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A reliable and efficient protocol for induction of hairy roots in *Agastache foeniculum*

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Abstract: Hairy root culture system is a valuable tool to study the characteristics of gene expression, gene function, root biology, biochemical properties and biosynthesis pathways of secondary metabolites. In the present study, hairy roots were established in Anise hyssop (*Agastache foeniculum*) via *Agrobacterium rhizogenes*. Three strains of *Agrobacterium rhizogenes* (A4, A7 and 9435), were used for induction of hairy roots in four various explants (hypocotyl, cotyledon, one-month-old leaf and five-month-old leaf) of Anise hyssop. The highest frequency of transformation was achieved using A4 strain in one-month-old leaves (51.1%). The transgenic states of hairy root lines were confirmed by PCR (Polymerase chain reaction) method. High performance liquid chromatography analysis revealed that the production of rosmarinic acid (RA) in transformed roots of *A. foeniculum* was almost 4-fold higher than that of the non-transformed roots. In a separate experiment, hairy roots obtained from one-month-old leaves inoculated with A4 strain, were grown in liquid medium and the effects of different concentrations of salicylic acid (0.0, 0.01, 0.1 and 1 mM) and chitosan (0, 50, 100 and 150 mg L^{-1}) (as elicitor) and sucrose (20, 30, 40 and 50 g L^{-1}) on the growth of hairy roots were evaluated. The results showed that, 30 g L^{-1} sucrose and 100 mg L^{-1}chitosan increased the biomass of hairy root cultures and application of salicylic acid reduced the growth of hairy roots compared with control roots.

Key words: *Agastache foeniculums*;*Agrobacterium rhizogenes*; elicitation; hairy roots; sucrose concentration

Introduction

Anise hyssop (*Agastache foeniculum*) is a perennial herbaceous plant, belonging to the Lamiaceae family. The origin of this plant has been reported in South America. The essential oil of *A. foeniculum* is used in food, pharmacy, cosmetics – sanitary and ice cream industries. Anise hyssop is also used in traditional medicine for treating lung diseases and cough (Omidbaigi 2007). *A. foeniculum* has many useful constituents including monoterpenes and phenyl propanoids. Rosmarinic acid, chlorogenic acid, rutin, apigenin and galangin, are the different phenolic compounds of *Anise hyssop* with many pharmacological properties. The antioxidant activity depends on total polyphenols, which is measured 98.6 mg g−1 in *Agastache foeniculum* (Matei 2012), 98.2 mg g^{-1} in *Allium sativum* (Eghdami et al. 2011) and 31.25 mg g^{-1} in *Mentha arvensis* (Choudhary et al. 2011).

Rosmarinic acid (RA) is a phenolic compound and is produced in the roots of *Agastache foeniculum*) Fig. 1). RA has shown antioxidant, anti-virus, anti-microbial, anti-allergic, anti- inflammatory and anti-cancer activities. It also possesses promising physiological actions related to cognitive performance such as prevention of Alzheimer's diseases, treatment of kidney disease, cardio protection and cancer chemo prevention

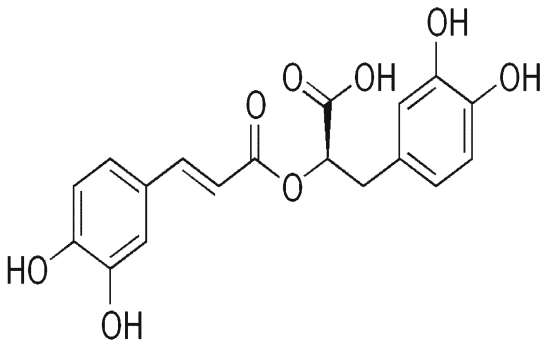

Fig. 1. Chemical structure of rosmarinic acid.

that are found in the plants of Lamiaceae family (Fesen et al. 1993).

Plant cell culture is known as an alternative method to agricultural processes and producing valuable phytochemical compounds. Hairy root cultures in hormone- free system is stable and has a high production rate (Hu & Du 2006). Rapid growth, low doubling time, easy maintenance and the ability to synthesize a wide variety of chemical compounds, are some of the advantages that have made hairy roots as an important source for the production of secondary metabolites. Genetic transformation by *Agrobacterium rhizogenes* is an

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effective method to increase the accumulation of secondary metabolites in plant cells (Gangopadhyay et al. 2010). *A. rhizogenes* is a gram- negative, soil bacterium. 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 (Christy & Braun 2005). Integration of this region in the host genome results in induction of hairy roots (Kayser & Quax 2007). Different *rol* gene products have various effects on the same target (Aoki 2004). Furthermore, *rol* genes affect the growth and development of transgenic roots and induce the synthesis of secondary metabolites by activating the transcription of genes (Ono et al. 2011). Creation of hairy root phenotype in transgenic plants may be the result of the synergistic action of *rol* genes (Lemcke & Schmulling 1998). Formation of hairy roots from infected cells is more likely regulated by *rolA*, *rolB* and *rolC*, among these, *rolB* locus play the most important role in the induction of hairy roots (Cardarelli et al. 1987). A proper expression level of *rolB* appears to be necessary for active growth of hairy roots because either a high or low expression level correlates with impaired growth of hairy roots (Palazon et al. 1997; Gorgiev et al. 2007). The *rolC* gene has a stimulatory effect on secondary metabolism, while the *rolD* gene stimulates root growth and the *rolA* encodes a protein which is suggested to act as a transcription factor and is implicated in the metabolism of gibberellins (Palazon et al. 1997). The strain and age of *A. rhizogenes*, type of explants and media composition are some of the effective factors in induction of hairy root cultures (Luo et al. 2004). Many studies have shown that various plant tissues have different responses to transformation with different strains of *A. rhizogenes* (Pirian et al. 2012; Sharafi et al. 2013; Samadi et al. 2013).

Source of carbon and its concentration are some of the effective factors in growth of hairy roots (Morgan et al. 2000). Sharafi et al. (2013) reported a stimulating effect of sucrose on increase of hairy root biomass in *Papaver bracteatum*.

