

## Hairy root induction and secondary metabolite production in *Perovskia abrotanoides* Karel

Sorayya Ebrahimi<sup>1</sup>, Arehzoo Zaker<sup>1</sup>, Parvaneh Abrishamchi<sup>1\*</sup>, Ahmad Reza Bahrami<sup>1</sup>, Ali Ganjeali<sup>1</sup> and Najme Sodagar<sup>2</sup>

<sup>1</sup>Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

<sup>2</sup>Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

(Received: 27/5/2016-Accepted: 01/10/2016)

### Abstract:

Induction of hairy roots in *Perovskia abrotanoides* Karel, a medicinally important member of the Lamiaceae family, was evaluated using different strains of *Agrobacterium rhizogenes* and various carbon sources. An efficient *Agrobacterium*-mediated transformation system was established for the first time for this plant species using the bacterial strains of ATCC15834, TR105, and R1000. Meanwhile, the effects of acetosyringone and carbon sources on both rooting and root biomass production were investigated and the potential of hairy root cultures for producing tanshinones was explored. Hairy root induction in the nodes of intact seedlings was found to be successful with all the bacterial strains tested but R1000 was found to be more effective than TR105. The highest transformation frequency belonged to the nodes infected with ATCC15834 (47.33%). Moreover, transformation frequency increased by up to 60.99% when 100  $\mu$ M of acetosyringone was added. The strain ATCC15834 recorded the highest root dry and fresh weights per 50 mL of the nutrient medium (0.149 and 1.996 g, respectively) and root length (34.45 cm) in a medium containing 3% sucrose. However, R1000 induced maximum lateral root density in hairy roots (7.1 branches  $\text{cm}^{-1}$ ) in a medium containing 3% glucose. PCR analysis using *rolC* specific primer with an amplicon of 612 bp confirmed the transgenic nature of the hairy roots. Finally, HPLC analysis revealed that the cryptotanshinone and tanshinone IIA levels in the hairy roots induced by TR105 (clone T7) were  $53.17 \pm 0.26$  and  $14.48 \pm 0.30 \mu\text{g g}^{-1}$  DW, respectively.

**Key words:** Acetosyringone, *Agrobacterium rhizogenes*, Carbon source, Hairy root, *Perovskia abrotanoides*, Tanshinone.

### Introduction:

Medicinal plants have of long been used as important therapeutic agents. Almost one fourth of the drugs presently manufactured contain plant extracts or active compounds obtained from the plants (Yaniv, 2005). *Perovskia abrotanoides* Karel (a perennial herb from the Lamiaceae family), grown in Iran, Afghanistan, Pakistan, and Turkmenistan (Rechinger, 1982), has been used in Iranian traditional medicine for the treatment of leishmaniasis (Moallem and Niapour, 2008). In recent years, the anti-plasmodial, anti-microbial, anti-nociceptive, anti-inflammatory, and cytotoxic activities of this species have also been reported (Nassiri-Asl *et al.*, 2002; Hosseinzadeh and Amel, 2001; Sairafianpour *et al.*, 2001; Ashraf *et al.*, 2014). However, while few scientific reports are available on the natural products from this species, most studies have focused on the essential oils obtained from their aerial parts (Mahboubi and Kazempour, 2009; Safaei-Ghomi and Batooli, 2010; Ashraf *et al.*, 2014). Moreover, phytochemical analysis has demonstrated that the roots of *P. abrotanoides* are rich sources of diterpenoid quinone compounds known as tanshinones (Sairafianpour *et al.*, 2001; Zaker *et al.*, 2015), which reportedly have such biological properties

as anti-microbial (Lee *et al.*, 1999), anti-inflammatory (Jang *et al.*, 2003), antioxidant (Cao *et al.*, 1996), anti-diabetes (Kim *et al.*, 2007), and anti-cancer (Liu *et al.*, 2009; Chiu *et al.*, 2010; Pan *et al.*, 2010) effects.

The commercially used secondary metabolites are generally higher-value, lower-volume products than the primary ones (Balandrin *et al.*, 1985). These metabolites are generally accumulated by plants in small quantities often at certain developmental stages and under given environmental conditions. The pharmaceutical substances of *P. abrotanoides* (e.g. tanshinones) are typically produced in plants roots; thus, hairy root cultures may enhance the production and accumulation of these metabolites.

Since chemical synthesis of natural products is not readily available, an alternative is to produce them through cell-tissue culture technology (Vanisree *et al.*, 2004). In this regard, particular emphasis is commonly laid on hairy root culture as a means of enhanced production of secondary metabolites for commercial applications (Kong *et al.*, 2003). These cultures generally have a higher capacity than cell suspension cultures or even roots of intact plants for synthesizing secondary metabolites (Giri and Narasu, 2000; Ono and

\*Corresponding Author, Email: [abrisham@um.ac.ir](mailto:abrisham@um.ac.ir)

Tian, 2010). Moreover, hairy root cultures are not only more reliable in terms of genetic stability but are characterized by fast growth and easy maintenance in a hormone-free medium.

Hairy root growth and biomass productivity are affected by different factors. For instance, it is established that addition of a carbon source to the cultivation medium is necessary for the growth of hairy roots. Study has shown that the production of most biologically active compounds through hairy roots is affected by the type and concentration of the carbon source present in the nutrient media (Kochan *et al.*, 2014). Moreover, plant growth, development, metabolism, and gene expression are reportedly affected by sugars (monosaccharide and disaccharide), as signal molecules (Praveen and Murthy, 2012; Petrova *et al.*, 2015).

To date, there have been no published reports on hairy root induction in *P. abrotanoides* and the effects of *Agrobacterium* strain and carbon sources on its hairy root growth. In this study, hairy root cultures of *P. abrotanoides* were established using three different strains of *Agrobacterium rhizogenes* and different carbon sources to determine the best carbon source for the production of tanshinones.

## Materials and methods:

**Plant material:** Mature seeds of *P. abrotanoides* Karel were collected in September from wild-growing plants in Kalat, Razavi Khorasan Province, Iran. The plant was identified in the herbarium of Ferdowsi University of Mashhad, Mashhad (No. 39299). The seeds were surface sterilized with 20% NaOCl (V/V) for 4 minutes and rinsed three times with sterilized water before they were aseptically germinated on the hormone-free half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 3% sucrose (W/V) and 0.7% agar (W/V) in the culture room at 25±1 °C under darkness. The pH of the medium was adjusted to 5.7 before autoclaving at 121 °C for 15 min. After germination, the cultures were kept at 25±1 °C during 16/8 h light/dark photoperiods with an irradiance level of 45 μmol m<sup>-2</sup> s<sup>-2</sup>.

