.029 g) were observed in the A4, A7 and ATCC11325 strains. The highest total phenolic and flavonoid contents were found at 74.05 mg g^{-1} DW and 11.80 µg g^{-1} DW in hairy roots induced by A7 strain.

Key words: *Agrobacterium rhizogenes*, Hairy roots, *Trigonella foenum– graecum* L.

INTRODUCTION

Herbs have been used extensively as natural food additives. Phenolic and flavonoid compounds are widespread in plant kingdom where they act as antioxidants and free radical scavengers. These properties are due to the presence of many active phytochemicals including vitamins, flavonoids, terpenoids, carotenoids, cumarins, curcumins, lignin, saponin, plant sterol etc (Lucia *et al*., 2002). Fenugreek (*Trigonella foenum-graecum* L.) belonging to the subfamily Papilionaceae, the family Fabaceae, is a valuable medicinal plant which is widely cultivated throughout the world (Bhagyasri *et al*., 2015). Fenugreek is an important legume used as vegetable, spice and medicinal plant in India, Pakistan, Argentina, Egypt, France, Spain, Turkey and China. Fenugreek has various properties such as anti-diabetic, anti-cancerous, anti-microbial and hypocholesterolemic. Fenugreek seeds contain polysaccharide, galactomannan, distinctive saponins, for example, diosgenin, yamogenin, adhesive, unstable oil and alkaloids, such as, choline and trigonelline (Aasim *et al*., 2010). Fenugreek seeds also contain high levels of iron and phosphorus and are used as insect and pest repellent in grain storage (Billaud and Adrian., 2001).

Transformed hairy roots are important in raising transgenic plants and molecular farming (Cao *et al*., 2009). Hairy roots are produced via the infection by *A. rhizogenes*, a soil-borne gram-negative bacterium, at the wounding sites. The bacterium transfers T-DNA of the Ri-plasmid into the nuclear genome of the host plant (Giri *et al*., 2001; Tzfira *et al*., 2004). Hairy roots offer great potential for the production of valuable secondary metabolites and the study of the associated secondary

metabolic pathways from many plants. The advantages of using hairy roots are their independence of plant growth regulators, high growth rates, and genetic and biosynthetic stability. Hairy roots are formed by genetic transformation of the plant cells using *A. rhizogenes*. Like most differentiated plant tissues; hairy roots exhibit a high degree of chromosomal stability over prolonged culture periods (Charlwood and Charlwood, 1991). Some researchers reported hairy root induction in *T. foenum-graecum* by different strains of *A. rhizogenes* (Al-Mahdawe *et al*., 2013). Previously, other studies have been also reported on the hairy root culture of *T. foenum- graecum* with A4 strain *A. rhizogenes* for diosgenin production (Merkli *et al*., 1997), sotolone production (Peraza-Luna *et al*., 2001). The result of successful production of morphinan alkaloid by hairy root cultures of *Papaver bracteatum* by over-expression of the salutaridinol 7-o-acetyltransferase gene (Sharafi *et al*., 2013a). In another article, Sharafi *et al*. (2013b) increased the amount of codeine and morphine in transgenic hairy root lines of *P. bracteatum* by over expression of codeinone reductase gene. In this study, the effects of six *A. rhizogenes* strains (including ATCC11325, ATCC15834, K599, A13, A7 and A4) and three explant ages (7, 14 and 21 days) in hairy root induction of fenugreek were evaluated. Finally, we evaluated the total phenolic and flavonoid contents in transgenic hairy roots and non- transgenic control. The main aim of the present research was the optimization of hairy root induction, growth, and production of secondary metabolites in *Trigonella foenum-graecum* L.

