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RESEARCH ARTICLE

Influence of *Agrobacterium rhizogenes* strains on hairy roots induction in *Trigonella foenum-graecum* L. and secondary metabolites production

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ABSTRACT: Fenugreek (*Trigonella foenum-graecum* L.) is a rich source of important medicinal metabolites. This plant belongs to the Fabaceae family. Induced hairy roots by *Agrobacterium rhizogenes* are a suitable tissue for the production of secondary metabolites, due to the stability and high production of roots without phytohormone in a short time. Different strains of *Agrobacterium rhizogenes* (A_4, ATCC11325 and ATCC15834) were evaluated for induction of transformed hairy roots in *T. foenum-graecum* L. using seedling explants. The application of hairy root culture may become an alternative method for increase secondary metabolites. Transgenic status of the roots was confirmed by PCR using *rol*B specific primers. All of the *A. rhizogenes* strains led to hairy roots induction. The maximum frequency of transformation (97.87%) was obtained using A_4 strain in 7-days-old seedling. The 7-days-old explants were inoculated using A4 strain result in highest fresh (0.166 g) and dry (0.080 g) weight of roots. The explants were inoculated by ATCC11325 strain produced hairy roots with highest amount of total phenol (8.113 mg/g DW) and flavonoid content (3.215 µg/g DW).

KEYWORDS: Fenugreek, Medicinal plant, Polymerase chain reaction, *rol*B

INTRODUCTION

Medicinal plants have been widely explored for hairy root culture and their secondary metabolites. Fenugreek (*Trigonella foenum-graecum* L.) belonging to the Fabaceae family, is an important endemic medicinal plant species found in Azarbaijan, Isfahan, Fars, Khorasan and Semnan. Fenugreek has been used as vegetable, spice and medicinal plant in different countries around the world. Fenugreek has various properties such as anti-diabetic, anti-cancerous, anti-microbial and hypocholesterolemic. Fenugreek seeds contain polysaccharide, galactomannan, distinctive saponins such as diosgenin, yamogenin, adhesive, volatile oil and alkaloids such as choline and trigonelline [1].

Agrobacterium rhizogenes is a soil gram- negative bacterium that induces hairy roots upon wounding. T-DNA fragment on Ri plasmid (root inducing plasmid) in this bacterium, contains *rol* genes encoding enzymes involved in the biosynthesis of auxin and cytokinin and are responsible for the formation of hairy roots [7]. Integration of this region in the host genome results in induction of hairy roots [12]. Transformed hairy roots obtained by infection of plants with *A. rhizogenes* strains are used as a tissue culture tool for secondary metabolite production [10, 24]. Hairy roots show a high growth rate in phytohormone-free media compared with untransformed root cultures. Several factors affect the rate of *A. rhizogenes* mediated transformation in different plants, including explant age, *A. rhizogenes* strain, and genetics [24]. *Agrobacterium* mediated hairy roots are fast-growing, genetically stable which can also be successfully cultured in large-scale bioreactors and

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have been intensively used to produce a stable and high production of secondary metabolites in several medicinally important plants [21]. Some researchers reported hairy root induction in *T. foenum-graecum* by different strains of *A. rhizogenes* [3]. In other study an efficient hairy root culture system for Diosgenin [16] and Sotolone [18] production in hairy roots induced by A4 strain of *A. rhizogenes* in *T. foenum- graecum*. The main goal of this study was the optimization of induction and growth of hairy roots and production of secondary metabolites in *T. foenum-graecum* L.

MATERIALS AND METHODS

Plant materials

Trigonella foenum-graecum L. seeds were surface sterilized utilizing 70% ethanol for 1 min, then sterilized with 2.5% sodium hypochlorite for 10 min. After that, the seeds were washed three times with sterile distilled water and germinated on MS medium [17]. The explants were obtained from 7, 14 and 21-days-old seedlings inoculated with *A. rhizogene*s suspension. The explants were maintained at 25±2 ºC in a growth chamber.

Preparation of *A. rhizogenes* strains

A. rhizogenes strains ATCC15834, ATCC11324 and A_4 were used in the experiments. Single colony of the bacterial strains was grown in LB medium [5] containing 50 mg/l rifampicin, with OD_{600}= 0.6 at 26 ºC, 120 rpm on a shaker incubator. The bacterial suspension was used for infection of explants and hairy roots induction.

