RESEARCH ARTICLE

Elmira Mohammadi[1, 2]
Khadijeh Bagheri[1]
Mina Beigmohamadi[1, 3]
Ali Sharafi[1, 4]

In vitro direct organogenesis and genetically transformed root cultures in *Artemisia sieberi* Besser as a source of pharmaceutical compound artemisinin

Authors' addresses:
[1]Zanjan Pharmaceutical Biotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran.
[2]Department of Plant production and Genetics, Faculty of Agriculture, University of Zanjan.
[3]University of Tabriz, Tabriz, Iran.
[4]Department of Pharmaceutical Biotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran.

Correspondence:
Ali Sharafi[1, 4]
[1]Zanjan Pharmaceutical Biotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran.
[4]Department of Pharmaceutical Biotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran.
Tel.: (+98) 24 33473635
e-mail: alisharafi@zums.ac.ir

Article info:
Received: 18 May 2020
Accepted: 02 July 2020

ABSTRACT
Artemisia sieberi has important pharmaceutical compound artemisinin which is recommended as the most effective active compound to treat malaria. In the present study, for *in vitro* shoot, organogenesis and plant regeneration, leaves from one-month-old plants were used as explants in MS media containing a different combination of plant growth regulators including 2, 4-D, IAA, NAA, BA, and TDZ. The highest indirect regeneration frequency was obtained in MS medium supplemented by 2 mg/l BA, 0.05 mg/l NAA and also MS medium supplemented by 2 mg/l BA, 0.5 mg/l IAA. The highest direct regeneration was observed in MS medium containing 0.1 mg/l TDZ. Also, an efficient transgenic root induction system for *A. sieberi* was developed through evaluating five different factors including *Agrobacterium rhizogenes* strain, explant type, pre-culture period (0, 24 and 48 hours), co-cultivation period (24, 48 and 72 hours) and absence or presence of acetosyringone. The highest roots induction was obtained by *A. rhizogenes* strains MSU440 (83.33%) and ATCC15834 (73%) and the 4-week-old leaf explants followed by a co-cultivation period of 2 days on a ½ MS medium containing 100 μM acetosyringone. To molecular confirmation of transgenic roots, PCR with specific primers of *rolB* gene was performed. Approximately 0.048 % of artemisinin was detected by HPLC analysis in root cultures. Our data suggest that transgenic roots can consider as a new alternative approach to artemisinin production.

Key words: *Artemisia sieberi*, Artemisinin, Direct regeneration, Genetically transformed root.

Introduction

Artemisia genus has some important pharmaceutical bioactive sesquiterpene lactones, such as artemisinin and α-santonin (Ivanescu et al. 2015; Sakipova et al. 2017). *A. sieberi* is an aromatic plant which has been naturally distributed in the semi-desert regions of Iran. Artemisinin, a sesquiterpene lactone endoperoxide, is a well-known and effective antimalarial drug isolated from *Artemisia* plants (Arab et al. 2006; Shi et al. 2013). Nahrevanian et al. (2012) reported the use of *A. sieberi* extracts in the treatment of murine malaria in mice (Nahrevanian et al. 2012). It is revealed that *A. sieberi* extracts have inhibitory effects on the β-Hematin formation (Akkawi et al. 2014; Jaber et al. 2015). On the other hand, several investigations introduced artemisinin as a potent anticancer agent (Buommino et al. 2009; Willoughby et al. 2009; Ferreira et al. 2010). Artemisinin is an effective drug against the chloroquine-resistant and other multidrug-resistant of *Plasmodium* species. In recent years, the global demand for artemisinin is considerably increasing. There is trace quantities of artemisinin in *A. annua* plants as the main source of this valuable active compound (Tang et al. 2014). As the chemical synthesis of artemisinin is not commercially promising (Paddon et al. 2013) thus, it is needed to introduce an efficient biotechnological system to increase artemisinin production. Some studies, including cell and tissue culture, plant genetic engineering, synthetic biology, and breeding of high artemisinin yielding plants have been investigated to increase the artemisinin production (Tang, Shen et al. 2014). The yield of this compound is necessary to be significantly enhanced for commercial utilization. Recent studies have already completed in revealing the biosynthetic pathway of artemisinin. Numerous key genes which expressed the enzymes in the artemisinin biosynthesis pathway have been cloned and functionally clarified (Chen et al. 2000; Liu et al. 2011; Mirzaee et al. 2016).