MATERIALS AND METHODS

Plant materials

The seeds of *T. foenum– graecum* were obtained from Pakanbazr Co. The seeds were washed with sterile distilled water and were surface sterilized in 70% (v v^{-1}) ethanol for one min. Then the seeds were washed with sterile distilled water, and were immersed in sodium hypochlorite 2.5% (w v^{-1}) for 10 min. After washing with sterile distilled water, the seeds were cultured in hormone-free MS medium (Murashige and Skoog, 1962) and maintained at 25±2 °C under a 16h photoperiod. For optimizing the hairy root induction, explant (cotyledon) and three explant ages (7, 14 and 21 days) were tested. Finally we evaluated the effects of explant age and strains on hairy root induction, hairy root number and length, fresh and dry weight and the total phenolic and flavonoid contents of hairy roots.

Bacterial strains and culture conditions

Six strains of *A. rhizogenes* ATCC15834, ATCC11325, A7, k599, A4 and A13 (MAFF-02-10266) were used for transformation. All strains were provided by the bank of microbes at the National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran. The *A. rhizogenes* strains were cultured for 24 h at 28 °C in LB liquid medium in the dark supplemented with 40 mg l^{-1} rifampicin.

Induction and culture of *T. foenum-graecum* L. hairy roots

The explants were wounded using injection method with bacterial culture of *A. rhizogenes* strains. Then, they were placed in sterilized filter paper to remove excess moisture in the surface of explants. After infection, the explants were transferred into B5 solid medium (Gamborg *et al*., 1968) for 3 days in the darkness for co-culture. Then, the cotyledons were washed with B5 basal medium with cefotaxime (500 mg l^{-1}). After washing, the explants were transferred into B5 solid medium supplemented with 500 mg L^{-1} cefotaxime. The explants were incubated under 16 h light/8 h dark conditions at 25±2 ˚C for 30 days for hairy root induction.

Polymerase chain reaction (PCR)

The isolation of genomic DNA was conducted using the CTAB method (Doyle and Doyle, 1987) from 100 mg of transformed hairy roots and control (non-transformed) roots. Amplification was performed in a final volume of 12 µl (1.75 µl of each primer (50 ng μl^{-1}), 1 µl of DNA (25 ng μl^{-1}), 6 µl of master mix (Sinna Gen, Iran), and 1.5 µl of distilled water). DNA from non-transformed adventitious roots and pRi ATCC11325 plasmids were used as negative and positive controls, respectively. The primers of *rolB* were 5'-ATGGATCCCAAATTGCTATTCCCCACGA-3'and 5'-TAGGCTTCTTTCATTCGGTTTACTGCAGC-3' (Banihashemi *et al*., 2015). The PCR condition for *rolB* amplification consisted of denaturation at 94 °C for 5 min followed by 35 cycles of 1 min denaturation at 94 °C, annealing at 55 °C for 45 s, then extention for 1 min at 72 °C and finally 7 min at 72 °C. The products were separated on a 0.8% agarose gel (w v^{-1}).

Hairy roots culture establishment

Hairy roots obtained from the solid B5 medium were cut and transferred into a 125 mL flask containing 20 mL of liquid B5 medium. Roots were kept in a growth chamber at 25±2 °C at 100 rpm rotation in the dark. The hairy roots were harvested after 4 weeks and their fresh and dry weights were recorded.

Determination of total phenolic and flavonoids content

Sample preparation

Ten mg of dried hairy roots were suspended in 10 ml 80% methanol then centrifuged in 4 °C at 8000 rpm

for 10 min. The clear supernatant was collected and stored in an amber tube for analysis.

Total phenolic content

The total phenolic of the extracts was determined using the Folin and Ciocalteu reagent. The absorbance of samples and standard was measured using a spectrophotometer at 720 nm against a blank. The test sample (0.1 ml) was mixed with 2.8 ml of water and 0.1 ml of Folin-Ciocalteu (50%) phenol reagent (1 : 1). After 5 min, 2 ml of saturated sodium carbonate solution (2% w v^{-1} in water) was added to the mixture and the volume was made up to 3 ml with distilled water. The reaction was kept in the dark for 30 min and after centrifugation the absorbance of yellow color from different samples was measured at 720 nm. The phenolic content was calculated as mg of gallic acid equivalents GAE/g of dry plant material on the basis of a standard curve of gallic acid (20–100 mg l^{-1}), (Meda *et al*., 2005).

