

## Improving Trigonelline Production in Hairy Root Culture of Fenugreek (*Trigonella foenum-graecum*)

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### Abstract

**Background:** *Trigonella foenum-graecum* L. commonly known as fenugreek is a rich source of important medicinal metabolite, i.e. trigonelline.

**Objective:** In this study, hairy roots culture as a novel method for trigonelline production was evaluated.

**Methods:** For optimizing the hairy roots culture of *Trigonella foenum-graecum*, three strains of *Agrobacterium rhizogenes* (ATCC15834, MSU440 and K599) via two inoculations methods including scotch and vacuum pump were used to agro-infiltration. Two elicitors including methyl jasmonate (0, 25, 50, 100 and 200  $\mu$ M) and chitosan (0, 50, 100, 150 and 200mg/l) were added to liquid medium as abiotic and biotic elicitors in various concentrations, respectively.

**Results:** The trigonelline content was increased via elicitation by methyl jasmonate and chitosan against control condition. The maximum trigonelline (36.7 and 37.3 mM/g D.W) were observed in 100  $\mu$ M of methyl jasmonate and 150 mg/l of chitosan, respectively.

**Conclusion:** All parts of the seedling (crown, stem and leaf) were able to produce the hairy roots. Also, the highest dry weight of hairy root was obtained by *A. rhizogenes* strain 15834. The transformation of fenugreek using *Agrobacterium rhizogenes* to form hairy root cultures has the potential benefits of fast growth and rates of secondary metabolite production equal to or greater than that found for the intact plant.

**Keywords:** *Trigonella foenum-graecum* L., Hairy Root Culture, Trigonelline, Chitosan, Methyl jasmonate

## Introduction

Fenugreek (*Trigonella foenum-graecum* L.) belonging to the subfamily *Papilionaceae*, family the *Fabaceae*, is a valuable medicinal plant which is widely cultivated throughout the world [2]. Fenugreek has various properties such as anti-diabetic, anti-cancerous, anti-microbial and hypocholesterolemic [13]. Therefore, it is necessary to find optimum methods for its metabolites production.

A new method of production of plant metabolites is hairy root cultures, and elicitation can be applied as an important strategy to improve their production. A wide variety of substances (biotic and abiotic) are able to act as elicitor which can trigger the production of many secondary metabolites in plants. As the elicitation process is mediated through different signal transduction pathways, many researchers have also used the signaling molecules as elicitor [3]. Methyl jasmonate (MeJa) derived from linolenic acid by the octadecanoid pathway has been shown to be a powerful inducer of secondary metabolites in various plants [9]. Chitosan, a polymer of  $\beta$ -(1, 4)-glucosamine, is known to be an effective inducer of secondary metabolites in plants and it is obtained by alkaline hydrolysis of shellfish chitin [15].

Trigonelline is a plant hormone, which is claimed to have anti-carcinogenic, anti-migraine, anti-septic, hypocholesterolemia, and hypoglycemic activities. Fenugreek (*Trigonella foenum-graecum*) is a plant which contains trigonelline [23]. Jayant [7] reported biosynthesis of trigonelline. A few investigations have done to produce diosgenin and trigonelline by tissue cultures of

*T. foenum-graecum*. The development of Fenugreek cell suspension culture has been achieved by Radwan and Kokate [18]. Hairy root culture of *T. foenum-graecum* has been established with *Agrobacterium rhizogenes* strain A4 for diosgenin production [11, 25]. Mathur and Yadav [10] studied the effect of salicylic acid on trigonelline production in *Trigonella foenum-graecum* cell suspension culture. The present study intended to evaluate the effects of two elicitors (including methyl jasmonate and chitosan) and a medium on trigonelline production in hairy root culture of fenugreek.

## Materials and Methods

**Plant material:** Persian fenugreek seeds (TF-925) were obtained from seed bank of the institute of Medicinal Plants, ACECR, Iran. Seeds surface were sterilized by sodium hypochlorite solution 2% (W/V) for 6 min. After washing with sterile distilled water, the seeds were cultured in hormone-free MS medium (Murashige and Skoog, 1962) and maintained at 25°C under light condition.