**Growth of *Agrobacterium rhizogenes*:** The wild type of *A. rhizogenes* strains including ATCC15834, TR105, and R1000 were cultured in a solid Yeast Mannitol Broth medium (YMB) containing 0.2 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 g L<sup>-1</sup> mannitol, 0.5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 0.4 g L<sup>-1</sup> yeast extract, and 0.1 g L<sup>-1</sup> NaCl adjusted to pH=7. Briefly, a single bacterial colony was inoculated in 15 mL of liquid YMB medium and grown overnight at 28 °C on a shaker incubator (100 rpm) to mid-log phase (OD<sub>600</sub>=0.6-0.8) (Vincent, 1970). The bacterial cells were then collected by centrifugation (Hermle Z230A, Germany) at 107g for 10 minutes and resuspended at a cell density of OD<sub>600</sub>=0.6-0.8 in a liquid inoculation medium (half-strength MS medium containing 3% sucrose).

**Induction of hairy roots in *P. abrotanoides* using *A. rhizogenes*:** *In vitro* grown (45-day-old) *P.*

*abrotanoides* seedlings were used for transformation with the three *A. rhizogenes* strains (ATCC15834, TR105, and R1000). In an early experiment, excised leaf and internode explants from the *in vitro* seedlings and nodes from intact seedlings were inoculated using insulin syringes with and without acetosyringone (100 μM) (Wawrosch *et al.*, 2014). Infected leaf and internode explants together with whole seedlings inoculated at their nodes were then cultured on a half-strength MS medium with 3% sucrose and 0.7% agar for 72 hours at 25±1 °C under 16 h day<sup>-1</sup> light. After 3 days of co-cultivation, the shoots were transferred to an agar-solidified half-strength MS medium containing 500 mg L<sup>-1</sup> cefotaxime to remove residual bacteria. Subsequently, they were sub-cultured in a half-strength MS medium containing 300 mg L<sup>-1</sup> cefotaxime after 10 days (Yao *et al.*, 2016). Controls consisted of explants and seedlings treated similarly, except that they were not co-cultivated with *A. rhizogenes*. Hairy roots emerged from the nodes of intact seedlings after 6 to 8 days. The percentage of hairy root induction was recorded after two months. In cases where no hairy roots initiated from the leaf and internode explants irrespective of the bacterial strain and the presence or absence of acetosyringone, they were excluded from the experiment.

**Establishment of hairy root cultures:** Induced hairy root lines produced with *A. rhizogenes* ATCC15834, TR105 and R1000 strains, were aseptically dissected in 2 cm lengths from the inoculated seedlings and cultured in 200 mL flasks containing 50 mL of a liquid half-strength MS medium with 200 mg L<sup>-1</sup> cefotaxime and 3% sucrose. All the cultures were maintained at 25±1 °C in the dark on a rotary shaker at 90 rpm. Isolated clones were sub-cultured every 4 days into a fresh liquid medium but with gradually reduced cefotaxime concentrations (from 200 to 100 and 50 μM) (Yao *et al.*, 2016). After complete removal of cefotaxime, the cultures were transferred into a liquid half-strength MS medium with 3% sucrose. Hairy roots were maintained by sub-culturing into a fresh medium every 14 days. The root lines A9, R5, and T7 already transformed with ATCC15834, R1000, and TR105 strains, respectively, were used for further experiments.

**PCR Analysis of hairy roots:** Genomic DNA was extracted from 200 mg of both fresh hairy and untransformed roots using Sharp's method (Sharp *et al.*, 1988). Polymerase chain reaction (PCR) was performed to detect the *rolC* gene using the following primer sets: forward-5'-CTCCTGACATCAAACCTCGTC-3' and reverse-5'-TGCTTCGAGTTATGGGTACA-3'. The reaction mixture consisted of 0.9 μl of 1 Unit Taq polymerase, 0.6 μl of 0.2 μM dNTP, 1 μl of 0.5 μM of each primer, 2 μl of 100 ng template DNA, 1.5 μl of 2 μM MgCl<sub>2</sub> and 2.5 μl of 10X reaction buffer; the mixture was replenished up to 25 μl with sterilizing distilled water. The following procedure was used in the PCR reaction: initial denaturation at 95 °C for 4 min followed by 95 °C for 1 min, annealing of the primer at 55 °C for 30 seconds and amplification at 72 °C for 1

min; the procedure was repeated for 35 cycles with a final extension at 72 °C for 7 min (Wang *et al.*, 1993). Amplification products were analyzed by electrophoresis on 1.2% agarose gel, stained with ethidium bromide, visualized, and documented using a Trans illuminator equipped with a gel documentation system (Major Science, USA). Plasmid DNA from the *A. rhizogenes* strain ATCC15834 was used as the positive control and DNA from the untransformed root served as the negative control.

#### Effect of carbon source on hairy root growth:

Hairy roots induced by three strains of *A. rhizogenes* (clones A9, R5, and T7 transformed with ATCC15834, R1000 and TR105 strains, respectively) were cultured in a half-strength MS medium containing 3% sucrose, glucose, or sorbitol to investigate the effects of carbon sources on hairy root growth. All the cultures were kept at 25±1 °C on a rotary shaker (90 rpm) in the dark. The hairy roots were harvested after 50 days and fresh and dry weights, root length, and lateral root density (i.e., number of lateral branches per 1 centimeter root) were recorded.

**Analysis of tanshinones in hairy roots:** One hairy root clone of *P. abrotanoides* induced by *A. rhizogenes* strain TR105 which grew vigorously with lateral branches in the medium containing 3% sucrose was selected to determine its tanshinones content. Briefly, dried hairy roots were ground into powder and extracted with methanol (0.5 g/30 mL) at room temperature in a sonic water-bath for one hour. The extract was evaporated to dryness and dissolved in 1 mL methanol, centrifuged at 5000 rpm for 5 minutes, and subjected to HPLC analysis. HPLC was performed on a C18 column (250×4 mm, 5 µm particle size) using gradient elutions of acetonitrile/water (20:80 V/V to 95:5 V/V) as the mobile phase at a flow rate of 1 mL min<sup>-1</sup>. UV detection was conducted at 254 nm. Cryptotanshinone and tanshinone IIA contents were detected and quantified against authentic standards obtained from Sigma.

**Statistical analysis:** A completely randomized design was used for all the experiments. Significance was determined by analysis of variance (ANOVA) using SPSS software and means were compared using the Duncan's Multiple Range Test at the 5% significance level. Data were reported as means ± standard error.

