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Evaluation of trigonelline production in *Trigonella foenum-* graecum hairy root cultures of two Iranian masses

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Abstract

The production of the trigonellin by hairy root cultures of *Trigonella foenum* was described in two Iranian masses (Zanjan and Borazjan). Different *Agrobacterium rhizogenes* strains (*A4*, *9126* and *15834*) and infection methods (co-coltivated and injection) were tested to investigate the ability for transformation and production of trigonellin in T. *foenum*. PCR analysis using *rolb* gene was used for identification of transformed hairy roots. All the strains of *A. rhizogenes* used in this study were able to produce hairy roots. The transformation efficiency was %26 in injection method for whole plants. The higher growth index was obtained in hairy roots induced by 2 strains, *15834* and *9126*, in Zanjan and Borazjan masses, respectively. Detection and identification of trigonellin for a 28 days period was carried out by high performance liquid chromatography method. The highest amounts of trigonellin in Borazjan and Zanjan hairy roots were 14.89 and 14.03 mM g^{-1} DW after 28 and 7 days, respectively. The selection of an effective *A. rhizogenes* strain and T. *foenum* genotype for the production of trigonellin in transformed root cultures is important. This successful production of trigonellin may be used as a useful system for comparative studies of production capacity of trigonellin in different genetic resources *T. foenum.*

Keywords: *Trigonella foenum* graecum, Hairy root, Trigonellin.
Abbreviations: PCR-polymerase chain reaction; HPLC-high performance liquid layer chromatography; MS-Murashige and Skoog.

Introduction

Trigonella foenum- graecum L. (fenugreek) is an annual plant from the family of papilionaceae, leguminosae and has been used for centuries as a natural remedy for many medicinal properties (antidiabetic, hypo cholesterolemic activity) (Khosla et al., 1995; Al-Habori et al., 1998; Motamedi et al., 2011). Fenugreek seeds have been extracted for polysaccharide, galactomannan, different saponins such as diosgenin, yamogenin, mucilage, volatile oil and alkaloids such as choline and trigonelline (Aasim et al., 2010). Trigonelline, coumarin and nicotinic acid have been isolated from Fenugreek seeds and shown to be useful in diabetes (Moorthy et al., 2010). Trigonelline is a hormone found naturally in plant products and is a vitamin B6 derivative. Trigonelline may also have several therapeutic properties, such as anti-migraine, anti-carcinogenic (cervix and liver), antiseptic, hypoglycemic and hypocholesterolemic, activities. There are various masses of this plant in Iran. Twenty masses of fenugreek native to Iran have been gathered from different parts of the Iran to assess the genetic variability of this plant (Moradi et al., 2010). The longest and the shortest whole chromosomal length (48.3 and 26.46 Microns) were measured in Zanjan and Borazjan, respectively. These results showed that Borazjan masses are different from the other masses and makes a suitable genetic resource for features research program. A few investigations have studied the production of diosgenin and trigonelline by tissue cultures of *T. foenum.* Brain and lockwwod (1976) have been reported variation in the levels and distribution of free and bound sapogenin with alteration in medium composition and culture age in suspension cultures of *T. foenum.* Time course studies were carried out on steroid levels during induction of callus from seedlings of *T. foenum* by lockwod and Brain (1976). The development of Fenugreek cell suspension culture has been achieved by Radwan and Kokate (1980). The content of trigonelline was appreciably higher than that of the calli. Oncina et al., (2000) reported the diosgenin accumulation in leaf, stem and root of Fenugreek calli. Hairy root culture of *T. foenum* has been stablished with *Agrobacterium rhizogenes* strain *A4* for diosgenin production (Merkli et al., 1997). The highest diosgenin production was observed in half- strength woody plant medium (0.040% dry weight) which represents almost twice the amount detected in non- transformed roots (0.024% dry weight). Stolone and its postulated precursors were detected in hairy root cultures of *T. foenum* (Paraza-luna et al., 2001). Hairy roots are formed by genetic transformation of plant cell using *Agrobacterium rhizogenes* like most differentiated plant tissues; hairy roots exhibit a high degree of chromosomal stability over prolonged culture periods (Charlwood and Charlwood, 1991). Hairy root cultures offer promise for production of valuable secondary metabolites in many plants, and large-scale cultures of hairy roots of several medicinal plant species have been reported so far (Yang et al., 2011).The aim of the present research was description of an efficient protocol for development of the hairy root cultures of *T. foenum* and production of trigonelline, as well as the evaluation of the trigonelline

production capacity and growth rate in hairy root cultures established from two Iranian masses of *T. foenum* (Zanjan and Borazjan).