In vitro plant cultures offer the possibility of obtaining desirable pharmaceutical compounds along with guaranteed sustainable conservation and rational use of biodiversity. Hairy root cultures might show significantly enhanced and stable production of secondary metabolites at high levels in

comparison to callus and cell suspension cultures and even to intact plants (Oksman-Caldentey & Inzé 2004). Hairy roots can also synthesis a range of secondary metabolites that are not present in the parent plant (Kim et al. 2002; Aberham et al. 2011). Also, it offers a great potential for metabolic engineering, elicitation, biotransformation, molecular farming, phytoremediation, and producing transgenic plants (Georgiev et al. 2007a). Several factors such as pre-culture, co-cultivation media, bacterial strain and type of explants can affect the rate of *A. rhizogenes* mediated transformation (Sharafi et al. 2014b; Beigmohamadi et al. 2019).

The artemisinin biosynthesis was described in *A. annua* shoot cultures and genetically modified root cultures (Ram et al. 2014). It is revealed that artemisinin was not detected in cell suspension cultures of *A. annua*, while trace quantity was produced in the multiple shoot cultures (Paniego & Giulietti 1994). Cell differentiation is important in the biosynthesis of secondary metabolites (Sharafi et al. 2013a). So, root culture can be used as an organ culture for the biosynthesis of this kind of secondary metabolites. In this study, we have introduced a rapid and efficient protocol for *in vitro* shoot organogenesis, regeneration, and *in vitro* artemisinin production in hairy root culture of medicinal plant *A. sieberi*.

Materials and methods

Plant materials and tissue culture conditions

Seeds of *A. sieberi* were provided from Forests, Range and Watershed Management Organization of Iran. The seeds were surface-sterilized with immersion in 70% (v/v) ethanol solution for 1.5 min and 5 % (v/v) sodium hypochlorite for 12 min and then rinsed six times with sterilized water. The seeds were germinated on agar solidified ½ MS medium (pH 5.8). The seeds were incubated in a growth chamber under light and dark period (16/8 h) at 23 ºC in 36 µmol s^{-1} m^{-2} light intensity. The leaves and stems from 4 weeks old seedlings were used as explants.

Tissue culture and shoot organogenesis media

The leaves from 4 weeks old plants were cut and cultured on solidified MS medium supplemented with different combination of BA, TDZ, NAA, IAA and 2, 4-D as follows: BA (0.5, 1 and 2 mg/l), TDZ (0.1, 0.5, 1 and 1.5 mg/l); NAA (0.05, 0.1, 0.5 and 1 mg/l), IAA (0.1 and 0.5 mg/l), 2, 4-D (0.5, 1 and 1.5 mg/l). The Petri dishes were incubated in a growth chamber. Explants were transferred to fresh medium every 2 weeks. After callus induction, explants were transferred to MS medium without hormones or supplemented with 0.5 or 1 mg/l BA for adventitious shoot induction. For root induction, obtained shoots were cut and transferred to MS medium supplemented with 0.1 mg/l IBA (Sharafi et al. 2014b). The plantlets were transferred to MS medium without hormone for more growth for at least 2 weeks. Finally, the obtained plantlets were acclimatized in the greenhouse.

Preparation of A. rhizogenes strains

Four strains of *A. rhizogenes* (ATCC 15834, ATCC 31798, MAFF-02-10266, and MSU440) were provided by Zanjan Pharmaceutical Biotechnology Research Center, Zanjan, Iran. From each strain, a sole bacterial colony was inoculated in liquid LB medium to an optical density of 0.7, at 28 °C, 140 rpm on a rotary shaker incubator. The bacterial suspensions were centrifuged at 4,000 rpm for 10 min. The pellets were re-suspended in 20 ml MS liquid medium supplemented with 100 µM acetocyringone after sterilization using 0.22 μm syringe filters at 28 ºC.

Induction *and establishment of transgenic root culture*

Leaf and stem explants were pre-cultured for one day on MS medium supplemented with 0.5 mg/l 6-benzyl-aminopurine (BA). The explants were immersed in a bacterial inoculation medium for 5 min and then blotted on sterile filter paper and incubated in a co-cultivation medium consisted of MS or ½ MS medium supplemented 50 mg/l sucrose and 100 µM acetocyringone. After 2 days of co-cultivation, the explants transferred to hormone-free MS media supplemented with 125 mg/l cefotaxime to eliminate the *A. rhizogenes*. Control explants were treated similarly, but without inoculation with *A. rhizogenes*. Under these conditions, explants were sub-cultured every week until *A. rhizogenes* colonies disappeared. After 2 to 3 weeks, hairy roots emerged on the explants. They were excised from the explants and transferred into a liquid MS medium containing antibiotics after 2 weeks. After six subcultures, hairy roots were transferred into an antibiotic-free liquid MS medium at the dark with shaking (120 rpm) at 25 ± 1°C. The effects of the *Agrobacterium* strain and explant type, pre-culture period (0, 24 and 48 hours), a co-cultivation condition during incubation, and co-cultivation on the induction rate of hairy root were evaluated.