**Bacterial strain:** Three *Agrobacterium rhizogenes* strains (ATCC15834, K599 and MSU440) were used to induce hairy roots. The bacteria were cultured into 250 ml liquid Luria-Bertani medium (LB) supplement with rifampicin antibiotic (50 $\mu$ g/ml) and held for 48h in 28°C under darkness with shaking. The optimal density monitored at 600 nm (OD<sub>600</sub>) was approximately 0.4 – 0.8 [25].

**Agro infiltration method and Induction of hairy root:** To induce hairy root, 100  $\mu$ l of *A. rhizogenes* suspension culture

transferred to MS medium supplemented with 100 $\mu$ M acetosyringone and 6% sucrose, (pH 5.8). After 10 minutes, the seedlings were infiltrated by two methods of scotch and vacuum pump. Then infiltrated explants were cultured in MS medium supplemented with cefotaxime (100 $\mu$ g/ml) under 16 h lights, 8 h darkness photoperiod at 25°C. For confirming the induced hairy root, the Polymerase Chain Reaction (PCR) analysis was performed by *rolB* (The forward primer was 5'-GCTCTTGCAAGTCTAGATTT-3', and reverse primer was 5'-GAAGGTGCAAGCTACCTCTC -3') and *virD* genes (The forward primer was 5'-ATGTCGCAAGGCAGTAAGCCC-3', and reverse primer was 5'-GGAGTCTTTCAGCATGGAGCAA-3') in 36 thermal cycles. Total genomic DNA was isolated according to CTAB method [1].

**Growth analysis:** The hairy roots were cultured into MS basal medium supplemented with 6% sucrose. To indicate the optimum time of elicitation the content of trigonelline and its growth index measured in 7, 14, 21 and 28 days.

**Elicitation and Trigonelline analysis:** Methyl jasmonate from sigma (cat no. 39-270-7) was dissolved in ethanol, filter-sterilized (Filter 0.22  $\mu$ M) [9] and Added to the cultures of hairy root at final concentrations of 0, 25, 50, 100 and 200  $\mu$ M. Chitosan was provided from Sigma (cat no. 44-886-9) as well. Chitosan was dissolved in 5% (v/v) 1 N hydrochloric acid (HCl) through gentle heating and continuous stirring. The pH was adjusted to 5 with 1 N sodium hydroxide (NaOH). The solution was stirred to dissolve chitosan

further and then autoclaved for 15 min at 121°C. The solution was kept at 4°C prior to use [15]. Chitosan was added to the cultures at final concentrations of 0, 50, 100, 150 and 200 mg/L. Based on the other researches, the highest dry weight was obtained in MS medium supplemented with 6% sucrose at 21 days 12.2 (mg/g DM) and the highest amount of trigonelline was obtained at MS medium supplemented with 6% sucrose at 7 days 15.29 (mM/g DM) respectively [16]. The hairy root cultures were elicited at 7<sup>th</sup> days after culturing. To analyze the trigonelline content, elicited and non-elicited hairy root were lyophilized. 10 mg of lyophilized samples were extracted in 1 ml ethanol: water (6:4) (24 hours, 25 °C, 100 rpm) and the supernatant was filtered (0.20  $\mu$ m) for HPLC. C18 column was used, the mobile phase consisted of acetonitrile-water (90:10) at a flow-rate of 1.2 ml/min, and detection wavelength was set at UV 265 nm. The column temperature was 30°C [24].

## Results

Several strains of *A. rhizogenes* are used to induction of hairy root in plants successfully. Also production of secondary metabolites in hairy root has reported [26]. All parts of the seedling including crown, stem and leaf were able to produce the hairy root (Figure 1). Also, the highest dry weight of hairy root was obtained by *A. rhizogenes* strain ATCC15834 (Figure 2). The verification of insertion T-DNA segments in root genome was done by PCR analysis with specific genes primers of *rolB* and *virD*, after emergence of the hairy roots (Figure 3).

The use of chitosan (150 mg/L) and MeJa (100  $\mu$ mol) as elicitors increased the amount of trigonelline to 37.3 and 35.43 mM/g DM, respectively (Figure 4, 5). The amount of the trigonelline alkaloid was increased with increasing chitosan concentration. Probably greater production of trigonelline was due to the effect of chitosan in the production cycle of methyl jasmonate. With increasing chitosan concentration to more than 150 mg/L, the

trigonelline content was reduced. Probably at this concentration, the cells were destroyed. With increasing concentration of methyl jasmonate to more than 100  $\mu$ mol, trigonelline production rate was decreased. This reduction was probably due to the burning of the cells. In general, the results revealed that *A. rhizogenes* strains, elicitors and the amount of sucrose are crucial in enhancing trigonelline production.