## Results:

**Effects of different *A. rhizogenes* strains on hairy root induction:** No hairy roots were induced from infected leaves or internodes regardless of the bacterial strain used and the presence or absence of acetosyringone. The fragments of leaves and internodes turned brown and gradually became necrotic and died. Results of *A. rhizogenes* inoculation on the nodes of intact *P. abrotanoides* seedlings after 60 days of *in vitro* culturing showed that all the tested strains were able to induce hairy roots even in the absence of acetosyringone. At the site of inoculation (nodes of *in*

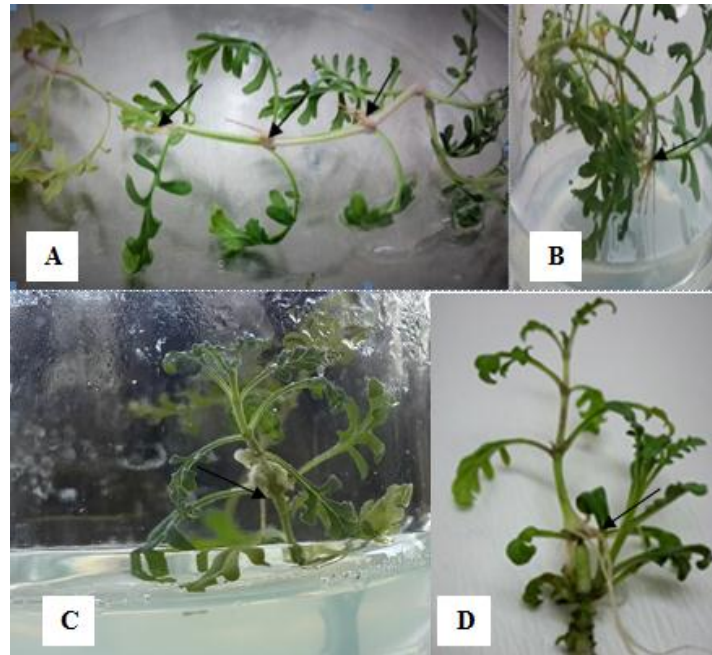
*vitro* *P. abrotanoides* seedlings), transformed hairy roots were successfully induced. The appearance and growth of hairy roots were monitored over a period of two months. The first ATCC15834-induced hairy roots emerged from the inoculated nodes after 6 days; in the case of R1000 and TR105-infected nodes, however, hairy roots appeared 8 days after inoculation (Figure 1). No hairy roots were induced in the control groups which had only been inoculated in a half-strength MS medium.

Hairy root formation percentages in the seedlings of *P. abrotanoides* transformed with ATCC15834, R1000, and TR105 strains of *A. rhizogenes* bacteria are reported in Table 1. It was found that bacterial strains had significant effects on hairy root formation ( $p < 0.05$ ). The three strains used varied in their ability to infect *P. abrotanoides* seedlings. In the absence of acetosyringone, the highest percentage of root induction was achieved with ATCC15834 (47.33%) followed by R1000 (38.16%) and TR105 (28.99%) (Table 1). Finally, the two strains ATCC15834 and TR105 recorded significantly ( $p < 0.05$ ) different capabilities for inducing hairy roots.

**Confirmation of transformation:** The transgenic nature of hairy roots was confirmed by PCR using *rolC* specific primers. A 612 bp *rolC* expected size fragment was present only in hairy roots but absent in normal roots (Figure 2).

**Effect of acetosyringone on hairy root induction:** According to Table 1, in the presence of acetosyringone, different strains of *A. rhizogenes* displayed varying degrees of ability to induce hairy roots on the nodes of *P. abrotanoides* seedlings. The presence of 100 µM acetosyringone increased hairy root induction on plants inoculated with the strain ATCC15834 from 47.33% to 60.99%. The use of acetosyringone with R1000 and TR105 strains also increased transformation efficiencies (from 38.16 to 49.49 in R1000 and from 28.99 to 36.33 in TR105) although the increments were not significant when compared with acetosyringone-free treatments (Table 1).

**Effect of carbon source on hairy root growth:** In the present study, application of sucrose, rather than glucose or sorbitol ( $p \leq 0.05$ ), in the culture medium resulted in increased dry weight, fresh weight, and length of transformed (hairy) roots of *P. abrotanoides* induced by all the strains of *A. rhizogenes*. It is evident from Figures 3, 4, 5, and 6 that not only were the highest fresh and dry weights (1.996 and 0.149 g, respectively) and maximum root length (34.45 cm) observed in the ATCC15834-transformed hairy roots grown in the half-strength MS medium supplemented with 3% sucrose but that glucose had a weaker effect on the growth (root length) and biomass (fresh and dry weights) of hairy roots than did sucrose ( $p \leq 0.05$ ). The transformed roots grown in the medium containing 3% sorbitol had significantly lower ( $p \leq 0.05$ ) weights, lengths, and lateral branchings in most cases. The lowest dry and fresh weights of hairy roots (0.222 and



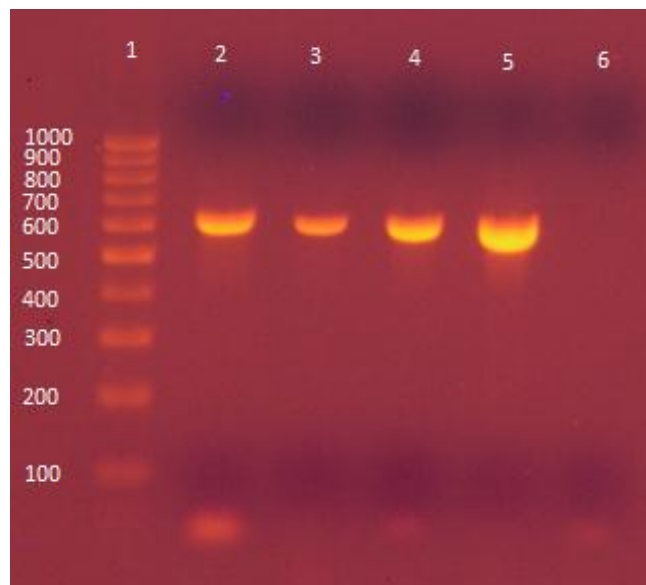
**Figure 1.** Hairy roots induced from the nodes of *Perovskia abrotanoides* seedlings after inoculation by different *Agrobacterium rhizogenes* strains. A: 12 days after inoculation with ATCC15834 strain; B: 2 weeks after inoculation with ATCC15834 strain (using acetosyringone); C: 9 days after inoculation with R1000 strain (using acetosyringone); D: 12 days after inoculation with TR105 strain (using acetosyringone). Black arrows indicate the hairy roots developed in the sites of inoculation.

**Table 1.** The effects of different strains of *Agrobacterium rhizogenes* and acetosyringone application on the percentage of hairy root formation in *Perovskia abrotanoides* seedlings, 60 days after inoculation.

<i>A. rhizogenes</i> strain	Percentage of hairy root induction	
	Without acetosyringone (100 $\mu$ M)	With acetosyringone (100 $\mu$ M)
ATCC15834	47.33 $\pm$ 5.06 <sup>bc</sup>	60.99 $\pm$ 4.12 <sup>a</sup>
R1000	38.16 $\pm$ 4.13 <sup>bcd</sup>	49.49 $\pm$ 4.75 <sup>b</sup>
TR105	28.99 $\pm$ 2.52 <sup>d</sup>	36.33 $\pm$ 2.77 <sup>cd</sup>

All data were represented as the means (n=20)  $\pm$  standard deviation.

Different script letters characterize significant differences in each column (or row) according to Duncan's Multiple Range Test at  $p \leq 0.05$ .