The use of biotic and abiotic elicitors is a common strategy that is used to increase the growth rate and production of secondary metabolite in hairy root systems (Soleimani et al. 2012). Elicitors are compositions that not only result in the accumulation of phytoalexins in plants, but also elicit pathways that are related to defensive response, resulting in synthesis of secondary metabolites in plants. They were classified in two groups: abiotic elicitors (mineral, chemical and physical agents) and biotic elicitors (agents with plant or pathogen origins) (Yoshikawa et al. 1978). Salicylic acid (SA) and chitosan are abiotic elicitors. SA is a phenolic compound that is produced in phenylpropanoids pathway in plants. SA is one of the effective molecules in stress signaling pathway. Its role in plant resistance against pathogens and other stressor agents is well known. Furthermore, adding SA in the culture medium could activate the defensive genes in plants. Moreover, SA inhibits the biosynthesis of ethylene and induce different compounds in plant (Ahmadian et al. 2010). Chitosan is a deacetylated derivative of chitin extracted from the fungal cell wall, crustacean exoskeletons, cuticles of insects and some algae (Sanford & Hutchings 1987).

Effects of different concentrations of salicylic acid on the growth rate of hairy roots in *Artemisia dubia* were examined; the increased concentration of salicylic acid decreased the growth rate of hairy roots (Ali et al. 2012).

Chitosan and jasmonic acid treatment increased the fresh and dry weights of hairy roots in *Artemisia annua* L. (Soleimani et al. 2012). In *Plumbago rosea*, chitosan showed the positive effect on fresh weight of cells and the highest amount of plumbagin accumulation (Komariah et al. 2002).

To our knowledge, this is the first report on the genetic transformation of *A. foeniculum*. Different strains of *A. rhizogenes* and four types of explants were evaluated for induction of hairy roots in *A. foeniculum*. The effects of different concentrations of sucrose, salicylic acid and chitosan were also evaluated for increasing the growth rate of hairy roots.

Material and methods

Seed culture and explants preparation

A. foeniculum seeds were obtained from the plants grown in research field of horticultural department, Urmia University, Iran. Seeds were surface sterilized using 70% ethanol for 1 min and 2.5% sodium hypochlorite for 10 min. Sterile seeds were cultured on MS (Murashige & Skoog 1962) media containing 7 g L^{-1} agar. Cultures were maintained in a growth chamber at 25 ℃ and 16/8 h (light/dark) photoperiod for germination. Hypocotyls, cotyledons, one-month-old and five-month-old leaves were prepared as explants for transformation.

Preparation of A. rhizogenes strains and plant transformation

A4, A7 and 9435 strains of *A. rhizogenes*, provided by bank of microbes at the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran, were used. Single clones of the bacterial strains were grown in Luria-Bertani (LB) medium (Bertani 1952) containing 50 mg L^{-1} rifampicin, at 28 ℃ for 48h on rotary shaker (180 rpm) in dark condition. The bacteria were collected from the liquid medium by centrifuging (3000 rpm) for 10 min and bacterial pellet were resuspended in MS liquid medium (*Agrobacterium* infection medium) containing 50 mg L^{-1} sucrose, pH = 5.5. Before using bacterial suspension for inoculation, its concentration was adjusted at OD_{600}= 0.4–0.5.

Different explants, hypocotyls, cotyledons, one-month-old and five-month-old leaves of *A. foeniculum* were prepared for co cultivation with different strains of *A. rhizogenes*. Explants were wounded randomly and inoculated by different strains of bacteria for 5 min. Explants were dried using sterile filter paper to remove their excessive bacteria. Inoculated explants were transferred to agar (7 g L^{-1}) solidified MS medium for co cultivation under dark conditions at 25 ℃ for 48h. Additionally, a few explants were immersed in sterile distilled water and were cultured at the same way as control samples. After 2 days, explants rinsed with sterile

distilled water containing 200 mg L^{-1} cefotaxime to remove the bacteria from the explants. The explants were transferred to MS medium supplemented with 200 mg L^{-1} cefotaxime, 30 g L^{-1} sucrose and 7 g L^{-1} agar. After emergence of hairy roots, they were separated from the explants tissue and were sub cultured every week onto the MS hormone-free medium and incubated at 25 ℃ in dark conditions. Cefotaxime concentration was declined gradually and finally eliminated from the culture medium after 2 months of culture. Transformation frequency is calculated by the number of pricks showing hairy root emergence/total number of pricks inoculated and DAI (days of hairy root emergence after bacterial infection) was recorded.

Establishment of hairy roots

The best strain and explant were A4 strain and one-month-old leaf, respectively. They were used to assess the effects of elicitors on the growth of hairy roots. Hairy roots obtained from one-month-old leaves, inoculated with A4 strain, after 2 months of co-culture on solid MS medium, and were transferred to 250 mL Erlenmeyer flasks containing 50 mL liquid 1/2 MS (half-strength MS) medium with 30 g L^{-1} sucrose and were placed on rotary shaker (100 rpm) at 25 ± 2 ℃ in dark for proliferation of hairy roots.

Polymerase chain reaction analysis

Genomic DNAs from transformed hairy roots and non-transformed roots (0.5 g FW), were isolated by CTAB method (Khan et al. 2007). PCR analysis was used to investigate the presence of *rolB* gene in hairy roots. The primer sequences to amplify a 780 bp fragment of the *rolB* gene were, F: 5'-TGGATCCCAAATTGCTATTCCACGA-3' and R: 5'-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3'. Amplified DNAs were analyzed by staining with ethidium bromide after 1% (w/v) agarose gel electrophoresis.

Rosmarinic acid extraction

Transformed and non-transformed roots of *A. foeniculum* after harvest were dried by 45 ℃ oven for 1 day. Then, approximately 0.5 g of dry tissue were powdered and transferred into tubes, 5 ml of extraction solution (2.5 mL methanol and 2.5 mL water) was added to each tube. Samples were placed in ultrasonator (art: Eurosonic4D – Euronda company – Italy) to disintegrated the cells by sound waves and mixed compounds with extraction solution for 20 min. The samples were centrifuged (2000 rpm) for 10 min. Then, 25 µL of the extract was used to determine the rosmarinic acid level.