Induction and culture of hairy roots

Explants were wounded with a sterile needle with *A. rhizogenes* suspension grown in LB medium. The inoculated explants were placed on B5 medium [9] at 25±2 ºC for 48 and 72 hours in darkness for co-culture. In the next step, the explants were placed on MS medium supplemented with 500 mg/ l cefotaxime. After 30 days, induction percentage, average number and length of hairy roots were investigated.

PCR analysis

Total DNA was extracted from 100 mg of fresh hairy root according to CTAB method [8]. Isolated DNA was used in PCR analysis for detecting the *rol*B gene. Non-transformed seedling roots' DNA and $pRiA_4$ plasmid's DNA were used as negative and positive controls, respectively. The designed primers to amplify *rol*B were 5-ATGGATCCCAAATTGCTATTCCCCACGA-3 and 5-TAGGCTTCTTTCATTCGGTTTACTGCAGC-3 [4]. The PCR was carried out under the following condition: 94 ºC for 5 min followed by 35 cycles of a 1 min denaturation at 94 ºC, annealing at 55 ºC for 45 s and then extended by 1 min at 72 ºC and then 7 min at 72 ºC. Finally, the products were separated by 0.8% agarose gels (w/v).

Hairy roots culture establishment

After 30 days, hairy roots were cut (1.5 cm) and transferred to 100 mL flask containing 50 mL of liquid B5-basal medium and incubated in a growth chamber at 25 ± 2ºC at 100 rpm rotation in the dark. The cultures were sub-cultured every 2-weeks and used for further analysis. The hairy roots were harvested after 30 days and their fresh and dry weight and secondary metabolites production were recorded.

Determination of total phenolic and flavonoids content

Sample preparation

For this purpose, 10 ml of 80% methanol was added to 10 mg of dried hairy roots. The solution was then subjected to extraction for 48-72 hours. Upon centrifuging at 8000 rpm for 10 min in 4ºC the clear supernatant was used for analysis.

Total phenolic content

The total phenolics of the extracts were determined using the Folin-Ciocalteu reagent. 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 sodium carbonate solution (2% w/v 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 centrifuging the absorbance of yellow color from different samples was measured at 760 nm. The phenolic content was calculated as mg gallic acid equivalents GAE/g of dry plant material on the basis of a standard curve of gallic acid (20–100 mg/l) [15].

Total flavonoids content

The aluminum chloride method was used for measurement of total flavonoid content in hairy roots. For this purpose, quercetin was used to make the

standard calibration curve. 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 M) 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. The total flavonoid content in the test samples was calculated from the calibration plot and expressed as mg quercetin equivalent (QE)/g of dried plant material [6].

Data analysis

Experiments were conducted with factorial experiment based on completely randomized design with four explants in each replication. Before the statistical analysis, the normality of the data was tested. Data were examined by analysis of variance (ANOVA) to detect significant differences between means and those differing significantly ($P \leq 0.05$) were compared using Duncan's multiple range test at the same (5%) probability level using SPSS 16.

RESULTS and DISCUSSION

Induction and culture of hairy roots

The hairy roots were apeared after 30 days from *T. foenum-graecum* L. with the three strains of *A. rhizogenes* (A_4, ATCC11325 and ATCC15834) (Fig 1). The analysis of variance (ANOVA) showed that there was no significant difference between effects of strains, explant ages and co-culture time (Table 1). In this plant, the highest percentage of roots formation (97.87%) was observed in 7-days-old seedlings by using A_4 strain. The minimum rate of transformation (31.25%) were observed in the ATCC15834 strain and 21-days-old explants (Fig 2).