Total flavonoids content

The aluminum chloride method was used for the determination of the total flavonoid content of the samples. For total flavonoid determination, quercetin was used to make the standard calibration curve. Stock quercetin solution was ready by dissolving 5.0 mg quercetin in 1.0 ml methanol, then the standard solutions of quercetin were prepared by serial dilutions using methanol (20–100 µg ml-1). An amount of 0.5 ml extracts was separately mixed with 1.5 ml methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml potassium acetate (1 molar) and 2.5 ml distilled water. After mixing, the solution was incubated for 30 min at room temperature. The absorbance of the reaction mixtures was measured against blank at 415 nm with a spectrophotometer. The total flavonoid content was calculated in the test samples using the calibration plot and expressed as mg quercetin equivalent (QE) g^{-1} of dried plant material (Chang *et al*., 2002).

Statistical analysis

Experiments were conducted with completely randomized design with four replications for each treatment. Before the statistical analysis, the normality of the data was tested. Significance of the difference between mean values was determined by one-way analysis of variance (ANOVA). Duncan's multiple range test wase used to compare mean of the treatments at $P \leq 0.01$ using SPSS 16.

RESULTS AND DISCUSSION

Induction and culture of hairy roots

The hairy roots appeared about 10-15 days after induction by six strains of *A. rhizogenes* (A4, A7, ATCC15834, ATCC11325, K599, and A13) (Figure 1). The control explants failed to develop any hairy roots. One-way analysis of variance (ANOVA) results indicated significant differences between the six strains of *A. rhizogenes* in hairy root induction (Table 1). Rashidi Asl *et al*. (2018) used two-week-

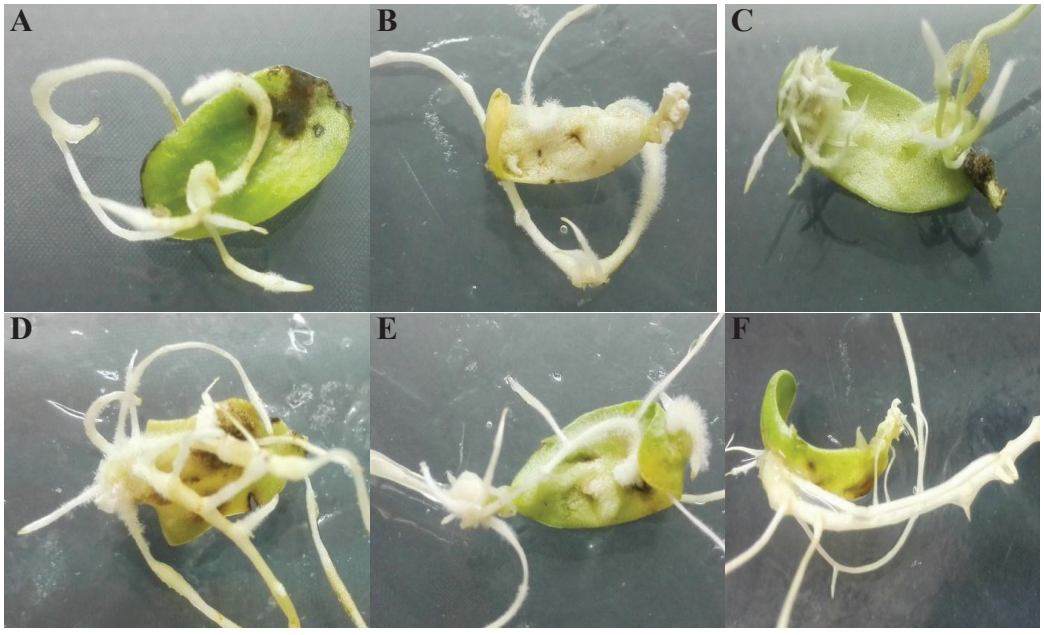

Figure 1. Hairy roots induction by *A. rhizogenes* in fenugreek, **A:** Hairy root induction in transformed explants by K599 strain, **B:** by ATCC15834 strain, **C:** by A4 strain, **D:** by ATCC11325 strain, **E:** by A7 strain, **F:** by A13 strain.