Determining the amount of rosmarinic acid

High performance liquid chromatography (HPLC) was used for determination of rosmarininc acid content. Chromatography system that used in this study was Agilent/1100 series HPLC system with DAD, model G1315B (USA), with a C18 reverse phase column (4.6 × 250 mm) at room temperature. The mobile phase (solution B) was acetonitrile / 5% acetic acid (7 : 3 v/v) with a linear gradient of 50% solution B to 30% solutions and extracts obtained from transformesd and non-transformed roots were injected into the device.

Rosmarinic acid was detected at 254 nm. Retention time of rosmarinic acid was 5.34 min. Chromatographic peaks were confirmed by retention time and UV spectra that are related to the reference standard. The retention time of the peak obtained with standard of rosmarinic acid was used to identify the corresponding peaks of root extracts.

Treatment of hairy roots in liquid media

In separate experiment, the effects of different concentrations of sucrose (20, 30, 40 and 50 g L^{-1}) on growth rate of hairy roots were evaluated. Approximately 2 g of hairy roots were incubated in 250 mL Erlenmeyer flasks containing 50 mL liquid 1/2 MS medium for 3 months on the rotary shaker (100 rpm) at 25 ℃. Fresh and dry weights of the roots were measured.

Additionally, the effects of different concentration of salicylic acid and chitosan as elicitors on formation and growth rate of hairy root cultures were investigated. Aqueous solutions of salicylic acid (0.01, 0.1 and 1 mM) were filter- sterilized with a 0.45 µM Millipore filter and then added to the culture medium. Chitosan (50, 100 and 150 mg L^{-1}) was dissolved in acetic acid at 60 ℃ within 10 min (final content 2%, v/v), and then the mixture was diluted with de-ionized water, adjusted to pH = 5.5, and then sterilized by autoclaving and added to the culture medium. Liquid 1/2 MS medium was used in all treatments as control sample. After 3 months, the roots were collected and their biomass (fresh and dry weight) was measured.

Statistical analysis

The experiments were based on a completely randomized design with 3 replications per treatment. The DAI experiment was carried out in factorial arrangement based on the randomized completely design (RCD) with two factors (strains and explants types) and three replicates. The analysis of variance (ANOVA, one-way analysis) was performed using SAS 9.1 (SAS Institute, Cary, North Carolina, USA) to detect the significance of differences among the treatment means and the means were compared using Duncan test at the 5% probability level.

Results and discussion

Effect of bacteria strains and explants type

The results showed that all *A. rhizogenes* strains used in the experiment had the ability of induction of hairy roots in *A. foeniculum* (Fig. 2). However, transformation rate and frequency of hairy roots induction significantly depended on the applied *A. rhizogenes* strains and explants types. One-month-old leaf was the best explant type for transformation by each of *A. rhizogenes* strains. The highest rate of hairy root induction (51.1 %) was obtained for one-month-old leaf explants by A4 strain, while 9435 strain induced hairy roots in only 6.66% of the five-month-old leaf explants. The hypocotyl explants showed necrosis with a low rate of hairy root induction. Moreover, hairy roots were not observed in control explants (Fig. 3).

Many studies have shown that *A. rhizogenes* strains and explants types have significant impacts on the induction of hairy roots (Danphitsanuparn et al. 2012; Soleimani et al. 2012; Pirian et al. 2012). The high percentage of transformation in one-month-old leaves might be due to the higher sensitivity of them to bacteria compared with other explants types and that this sensitivity depends on the physiological state of tissues (Pawar & Matheshwari 2003). The key effect of *rol* genes on the inactivation of auxin and cytoknin pathway has been reported to be the reason for the differ-