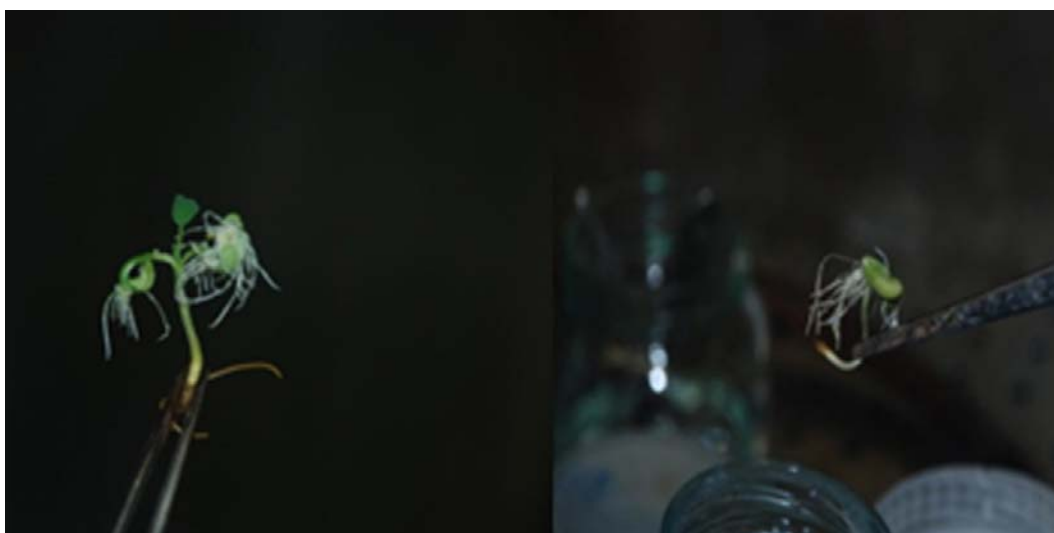


Figure 1- The hairy root induced on all part of plantlet

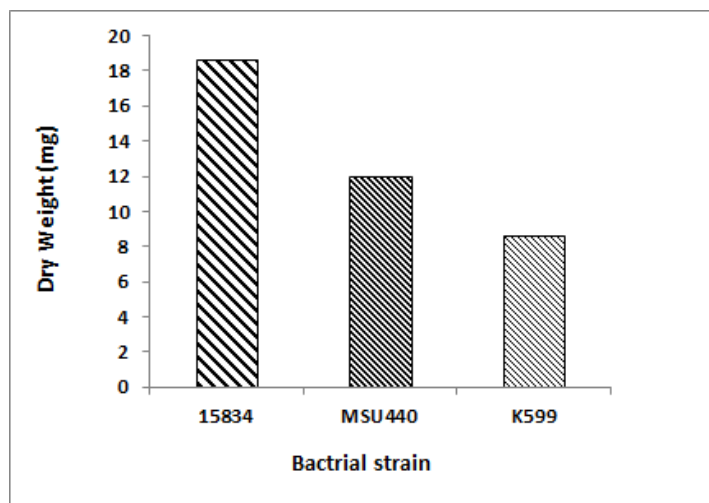


Figure 2- The effect of three strains of *A. rhizogenes* on dry matter production of *T. foenum* hairy roots