Detection of rol B gene in transgenic roots by polymerase chain reaction (PCR)

Total DNA was isolated from the hairy roots (100 mg) of each clone and control roots using a genomic plant DNA extraction kit. The pellets of DNA were air-dried, dissolved in 100 µL of deionized water, and then stored at –20 °C until use. Isolated DNA was used in PCR analysis for detecting the *rol* B gene. PCR was performed by *rol B* gene-specific primer pairs: 5'-GCTCTTGCAGTGCTAGATTT-3' and 5'-GAAGGTGCAAGCTACCTCTC-3'.

The PCR was carried out under the following conditions: 94 ºC for 5 min, 30 cycles of three steps [94 ºC for 1 min (denaturation), 57 ºC for 1 min (annealing) and 72 ºC for 1 min (elongation)], and 72 ºC for 10 min for the final

extension. PCR products were separated by electrophoresis on 0.8 % agarose gel in 0.5 × TBE buffer, stained with ethidium bromide, and visualized under UV transilluminator.

Sample extraction and high performance liquid chromatography (HPLC)

Hairy roots and control samples were dried in the oven at 60 °C for 24 h. The dried leaves were refluxed by 30 ml ethanol at 70 °C for 10 h. The soup was filtrated and evaporated; the obtained sediment materials were dissolved in 5 ml HPLC grade ethanol (Merck Co.). For HPLC analysis Simth's method (Smith et al., 1997) was used in our experiment. The mobile phase consisted of acetonitrile and water 60:40 (% v/v) and was delivered at a flow rate of 1.0 ml/min using a double-reciprocating pump and the analysis wavelength was at 210 nm (Waters, MA, USA, model Breeze).4.6mm, particle size 5µm; Perfect sill, MZ-Analysen. The sample was injected through a 20 µL sample loop. A C8 analytical column (150mm Technik, Germany) equipped with a guard column of the same packing was used.

Statistical analysis

The experiments were laid on a completely randomized design (CRD) with six replications and nine explants cultured in each Petri dish. The data collected were subjected to analysis of variance test. The means were compared using Duncan's multiple range tests. The data expressed as percentages were subjected to arcsine transformation before the statistical analysis.

Results

In vitro tissue culture and organogenesis of A. sieberi

The effect of plant growth regulators (IAA, NAA, 2, 4-D, BA and TDZ) on shoot organogenesis were investigated. Callus appeared on most of the treatment containing different concentrations and combinations of IAA, NAA, 2, 4-D, BA after two sub-cultures (Figure. 1). Obtained calli were transferred to free plant growth regulators MS medium or supplemented with 0.5 mg/l BA for shoot induction. In these media, shoot induction appeared in three weeks. A high amount of shoot organogenesis was observed in MS medium containing 2 mg/l BA and 0.05 mg/l NAA (96.3 %) (Figure. 2). Also, the calli originated from the combination of 2 mg/l BA and 0.5 mg/l IAA showed a high frequency of shoot induction (88.3 %). A low rate of shoot organogenesis was observed in medium supplemented with 1 mg/l BA and 0.05 mg/ l NAA and medium supplemented with a combination of BA and 2, 4-D. While shoot organogenesis was induced from the leaf stalks in *A. sieberi* (Figure. 1), whole leaf blades produced callus and shoot organogenesis in *A. annua*. In contrast, excised leaf stalks of *A. sieberi* induced higher adventitious shoot organogenesis in comparison with a complete leaf blade. Interestingly, direct shoot induction was obtained in MS medium containing 2 mg/l BA, and 0.1 mg/l NAA, 1 mg/l BA and 0.1 mg/l IAA and MS media containing 2 mg/l BA (Figure 2).