Results and discussion

Various species of bacteria can transfer genes to higher plants (Broothaerts et al., 2005). *Agrobacterium rhizogenes,* a gram negative soil bacterium, is one of the most widely studied among them. It infects the plant cell and leads to the formation of hairy roots (Guillon et al., 2006). There are some reports that suggest the successful use of *A. rhizogenes* harboring binary vectors with desired gene constructs for plant genetic transformation (Christey, 2005). *A. rhizogenes 15834, 9126 and A4* are the most common strains used for hairy root induction. Recently, several other studies have also reported the production of secondary metabolites from hairy root cultures induced by these strain. In this study different *Agrobacterium rhizogenes* strains were tested to investigate the ability for transformation and production of secondary metabolites in *T. foenum.* The hairy roots were obtained after 3 weeks past transformation of *T. foenum* with the 3 strains of *A. rhizogenes* (*AR15834, 9126 and A4*). The transformation efficiency was 6% in co-cultivated explants (cotyledon) with bacterial suspension and 26% for whole plants by injection method. The transformed nature of the six hairy root clones was confirmed through PCR analysis by the presence of *rolb* (Fig 1). The hairy roots were long, well branching and formed typical transformed root cultures. Because of higher transformation efficiency, we proposed the injection method of cotyledon and leaf explants for induction of hairy roots in *T. foenum* (Fig 2). Rahnama et al, (2008) studied hairy root induction in *Silybum marianum* by injection or inoculation of explants with *A. rhizogenes*. They proposed inoculation method of cotyledon and leaf explants for induction of hairy roots. This implies that different types of hairy root induction have great influence on the induction of hairy roots. The productivity potentials of the clones after 28 days, in terms of dry weight, are presented in Fig 3. It was observed that the lowest biomass was obtained in *T. foenum* hairy roots induced by *A4* strain. Over a 28- day period, the dry weights of hairy roots from Zanjan and Borazjan were 0.055 and 0.021 g, respectively. The dry weights of hairy roots induced by *9126* strain from Zanjan and Borazjan sources were 0.085 and 0.1445 g, respectively. The highest dry weight (0.219 g) was achieved in Zanjan hairy roots induced by *15834* strain of *A. rhizogenes* that was 7.06- fold greater than the Borazjan hairy roots obtained with the same strain of *A. rhizogenes.* This is in agreement with earlier reports that bacterial strain specificity was found to play a determine role in establishing hairy roots (Chandra Verma et al., 2007) Our results showed that the sources of hairy root lines have a significant role in growth behavior of hairy roots. Based on these results, hairy roots established with *15834* and *9126* strains for Zanjan and Borazjan sources were chosen for further experiments. The induction of hairy roots is genotype dependent in T. *foenum.* In agreement with these observation, Michel et al., (2008) reported that ten cotton genotypes was different in callus initiation.The next experiment was based on the selected hairy roots. The results of time course study for trigonellin production and dry matter in hairy root cultures resulting from Zanjan mass are shown in Fig 4A. The DW of hairy roots was not significantly changed after 7 days (0.005 g). A significant increase in the DW of cultures was observed after 14 days (0.01 g) that was 2- fold above the 7 days cultures. A

Fig 1. PCR analysis of *T. foenum* hairy roots for *rolb* transgenes with three strains of *Agrobacterium rhizogenes* (*A4*, *9126* and *15834*). Molecular weight marker (left and right), 1, 2 and 3: Positive control (*A4*, *9126* and *15834* strains, respectively); 4: Non transgenic root, 5, 6 and 7: Transgenic hairy roots (Zanjan), 8, 9 and 10: Transgenic hairy roots (Borazjan).

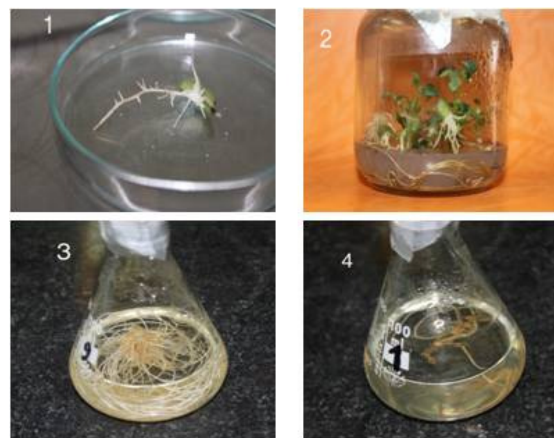

Fig 2. Hairy root induction in *T. foenum* in cotyledon (1) and whole plant (2), with injection method. Hairy roots in liquid culture: Zanjan (3) and Borazjan (4) on hormone- free MS medium after 28 days of culture.