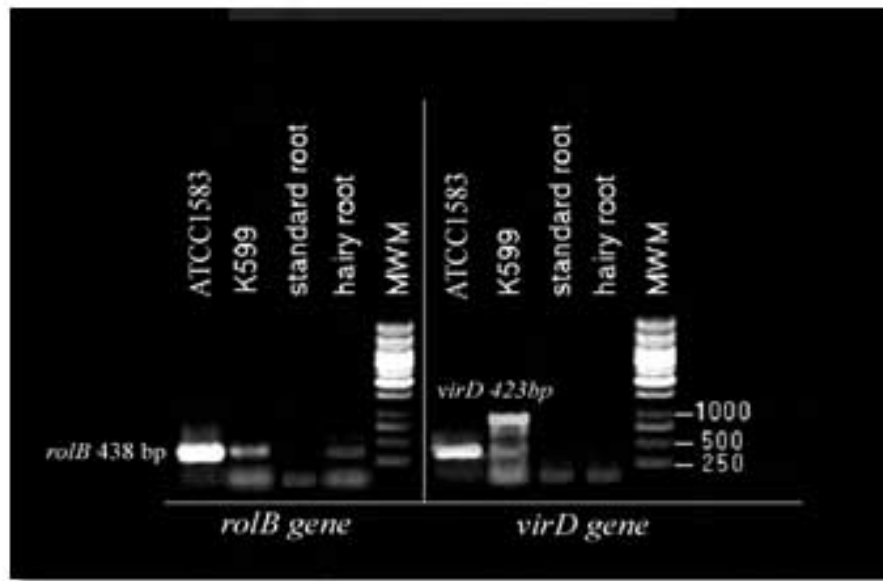


Figure 3- PCR analysis of *T. foenum* hairy roots for *rolB* transgenes with three strains of *Agrobacterium rhizogenes*

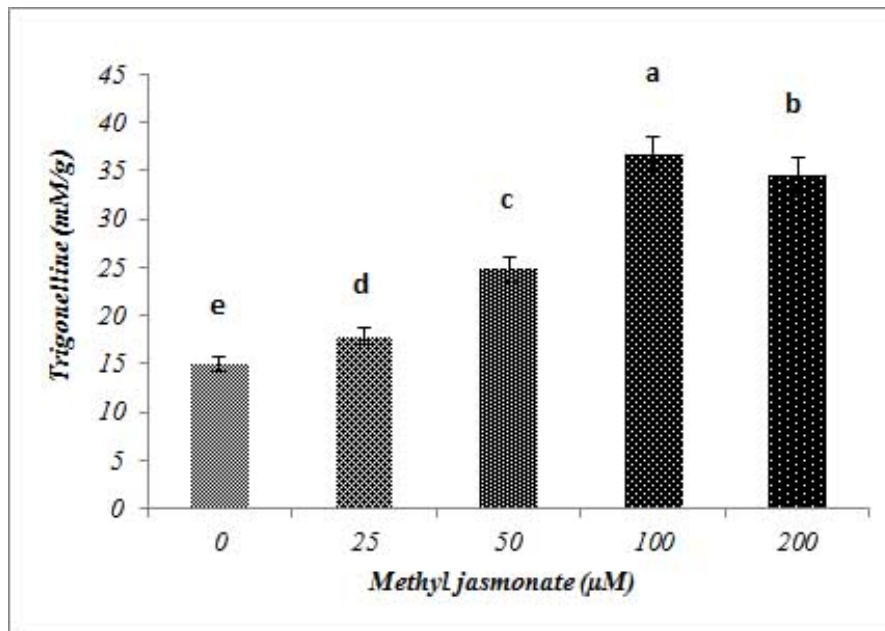
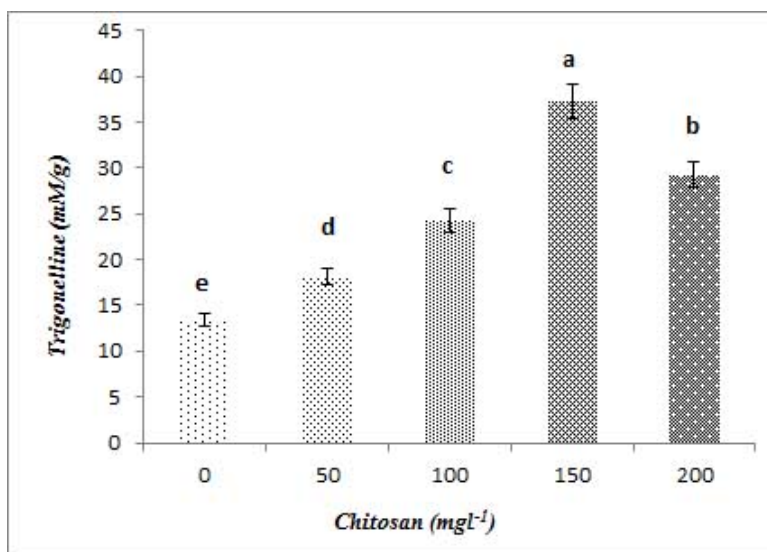