**Figure 2.** PCR analysis of a 612 bp fragment of a *rolC* gene in *Agrobacterium rhizogenes*. Lane 1: molecular weight marker (1000 bp ladder); Lane 2: amplified band from the DNA of strain ATCC15834 (positive control); Lane 3: amplified band from the DNA of hairy roots induced by TR105 strain (clone T7); Lane 4: amplified band from the DNA of hairy roots induced by R1000 strain (clone R5); Lane 5: amplified band from the DNA of hairy roots induced by ATCC15834 strain (clone A9); Lane 6: the band is absent in non-transformed root (negative control).

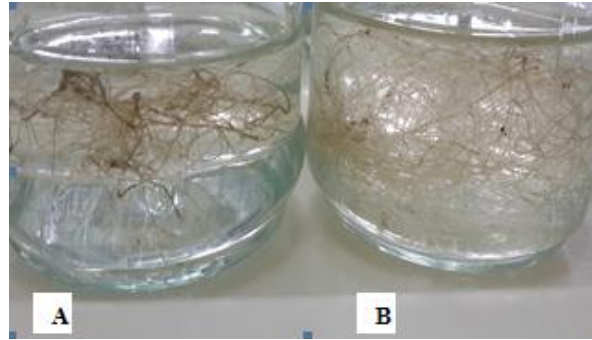


Figure 3. Growth of hairy roots induced by the ATCC15834 strain of *Agrobacterium rhizogenes* in  $\frac{1}{2}$  MS medium after 50 days culture under the dark condition. A: in the medium containing 3% (W/V) glucose; B: in the medium containing 3% (W/V) sucrose.

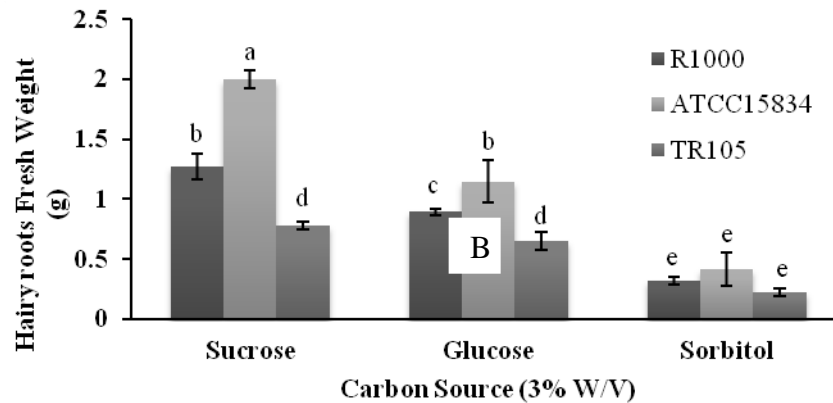


Figure 4. The effect of carbon sources on the fresh weight of hairy roots in *Perovskia abrotanoides*, induced by different strains of *Agrobacterium rhizogenes* after 50 days of culture. Data are means ( $n=3$ )  $\pm$  standard deviation. Bars with different letters are significantly different from one another (Duncan's Multiple Range Test at  $P \leq 0.05$ ).

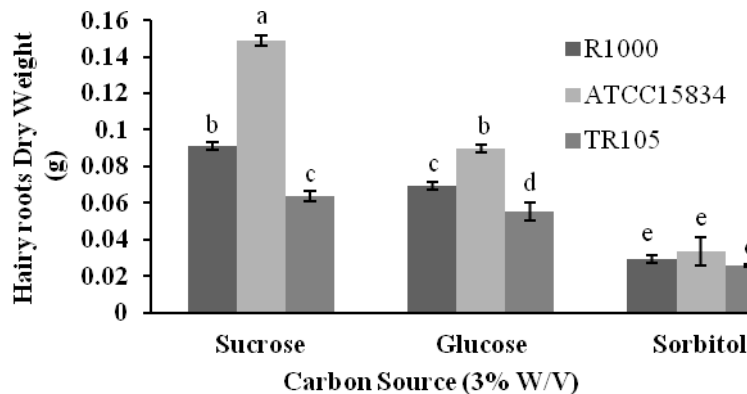


Figure 5. The effect of carbon sources on the dry weight of hairy roots in *Perovskia abrotanoides*, induced by different strains of *Agrobacterium rhizogenes* after 50 days of culture. Data are means ( $n=3$ )  $\pm$  standard deviation. Bars with different letters are significantly different from one another (Duncan's Multiple Range Test at  $P \leq 0.05$ ).

0.025 g, respectively) were obtained when roots induced by TR105 were cultured in the medium supplemented with 3% sorbitol (Figures 4, 5, and 6). The highest lateral root density ( $7.1 \text{ branches cm}^{-1}$ ) belonged to the roots induced by R1000 and grown in the medium containing 3% glucose. The least lateral root branching ( $1 \text{ branch cm}^{-1}$ ) was recorded for TR105-transformed roots grown in a medium containing 3% sorbitol (Figure 7).

**HPLC Analysis of tanshinones in hairy roots:** HPLC analysis revealed cryptotanshinone and tanshinone IIA contents of  $53.17 \pm 0.26$  and  $14.48 \pm 0.30 \mu\text{g g}^{-1}$  DW, respectively, in the TR105-induced hairy roots (clone T7). These data cannot be compared with those from the

control seedlings (not inoculated with *A. rhizogenes*) since they did not produce any adventitious roots in the half-strength MS medium.

### Discussion:

It has been well established that hairy root formation in different plant species greatly depends on the type of explant. Wound response has been found to be the most important factor affecting successful transformation. Different explants reportedly vary in their wound response (i.e., in the number of competent cells produced for transformation) (Potrykus, 1991). This is because explant cells differ in their DNA synthesis and

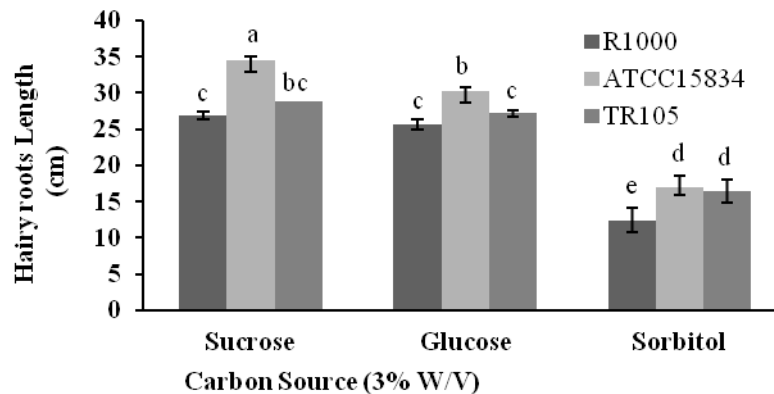


Figure 6. The effect of carbon sources on the length of hairy roots in *Perovskia abrotanoides*, induced by different strains of *Agrobacterium rhizogenes* after 50 days of culture. Data are means ( $n=3$ )  $\pm$  standard deviation. Bars with different letters are significantly different from one another (Duncan's Multiple Range Test at  $p \leq 0.05$ ).