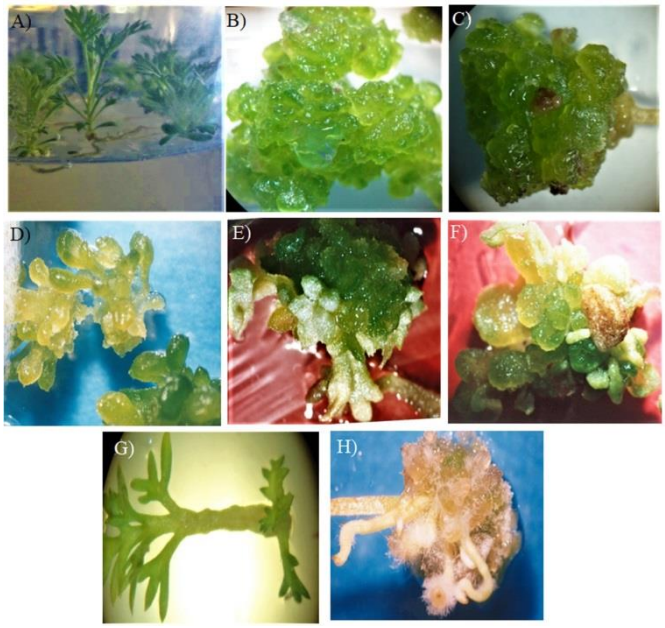

Figure 1. *Indirect regeneration A. Siberi. Callus induction in MS medium containing 2 mg/L BA and 0.5 mg/L IAA (B-C); shoot induction in medium containing 2 mg/L BA and 1 mg/l 2,4-D (D, E, F, G); root induction in 1.5 mg/l 2,4-D (I).*

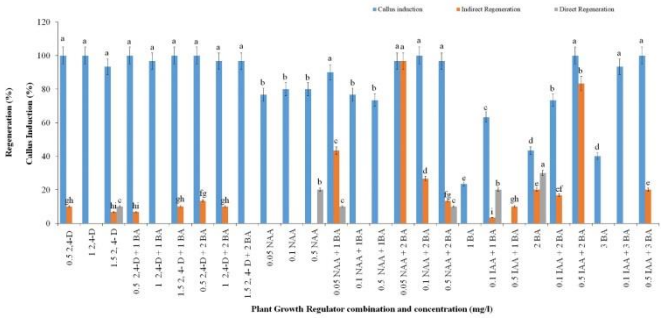

Figure 2. *Effect of different Plant growth regulator combination (BA + NAA, BA + IAA AND BA + 2,4-D) on callus induction and regeneration of A. sieberi.*

In treatments containing TDZ, all of the regeneration was a direct form (Figures 3A-F). It was observed that explants in TDZ treatments produce more branches. MS medium containing 0.5 mg/l GA3 was used for shoot elongation of obtained induces shoots. The elongated shoots were transferred to the root induction medium for two weeks (Figure 3G). MS medium considered for more growth of plantlets. Finally, the plantlets were acclimatized in the greenhouse (Figure 3H).

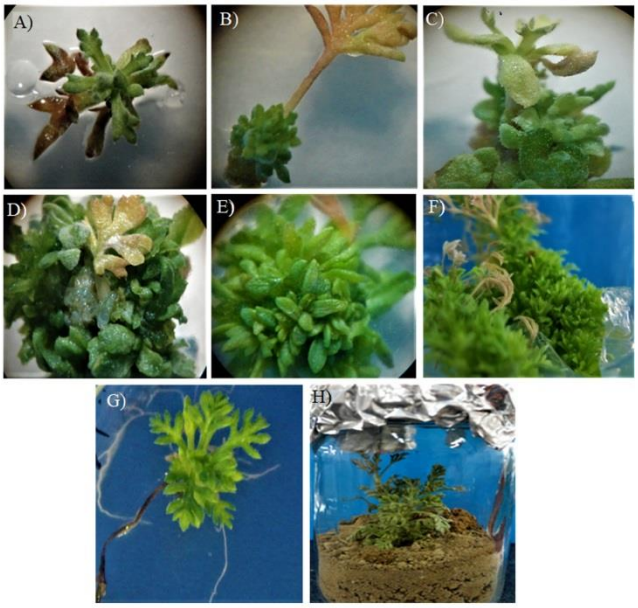

Figure 3. *Direct regeneration of A. siberi. MS medium containing 2 mg/l BA 0.1mg/l NAA (10%) (A) MS medium containing 1 mg/l BA, 0.1mg/l IAA (20%) (B) MS medium containing 2 mg/l BA, mg/l IAA and 2 mg/l BA (C) MS medium containing 0.1 mg/L TDZ (D-F) Root development following subculture on MS medium containing 0.5 mg/l IBA (G) transferring regenerated plant to soil (H).*

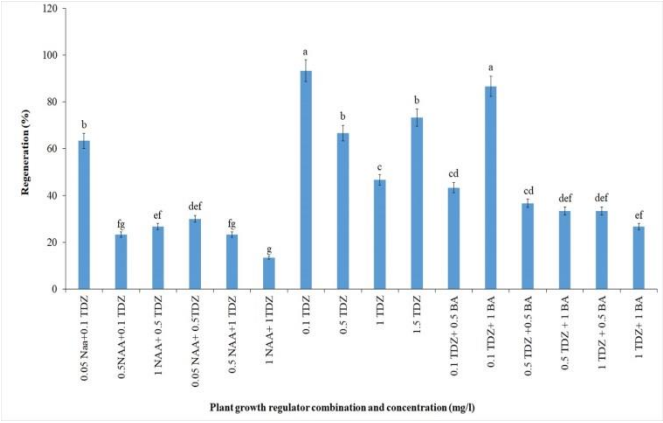

Figure 4. *Effect of treatment containing TDZ on callus induction and regeneration of A. sieberi.*