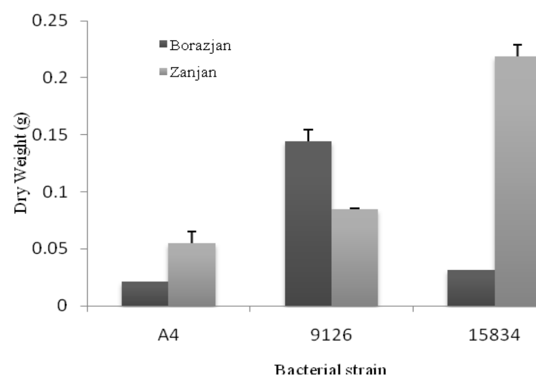

Fig 3. Comparative analysis of dry matter (g) production potential of six *T. foenum* hairy roots (Zanjan and Borazjan) transformed with three strains of *A. rhizogenes* (*A4*, *9126* and *15834*) after 28 days. Data are the average of three experiments; each in triplicate (means ± SD).

reduction in DW (0.007 g) was observed after refreshment of medium in 21 day for a 28-day culture period. The DW of non- transformed hairy roots was 0.01 g in 28 days cultures. The highest trigonellin content was obtained after 7 days that was 2.22- fold greater than the primary hairy root cultures (7.64 mM g^{-1} DW). The trigonellin content in the 28 days hairy root cultures was 7.56 mM g^{-1} DW while in the 21 days hairy root cultures was 4.75 mM g^{-1} DW. Fig 4B indicates trigonellin and dry matter in hairy root cultures resulting from Borazjan mass. The DW of hairy roots was significantly increased in culture period and reached an extremely high level (0.009 g) after 21 days. The DW of subcultured hairy roots did not change significantly. As an overall trend, it is quite obvious that the content of trigonellin dramatically rose, hitting a peak (9.41 mM g^{-1} DW) from 0 to 21 days that was 2.18- times that of the primary cultures (4.30 mM g^{-1} DW). There was a significant increase in trigonellin content (14.89 mM g^{-1} DW) in 28 days hairy root culture that we had refreshed their media after 21 days. As shown in Fig 4B the changes of total trigonellin in hairy root cultures (Borazjan) was similar to those of DW content. The trigonellin content in non-transgenic root cultures was 2.97 mM g^{-1} DW after 28 days of culture, which was 5.01 times less than of the 28 days transgenic hairy root cultures.

The selection of an effective *A. rhizogenes* strain for the production of transformed root cultures is important (Lee et al., 2010), and must be determined for each experiment. Therefore, three different *A. rhizogenes* strains: *A4*, *15834* and *9126* were investigated to check their ability of transformation of *T. foenum* explants. After 10 min of co-cultivation with *A. rhizogenes*, explant tissues were transferred to agar-solidified MS medium containing 250 mg l^{-1} cefotaxime, to eliminate *A. rhizogenes.* Hairy roots emerged from wound sites on *T. foenum* explant within 10 days after inoculation. After one week, hairy root began to grow more rapidly. About three weeks hairy roots were excised from the explant tissues and subcultured on agar-solidified medium containing 250 mg l^{-1} cefotaxime. Wounded *T. foenum* leaves were susceptible to infection by each strain of *A. rhizogenes.* The transformation frequency for injection method was higher than co-cultivation of explants (cotyledon) with bacterial suspension. The highest DW was found in Borazjan and Zanjan hairy roots induced by *9126* and *15834* strains, respectively, by injection method. We found that there was difference in the morphological characteristics between Borazjan and Zanjan hairy roots.Our results indicate an efficient *A. rhizogenes*-mediated transformation protocol for the establishment of *T. foenum* hairy root cultures using three different *A. rhizogenes* strains. We were successful to introduce the best strain of *A. rhizogenes* for establishment of *T. foenum* hairy root cultures. The genetic transformation mediated by *Agrobacterium* is affected by explant genotype and structure, chemical and physical factors, bacterial strains and signal molecules (Tao and Li, 2006). Different strains of *A. rhizogenes* vary in their transforming ability (Krolicka et al. 2001; Zehra et al., 1998; Vanhala et al., 1995). This also was confirmed in our study that bacterial strains used in the experiment demonstrated the significant differences in virulence. Strain specificity observed in the present study agrees with the hypothesis that, the ability for infection of the different *A. rhizogenes* strains in a given species was different. The different nature of the induced hairy roots based on specific insertion of Ri T-DNA is responsible for such variation in hairy root potentials in the metabolites biosynthesis and growth index. Growth- related production behavior of the active constituents has been noted in a number of other plant species by several investigators

