RESEARCH ARTICLE

Antioxidative enzymes activities and accumulation of steroids in hairy roots of *Trigonella*

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Abstract Steroidal sapogenins and phytosterols are a group of secondary metabolites which are very considerable in the pharmaceutical industry. Fenugreek (*Trigonella foenum-graecum* L.) is the good source of these compounds. In recent decades, there is a great interest to production of these compounds by cultivation of transformed roots. In present study, hairy roots induction in two *Trigonella* species (*T. foenum-graecum*, *T. monantha*) with three strains of *Agrobacterium rhizogenes* (15,834, A4 and wt) was investigated. Transgenic status of roots was confirmed by PCR using *rolB* specific primers. Virulence of these strains was examined on explants of leaf, leaf cotyledone and hypocotyle in both species. The best strain was wt for hairy root induction in hypocotyle and leaf explants of *T. foenum-graecum* and *T. monantha*. Significant quantitative differences were showed between shoot, root and hairy roots in both species. Protein content in root and hairy root of both species was significantly lower in comparison with shoot. Superoxide dismutase (SOD) and peroxidase (POX) activities in hairy roots of both species were higher as compared to other organs. The hairy roots of both species showed an ability to synthesize steroidal sapogenins. These results presented that hairy roots could be a suitable procedure for producing sapogenins compounds that have medicinal value in *Trigonella*.

Keywords *Trigonella* · *Agrobacterium rhizogenes* · Hairy root · Steroidal sapogenins · Antioxidative enzymes

Introduction

Trigonella L. is annual plant of Fabaceae that include 135 species. Most of *Trigonella* species exist in arid regions. *Trigonella foenum-graecum* (Fenugreek) and *Trigonella monantha* are important crops of this genus. Fenugreek has medicinal value and cultured in Ukraine, Iran, India, China and Turkey. Fenugreek applies to treat inflammation, infection, breast augmentation, epilepsy, diabetes and lung inflammation. Moreover, this plant have anticancer, anti-fertility; anti-aging, antibacterial and anti-parasite characterizes (Habori and Raman 2002; Mullaicharam et al. 2013).

Saponins are a group of glycosylated triterpenes which have been detected in fenugreek and have antibacterial and antiviral properties. Diosgenin is a sapogenin compound that is found in fenugreek and has anti-aging and anticancer characterizes and use for treatment of cardiovascular disease (Freeman and Beattie 2008). Diosgenin is used as a precursor for steroidal drugs production. One of sources for diosgenin is *T. foenum-graecum* (Evans 1996).

One of methods that have been applied for secondary metabolites production is cell and tissue culture. Hairy root is one of the techniques that used to manufacture secondary metabolites in plants. Hairy roots are transformed roots that produce injection of *Agrobacterium rhizogenes* into the injured tissue of the plant. *Agrobacterium rhizogenes* belongs to the genus *Agrobacterium* and is a gram negative and rod-type bacterium (Conn 1942). Hairy roots are caused via the insert of a part of the DNA of the bacterium (T-DNA) into the plant cell chromosome. Hairy roots differ

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from wild roots and are more spacious and show rapid growth in the absence of growth regulators (Veena and Taylor 2007).

Diosgenin and phytosterols as raw materials are very important for steroid hormones production. Therefore, this research was conducted to determine the best condition for hairy root induction in both species of *Trigonella* by infecting leaf, leaf cotyledone and hypocotyle explants with three strains of *A. rhizogenes*. Also, the changes of proteins and antioxidant enzymes activities in shoot, root, and hairy root were surveyed.

Materials and methods

Plant material

The seeds of two *Trigonella* species (*T. foenum-graeceum*, *T. monantha*) were sterilized with 96% ethanol for 1 min and 15% sodium hypochlorite solution for 15 min and washed four times with sterile distilled water. They were transferred on solid 1/2 MS medium including 3% sucrose and were maintained at 25 °C in the light.

Bacterial strains

Three strains of *A. rhizogenes* including 15,834, wt, and A4 were used to determine the transformation efficiency. All strains obtained by Tarbiat Modares University. The medium used for culture of bacteria was LB with 1/5% agar. Prior to infection, the bacterial strains were grown for 36 h in LB liquid medium (40 ml in 250-ml conical flasks) at 28 °C on a rotary shaker at 110 rpm.

Induction of hairy roots

The sterile 4 to 5-week-old seedlings were cut into leaf, leaf cotyledons and hypocotyle explants. Then, explants were immersed in bacterial suspension for 2–3 min. The explants were blotted on sterile filter-paper to eliminate excess bacteria and were transferred on MS medium with 0.75% agar and 3% sucrose and then were kept at 25 °C in the dark. After 60–72 h, the explants were transferred to liquid MS medium comprising 500 ppm cefotaxime (to remove bacteria) and incubated at 25 °C under 16 h light and 8 h dark photoperiod. Then, ability of hairy root induction was examined by different strains of *A. rhizogenes* in two *Trigonella* species. Finally, in order to investigate the quantitative and qualitative levels of steroidal saponins, sterols, and antioxidative enzymes, hairy roots were propagated in 1/2 B5 medium without hormone.