Effect of explants and the virulence of Agrobacterium strains on hairy root induction

Hairy roots of A. sieberi were initiated from stem and leaf explants after two to three weeks (Figures 5 A-C). Obtained hairy roots were excised and cultivated on agar solidified MS medium for further growth. They were moved to ½ MS liquid medium for more growth after one month (Figures 5D-I). Hairy root lines showed a lack of geotropism (plagiogeotropism) in liquid medium as expected. All of the A. rhizogenes strains directed to the induction of hairy root at the wound site of explants and in some cases produced the tumorigenic calli. Tumor formation occurred in all strains with different rates.

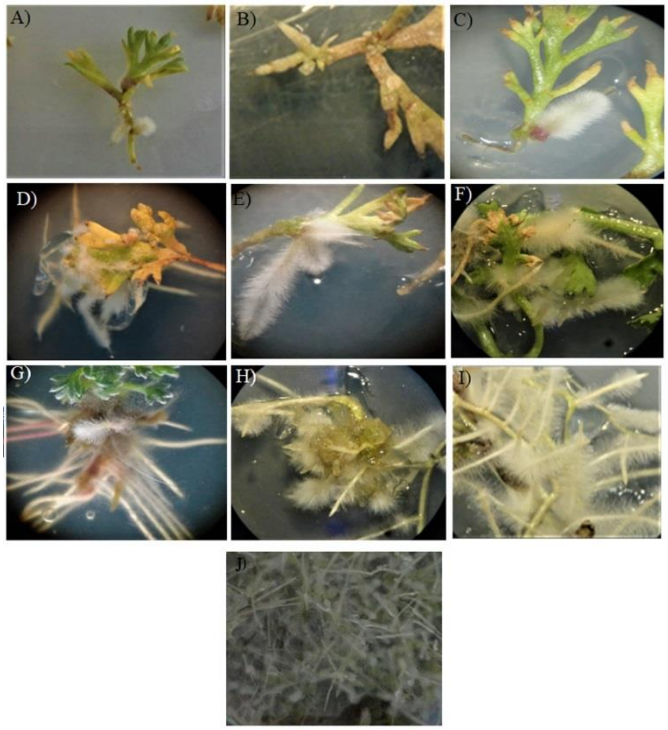

Figure 5. *A. rhizogenes mediated transformation of A. sieberi hairy root induction after 4 weeks of inoculation hairy root culture after 6 weeks of inoculation using A4 (A, B) ; Hairy root culture after 6 weeks of inoculation using A. rhizogenes A13 (C,D); hairy root culture after 6 weeks of inoculation using A. rhisogenes ATCC15834 (E,F)) hairy root culture after 6 weeks of inoculation using A. rhisogenes MSU440 (G-J).*

The results showed that the best explant for the transformation of *A. sieberi* by *A. rhizogenes* is leaf explant which is significantly susceptible to infection by all strains of *A. rhizogenes* used in this study. The maximum frequency of hairy root induction occurred by *A. rhizogenes* strains ATCC15834 and MSU440 on the leaf explants with a transformation frequency of 85% and 73%, respectively (Figure 6); while *A. rhizogenes* strain MAFF-02-10266 was infected only 30% of explants (Figure 6). Excised shoot explants showed a lower frequency of transformation in comparison to leave explants.

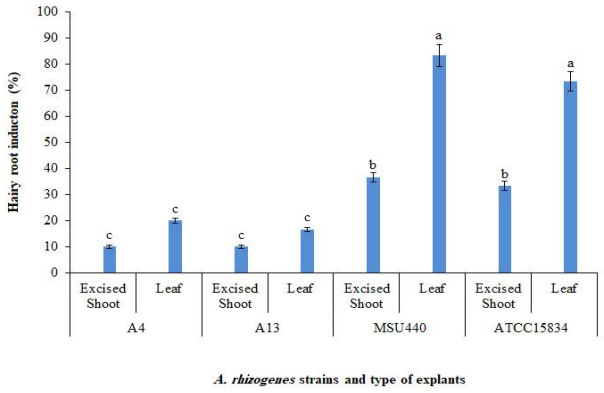

Figure 6. *Effect of bacterial strains and Explant on frequency hairy root induction of A. sieberi. Bacterial strains (A4, A13, MSU440, and ATCC15834), Explant (Leaf, Excited Shoot).*

Effect of preculture period on hairy root induction

Results indicated that the highest percentage of hairy root induction was observed in MSC440 and (85% %) and in ATCC15834 (70%) with a pre-culture period (Figure 7). Overall, it seems that pre-culture can enhance transformation frequency in *A. sieberi*.