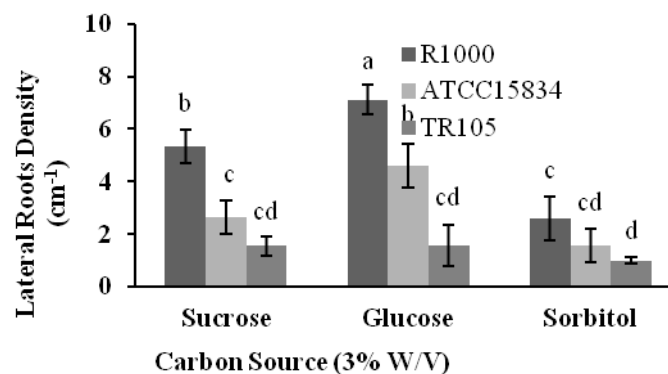


Figure 7. The effects of carbon sources on the density of lateral hairy roots in *Perovskia abrotanoides*, induced by different strains of *Agrobacterium rhizogenes* after 50 days of culture. Data are means ( $n=3$ )  $\pm$  standard deviation. Bars with different letters are significantly different from one another (Duncan's Multiple Range Test at  $p \leq 0.05$ ).

cell division due to differences in the physiological maturity of the cells (Karmarkar *et al.*, 2001). The specificity of various strains of *A. rhizogenes* to induce hairy roots in different explants and tissues is related to their age, cell division ability, and hormonal status (Karmarkar and Keshavachandran, 2001).

Our results revealed that neither the leaves nor the internodes of *P. abrotanoides* were capable of forming hairy roots. Consistent with this finding, no hairy roots reportedly formed in the leaves or internodes of *Hollostemma ada-kodien* K. Schum (Karmarkar and Keshavachandran, 2001) and *Salvia officinalis* L. (Grzegorzczak *et al.*, 2006) transformed with *A. rhizogenes*. The observation by Karmarkar *et al.* (2001) that, contrary to leaves and internodes, seedling hypocotyls and shoot buds induced hairy roots might have been due to their ability to produce greater numbers of wound-adjacent cells competent for regeneration and transformation. To this may be added the fact that wounds induce the production of certain inhibitory compounds and that not all the *A. rhizogenes* strains are capable of degrading these inhibitory compounds (Karmarkar *et al.*, 2001). It has been accepted that leaves or plant aerial parts contain compounds that might affect the transformation process in a positive and/or negative way (Bivadi *et al.*, 2014).

Transformation efficiency has been shown to be greatly influenced by bacterial strain (Jain and Sing, 2015). In our investigation, all the three strains of *A. rhizogenes* were able to induce hairy roots in the nodes of intact *P. abrotanoides* seedlings albeit they differed significantly in the percentage of hairy roots they induced. ATCC15834 was the most efficient *Agrobacterium* strain in inducing hairy roots. Similar results have indicated the higher susceptibility of certain explants from various plant species to the strain ATCC15834. Some of these include *Helicteres isora* (Kumar *et al.*, 2014), *Hypericum perforatum* (Bivadi *et al.*, 2014), *Plumbago indica* (Gangopadhyay *et al.*, 2010), *Solenostemon scutellarioides* (Saleh and Thuc, 2009), *Ipomoea batatas* (Chandran and Potty, 2008) and *S. officinalis* (Grzegorzczak *et al.*, 2006). Virulence of *A. rhizogenes* varies depending on its strain, which thereby influences its transformation efficiency (Brijwal and Tamta, 2015). Moreover, the hairy roots obtained by different strains of *A. rhizogenes* vary with respect to their morphology, growth rate, and potential for the production of secondary metabolites, which may be attributed to the different plasmids the *Agrobacterium* harbor (Giri and Narasu, 2000; Kim *et al.*, 2002; Rhodes *et al.*, 1989). It seems that agropine strains such as ATCC15834, R1000, TR105, LBA9402, and A4, whose

plasmids contain both TR and TL regions, are more effective than mannopine strains in transforming a wide range of plant species (Otani *et al.*, 1993). The TR fragment of T-DNA delivers genes for auxin synthesis that triggers cellular division; hence, the ability of these strains to transform a wide range of species.

Wounding is a precondition for the genetic transformation process through *Agrobacteria*. It may not only help in the production of signal phenolic molecules such as acetosyringone but also enhance the availability of assumed cell-wall binding factors to the bacterium (Gelvin, 2000). Acetosyringone, as a phenolic inducer molecule, has been successfully applied to elevate transformation in *Agrobacterium*-mediated genetic transformation of plants due to the activation of bacterial virulence genes which is necessary for the delivery of T-DNA to plant tissues (Brijwal and Tamta, 2015; Kumar *et al.*, 2006). This was confirmed by our results as acetosyringone treatment was found to lead to a significant enhancement in transformation frequency (nearly 1.3 fold) for all the bacterial strains tested. Similar observations have been documented in *Solanum melongena* (Jain and Singh, 2015), *Cucumis melo* (Mohiuddin *et al.*, 2012), *S. scutellarioides* (Saleh and Thuc, 2009), *S. officinalis* (Grzegorzczak *et al.*, 2006), wheat (Rashid *et al.*, 2011), rice (Tripathi *et al.*, 2010), and cotton (Afolabi-Balogun *et al.*, 2014).

Carbohydrates, both as sources of energy and carbon and osmotic regulators, play a crucial role in *in vitro* cultures. In addition, sugars can act as signaling molecules and/or regulators of gene expression regulating plant growth and development (Rolland *et al.*, 2006; Smeekense *et al.*, 2010). Recent work has shown that exogenous sucrose can compensate for the regulators needed for root meristem maintenance (Wahl *et al.*, 2010). Expression of a homeobox gene, which maintains localized auxin maxima in the root apical meristem, is induced by a non-metabolizing sucrose analogue (Gonzali *et al.*, 2005).

Many studies have shown that different sugars significantly affect hairy root growth (Jain and Singh, 2015; Petrova *et al.*, 2015). The results of the present study showed that carbon source had significant effects on root dry and fresh weights, root length, and lateral root density. In general, sucrose was identified as the best carbon source for *P. abrotanoides* hairy root growth while sorbitol was the weakest. Maximum growth of hairy roots was achieved in a 1/2 MS medium containing 3% sucrose. In agreement with our findings, Sivanesan and Jeong (2009) found that fructose or glucose (3%, w/v) used instead of sucrose in the culture medium led to a significantly slower growth rate of *Plumbago zeylanica* L. hairy roots.

Sucrose is the most widely used carbon source in plant tissue cultures. This is due to its efficient absorption through the cell membrane (Petrova *et al.*, 2015). This is also confirmed by the findings of Sivakumar *et al.* (2005) who reported that sucrose

effectively enhanced the growth of ginseng hairy roots. The transgenic hairy roots of *Arnica montana* (Petrova *et al.*, 2015), *S. melongena* (Jain and Singh, 2015), *Solanum trilobatum* (Shilpha *et al.*, 2015), *Momordica charantia* L. (Thiruvengadam *et al.*, 2014), *Withania somnifera* (L.), and Dunal (Praveen and Morthy, 2012) have also achieved their best growth and highest biomass in an MS medium containing 3% sucrose.