old cotyledonary explants of *Hyoscyamus reticulatus* L. and A7 strain of *A. rhizogenes* for successful hairy root induction. In the present study, the highest percentage of transformation (87.5%) was observed in 7-day-old explants induced by A4 and ATCC11325 strains (Figure 2). In addition, the highest root number (4.25) and root length (2.1) were observed in the 14-day-old explants infected by ATCC11325 strain (Figure 3 and 4). After 4 weeks of culture, the hairy roots were transferred to B5 liquid medium (Figure 5). Sharafi *et al*. (2014a) suggested an efficient hairy root induction system for *Dracocephalum kotschyi*, through *A. rhizogenes*-mediated transformation by modifying the co-cultivation in MS medium using five bacterial strains, A4, ATCC15834, LBA9402, MSU440, and A13. A drastic increase in transformation frequency was observed when an MS medium lacking NH_4NO_3 KH_2PO_4, KNO_3 and $CaCl_2$ was used. In another study, Sharafi *et al*. (2014b) reported the maximum frequency of hairy root induction in *Artemisia aucheri* Boiss using MSU440 (93%) and ATCC15834 (89%) bacterial strains. Influence of bacterial strains on transformation frequency has been documented earlier in different plant species. A4 strain has been widely used for hairy root induction in many plants such as *Hypericum perforatum* (Vinterhalter *et al*., 2006), *Catharanthus roseus* (Batra *et al.,* 2004), *Gentiana macrophylla* (Tiwari *et al*., 2007). In another report, Akbarian *et al*. (2011) evaluated hairy root cultures of two fenugreek ecotypes via cotyledonary explants infected with *A. rhizogenes* (OD_{600}=0.4) and transgenic roots were identified using the *rolB* gene as a marker in PCR analysis. Hairy roots were developed in *Trigonella foenum- graceum* using three concentrations of OD_{600}=0.8, 1.2, and 1.6 of *A. rhizogenes* strain K599 harboring a GFP gene. The highest transgenic hairy root (8.76), the transformation frequency (79.76%), and the growth rate of transgenic roots (0.77 d) were obtained from infection with K599 at OD600=1.2 (Shahabzadeh *et al*., 2013). Juvenility and nature of explant influences the *Agrobacterium* mediated transformation process and *Agrobacterium* transformation is closely related with the age of the host tissue (Kabirnataj *et al*., 2013).

Table 1. Analysis of variance of the effect of explant age and strain type on hairy root characteristics.

Source of variation	df	Mean of square		
		Percentage of hairy root induction	Roots number	Root length
Strain	5	5862.847**	2.504**	1.409**
Explant age	2	10842.014**	2.552**	1.845**
Strain×Explant age	10	685.764**	0.800**	0.460**
Error	54	101.273	0.21	0.20

*, **: significant at 5% and 1% probability level, respectively.