PCR confirmation of hairy roots

Genomic DNA was isolated from hairy root samples (500 mg fresh weight) by CTAB method (Doyle et al. 1999) and PCR analysis was used for identifying the presence of *A. rhizogenes* rolB gene in hairy roots. The PCR was carried out under the following conditions: 94 °C for 4 min, 35 cycles of three steps [94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1 min (elongation)], and 72 °C for 7 min. After PCR, the samples were separated on 0.8% agarose gel and stained with ethidium bromide. Gel pictures were gained using Gel-Doc Transilluminator.

Antioxidant enzyme activities

For estimation of total protein content and enzyme activity, shoot, root and hairy root were extracted with ice-cold 1 M Tris–HCl (pH 6.8) buffer. The homogenates were centrifuged at $13,000 \times g$ for 30 min at 4 °C and supernatants were kept in – 70 °C and used for protein determination and enzyme assays. The protein content was determined based on the method of Bradford (1976) using bovine serum albumin as a standard.

The activity of superoxide dismutase (SOD) was assayed based on Giannopolitis and Ries (1977). Reaction solution included sodium phosphate buffer (50 mM, pH 7.5), 0.1 mM EDTA, 13 mM methionine, 75 µM NBT, 75 µM riboflavin and 100 µl of enzyme extract. The reaction solution was placed in front of the light for 18 min and then absorbance was measured at 560 nm.

For measurement of peroxidase (POX) activity, reaction mixture consisted of 0.1 ml benzidine (40 mM), 0.2 ml H₂O₂ (3%), 2 ml of 0.2 M acetate buffer (pH 4.8) and 50 µl of enzyme extract. Activity of this enzyme was recorded at 530 nm (Abeles and Biles 1991).

Poly phenol oxidase (PPO) activity was measured by Raymond et al. (1993). The reaction mixture contained 0.2 ml of pyrogallol (20 mM), 2.5 ml of 200 mM sodium phosphate buffer (pH 6.8) and 100 µl of enzyme extract. The enzyme activity was recorded at 430 nm.

Gel electrophoresis

For determination of protein profiles in shoots, root, and hairy roots, discontinuous sodium dodecyl sulfate polyacrylamide gel electro-phoresis (SDS-PAGE) was done via Laemmli (1970). PAGE was carried out for isoenzymes identification via Davis (1964). For determination of SOD isoenzymes, gels were placed in 0.05 M Tris–HCl (pH 8.0) having 2 mg EDTA, 4 mg riboflavin and 20 mg MTT for 40 min (Wendel and Weeden 1989). Then the gels were kept under one 15 W fluorescent tubes and shaken till

Fig. 1 Seedling of *T. foenum-graceum* (a) and *T. monantha* (b) in MS 1/2 medium, c (*T. foenum-graceum*), d (*T. monantha*): induction of hairy roots from leaf explants of two species, e (*T. foenum-graceum*), f (*T. monantha*): hairy root culture established on B5 solid medium

Table 1 Effect of different bacterial strains on induction of hairy root (%) in both species of *Trigonella*

Bacterial strains	Species	Leaf	Hypocotyle
wt	<i>T. foenum-graceum</i>	27.66 ± 2.66 a	40 ± 5.13 a
15,834		10.66 ± 0.66 b	34.33 ± 1.33 b
A4		–	39 ± 3 a
wt	<i>T. monantha</i>	17 ± 1.52 a	91.33 ± 4.91 a
15,834		–	47.33 ± 2.66 b
A4		–	58.33 ± 5.33 b

Values are mean ± SE of three replicates

Different letters indicated significant ($p < 0.05$) differences

white bands appeared in violet background. POX isoenzymes were gained by Van Loon and Geelen (1971). The gels were incubated in 0.2 M acetate buffer (pH 4.8) containing 3% H_2O_2 and 4 ml benzidine at room

temperature till the appearance of brown color. For PPO isoforms determination, the gels were incubated in 0.2 M phosphate buffer (pH 6.8) containing 20 mg 3,4

Fig. 2 PCR analysis of *rolB* on agarose gel: M: DNA marker; 1 and 2: negative control; 3 and 4: positive control; 5: transgenic hairy roots

dihydroxyphenylalanine (L-Dopa) and 700 μl CaCl_2 (Van Loon and Geelen 1971).