Hairy root cultures aimed at producing secondary metabolites have been employed with different plant species including *Ophiorrhiza pumila* (Kitajima *et al.*, 2002; Asano *et al.*, 2013), *Panax ginseng* (Yu *et al.*, 2005), *Ruta graveolens* (Kuzovkina *et al.*, 2004), *Scutellaria lateriflora* (Wilczanska-Barska *et al.*, 2012), and *Lithospermum canescens* (Sykłowska-Baranek *et al.*, 2012). Successful production of tanshinones in hairy root cultures of *Salvia miltiorrhiza* Bunge (Hu and Alfermann, 1993; Wang and Wu, 2010) and enhanced production of these secondary metabolites by elicitation have also been reported (Ge and Wu, 2005; Yan *et al.*, 2005; Wu and Shi, 2008; Wang and Wu, 2010; Gupta *et al.*, 2011).

Hu and Alfermann (1993) first reported on hairy root culture of *S. miltiorrhiza* induced by infecting explants from sterile plants with the ATCC15834 and TR105 strains of *A. rhizogenes*. The authors cultured the hairy roots in a liquid MS medium without ammonium nitrate and detected seven types of tanshinones (containing tanshinone IIA and cryptotanshinone) in the hairy roots thus produced. They also noted a 22-fold increase in the dry weight of the hairy roots in 20 days and a total root tanshinone content of 43 mg g<sup>-1</sup> DW (20 mg g<sup>-1</sup> DW as cryptotanshinone). In a later study by Chen *et al.* (2001), hairy roots of *S. miltiorrhiza* were induced from plantlets using *A. rhizogenes* ATCC15834 with only trace amounts of cryptotanshinone (0.001% DW) and other tanshinones (tanshinone IIA, tanshinone IIB, and tanshinone I) produced in the MS medium lacking ammonium nitrate. Upon elicitation with yeast elicitor, not only was the intracellular content of cryptotanshinone observed to increase from 0.001% to as much as 0.096% of the dry weight but the growth of hairy roots also improved. Our HPLC analysis results indicated measurable presence of cryptotanshinone and tanshinone IIA in the hairy root clone T7 of *P. abrotanoides* after less than two months of culturing in a liquid medium. The amount of cryptotanshinone (53.17±0.26 µg g<sup>-1</sup> DW or 0.005% DW) was 5 times higher than that recorded by Chen *et al.* (2001) for *S. miltiorrhiza*. However, the plant species, bacterial strains, and culture conditions in our experiment with no elicitors used were not exactly the same as those in Chen *et al.*'s. Hairy root culture of *P. abrotanoides* coupled with techniques for increasing secondary metabolite production seems to provide evidence for the useful application of biotechnology to enhance tanshinones production.

### Conclusion:

Transgenic hairy roots of *Perovskia abrotanoides* were

successfully induced and established using three different strains of *A. rhizogenes* (ATCC15834, R1000, and TR105). Whole *in vitro* seedling nodes were found to be explants most favorable to hairy root induction. Also, the half-strength MS medium containing 3% sucrose was identified appropriate for the growth and biomass accumulation of hairy roots. The hairy roots thus produced were found capable of growing in the nutrient medium in the absence of growth regulators. Moreover, the hairy roots of *P. abrotanoides* were found to possess the potential for producing such medicinally important components as tanshinones. The findings of the present study can be used for further

investigation of the metabolite profiles and production of pharmaceutically valuable compounds such as tanshinones in the hairy roots of the species.

#### Conflict of interest:

The authors declare that they have no conflict of interests.

#### Acknowledgments:

This research was supported financially through Grant No. 30118 by Ferdowsi University of Mashhad, Mashhad, Iran.

#### Reference:

- Afolabi-Balogun, N. B., Inuwa, H. M., Ishiyaku, M. F., Bakare-Odunola, M. T., Nok, A. J. and Adebola, P. A. (2014) Effect of acetodyringone on *Agrobacterium*-mediated transformation of cotton. *ARNP Journal of Agricultural and Biological Science* 9: 284-286.
- Asano, T., Kobayashi, K., Kashihara, E., Sudo, H., Sasaki, R., Iijima, Y., Aoki, K., Shibata, D., Saito, K. and Yamazaki, M. (2013) Suppression of camptothecin biosynthetic genes results in metabolic modification of secondary products in hairy roots of *Ophiorrhiza pumila*. *Phytochemistry* 91: 128-139.
- Ashraf, S. N., Zubair, M., Rizwan, K., Tareen, R. B., Rasool, N., Zia-Ul-Haq, M. and Ercisli, S. (2014) Compositional studies and biological activities of *Perovskia abrotanoides* Kar. oils. *Biological Research* 47: 12.
- Bivadi, V., Asghari-Zakari, R., Zare, N. and Yazdani, B. (2014) Effects of different tissue culture conditions in hairy roots induction in *Hypericum perforatum* L. *International Journal of Agriculture and Crop Sciences* 7: 646-653.
- Brijwal, L. and Tamta, S. (2015) *Agrobacterium rhizogenes* mediated hairy root induction in endangered *Berberis aristata* DC. *SpringerPlus* 4: 443.
- Cao, E. N., Liu, X. Q., Wang, J. J. and Xu, N. F. (1996) Effect of natural antioxidant tanshinone II-A on DNA damage by lipid peroxidation in liver cells. *Free Radical Biology and Medicine Journal* 20: 801-806.
- Chandran, P. P. and Potty, V. P. (2008) Induction of hairy roots through the mediation of four strains of *Agrobacterium rhizogenes* on five host plants. *Indian journal of Biotechnology* 7: 122-128.
- Chiu, T. L. and Su, C. C. (2010) Tanshinone IIA induces apoptosis in human lung cancer A549 cells through the induction of reactive oxygen species and decreasing the mitochondrial membrane potential. *International Journal of Molecular Medicine* 25: 231-236.
- Gangopadhyay, M., Chakraborty, D., Bhattacharyya, S. and Bhattacharyya, S. (2010) Regeneration of transformed plants from hairy roots of *Plumbago indica*. *Plant Cell, Tissue and Organ Culture* 102: 109-114.
- Ge, X. and Wu, J. (2005) Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag<sup>+</sup> and yeast elicitor. *Plant Science* 168: 487-491.
- Gelvin, S. B. (2000) *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology* 51: 223-256.
- Giri, A. and Narasu, L. M. (2000) Transgenic hairy roots: recent trends and application. *Biotechnology Advances* 18: 1-22.
- Gonzali, S., Novi, G., Loreti, E., Paolicchi, F., Poggi, A., Alpi, A. and Perata, P. (2005) A turanose-insensitive mutant suggests a role for WOX5 in auxin homeostasis in *Arabidopsis thaliana*. *The Plant Journal* 44: 633-45.
- Grzegorzcyk, I., Krollicka, A. and Wysokinska, H. (2006) Establishment of *Salvia officinalis* L. hairy root cultures for the production of rosmarinic acid. *Zeitschrift fur Naturforschung C* 61: 351-356.
- Gupta, S. K., Liu, R. B., Liaw, S. Y., Chan, H. S. and Tsay, H. S. (2011) Enhanced tanshinone production in hairy roots of *Salvia miltiorrhiza* Bunge under the influence of plant growth regulators in liquid culture. *Botanical Studies* 52: 435-443.
- Hosseinzadeh, H. and Amel, S. (2001) Antinociceptive effects of the aerial parts of *Perovskia abrotanoides* extracts in mice. *Iranian Red Crescent Medical Journal* 4: 15-17.
- Hu, Z. B. and Alfermann, A. W. (1993) Diterpene production in hairy root cultures of *Salvia miltiorrhiza*. *Phytochemistry* 32: 699-703.
- Jang, S. I., Jeong, S. I., Kim, K. J., Kim, H. J., Yu, H. H., Park, R. and You, Y. O. (2003) Tanshinone IIA from *Salvia miltiorrhiza* inhibits expression of inducible nitric oxide synthase and production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in activated RAW 264.7 cells. *Planta Medica* 69: 1057-1059.
- Jain, A. and Singh, S. (2015) Effect of growth regulators and elicitors for the enhanced production of solasodine in hairy root cultures of *Solanum melongena* (L.). *Journal of the Indian Botanical Society* 94: 23-39.