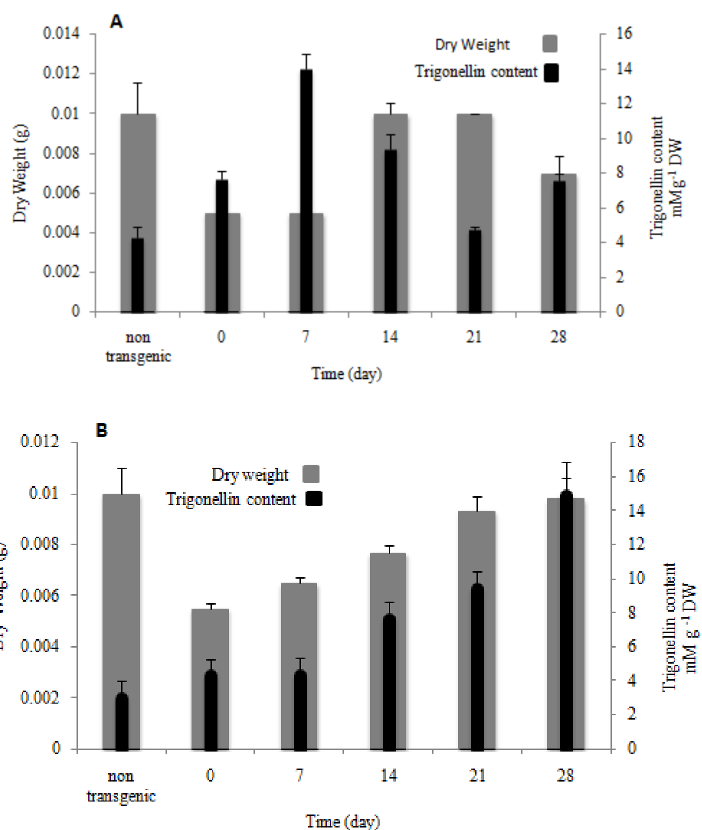

Fig 4. Time course of dry matter (g) and trigonellin content (mM g-1 DW) in T. foenum hairy roots (Zanjan (A) and Borazjan (B)) transformed with A. rhizogenes (15834 (A) and 9126 (B)). Data are the average of three experiments; each in triplicate (means ± SD).

(Chandra et al., 2007; Lee et al., 2010). In addition, extensive morphological variation in individual hairy root cultures can be possibly due to differential expression of T-DNA genes present in the transformed roots, variable copy numbers of T-DNA inserts and positional integration effects of the T-DNA in the host genome (Cho et al., 1998). It is interesting to note that the biosynthetic potential of the two types of hairy roots belong to different masses (Zanjan and Borazjan), in terms of trigonellin content, indicated various behavior. Additionally, this selected root cultures of *T. foenum* produced almost three and five times higher amounts of trigonellin compare to that of the non- transformed roots at their respective optimum production times. Moreover, the origin of hairy root lines and differences in chromosome size may offer a good opportunity to obtain hairy root cultures with increased productivity of secondary metabolites. In most plant species artificial polyploidy has enhanced the vigor of determinate plant parts. Thus, artificial polyploidy may prove useful in achieving rapid genetic improvement of plants. Although the effects of polyploidy are not generally predictable, and each species must be generally examined individually, doubling the chromosome number of plant species may offer a good opportunity to obtain plants with increased productivity of secondary compounds. Several investigator suggested polyploid induction of hairy roots to increase metabolites production (Wallaart et al., 1999).

Materials and methods

Plant materials

Fenugreek seeds (Zanjan (48º29´ N, 36º41´ E, 1662 m) and Borazjan (51º34´ N, 29º35´ E, 632 m) were obtained from the Faculty of horticulture, Islamic Azad University, Science and Research Branch, Tehran, IRAN. The seeds were sterilized by dipping in sodium hypochlorite 2% (W/V), Tween 20, 0.1% (V/V), and rinsed exhaustively with sterile distilled water. The seeds were cultured in hormone-free Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium and incubated in the dark at 26 ± 1°C.