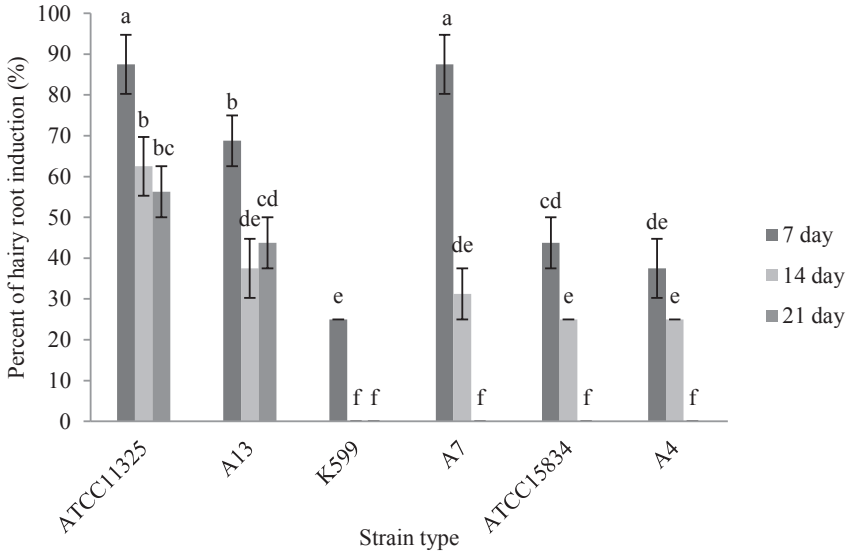

Figure 2. Mean comparisons of the effects of explant age and strain type on hairy roots induction.

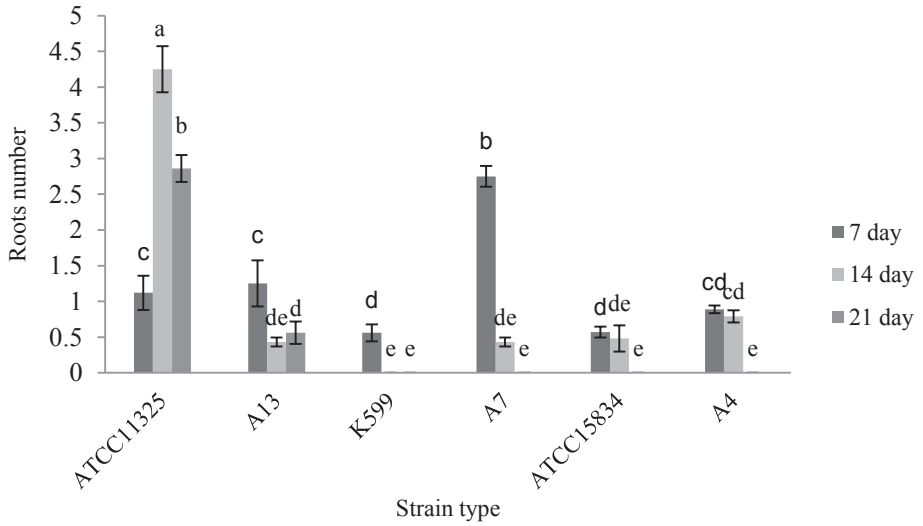

Figure 3. Mean comparisons of the effects of explant age and strain type on hairy roots number.

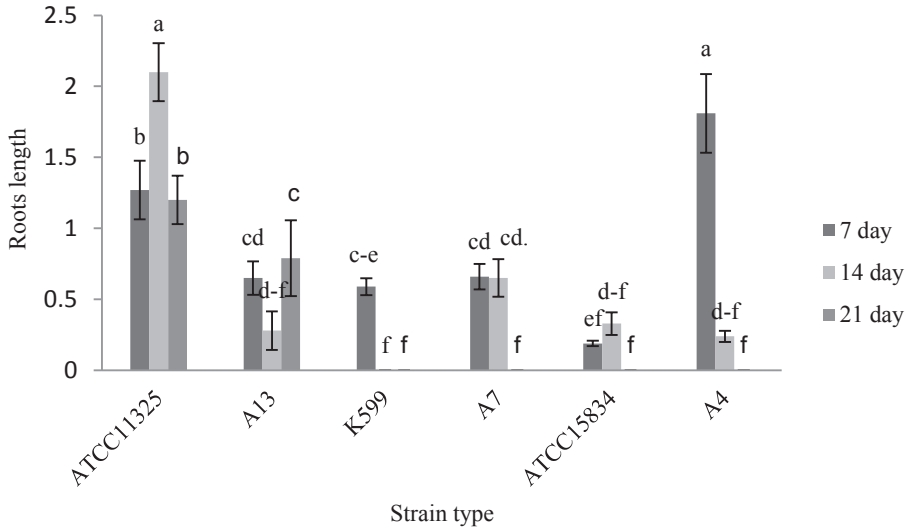

Figure 4. Mean comparisons of the effects of explant age and strain type on hairy roots length.