Assay of steroidal sapogenin

Powdered material (0.2 g) was extracted with 200 ml of petroleum ether for 6 h using the soxhlet apparatus. Then

the residue of plant material was extracted with methanol. The resulting petroleum ether and methanol extracts were reduced in rotary evaporator and stored at 4 °C until further use in the experiment. Steroidal sapogenin analyzed by gas liquid chromatography (Ebrahimzadeh and Niknam 1999). A Shimadzu GC-16A equipped with a flame ionization detector and 1.6 m $\times$ 3.2 mm i.d. glass column packed with 5% SE-30 was used. Operation temperatures were 280 °C for the oven and 290 °C for both the injector and detector block. Nitrogen was used as carrier gas at a flow rate of 50 ml min^{-1} . Flow rates of hydrogen and air were 55 and 400 ml min^{-1} , respectively.

Statistical analysis

All the presented data are the mean value of three different experiments. The data were analyzed by using one-way analysis of variance using SPSS and means were compared by Duncan's test at the 0.05 level of confidence.

Fig. 3 Protein content in *T. foenum-graceum* (a) and *T. monantha* (b). SDS-PAGE pattern of proteins in both species: shoot, root and hairy root in *T. foenum-graceum* (1 to 3), shoot, root and hairy root in

T. monantha (4 to 6) and protein marker (M). Arrows indicate the new and changed bands. Equal amounts of protein (100 μg) were loaded in each lane

Fig. 4 Changes in specific activities of SOD and POX in *T. foenum-graceum* (a, c) and *T. monantha* (b, d). Columns indicate mean $\pm$ SE based on three replicates. Means with different letters for each cultivar indicate a significant difference at $P < 0.05$ using Duncan multiple range test

Results and discussion

Characterization of hairy root

Three strains of *A. rhizogenes* were utilized to infect leaf, leaf cotyledon and hypocotyle of two *Trigonella* species (Fig. 1). Hairy roots were formed at the site of wound. Hairy root was not observed in control explants. Used bacterial strains had different effects on the hairy root induction. In this study, wt strain was more efficient than 15,834 and A4 strains in two *Trigonella* species (Table 1). The highest percentage of rooting was in the hypocotyle, and the percentage of rooting in the leaf was lower but no root was found in the leaf cotyledon. The presence of the *rolB* gene in the hairy roots was determined through PCR of the DNA using *rolB* forward and reverse primers. *Agrobacterium rhizogenes* used as the positive control and DNA from the non-transformed roots was used as the negative control. All hairy roots showed existence of the *rolB* gene (Fig. 2).

Various factors such as genotype, the type of plant tissue, compounds of the culture medium and the type of bacterial strain influence on the transformation of plant tissues. Morphology difference in hairy roots may be due to different of T-DNA, the number of copies of T-DNA, and the site of T-DNA in the host cell genome. Merkli et al. (1997) showed that A4 strain caused the hairy roots formation in stem explants of *T. foenum-graceum*. According to our data, bacterial strains had the significant differences in virulence.

Antioxidative enzymes and isozyme profile

Protein content in hairy roots was lower as compared to root and shoot in two species.

The protein content of *T. foenum-graceum* was higher in the three studied organs than other species (Fig. 3a, b). Based on the SDS-PAGE analysis (Fig. 3c), protein patterns of three organs were different, so the type and amount of protein depends on the organ type.

Fig. 5 PPO activity in *T. foenum-graceum* (a) and *T. monantha* (b). Columns indicate mean $\pm$ SE based on three replicates. Means with different letters for each cultivar indicate a significant difference at $P < 0.05$ using Duncan multiple range test

Overall, the POX and PPO activities were more in *T. monantha*, but SOD activity was higher in *T. foenum-graceum*. The SOD activity in hairy roots of both species was higher than other organs (Fig. 4a, b). According to non-denaturing PAGE (Fig. 6a, b), seven and five SOD isoforms were detected in *T. foenum-graceum* and *T. monantha*, respectively. SOD3, SOD4, SOD5 and SOD7 were identified in hairy roots derived of *T. foenum-graceum*. All SOD isoforms were showed in hairy root produced of *T. monantha* and SOD5 had stronger band. Hairy roots had the highest POX activity in both species and its activity was more pronounced in *T. monantha* (Fig. 4c, d). The POX1 was detected only in hairy root derived of *T. monantha*. The POX 2,4,5 isoforms activity were higher in hairy root created of *T. monantha* in comparison with root (Fig. 6d). In contrast to other enzymes, PPO activity in the root was more than hairy root of both species (Fig. 5). Four and six PPO isoforms were detected in *T. foenum-graceum* and *T. monantha*, respectively. The PPO4 was showed only in hairy roots derived from *T. foenum-graceum* (Fig. 6e, f).