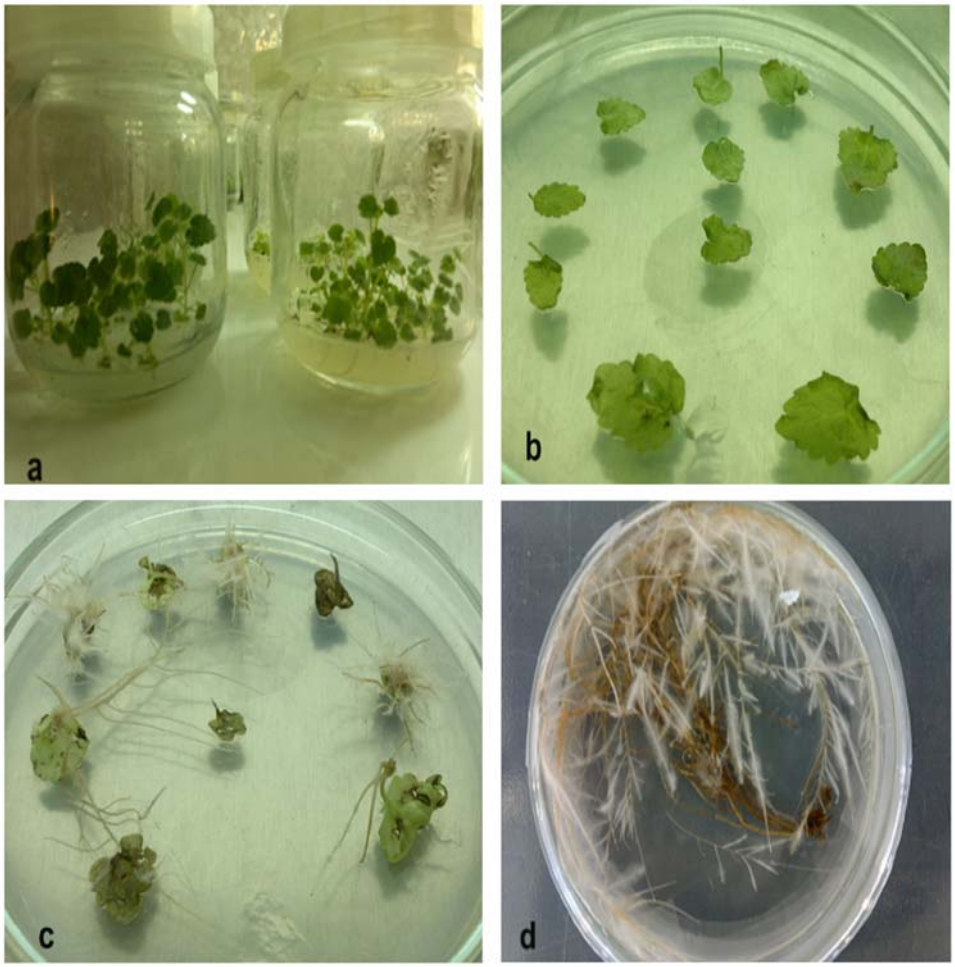

Fig. 2. *A. rhizogenes*-mediated transformation of *A. foeniculum*. a – *In vitro A. foeniculum* seedlings germinated from seeds; b – Culture of one-month-old leaves on MS media after inoculation with bacteria; c – Initiation and growth of hairy roots in phytohormone-free MS medium after 2 weeks of co-culture; d – Well development hairy roots after 2 months of sub-culturingon to the same medium containing 200 mg L^{-1} cefotaxime.

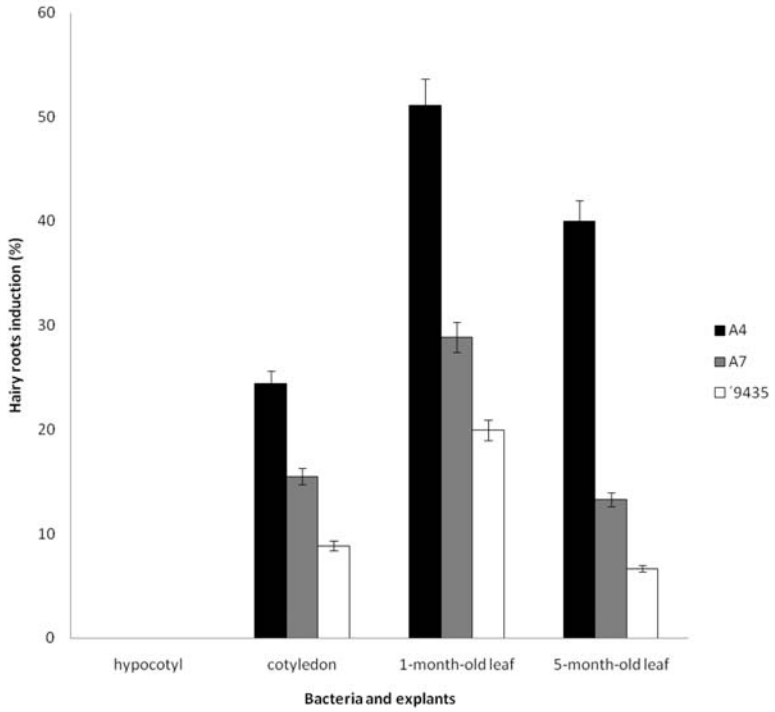

Fig. 3. Comparison of the ability of different strains of *A. rhizogenes* for induction of hairy roots on different explants of *A. foeniculum*. Transformation frequency was calculated as: number of explants showing hairy root/total number of explants inoculated. Vertical lines represent SE.

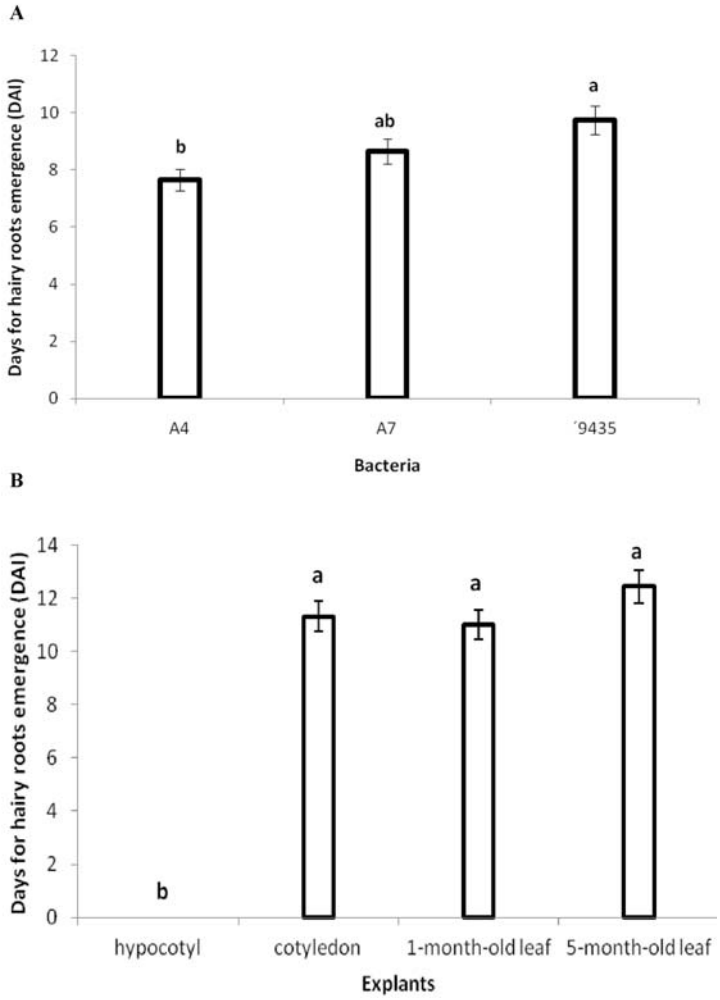

Fig. 4. Comparison of days for hairy root emergence after bacterial infection (DAI) in *A. foeniculum* explants. A – Effect of strain types of *A. rhizogenes* on DAI; B – Effect of explant types of *A. foeniculum* on DAI. The data were obtained as mean of three replications. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent SE.

ences between numbers of transgenic explants in a plant (Hamill 1993).