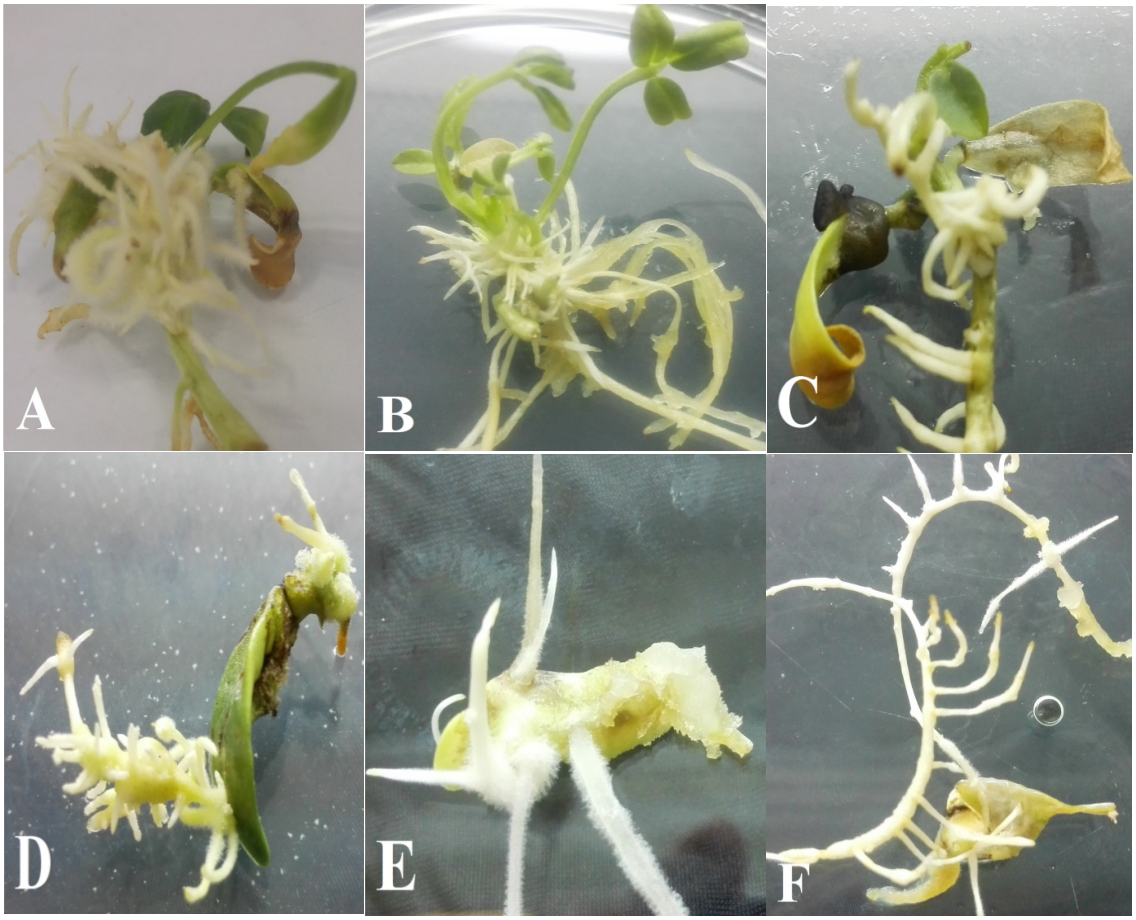

Fig 1. Hairy root induction in *T. foenum-graecum* A and D: Hairy root induction in transformed seedling explants by A_4 strain, B and E: ATCC11325 strain, C and F: ATCC15834 strain after 30 days.

Table1. Analysis of variance of the effects of strain and explant age and co-culture time on hairy root induction characteristic

Source of variation	df	Means of square				
		Hairy root induction (%)	Root number	Root length (cm)	Fresh weight	Dry weight
Strain and Explant age	8	21.509*	77.305**	30.532**	0.077**	0.033**
co-culture	1	7.146ns	89.445**	60.189**	0.035*	0.028**
Strain and Explant age × co-culture	8	1.366ns	9.596ns	4.777ns	0.007ns	0.006ns
Error	54	8.393	10.227	5.880	0.006	0.003

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

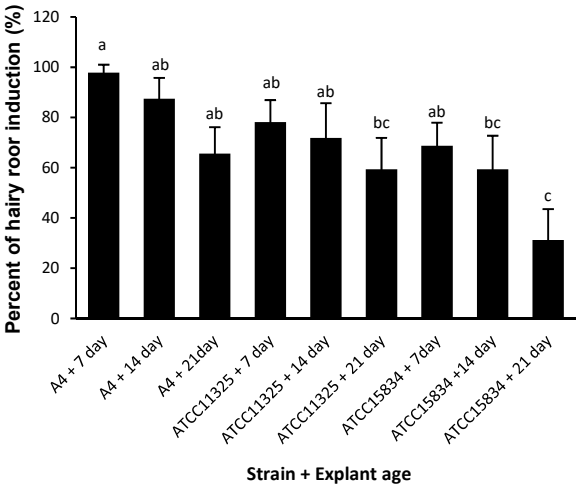

Fig 2. Mean comparisons of the effects of strain type and explant age on hairy roots induction.