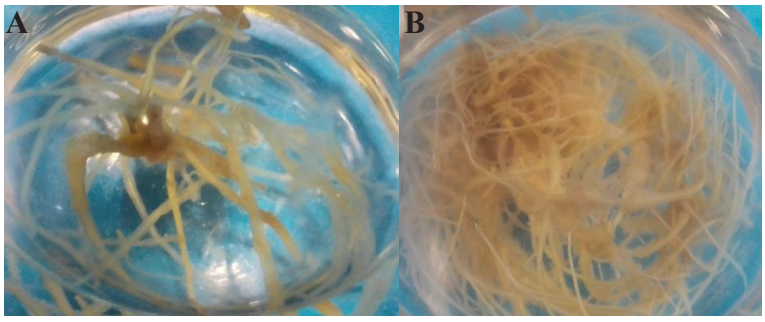

Figure 5. A: Hairy root production by *A. rhizogenes* in fenugreek, **B:** Hairy root growth in liquid medium.

PCR analysis of transgenic roots

PCR was performed using specific primers to determine the presence of T-DNA segment of Ri plasmid in the genomic DNA of T. *foenum– graecum* hairy roots. The PCR with primers specific for *rolB* gene and template DNA from hairy roots amplified the expected band of 760 bp (Figure 6) confirming the successful integration of T-DNA, while DNA templates from untransformed roots (used as control) did not show any amplification.

Hairy root establishment

Among different strains and explant ages evaluated, the ATCC11325 and A13 strains were found to be suitable for enhancement of fresh weight of hairy roots in all explant ages. Other strains were suitable in earlier age of explants. The highest biomass (0.203 g FW) was obtained in the hairy root culture induced by A4 strain in 14-day-old explants. Maximum dry weight was obtained by ATCC11325 and A7 strains and 7-day- old explants and growth index of hairy roots (5.375) was obtained with ATCC11325 strain (Table 2 and 3). In a research, three strains of *Agrobacterium rhizogenes* (ATCC15834, MSU440 and K599) were used for optimization of hairy root development. All parts of the seedlings were able to produce hairy roots. Also, the highest dry weight of hairy root was obtained by *A. rhizogenes* strain ATCC15834 (Qaderi *et al*., 2016). In another study, hairy root culture system of *Gentiana scabra* and influence of different plant growth regulators on the production of secondary metabolites were described. In this study, leaf explants were infected with *A. rhizogenes*, which induced hairy roots up to 21%. Among various solid and liquid media, B5 liquid medium resulted in maximum root biomass (36-fold higher) in 4-weeks. Quantitative analysis showed that secondary metabolite accumulation was 1.8-fold higher in the presence of naphthalene acetic acid (NAA, 1 mg l^{-1}). The use of hairy root cultures is an excellent alternative for harvesting natural or *in vitro* grown plants to produce pharmaceutically important metabolites in less time with ensured quality (Ooi *et al*., 2013; Huang *et al*., 2014). Sharafi *et al*. (2012) used three explant types, hypocotyls, leaves and excised shoots of *Papaver bracteatum* for hairy root induction with strains A4, ATCC15834, LBA9402, MSU440 and A13. The highest frequency of transformation was