The difference in the induction of hairy roots between strains could be due to the difference in virulence of various strains of *A. rhizogenes* (Porter 1991). In *Artemisia annua* L. the highest percentage of root formation (75.6%) was observed in 2-week-old seedlings by using AR15834 *A. rhizogenes* strain (Soleimani et al. 2012). Induction of hairy roots in leaf, stem and root explants of *Portulaca oleracea* by different strains of *A. rhizogenes* (AR15834, A4, 9435 and C318) showed that the highest transgenic rate was obtained in leaf explants inoculated with AR15834 strain(Pirian et al. 2012). In *Papaver bracteatum*, five strains (A4, ATCC15834, LBA9402, MSU440 and A13) and three explants (hypocotyls, leaves and excised shoots) were examined. Results showed that the highest frequency of transformation was achieved using LBA9402 strain in the excised shoots (Sharafi et al. 2013).

In addition, the number of days after inoculation (DAI) that hairy roots emerged was recorded. Hairy roots emerged after 2–3 weeks of culture in selection medium. Average of DAI was less than 8 in A4 and 10 in 9435 strains. One-month-old leaf explants showed low DAI (11 days). On the other hand, the hypocotyls explant showed necrosis (Fig. 4). Cao et al. (2009) investigated the DAI to determine the best genotypes for inoculation with K599 strain of *A. rhizogenes* in the soybean hairy root transformation system. Acquiring a short DAI is very important because it results in saving space and time (Cao et al. 2009; Weber and Bodanese-Zanettini 2011).

Overall results showed that, strain A4 *Agrobacterium rhizogenes* was successful for induction of hairy roots in one-month-old leaf explants of *A. foeniculum*.

Rosmarinic acid production in hairy roots

The HPLC analysis confirmed the presence of RA in tested samples according to the same retention time of 5.34 min as standard. The highest amount of RA (213.42 μg g^{-1} dry weight) was produced in transformed roots (Fig. 5); the level was almost 4-fold higher than RA content of non-transformed roots

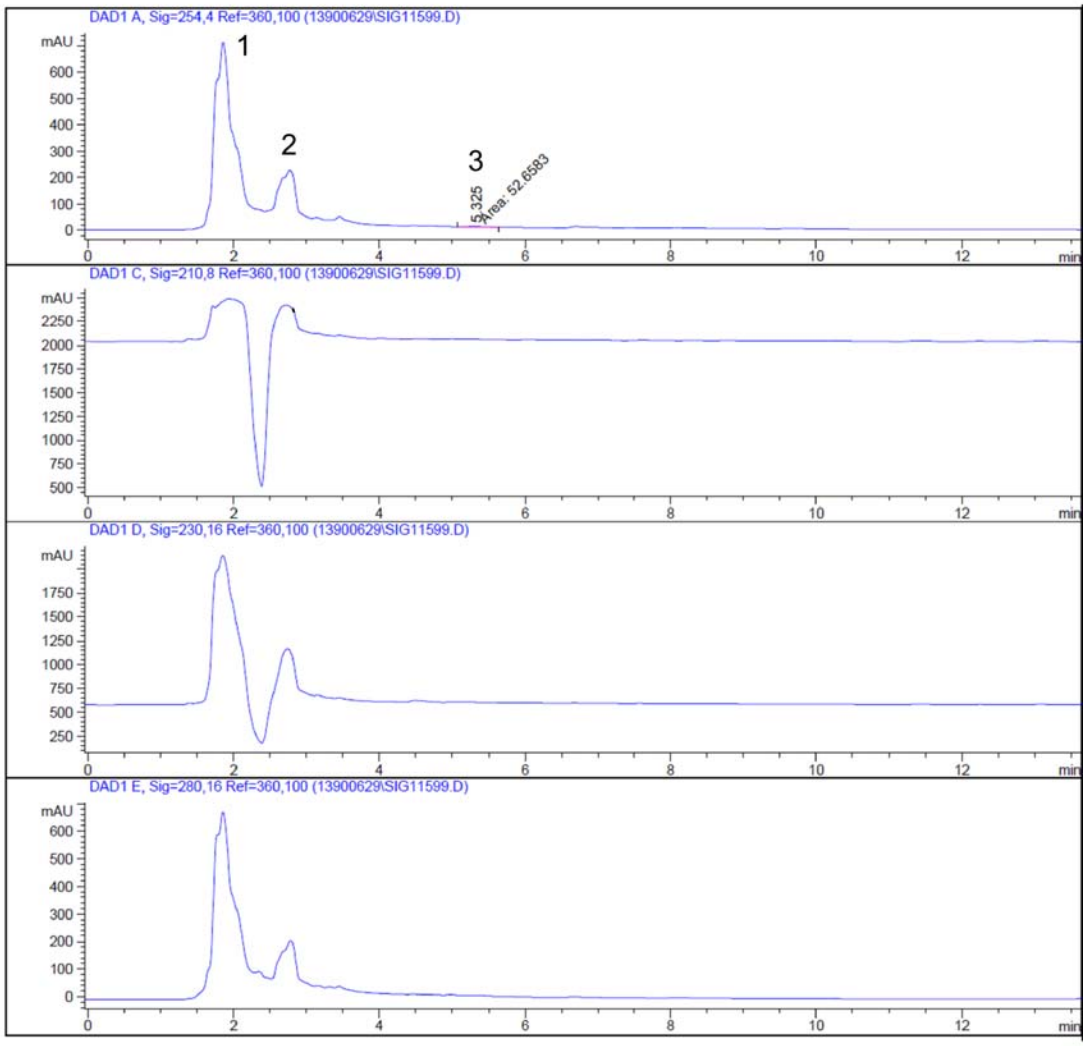

Fig. 5. Rosmarinic acid content of hairy root cultures of *A. foeniculum,* grown on1/2 MS medium. Values represent the mean ± SD of three independent measurements.

(52.28 μg g^{-1} dry weight) under the same conditions (Fig. 6).