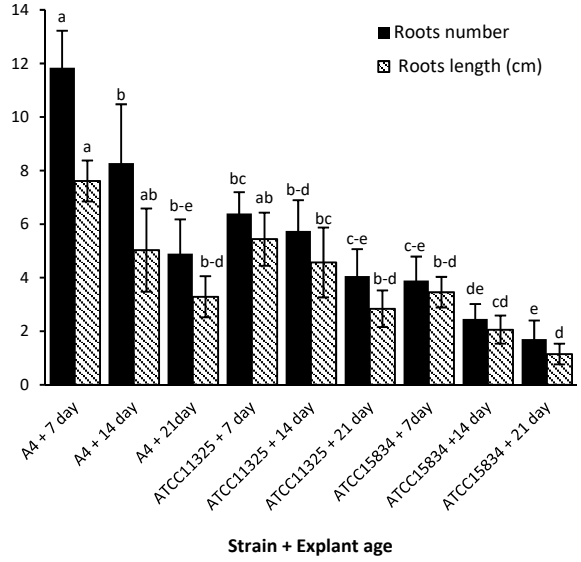

Fig 3. Mean comparisons of the effects of strain type and explant age on hairy roots number and length.

Kabirnataj et al. (2013) reported that A4, A13 and ATCC15834 strains of *A. rhizogenes* able to hairy roots induction in *C. intybus* and MS medium was considered as the best culture medium for co-culture of explants with bacteria [11]. The maximum frequency of hairy root induction in *T. foenum-graecum* L. was obtained with K599 at OD_{600}= 1.2 (79.76%) bacterial strains [20] and also the highest rate of transformation in *Artemisia aucheri* Boiss were obtained using MSU440 (93 %) and ATCC15834 (89 %) bacterial strains [22]. Akbarian et al. (2011) evaluated hairy root cultures of two fenugreek ecotypes via cotyledon explants infected with OD_{600}= 0.4 of *A. rhizogenes* and transgenic roots were identified using the *rol*B gene as a marker in PCR analysis [2]. Any hairy roots were not observed in untransformed explants also, the most roots number (11.84) and root length (7.61 cm) were recorded in A4 strain, 7-days-old explant and 72 h co-culture time (Fig 3 and 4). Overall results showed that, A4 strain, 7-days-old explants and 72 hours' co-culture time were found to be the best treatment which was able to show highest percentage of transformation. After 30 days of culture, the hairy roots transferred to B5 liquid medium (Fig 5).

Molecular analysis of transgenic roots

PCR reaction with primers for *rol*B genes was used to investigate integrated T-DNA into isolated DNA from hairy roots of *T. foenum-graecum L.* PCR analysis confirmed the transfer of T-DNA region of Ri plasmid of bacteria into hairy roots genome (760 bp), while DNA isolated from non-transformed adventitious roots (negative control) was indicative of no amplification (Fig 6).

Hairy roots culture establishment

The hairy roots can be growing in B5 liquid medium without growth regulators. Fresh and dry weight of hairy

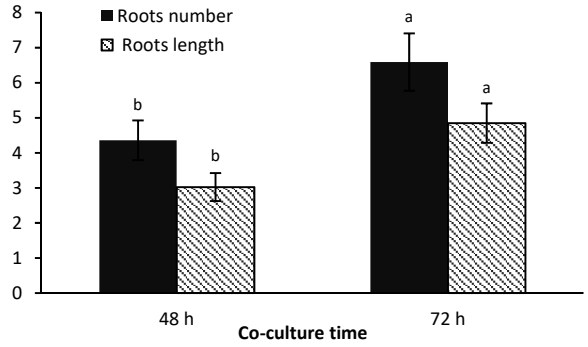

Fig 4. Mean comparisons of the effect of co-culture time on hairy roots number and length