Figure 4- The effect of methyl jasmonate on trigonelline production in hairy roots. Methyl jasmonate (0, 25, 50, 100 and 200  $\mu\text{M}$ ) was added to 7day



**Figure 5-** The effect of chitosan on trigonelline production in hairy roots. Chitosan (0, 50, 100, 150 and 200mg l<sup>-1</sup>) was added to 7 day

## Discussion

In many studies, the important roles of elicitors on over-production of secondary metabolites in medicinal plants have verified such as artemisinin production in hairy root cultures of *Artemisia annua* [21], production of diosgenine by hairy root cultures of *Trigonella foenum-graecum* [11] and elicitation of diosgenin production in *Trigonella foenum-graecum* seedling by heavy metals and signaling molecules [3].

Previously, other studies have also reported the different efficiency of various *A. rhizogenes* strains in promoting the induction, growth and secondary metabolite production of hairy roots. For example, different *A. rhizogenes* strains affected growth rate, saponin production and the ratio of different astragal sides in transgenic root cultures of *Astragalus mongholicus* [6]. The strain of *Agrobacterium* also influenced the development, growth rate and tropane

alkaloids production in transformed root cultures of *Hyoscyamus muticus* [22, 8]. Hairy root cultures of *Gentiana macrophylla* were established by infecting with four *A. rhizogenes* strains and each hairy root lines showed different response regarding growth and production of secoiridoid glucoside gentiopicoside in transformed hairy root cultures [19]. Clearly, 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. A three-fold increase in podophyllotoxin content in comparison with controls was obtained in transformed calli of *Podophyllum hexandrum* developed by transformation of embryo using different strains of *Agrobacterium rhizogenes* viz. A4, 15834, and K599 [5].

The *Agrobacterium rhizogenes* plasmid Ti section with its three genes, *Rol A*, *B*, and *C*, is important for root induction and growth. Once

the roots have grown for a sufficient period of time, they can be excised from the explants tissue and then cultured in a growth medium containing an antibiotic, ultimately to free the cultures of residual *Agrobacterium*.

## Conclusion

The present research is the first study on the effects of two elicitors (including methyl jasmonate and chitosan) and medium on trigonelline production in hairy root culture of fenugreek. Interestingly the trigonelline production positively correlated to elicitors' concentration. All parts of the seedling that is crown, stem and leaf were able to produce the

hairy root. Also, the highest dry weight of hairy root was obtained by *A. rhizogenes* strain 15834. The transformation of fenugreek using *Agrobacterium rhizogenes* to form hairy root cultures has the potential benefits of fast growth and rates of secondary metabolite production equal to or greater than that found for the intact plant.

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## References

1. Akbari Z, Qaderi A, Kalatehjari S, Mehrafarin A and Naghdibadi H. Effect of nitrogen compounds on production of trigonelline in hairy root culture of Iranian fenugreek (*Trigonella foenum-graecum* L.) *J. Medicinal Plants* 2012; 42: 128 - 35.
2. Bhagyasri Y, Lavakumar V, Divya Sree MS and Ashok Kumar CK. An overview on anti-inflammatory activity of Indian herbal plants. *International J. Res. In Pharmaceutical and Nano Sci.* 2015; 4 (1): 1 - 9.
3. Debjani D and Bratati De. Elicitation of Diosgenine production in Fenugreek (*Trigonella foenum-graecum* L.) seedling by Heavy metals and signaling molecules', *J. Acta Physiol. Plant* 2011; 10: 1 - 7.
4. Errol Z. Trigonelline: Review of Toxicological Literature, National Institute of Environmental Health Sciences. 1997, pp: 1 - 27.
5. Giri A, Giri CC, Dhingra V and Narasu ML. Enhanced podophyllotoxin production from *Agrobacterium rhizogenes* transformed cultures of *Podophyllum hexandrum*, *Natural Product Letters* 2001; 15: 229 - 35.
6. Ionkova I, Kartnig T and Alfermann W. Cycloartanesaponin production in hairy root cultures of *Astragalus mongholicus*, *Phytochem.* 1997; 45: 1597 - 1600.
7. Jayant G, Joshi and Handler P. Biosynthesis of Trigonelline', *J. Biological Chem.* 1960; 235: 81 - 83.
8. Mateus L, Cherkaoui S, Christen P and Oksman-Caldentey K. Simultaneous determination of scopolamine, hyoscyamine and littorine in plants and different hairy root clones of *Hyoscyamus muticus* by micellar electro kinetic chromatography, *Phytochem.* 2000; 54: 517 - 23.
9. Mathew R and Sanker P. Effect of methyl jasmonate and chitosan on growth characteristics of *Ocimum basilicum* L., *Ocimum sanctum* L. and *Ocimum gratissimum* L. cell suspension cultures, *African Journal of Biotechnol.* 2012; 11: 4759 - 66.
10. Mathur I and Yadav R. Studied effect of salicylic acid on trigonelline production in