Production of rosmarinic acid in hairy root cultures was observed by Lee et al. (2008) in *Agastache rugsa* and by Lee at al. (2010) in *Nepeta cataria* L. Many studies have pointed out that hairy root growth and metabolite production depend on '*rol*' gene expression (Bulgakov 2008). Recently, an inhibitory effect of the *rolC* of *A. rhizogenes* has been demonstrated on rosmarinic acid production (Bulgakov et al. 2005). The *rol* genes could activate expression of defense genes and thus the production of secondary metabolites (phytoalexin production) in plant cells (Bulgakov 2008).The ability of secondary metabolite synthesis in hairy roots is much higher than the wild type roots and production of secondary metabolites from hairy roots is more stable compared to other types of cell cultures (Guillun et al. 2006).

PCR analysis

PCR method can be used simply for detecting T-DNA sequences in putative transformants (Palazon et al. 2003b). Ri plasmid is a diagnostic tool that is used to confirm the integration of T-DNA into host genome (Soleimani et al. 2012). The *rolB* gene is absolutely essential for the induction of hairy roots (Samadi et al. 2012). In this study, the integration T-DNA of *A. rhizogenes* Ri plasmid into *A. foeniculum* genome was confirmed by PCR analysis using primers specific to *rolB*. The 1% agarose gel Electrophoresis revealed the presence of 780 bp bands in production of hairy roots but no such amplicon was observed in the untransformed root (negative control) sample. *A. rhizogenes* plasmid was used as positive control (Fig. 7).

Effect of sucrose concentration on the growth rate of hairy roots

In vitro hairy root cultures require the supply of carbon sources in order to satisfy energy demands. The results of present study revealed that, sucrose concentration could affect the growth rate of hairy roots. The growth rate of hairy roots was increased by increasing the concentration of sucrose up to 30 g L^{-1}. However, concentration of higher than 30 g L^{-1} sucrose reduced the growth of hairy root lines (Fig. 8). The growth rate of hairy roots is usually high but some fac-

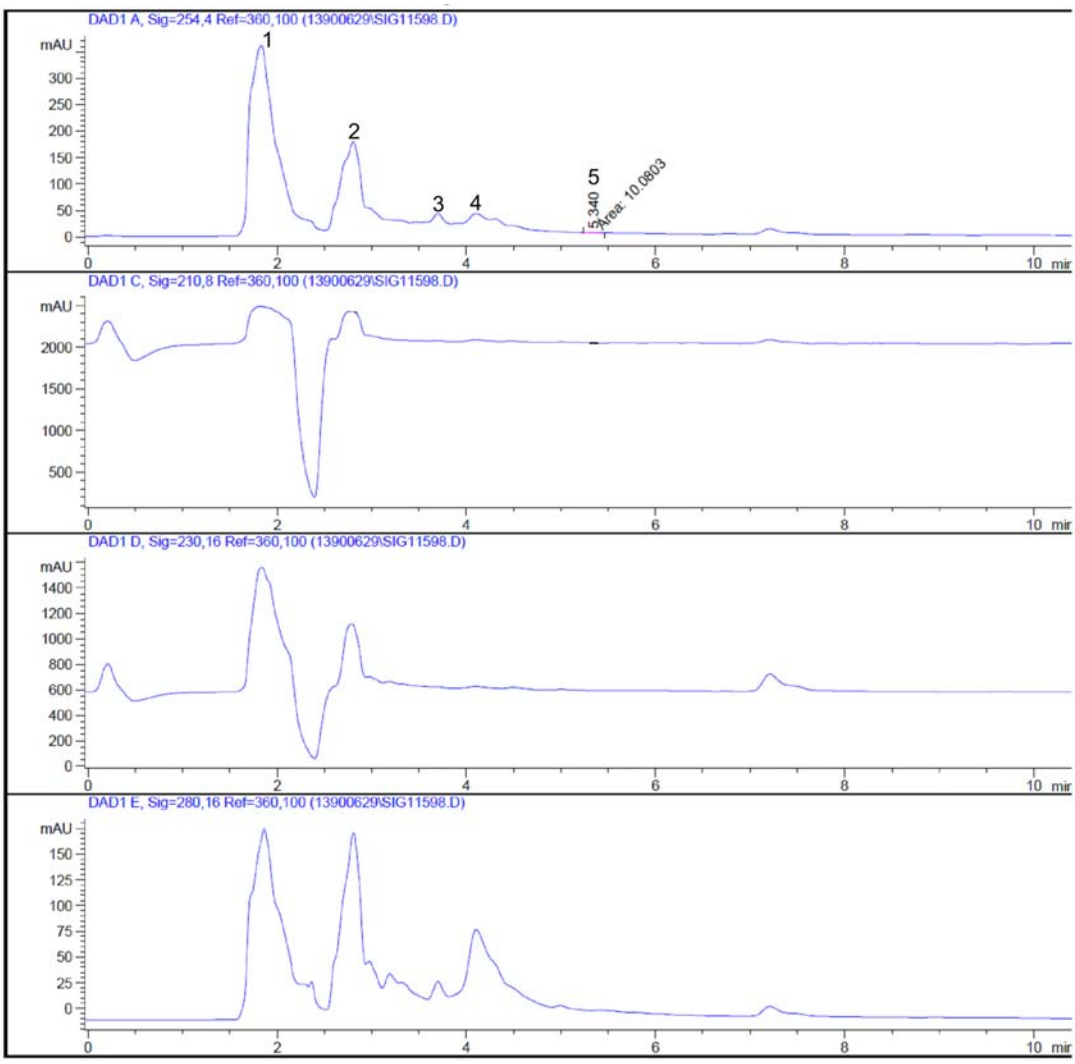

Fig. 6. Rosmarinic acid content of non-transformed roots of *A. foeniculum*, grown on 1/2 MS medium. Values represent the mean ± SD of three independent measurements.

tors are able to change the growth rate of hairy roots. Accordingly, in *Papaver bracteatum* hairy root cultures, growth rate of hairy roots was reduced in higher and lower concentrations of 30 g L^{-1} sucrose (Sharafi et al. 2013). Optimization of medium compounds often increases both the growth rate of hairy roots and accumulation of secondary metabolites. Application of the modified medium is generally required, that these changes are generally in sources of carbon, nitrogen and phosphorus (Bensaddek et al. 2008). High concentration of sucrose reduced considerably the growth rate of *Psoralea* species hairy roots that is probably due to osmotic stress (Nguyen et al. 1992). Additionally, the experiments carried out on *Angelica gigas* Nakai, for investigating the effects of different concentrations of sucrose (10, 20, 30, 40, 50 g L^{-1}), showed that the growth increased up to 40 g L^{-1} sucrose concentration and further increment of the concentration beyond 50 g L^{-1} decreased the growth rate (Xu et al. 2009). Putalun et al. (2006) reported that the growth rate of *Senna alata* hairy roots was increased in dark condition in a 1/2 MS medium containing 50 g L^{-1} sucrose.