In plants, active oxygen species are continuously produced, and hydrogen peroxides (H_2O_2) affects growth and

Fig. 6 Changes in the isoform patterns of SOD, POX and PPO in *T. foenum-graceum* (a, c, e) and *T. monantha* (b, d, f). 1: shoot; 2: root; 3: hairy root. Arrows indicate the new and changed bands. Equal amounts of protein (100 μ g) were loaded in each lane

Table 2 The changes (μ g g^{-1} DW) in sterols and steroidal sapogenins in *T. foenum-graceum*

Compounds	Shoot	Root	Hairy root
Cholesterol	–	3.029 $\pm$ 0.9 f	–
Campesterol	–	8.34 $\pm$ 0.78 d	–
β -sitosterol	–	28.21 $\pm$ 2.12 b	–
Stigmasterol	8.67 $\pm$ 0.9 b	23.97 $\pm$ 5.23 c	–
Diosgenin	76.7 $\pm$ 4 a	–	26.3 $\pm$ 1.23 b
Tigogenin	–	32.629 $\pm$ 2.56 ^a	41.86 $\pm$ 3.6 a

Values are mean $\pm$ SE (n = 3) of three replicates

Different letters indicated significant ($p < 0.05$) differences

cell processes, but their high production can cause destruction in cell function. Plants use antioxidant systems to overcome oxidative damage. According to our results,

Table 3 The changes ($\mu\text{g g}^{-1}\text{DW}$) of sterols and steroidal sapogenins in *T. monantha*

Compounds	Shoot	Root	Hairy root
Cholesterol	2.78 $\pm$.34 c	–	–
Campesterol	–	–	–
β -sitosterol	–	–	–
Stigmasterol	12.62 $\pm$ 1.76 b	–	–
Diosgenin	74.37 $\pm$ 6 a	43.91 $\pm$ 3.87 b	30.09 $\pm$ 2.456 b
Tigogenin	–	56.08 $\pm$ 4.56 a	69.90 $\pm$ 5.6 a

Values are mean $\pm$ SE (n = 3) of three replicates

Different letters indicated significant ($p < 0.05$) differences

SOD and POX activity increased during hairy root formation, while PPO activity reduced. SOD enzyme is a metalloprotein that causes dismutation of superoxide radicals to H_2O_2 and O_2 . H_2O_2 influences genes expression, protein synthesis and somatic embryogenesis (Apel and Hirt 2004). Tian et al. (2003) demonstrated that antioxidant enzymes affected shoot organogenesis in strawberry callus. The POX plays an important role in growth and differentiation, thus its high activity is linked with differentiation that occurs in the hairy root induction (Gaspar 1995). According to previous studies, POX enzyme and its isoforms were indicators of somatic embryogenesis (Krsnik-Rasol et al. 1982; Joersbo et al. 1989). According to previous studies, expression of *rol* genes is the main factor in metabolites production, and *rolC* may activate defense mechanisms that decreased ROS level and detoxifies the oxidative damage in transformed cell (Bulgakov et al. 2008; Nikraves et al. 2012).

Steroids production in hairy roots

In general, the content of sterols in *T. foenum-graceum* was more than other species, but *T. monantha* had more steroidal sapogenins (Tables 2 and 3). All sterols contain cholesterol, campesterol, β -sitosterol and stigmasterol were identified in roots of *T. foenum-graceum*, but only stigmasterol was detected in the shoot. None of the four sterols was detected in the hairy roots derived of *T. foenum-graceum*. Diosgenin and tigogenin were shown as steroidal sapogenins in the hairy roots derived of *T. foenum-graceum*. Campesterol and β -sitosterol were not detected in three organs of *T. monantha*. Cholesterol and stigmasterol only were in shoot of *T. monantha*. Diosgenin and tigogenin were in root and hairy root derived of *T. monantha* that the content of tigogenin was higher in hairy root.

All tissues of the plants include leaves, roots and stems, flowers, fruits and seeds are capable producing sterols, but their synthesis amount depends on the type and age of plant

tissue. Based on previous studies, hairy roots can produce secondary metabolites, which is consistent with our results (Dhakulkar et al. 2005; Medina-Bolivar et al. 2007). Binesh and Gnanam (2009) found that diosgenin content enhanced in hairy root cultures of *T. foenum-graceum*. Diosgenin belongs to steroidal sapogenins and is used to produce sterols, corticosteroids, contraceptives and sex hormones (Niño et al. 2007). Production of valuable metabolites have been developed from several plant species (Gabr et al. 2019; Nikraves et al. 2012).

In conclusion, transgenic roots derived from *Trigonella* could be considered as a potent and abundant source of steroidal sapogenins that have pharmaceutical properties. Investigation of different aspects of sapogenins biosynthesis pathway in hairy roots, application of various elicitors to increase production of useful compounds and effect of ROS production on transformation efficiency can be further objectives.

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Authors' contribution This research paper was accomplished with the collaboration of authors. SK performed the experiment and MR wrote the manuscript. VN and MM designed the study.

Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

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