*Trigonella foenum-graecum* L. cell suspension culture', *International Referred Research J.* 2011; 1: 137 - 38.

**11.** Merkli A, Christen P and Kapetanidis I. Production of diosgenine by hairy root cultures of Fenugreek (*Trigonella foenum-graecum* L.), *Plant Cell Reports* 1997; 16: 632 - 36.

**12.** Micheal A and Spena A. The plant oncogenes *rol* A, B and C from *Agrobacterium rhizogenes*. *Methods Molecular Biol.* 1995; 44: 207 - 22.

**13.** Murashige T and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures', *Physiol. Plantarum.* 1962; 15: 473 - 97.

**14.** Nagananda GS, Das A, Bhattacharya S and Kalpana T. In vitro Studies on the effect of bio-fertilizers (Azotobacter and Rhizobium) on seed germination and development of *Trigonella foenum-graecum* L. using a Novel Glass Marble containing liquid medium', *International J. of Botany* 2010; 6: 1 - 10.

**15.** Nagata T and Ebizuka Y. Biotechnology in Agriculture and Forestry (Medicinal and Aromatic Plants) Japan. University of Tokyo. 2002, p: 123.

**16.** MA Abd-El Mawla, Ahmed and Eldien H. Osman. Elicitation of Trigonelline and 4-Hydroxyisoleucine with Hypoglycemic Activity in Cell Suspension Cultures of *Trigonella foenum graecum* L. In The open conference proceedings *J.* 2011; 2 (1): 80 - 7.

**17.** Oncina R, Boto A, Del RJA and Ortun AA. Bioproduction of diosgenin in callus cultures of *Trigonella foenum-graecum* L. *Food Chem.* 2000; 70: 489 - 92.

**18.** Radwan S and Kokate C. Production of higher levels of Trigonelline by cell cultures of Fenugreek (*Trigonella foenum-graecum* L.) than by the differentiated plant, *J. Planta.* 1980; 147: 340 - 44.

**19.** Tiwari RK, Trivedi M, Guang ZC, Guo GQ

and Zheng GC. Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicoside in transformed hairy root cultures', *Plant Cell Reports* 2007; 26: 199 - 210.

**20.** Toppo FA and Akhand R. Pharmacological actions and potential uses of *Trigonella foenum-graecum* L. *Asian J. Pharmaceutical and Clinical Res.* 2009; 2: 29 - 38.

**21.** Udomsuk L, Jarukamjorn K, Tanaka H and Putalun W. Improved isoflavonoid production in *Puerariacandollei* hairy root cultures using elicitation, *Biotechnol. Lett.* 2011; 33: 369 - 74.

**22.** Vanhala L, Hiltunen R and Oksman-Caldentey K. Virulence of different *Agrobacterium* strains on hairy root formation of *Hyoscyamus muticus*, *Plant Cell Reports* 1995; 14: 236 - 40.

**23.** Zeiger E and Tice R. Trigonelline - Review of Toxicological Literature. Research Triangle Park, North Carolina: National Institute of Environmental Health Sciences and Integrated Laboratory Systems. 1997, p: 27.

**24.** Zhao H, Qu Y, Wang X, Zhang H, Li F and Masao H. Determination of Trigonelline in Fenugreek (*Trigonella foenum-graecum* L.) by HPLC, *J. Material Medica.* 2003; 27: 194 - 96.

**25.** Akbarian R, Hasanloo H and Mahmud KH. Evaluation of trigonelline production in *Trigonella foenum- graecum* hairy root cultures of two Iranian masses. 2011, *POJ* 4 (7): 408 - 12.

**26.** Christey MC and Braun RH. Production of hairy root cultures and transgenic plants by *Agrobacterium rhizogenes* mediated transformation. *Methods Mol. Biol.* 2005; 286: 1283 - 88.