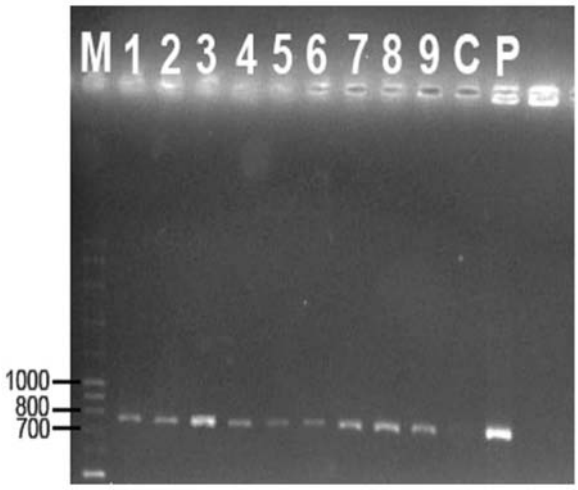

Fig. 7. PCR amplification of *rolB* gene in hairy roots of *A. foeniculum*. *Lane M* – Molecular size marker (1 kb ladder Fermentase); *lanes 1–9* – Transgenic hairy roots induced on cotyledon, one-month-old leaf and five-month-old leaf explants infected by *A. rhizogenes* strains; *lane C* – Non transformed roots as negative control; *lane P* – Ri plasmid from *A. rhizogenes* strain as a positive control.

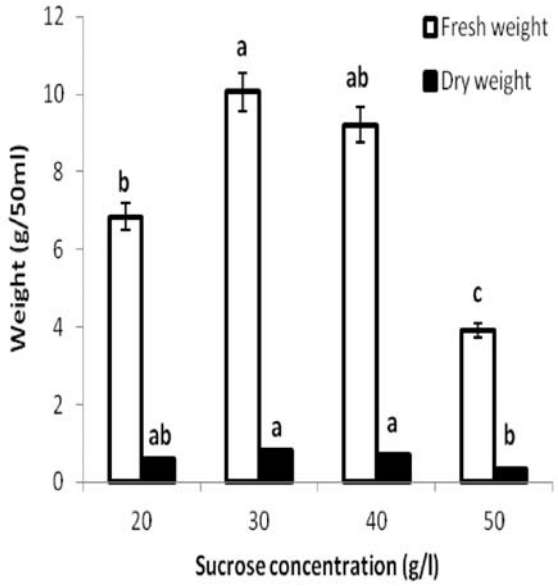

Fig. 8. Effect of different concentrations of sucrose (20–50 g L^{-1}) on the fresh and dry weight of hairy roots in one-month-old leaf explants of *A. foeniculum* inoculated with *A. rhizogenes* A4. The data presented as a mean of three replications. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent SE.

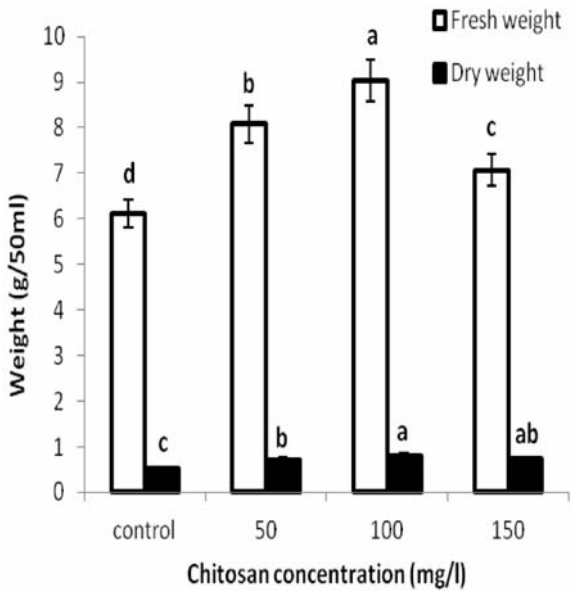

Fig. 10. Effect of chitosan concentration on growth rate of hairy roots in *A. foeniculum*. Fresh weight (g) and dry weight (g) after elicitation with chitosan. Each value is the average of 3 replicates. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent SE.

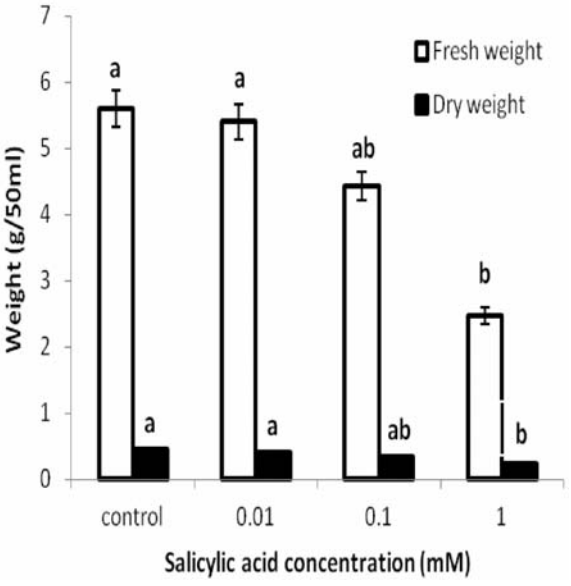

Fig. 9. Effect of SA on growth rate of hairy roots in *A. foeniculum*. Fresh weight (g) and dry weight (g) after elicitation with SA. Each value is the average of 3 replicates. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range test. Vertical lines represent SE.