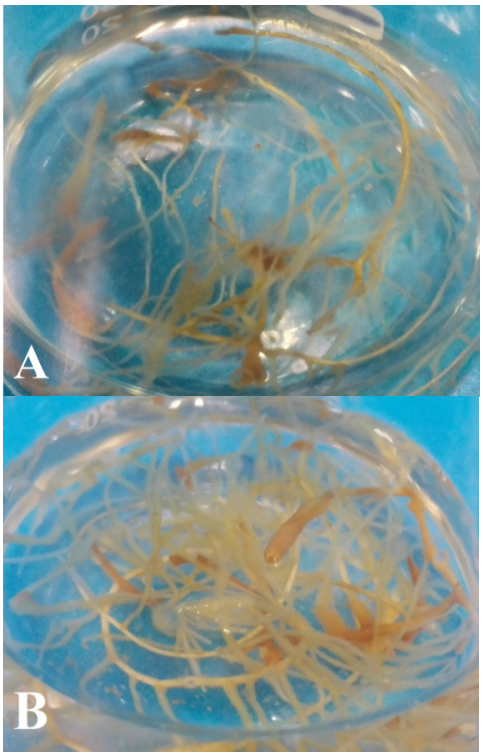

Fig 5. Hairy roots induction by *A. rhizogenes* in fenugreek, A and B: Growth of hairy roots in B5 liquid medium

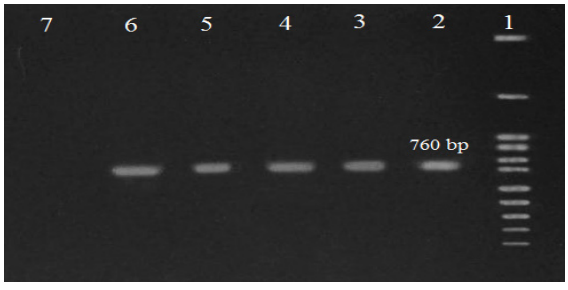

Fig 6: PCR amplification of *rol*B gene, 1: Molecular marker (100 bp DNA ladder from sinagen company), 2. positive control (plasmid DNA from A4 strain), 3-6: DNA from hairy roots, 7.DNA from normal plant root.

Table 2. Mean comparisons of the effects of strain and explant ages on hairy roots growth rate

Strain + Explant age	Fresh weight (g)	Dry weight (g)
A_4 + 7 day	0.166[a]	0.080[a]
A_4 + 14 day	0.075[c]	0.032[cd]
A_4 + 21 day	0.053[cd]	0.025[cd]
ATCC11325 + 7 day	0.156[ab]	0.064[ab]
ATCC11325 + 14 day	0.063[c]	0.028[cd]
ATCC11325 + 21 day	0.037[cd]	0.015[d]
ATCC15834 + 7 day	0.124[b]	0.051[bc]
ATCC15834 + 14 day	0.053[cd]	0.022[d]
ATCC15834 + 21 day	0.023[d]	0.008[d]

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

Table 3. Mean comparisons of the effect of co-culture time on hairy roots growth rate

co-culture time	Fresh weight (g)	Dry weight (g)
48 h	0.066[b]	0.025[b]
72 h	0.101[a]	0.047[a]

roots were recorded after 30 days. It was observed that the lowest biomass was obtained in *T. foenum-graecum* L. hairy roots induced by 21-days-old and 48h co-culture time explants also, the highest Fresh weight (0.166 g) and dry weight (0.080 g) of hairy roots were obtained by A_4 strains and 7-day-old explants (Table 2 and 3). In one study, three strains of *A. rhizogenes* (ATCC15834, MSU440 and K599) were used for hairy roots induction in *Trigonella foenum-graecum* L. All parts of the seedling were able to produce the hairy roots also, the highest dry weight of hairy root was obtained by ATCC15834 strain [19]. The integration sites of T-DNA within plant genomes are largely randomly and each transformed cell produces a hairy root line, therefore each line shows a different rate of growth and development [13]. Sharafi et al. (2012) examined different explants of *Papaver bracteatum* for hairy roots induction with A_4, ATCC15834, LBA9402, MSU440 and A_{13} strains and the highest frequency of transformation was achieved using LBA9402 strain in the excised shoots [23]. 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 [14]. In another report, Valimehr et al. (2014) two explant types, several co-culture media and

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

Source of variation	df	Means of square	
		Phenolic Content (mg/g DW)	Flavonoid 415 nm (µg/gDW)
Strain	3	1.952**	1.692**
Error	12	0.278	0.163

**: significant at 1% probability level.

Table 5. Mean comparisons of the effects of strain on total phenol and flavonoid in hairy roots