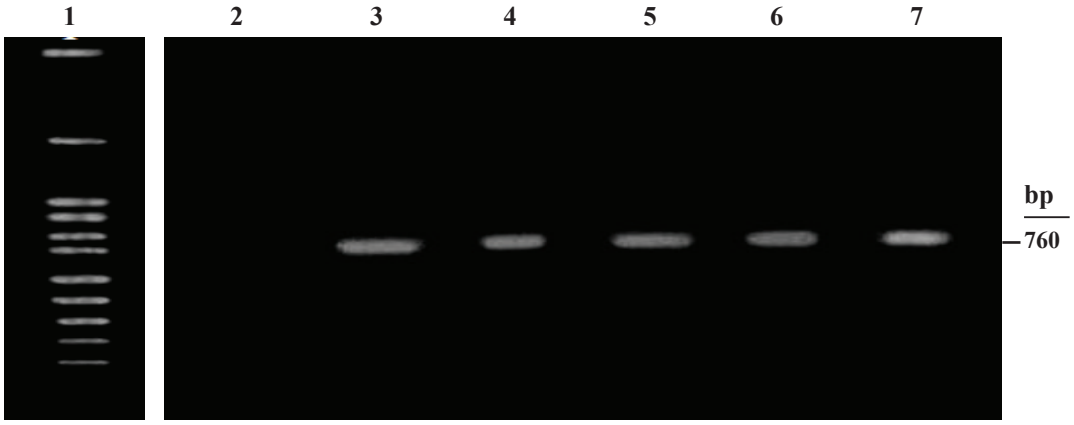

Figure 6. PCR Figure amplified DNA fragments (760 bp) using specific primers for the *rolB* gene of *A. rhizogenes* in *fenugreek* hairy root DNA. 1: 100 bp DNA Ladder, 2: Adventitious root raised form non-transformed explant as a negative control, 3- 6: hairy root lines, 7: *A. rhizogenes* plasmid ATCC11325 strain as a positive control.

Table 2. Analysis of variance of the effect of strains and explant ages on hairy roots growth.

Source of variation	df	Mean of square		
		Fresh weight	Dry weight	Growth index
Strain	5	0.199**	0.025**	772.856**
Explant age	2	0.111**	0.031**	235.381**
Strain×Explant age	10	0.035*	0.005*	57.204*
Error	54	0.015	0.002	28.361

*, **: significant at 5% and 1% probability level, respectively.

Table 3. Mean comparisons of the effects of strains and explant ages on hairy roots growth rate.

Strain	Explant age (day)	Fresh weight (g)	Dry weight (g)	Growth index
ATCC11325	7	0.056^{ab}	0.029^{a}	5.375^{a}
	14	0.120^{ab}	0.017^{ab}	2.500^{b-c}
	21	0.057^{ab}	0.019^{ab}	4.000^{a-c}
A13	7	0.117^{ab}	0.015^{ab}	4.136^{ab}
	14	0.052^{ab}	0.011^{ab}	3.937^{a-c}
	21	0.118^{ab}	0.015^{ab}	3.950^{a-c}
K599	7	0.048^{b}	0.005^{ab}	1.66^{b-d}
	14	0^{b}	0^{b}	0^{d}
	21	0^{b}	0^{b}	0^{d}
A7	7	0.096^{ab}	0.029^{a}	1.973^{b-d}
	14	0.018^{b}	0.009^{ab}	1.194^{d}
	21	0^{b}	0^{b}	0^{d}
ATCC15834	7	0.010^{b}	0.002^{b}	0^{d}
	14	0.007^{b}	0^{b}	0^{d}
	21	0^{b}	0^{b}	0^{d}
A4	7	0.069^{ab}	0.017^{ab}	1.35^{cd}
	14	0.203^{a}	0.023^{ab}	1.617^{cd}
	21	0^{b}	0^{b}	0^{d}

Numbers followed by the same letter are not significantly differents ($P \leq 0.05$).