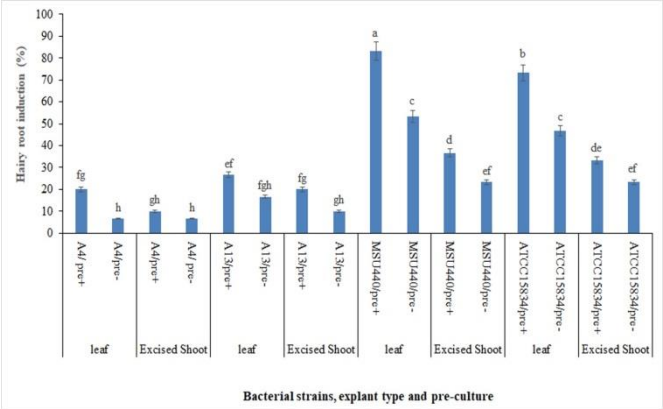

Figure 7. *Effect of bacterial strains, explant and pre culture media on frequency hairy root induction of A. sieberi. Bacterial strains (A4, A13, MSU440, ATCC15834), Explant (Leaf, Excited Shoot), Pre culture Media (Pre$^+$, Pre$^-$).*

Effect of co-culture condition on transformation frequency

We used acetosyringone to stimulate *A. rhizogenes* T-DNA transduction in all the experiments. Our results showed that acetosyringone in co-cultivation and inoculation medium improved the transformation rate (Figure 8). Full MS and ½ MS were evaluated as co-cultivation media to determine the optimal co-cultivation medium for *A. rhizogenes* mediated transformation of *A. sieberi*. In the full strength MS medium as a co-cultivation medium, the rate of transformation was 52% and in the ½ MS co- cultivation medium was 83.5% in *A. rhizogenes* MSU440 (Figure 9).

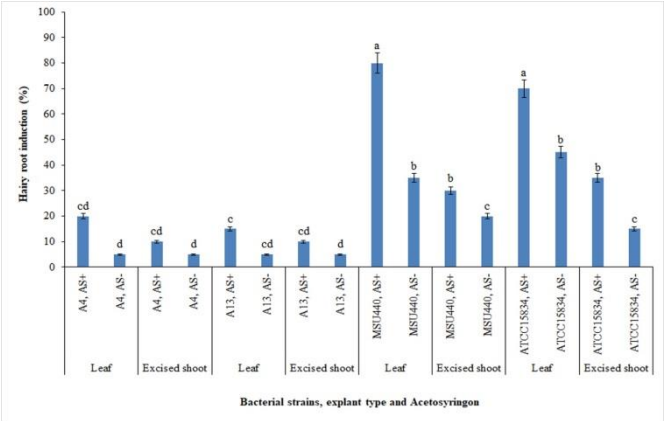

Figure 8. *Effect of Bacterial strains, Explant and Acetosyringone on frequency hairy root induction of A. sieberi. Bacterial strains (A4, A13, MSU440, and ATCC15834), Explant (Leaf, Excited Shoot), Acetosyringone (AS$^+$: presence of acetosyringone, AS$^-$: absence of acetosyringone).*

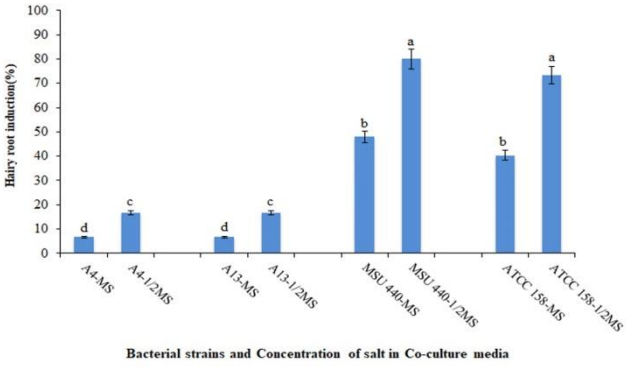

Figure 9. *Effect of bacterial strains and concentration of Salt in Co-culture Media on frequency hairy root induction of A. sieberi. Bacterial strains (A4, A13, MSU440, ATCC15834), concentration of Salt in Co-culture Media (MS, ½ MS).*

Effect of cefotaxim concentration on hairy root induction

After the co-cultivation step, explants inoculated with *Agrobacterium* strains were separately cultivated with four concentrations of cefotaxime (125, 250, 500 mg/l). As shown in Figure 10, the effect of concentration of cefotaxime on the hairy root induction was remarkable. According to the results, the highest percentage of hairy root induction was observed in transgenic explants with MSU440 strain and antibiotic concentration of 125 mg/l. Also, a higher concentration of cefotaxime caused necrosis and the disappearance of explants. Cefotaxime has been shown to be more potent against this bacterium (Han et al. 2005).