Strain	Phenol 760 nm (mg/g DW)	Flavonoid 415 nm (µg/g DW)
A_4	8.017[a]	3.852[a]
ATCC11325	8.113[a]	3.215[a]
ATCC15834	6.595[b]	2.375[b]
Control	7.730[a]	3.62[a]

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

different bacterial strains of *Nepeta pogonosperma* examined to hairy roots induction with A_4, ATCC15834, LBA9402, MSU440 and A_{13} strains. The maximum rate of hairy root induction was obtained from stem explants using MSU440 and ATCC15834 bacterial strains [25]. Akbarian et al. (2011) reported induction of hairy roots from *T. foenum-graecum* L. The highest dry weight (0.219 g) achieved in hairy roots induced by ATCC15834 strain [2].

Measurement of total phenolic and flavonoid content

Results showed significant ($P \leq 0.01$) differences between the phenolic content of dried methanol extract in hairy roots resulted from different treatments (Table 4). Many secondary metabolites are derivatives of the phenolic compounds and may also be increased by enhancing phenol production.

It was found that the content of total phenolic compounds in extracts of hairy roots induced by ATCC11325 and A4 strains and control were not statistically significant. The analysis of extracts showed that the maximum and minimum content of total phenolic compounds was 8.11mg/g and 6.59 mg/g in hairy roots inoculated by ATCC11325 and ATCC15834 strains, respectively. Highest Flavonoid content (3.85 µg/g DW) observed in hairy roots induced by A4 strain (Table 5).

CONCLUSION

A. rhizogenes has been used for the production of hairy roots cultures from many plants. Hairy roots induction is a result of integration and subsequent expression of Transferred DNA (T-DNA) from the bacterial Ri (Root inducing) plasmid of *A. rhizogenes* in the plant genome. To our knowledge, no previous study has surveyed the influence of explant age and different co-culture times on enhancement of hairy roots induction, growth rate and secondary metabolites productivity in *T. foenum-graecum*. The maximum percentage of hairy root induction and fresh and dry weight were achieved using A_4 strain and 7-days-old. The highest total phenolic and flavonoid content were found in hairy roots induced by ATCC11325 strain.

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تأثیر سویه‌های آگروباکتریوم رایزوژنز بر القاء ریشه‌های مویین در شنبلیله (*Trigonella foenum-graecum* L.) و تولید متابولیت‌های ثانویه

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چکیده

شنبلیله یک منبع غنی از متابولیت‌های مهم دارویی و از تیره‌ی Fabaceae بوده و دارای خواص سودمند زیادی است. سویه‌های مختلف اگروباکتریوم رایزوژنز (ATCC15834 و A_4, ATCC11325) برای القای ریشه‌های موئین تراریخته در گیاه شنبلیله با استفاده از ریزنمونه گیاهچه مورد استفاده قرار گرفتند. کشت ریشه‌های موئین با افزایش متابولیت‌های ثانویه، بهبود پایداری ژنتیکی و بیوشیمیایی می‌تواند به‌عنوان یک روش جایگزین برای بهبود وضعیت این گونه‌ها در مقایسه با گیاه معمولی باشد. تراریخته بودن ریشه‌ها با استفاده از PCR و پرایمرهای خاص *rol*B تأیید شد. تجزیه و تحلیل PCR نشان داد که ژن *rol*B از پلاسمید Ri از اگروباکتریوم در داخل ژنوم ریشه‌های موئین تراریخته قرار گرفته است. همه‌ی سویه‌های اگروباکتریوم رایزوژنز منجر به القای ریشه موئین شدند. بالاترین میزان تراریختی (۹۷/۸۴درصد) توسط سویه‌های A_4 در ریزنمونه‌ی ۷ روزه بدست آمد. در ریشه‌های موئین کشت شده در محیط مایع، بیشترین وزن تر (۰/۱۶۶ گرم) و وزن خشک (۰/۰۸۰ گرم) در سویه A_4، ریزنمونه ۱۰ روزه مشاهده شد و بالاترین محتوای فنل کل (۸/۱۱۳ میلی‌گرم بر گرم وزن خشک ریشه) و فلاونویید کل (۳/۲۱۵ میکروگرم برگرم وزن خشک ریشه) از سویه ATCC11325 حاصل شد.

کلمات کلیدی: اگروباکتریوم رایزوژنز، ریشه موئین، شنبلیله و *rol*B.