- Karmarkar, S. H. and Keshavachandran, R. (2001) Genetic transformation and hairy root induction in *Holostemma adakodien* K. Schum (a vulnerable medicinal plant). *Indian Journal of Experimental Biology* 39: 1263-1267.
- Karmarkar, S. H., Keshavachandran, R., Nazeem, P. A. and Girija, D. (2001) Hairy root induction in adapathiyian (*Holostemma adakodien* K. Schum.). *Journal of Tropical Agriculture* 39: 102-107.
- Kim, E. J., Jung, S. N., Son, K. H., Kim, S. R., Ha, T. Y., Park, M. G., Jo, I. G., Park, J. G., Choe, W., Kim, S. S. and Ha, J. (2007) Antidiabetes and antiobesity effect of cryptotanshinone via activation of AMP-activated protein kinase. *Molecular Pharmacology* 72: 62-72.
- Kim, Y., Wyslouzil, B. E. and Weathers, P. J. (2002) Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cellular and Developmental Biology-Plant* 38: 1-10.
- Kochan, E., Szyman'ska, G. and Szymczyk, P. (2014) Effect of sugar concentration on ginsenoside biosynthesis in hairy root cultures of *Panax quinquefolium* cultivated in shake flasks and nutrient sprinkle bioreactor. *Acta Physiologiae Plantarum* 36: 613-619.
- Kong, G. M., Goh, N. K., Chia, L. S. and Chia, T. F. (2003) Recent advances in traditional plant drugs and orchids. *Acta Pharmacologica Sinica* 24: 7-21.
- Kitajima, M., Yoshida, S., Yamagata, K., Nakamura, M., Takayama, H., Saito, K., Sekib, H. and Aimi, N. (2002) Camptothecin-related alkaloids from hairy roots of *Ophiorrhiza pumila*. *Tetrahedron* 58: 9169-9178.
- Kumar, V., Sharma, A., Prasad, B. C. N., Gururaj, H. B. and Ravishankar, G. A. (2006) *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone treatment. *Electronic Journal of Biotechnology* 9: 349-357.
- Kumar, V., Desai, D. and Shriram, V. (2014) Hairy Root induction in *Helicteres isora* L. and production of diosgenin in hairy roots. *Natural Products and Bioprospecting* 4: 107-112.
- Kuzovkina, I., Al'terman, I. and Schneider, B. (2004) Specific accumulation and revised structures of acridone alkaloid glucosides in the tips of transformed roots of *Ruta graveolens*. *Phytochemistry* 65: 1095-1100.
- Lee, D. S., Lee, S. H., Noh, J. G. and Hong, S. D. (1999) Antibacterial activities of cryptotanshinone and dihydrotanshinone I from a medicinal herb, *Salvia miltiorrhiza* Bunge. *Bioscience, Biotechnology and Biochemistry* 63: 2236-2239.
- Liu, J. J., Zhang, Y., Lin, D. J. and Xiao, R. Z. (2009) Tanshinone IIA inhibits leukemia THP-1 cell growth by induction of apoptosis. *Oncology Reports Journal* 21: 1075-1081.
- Mahboubi, M. and Kazempour, N. (2009) The antimicrobial activity of essential oil from *Perovskia abrotanoides* Karel and its Main components. *Indian Journal of Pharmaceutical Sciences* 71: 343-347.
- Mohiuddin, A. K. M., Abdullah, Z. C., Chowdhury, K., Harikishna, K. and Napis, S. (2011) Enhanced virulence gene activity of *Agrobacterium* in muskmelon (*Cucumis melo* L.) cv. 'Birdie'. *Notulae Scientia Biologicae* 3: 71-79.
- Moallem, S. A. and Niapour, M. (2008) Study of embryo toxicity of *Perovskia abrotanoides*, an adulterant in folk-medicine, during organogenesis in mice. *Journal of Ethnopharmacology* 117: 108-114.
- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-479.
- Nassiri-Asl, M., Parvardeh, S., Niapour, M. and Hosseinzadeh, H. (2002) Antinociceptive and anti-inflammatory effects of *Perovskia abrotanoides* aerial part extract in mice and rats *Journal of Medicinal Plants Research* 3: 25-33.
- Ono, N. N. and Tian, L. (2010) The multiplicity of hairy root cultures: Prolific possibilities. *Journal of Plant Sciences* 180: 439-446.
- Otani, M., Mii, M., Handa, T., Kamada, H. and Shimada, T. (1993) Transformation of sweet potato (*Ipomoea batatas* (L.) Lam.) plants by *Agrobacterium rhizogenes*. *Plant Science* 94: 151-159.
- Pan, T. L., Hung, Y. C., Wang, P. W., Chen, S. T., Hsu, T. K., Sintupisut, N., Cheng, C. S. and Lyu, P. C. (2010) Functional proteomic and structural insights into molecular targets related to the growth inhibitory effect of tanshinone IIA on HeLa cells. *Proteomics* 10: 914-929.
- Petrova, M., Zayova, E., Dincheva, I., Badjakov, I. and Vlahova, M. (2015) Influence of carbon sources on growth and GC-MS based metabolite profiling of *Arnica montana* L. hairy roots. *Turkish Journal of Biology* 39: 469-478.
- Potrykus, I. (1991) Gene transfer to plants: assessment of published approaches and results. *Annual Review of Plant Physiology and Plant Molecular Biology* 42: 205-225.
- Praveen, N. and Murthy, H. N. (2012) Synthesis of withanolide A depends on carbon source and medium pH in hairy root culture of *Withania somnifera*. *Industrial Crops and Products* 35: 241-3.
- Rashid, R., Caudhry, Z. and HaroonKhan, M. (2011) Effect of explant plant source and acetosyringone concentration on transformation efficiency of wheat cultivars. *African Journal of Biotechnology* 10: 8737-8740.
- Rechinger, K. H. (1982) *Flora Iranica*. Akademische Druck und Verlagsanstalt, Graz- Austria.
- Rhodes, M. J. C., Robins, R. J., Lindsay, E., Arid, M., Payne, J., Pare, A.J. and Walton, N.J. (1989) Primary and Secondary Metabolism of Plant Cell Cultures. Pp. 58-73. Springer-Verlag, New York.
- Rolland, F., Baena-Gonzalez, E. and Sheen, J. (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review of Plant Physiology* 57: 675-709.