Growth and elicitation of hairy root cultures

Investigation the effects of different concentrations of salicylic acid and chitosan on the growth rate of *A. foeniculum* hairy roots showed that application of salicylic acid reduced the growth of hairy roots compared with the control explants. Fresh and dry weight of hairy roots declined sharply in 1 mM of SA (Fig. 9).

Amongst the various concentrations of chitosan (0, 50, 100, 150 mg L^{-1}), the 100 mg L^{-1} concentration increased the fresh weight (9.03 g) and dry weight (0.81 g) of the hairy roots (Fig. 10 and 11).

Effect of elicitation depends on the elicitor type, environmental conditions, the concentration of elicitor and the interaction between the elicitor and plant cells (Smetanska 2008). In the current study, reduction of biomass of typical hairy roots was observed after treatment with different concentrations of SA. Similar results have been reported, previously. Application of SA in *Stemona* sp., reduced the hairy roots growth at all concentrations (0.1, 0.3, 0.5 and 1 mM), especially at concentration of 1 mM (Chichana and Dheeranupattana 2012). All concentrations (100, 200, 300, 500 mg L^{-1}) of SA had a negative effect on the growth of *Stemona curtisii* Hook hairy roots (Chotikadachanarong et al. 2011). In an experiment on induction of hairy roots in *Artemisia dubia*, comparing three different concentrations of SA (0.138, 1.38 and 13.8 mg L^{-1}), the 0.138 mg L^{-1}SA concentration showed the highest growth response and by increasing the concentration of SA, the growth rate of hairy roots was reduced (Ali et al. 2012). The effect of salicylic acid, jasmonic acid, chitosan and fungal cell wall elicitors on growth and accumulation of rosmarinic acid in *Ocimum basilicum* hairy roots was examined by Bais et al. (2002), the results showed that hairy roots, treated with salicylic acid, jasmonic acid and chitosan, gradually showed necrotic status and their biomass decreased in comparison to the control samples. According to the results, the effects of various concentrations of SA on the growth rate of hairy roots were different, which is due to its role in cellular signaling pathway. At low concentration, SA is

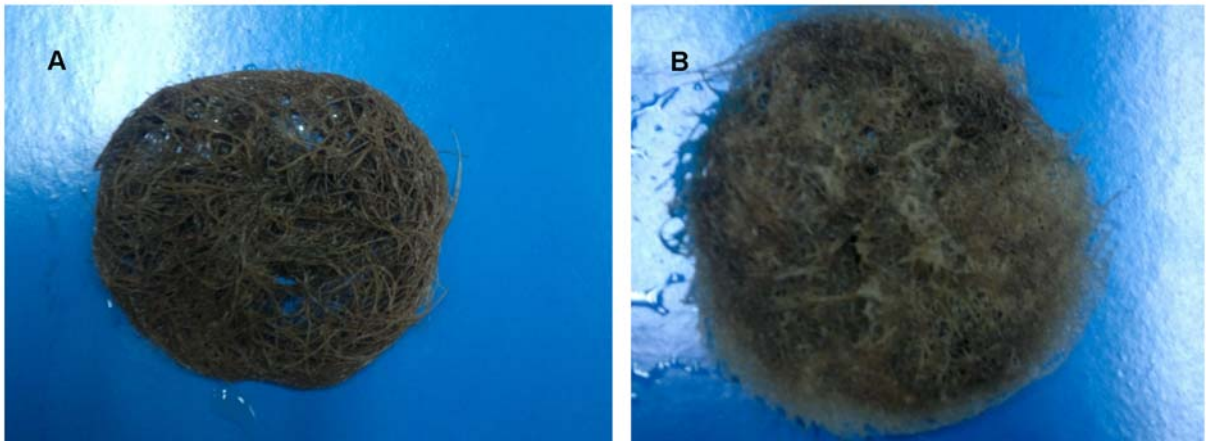

Fig. 11. Harvested hairy roots. A – *A. foeniculum* hairy roots without using elicitors; B – A hairy root clone elicited with 100 mg L^{-1} chitosan.

useful for cell signaling but its high concentration can cause impaired growth (Ahmadian et al. 2010). A high concentration of SA causes stress and severe damage to the root tissue and prevents the growth and reduces the cell metabolism (Ahmadian et al. 2010).

Chitosan is another elicitor used in this experiment. According to the results, adding of chitosan to the culture medium increased the growth rate of hairy roots. In particular, the root biomass was highest in the presence of 100 mg $L^{-1}$of chitosan. Chitosan is a growth promoter in some plant species and its derivatives are natural elicitors for defensive response in plants (Sanford & Hutchings 1987). Similarly the presence of chitosan increased the growth rate of *Hyoscyamus muticus* hairy roots (Sevonetal. 1992). Increasing the concentration of chitosan, increased the growth of *Withania somnifera* L. hairy roots, and growth of roots was higher in 150 mg $L^{-1}$of chitosan (Doma et al. 2012). However, addition of chitosan inhibited the root growth and production of ginsenosides in hairy root cultures of *Panax ginseng* (Palazon et al. 2003).These results indicated that chitosan responds differently in different plant cultures. High concentrations of chitosan may be detrimental to the cells by increasing the permeability of cell membranes (Jin et al. 1999).

Our results indicate that formation of hairy roots in *A. foeniculum* was strongly affected by type of explants and bacterial strain. For optimum production of hairy roots, the cultures should be grown in phytohormone-free MS basal medium with 30 g L^{-1} sucrose and 100 mg L^{-1} chitosan. It is possible to generate a transformed root line with significantly higher RA content than that of roots of field-grown plants of *A. foeniculum*. For induction of hairy roots, it is necessary to carry out many experiments on various plant species and examine the effects of the environment, selection of best medium culture and culture conditions.

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