achieved using LBA9402 strain in the excised shoots. Also the effect of sucrose concentration and the ratio of NH4: NO3 on hairy root biomass was examined. Maximum biomass was obtained in 30 g/l sucrose and 20:10 mM ratio of NH_4 to NO_3 on MS medium. Valimehr *et al*. (2014) used two explant types, several co-culture media and different bacterial strains of *Nepeta pogonosperma* for hairy root induction with strains, A4, ATCC15834, LBA9402, MSU440 and A13. The maximum rate of hairy root induction was obtained from stem explants using MSU440 and ATCC15834 bacterial strains. Akbarian *et al*. (2011) reported successful induction of hairy roots from *Trigonella foenum-graecum* L. The highest dry weight (0.219 g) was achieved in Zanjan hairy roots induced by 15834 strain of *A. rhizogenes*. In another study, the abilities and efficiencies of five different strains of *A. rhizogenes* (strain ATCC 31798, ATCC 43057, AR12, A4 and A13) were tested for hairy root induction in *Solanum mammosum* (Ooi *et al*., 2013). Subsequently, cultures were established for five different hairy root lines generated by five different strains of bacteria. However, different hairy root lines showed different growth indices under the same culture condition. The hairy root lines induced by *A. rhizogenes* strain ATCC 31798 exhibited largest increase in fresh biomass at 45 days of culture under 16 h light/8 h dark photoperiod in half-strength MS medium.

Total phenolic and flavonoid content

Phenolic compounds show a range of physiological properties, such as anti-inflammatory, antimicrobial and antioxidant effects (Balasundram *et al*., 2006). The qualitative and quantitative analyses of phenolic compounds from *T. foenum–graecum* hairy roots and non-transformed (roots from *in vitro* seedling) root extracts were studied. Results showed significant ($P \leq 0.01$) differences between the phenolic content of dried methanol and ethanol extracts in the hairy roots resulted from different treatments (Table 4). The total phenolic content was calculated using the Folin-Ciocalteu method. The total phenolic content was highest (74.05 mg g^{-1} DW) in the hairy roots obtained from the A7 strain. The results of flavonoid measurement showed significant differences between flavonoid content in hairy root in comparison with control culture. Flavonoid content was maximum (11.80 µg g^{-1} DW) in the hairy roots obtained from A7 strain (Table 5). Previously, it was reported that *rol* gene from *A. rhizogenes* T-DNA stimulated the production of secondary metabolites in the transformed plant cells of different plants. It was revealed that hairy roots enhanced the amount of flavonoid alkaloids in *Daucus carota* (Bel- Rhlid *et al*., 1993), polyphenols in *Momordica charantia*

(Thiruvengadam *et al*., 2014) and saponin in *Bacopa monnieri* (Majumdar *et al*., 2011).

Table 4. Analysis of variance of the effect of strain on hairy roots growth.

Source of variation	df	Mean of square	
		Phenolic content	Flavonoid 415 nm
Strain	6	189.027**	29.594**
Error	14	29.317	0.223

*, **: significant at 5% and 1% probability level, respectively.

Table 5. Mean comparisons of the effects of strain on hairy roots Characteristics.

Strain	Phenol 720 nm (mg g-1 DW)	Flavonoid 415 nm (µg g-1 DW)
ATCC11325	60.63^{b}	3.18^{cd}
A13	61.57^{b}	4.98^{b}
K599	60.09^{b}	3.95^{c}
A7	74.05^{a}	11.80^{a}
ATCC15834	48.33^{c}	2.55^{d}
A4	59.73^{b}	3.80^{c}
Control	53.52^{c}	3.89^{c}

Numbers followed by the same letter are not significantly differents ($P \leq 0.05$).

CONCLUSION

The present study describes the successful genetic transformation and establishment of a highly productive hairy root culture of *T. foenum–graecum*. This is the first report of hairy root induction of medicinal plant *T. foenum– graecum* L. by A13, A7 and ATCC11325 *A. rhizogenes* strains. The *A. rhizogenes* plasmid Ti section with its gene *rolB* is important for root induction and growth. The results showed that the use of different bacterial strains and explant ages were effective factors for enhancement of growth and secondary metabolite production in hairy root culture of *T. foenum–graecum.*

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