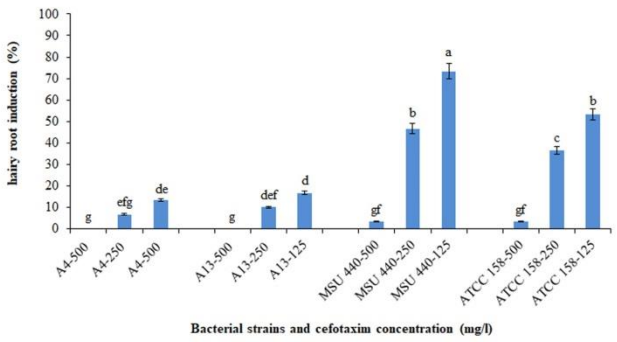

Figure 10. *Effect of bacterial strains and concentration of Cefotaxime on frequency hairy root induction of A. sieberi. Bacterial strains (A4, A13, MSU440, ATCC15834), concentration of Cefotaxime (500, 250, 125 mg/l).*

Molecular analysis

Molecular analysis of transformed roots and controls was performed with the help of PCR for *rol B* gene and the expected fragment was observed in hairy roots to confirm transformation similar to that found in the positive control. There was no amplification in the DNA isolated from normal roots (Figure 11). Five transgenic hairy root lines presented in Figure 11. PCR analysis using specific primers for *vir*G was done to confirm that hairy roots were not contaminated by *A. rhizogenes*, and amplification of *vir*G was not observed (data not shown). These results indicated transferring of T-DNA from Ri plasmids to hairy roots lines.

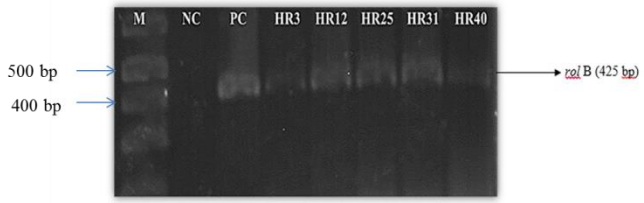

Figure 11. *PCR analysis for detection of the rolB gene in 5 hairy root lines of A. sieberi; M: Molecular size marker (100 bp); HR: hairy root lines, NC: negative control (non-transformed root); PC: positive control (Ri plasmid).*

HPLC analysis

HPLC analysis was performed to confirm the production and quantification of artemisinin in hairy roots; artemisinin in hairy roots was calculated 0.048 mg dry matter (Figure 12).