Induction and establishment of hairy root cultures

Hairy root cultures of *T. foenum* were established using three strains of *Agrobacterium rhizogenes*: *AR15834, 9126 and A4*. All explants were precultured for 3 days on MS basal medium. The explants were co-cultivated with bacterial suspension for 10 min (OD_{600} = 0.4). After three days, they were transferred to MS medium supplemented with cefotaxime (250 mg l^{-1}). In the second method, the hairy roots were induced by injection of *A. rhizogenes* suspension culture to cotyledon of whole seedling. Hairy roots which arose mainly from the cut surfaces of the explants were separated, when they attained a length of 1-2 cm, and subcultured in the dark on above media. Wiled- type root cultures were established in hormone- free MS liquid medium as control. The hairy roots were maintained by subculture of 6 pieces (1 cm) on MS liquid medium on rotary shaker (120 rpm) at 25 ºC in complete darkness. Cultures were subcultured every 2 weeks.

PCR analysis

Genomic DNA from root was extracted for PCR analysis as described by Khan et al. (2007) and Rahnama et al. (2008). The PCR was performed for 35 thermal cycles (denaturizing at 94°C for 1 min, primer annealing at 53 °C for 1 min, and primer extension at 72 °C for 1 min) for *rol*b (5´-ATGGATCCCAAATTGCTATTCCCCACGA-3' and 5'-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3') specific primers.

Growth studies

The growth characteristic of 6 independently generated hairy roots established from two masses and three strains of *Agrobacterium rhizogenes*: *AR15834, 9126 and A4*) were evaluated on the basis of total dry weight (DW) after 28 days on MS liquid medium on rotary shaker (120 rpm) at 25 ºC in complete darkness. On the basis of the growth behaviors, 2 individual roots from 2 masses (Zanjan and Borazjan) were selected for further studies. The selected hairy roots (six 1 cm pieces from 28- day- old culture) were inoculated into 50 ml liquid MS- hormone free media containing 3% (w/v) sucrose in 250 ml Erlenmeyer flasks on a rotary shaker at 120 rpm, 25°C and cultured in darkness for 21 days. The hairy root cultures media was refreshed after 21 days. For a time-course study, hairy roots were harvested after 7, 14, 21 and 28 days. A non- transgenic root cultures was harvested after 28 days for biomass and metabolite content. Each experiment was repeated twice with 3 replicates each.

Extraction and determination of Trigonelline

The hairy roots were harvested from the shake-flasks and dried by tissue paper. Lyophilized powdered hairy root samples were measured in terms of dry weight (DW). Dry tissue 50 mg was powdered and extracted with methanol 2.5 ml by 30 min ultrasonication. After centrifugation for 15 min at 3000 rpm and at 4 ºC, the supernatant was collected. The residue was reextracted with methanol 2.5 ml and ultrasonicated for 15 min. The two supernatants were combined and evaporated to dryness. The dry residue was reconstituted in 5 ml methanol and kept at 4 °C in darkness. A method of high performance liquid chromatography (HPLC) was established for the quantitative determination of trigonelline (Rongjie et al., 2010). A knauer liquid chromatography equipped with a knauer injector with a 20 µl loop, a YMC_ Pack polyamine Π, 250× 4.6 mm, l.d.S_5µm, 12 nm column, knauer K2600A UV detector and Chromgate software for peak integration. Mobil phase consisted of the solvents; acetonitrile: water (10:90). All solvents and chemicals were of HPLC grade (Merck). The elution time and flow rate were 6 min and 1 ml min^{-1} and peaks detected at 263 nm. Identification was achieved by comparison of retention times (R_t) of standard of trigonelline. The trigonelline content of the root was expressed as mM per g DW and accomplished using a known concentration of standard and peak areas. The data obtained from the analysis of each sample allowed to plotting a calibration curve showing a good linearity (r^2 = 0.999). The data were given as the mean of at least three replicates. Statistical significance was calculated using Duncan test for unpaired data (ά≤ 0.05) and ANOVA method was used for comparisons of means. Statistical analysis was made by SAS software (Version 6.2). Standard of trigonelline was from sigma.

Conclusions

The availability of a protocol for the production of trigonelline similar to those described above provides a powerful system to study
various aspects of the metabolic and molecular regulation of trigonelline biosynthesis and it is necessary to evaluate and screen various elicitors with different mechanisms on the production and accumulation of trigonelline for pharmaceutical industries.

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