- Safaei-Ghomi, J. and Batooli, H. (2010) Determination of bioactive molecules from flowers, leaves, stems and roots of *Perovskia abrotanoides* Karel growing in central Iran by nano scale injection. *Digest Journal of Nanomaterials and Biostructures* 5: 551-556.
- Sairafianpour, M., Christensen, J., Staerk, D., Budnik, B. A., Kharazmi, A., Bagherzadeh, K. and Jaroszewski, G. W. (2001) Leishmanicidal, antiplasmodial, and cytotoxic activity of novel diterpenoid 1,2-quinones from *Perovskia abrotanoides*: new source of tanshinones. *Journal of Natural Product and Plant Resources* 64: 1398-1403.
- Saleh, N. M. and Thuc, L. V. (2009) Assessment of hairy roots induction in *Solenostemon scutellarioides* leaves by different strains of *Agrobacterium rhizogenes*. *African Journal of Biotechnology* 8: 3519-3523.
- Sharp, P. J., Kreis, M., Shewry, P. R. and Gale, M. D. (1988) Location of  $\beta$ -amylase sequences in wheat and its relatives. *Theoretical and Applied Genetics* 75: 286-290.
- Shilpha, J., Satish, L., Kavikkuil, M., Joe Virgin Largia, M. and Ramesh, M. (2015) Methyl jasmonate elicits the solasodine production and anti-oxidant activity in hairy root cultures of *Solanum trilobatum* L. *Industrial Crops and Products* 71: 54-64.
- Sivakumar, G., Yu, K. W., Hahn, V. and Paek, K. Y. (2005) Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors. *Current Science* 89: 641-649.
- Sivanesan, I. and Jeong, B. R. (2009) Induction and establishment of adventitious and hairy root cultures of *Plumbago zeylanica* L. *African Journal of Biotechnology* 8: 5294-5300.
- Smeekens, S., Ma, J., Hanson, J. and Rolland, F. (2010) Sugar signals and molecular networks controlling plant growth. *Current Opinion in Plant Biology* 13: 274-279.
- Syklowska-Baranek, K., Pietrosiuk, A., Gawron, A., Kawiak, A., Łojkowska, E., Jeziorek, M. and Chinou, I. (2012) Enhanced production of antitumor naphthoquinones in transgenic hairy root lines of *Lithospermum canescens*. *Plant Cell, Tissue and Organ Culture* 108: 213-219.
- Thiruvengadam, M., Praveen, N., John, K. M., Yang, Y. S., Kim, S. H. and Chung, I. M. (2014) Establishment of *Momordica charantia* hairy root cultures for the production of phenolic compounds and determination of their biological activities. *Plant Cell, Tissue and Organ Culture* 118: 545-557.
- Tripathi, R. M., Bisht, H. S. and Singh, R. P. (2010) Effect of acetosyringone and callus age on transformation for scutellum-derived callus of rice. *International Journal of Pharma and Bio Sciences* 1: 163-171.
- Vincent, J. M. (1970) *A Manual for the Practical Study of Root-nodule Bacteria*. Blackwell Scientific Publications, London.
- Wahl, V., Brand, L. H., Guo, Y. L. and Schmid, M. (2010) The Fantastic Four proteins influence shoot meristem size in *Arabidopsis thaliana*. *BMC Plant Biology* 10: 285.
- Wan, X., Tian, M. and Row, K. H. (2009) Extraction and HPLC analysis of tanshinone I, tanshinone IIA and cryptotanshinone from *Salvia miltiorrhiza* Bunge. *Asian Journal of Chemistry* 21: 5999-6004.
- Wang, H., Qi, M. and Cutler, A. J. (1993) A simple method of preparing plant samples for PCR. *Nucleic Acids Research* 21: 4153-4154.
- Wang, W. J. and Wu, J. Y. (2010) Tanshinone biosynthesis in *Salvia miltiorrhiza* and production in plant tissue cultures. *Applied Microbiology and Biotechnology* 88: 437-449.
- Wawrosch, C., Schwaiger, S., Stuppner, H. and Kopp, B. (2014) Lignan formation in hairy root cultures of Edelweiss (*Leontopodium nivale* ssp. alpinum (Cass.) Greuter). *Fitoterapia* 97: 219-223.
- Wilczanska-Barska, A., Krolicka, A., Glod, D., Majdan, M., Kawiak, A. and Krauze-Baranowska, M. (2012) Enhanced accumulation of secondary metabolites in hairy root cultures of *Scutellaria lateriflora* following elicitation. *Biotechnology Letters* 34: 1757-1763.
- Wu, J. Y. and Shi, M. (2008) Ultrahigh diterpenoid tanshinone production through repeated osmotic stress and elicitor stimulation in fed-batch culture of *Salvia miltiorrhiza* hairy roots. *Applied Microbiology and Biotechnology* 78: 441-448.
- Yao, S. C., Bai, L. H., Lan, Z. Z., Tang, M. Q., Zhai, Y. J., Huang, H. and Wei, R. C. (2016) Hairy root induction and polysaccharide production of medicinal plant *Callerya speciosa* Champ. *Plant Cell, Tissue and Organ Culture* 126: 177-186.
- Yan, Q., Hu, Z., Tan, R. X. and Wu, J. (2005) Efficient production and recovery of diterpenoid tanshinones in *Salvia miltiorrhiza* hairy root culture with *in situ* adsorption, elicitation and semi-continuous operation. *Journal of Biotechnology* 119: 416-424.
- Yaniv, Z. (2005) *Handbook of Medicinal Plant*. 7<sup>th</sup> Ed, Haworth Medical Press, London.
- Yu, K. W., Murthy, H. N., Hahn, E. J. and Paek, K. Y. (2005) Ginsenoside production by hairy root cultures of *Panax ginseng*: influence of temperature and light quality. *Biochemical Engineering Journal* 23: 53-56.
- Zaker, A., Sykora, C., Gossnitzer, F., Abrishamchi, P., Asili, J., Mousavi, S. H. and Wwarosch, C. (2015) Effects of some elicitors on tanshinone production in adventitious root cultures of *Perovskia abrotanoides* Karel. *Industrial Crops and Products* 67: 97-102.