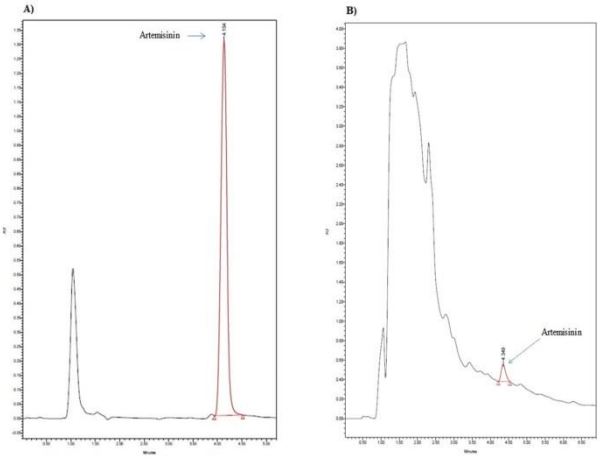

Figure 12. *HPLC analysis; Standard (A) Hairy root sample (B).*

Discussion

Artemisia sieberi has important antimalarial compound artemisinin. In this study, for *in vitro* shoot organogenesis and plant regeneration, leaves from one-month-old sterile plants as explants were used in plant media containing different combination hormones, including 2, 4-D, IAA, NAA, BA, and TDZ. Interestingly, direct shoot induction was obtained in MS medium containing 2 mg/l BA and 0.1 mg/l NAA, 1 mg/l BA and 0.1 mg/l IAA and MS media containing 2 mg/l BA (Figure 2). Sharafi et al. (2014) reported the shoot organogenesis from *A. sieberi* explants on MS medium supplemented with BA plus NAA from induced callus, but they did not report direct organogenesis (Sharafi et al. 2014a). Another study on medicinal plant *A. judaica* L. reported shoot organogenesis by adding of BA growth regulator (Liu et al. 2003). Media containing 0.1 mg/l TDZ showed the highest frequency of direct organogenesis with 93.3 (Figure 4). Among plant growth regulators, cytokinins have proven to be the most important hormone affecting shoot regeneration (Magyar-Tábori et al. 2010). The present study showed that low concentrations of TDZ can be effective for direct shoot organogenesis in *A. sieberi*. This result is in accordance with Faisal and Anis (2006); efficient *in vitro* plant regeneration occurred in low concentrations of TDZ (Faisal & Anis 2006). TDZ is the most active cytokinin-like substances and it induces greater *in vitro* shoot proliferation than many other cytokinins in many plant species (Khawar et al. 2005; Hosseini et al. 2016). TDZ appears less susceptible to enzymatic degradation *in vivo* than other naturally occurring amino purine cytokinins, and therefore at low concentrations, it can be more efficient than other cytokinins. Numerous studies had previously reported that an optimal concentration of cytokinins, alone or in combination with auxin, is necessary for *in vitro* shoot organogenesis of plants (Magyar-Tábori, Dobránszki et al. 2010; Jana et al. 2013). This approach could be effective and

simple *in vitro* tissue culture method for obtaining a high frequency of *A. sieberi* regeneration in a short period, especially for direct shoot organogenesis. *In vitro* direct shoot organogenesis considered as an effective procedure for producing of genetically alike plants without somaclonal variation which occurred after callus induction (Sharafi et al. 2014c).

To increase biomass and the secondary production in hairy root culture of the medicinal plants, studies of *A. rhizogenes* transformation were interested (Nguyen et al. ; Manuhara et al. 2015; Chen et al. 2018). The highest roots induction was obtained by MSU440 (83.33%) and ATCC15834 (73%) onto the 4-week-old leaf explants followed by a co-cultivation period of 2 days on a ½ MS medium containing 100 µM acetosyringone. Tumorigenic calli (galls) appeared in some explants. Scientists believe that formation of these galls may be due to T_R-DNA transferring *iaa*M and *iaa*H genes which are responsible for the auxins biosynthesis (Georgiev et al. 2007b). Also, this phenotype may be because of the interaction between explant and bacterial strain in response to the *rol* genes products coupled with the special properties of endogenous hormone(Sudha et al. 2013). For large scale pharmaceutical secondary metabolite productions, tumorigenic calli are not suitable; so, the results indicated that strain MSU440 inducing low rate tumorigenic calli is more capable than the other strains used in this study. The difference in virulence power of bacteria, morphology and growth rate of hairy root lines can be moderately clarified by the plasmids variety which harbored by the different strain of *A. rhizogenesis* (Georgiev, Pavlov et al. 2007b).

Overall, previous reports indicated that some factors such as *A. rhizogenes* strain, type of explant, pre-culturing and co-culturing condition can significantly effect on the frequency of transformation (Danphitsanuparn et al. 2012; Sudha, Sherina et al. 2013; Thiruvengadam et al. 2016). In this study, it seems that pre-culturing enhance hairy root induction in *A. sieberi*. In contrast with our result, in *Citrullus colocynthis*, the pre-culture period has a negative influence on hairy root induction (Beigmohammadi et al. 2019). We can conclude that it depends on plant species. As previous studies described that adding phenolic compound acetosyringone in co-cultivation medium can promote T-DNA transferring process (Georgiev et al. 2012; Nakano 2017; Srinivasan et al. 2017). Our results showed that acetosyringone in co-cultivation and inoculation medium can improve the transformation rate (Figure 8). It proposed that a medium with lacking mineral compounds is an effective co-cultivation medium to reach the maximum transformation frequency. Parallel results were described in the case of *Lilium* transformation by *A. tumefaciens* and genetically transformed root induction in *Papaver bracteatum*, *Dracocephalum kotschyi*, *Nepeta pogonosperma* and *Artemisia aucheri* (Azadi et al. 2010; Sharafi et al. 2013b; Sharafiet al. 2014b; Sharafi et al. 2014c; Valimehr et al. 2014). As the effectiveness of genetic transformation by *A. rhizogenes* was significantly improved by reducing MS salts, the outcomes proposed that extra mineral salts in the co-cultivation medium can have an inhibitory effect on the transformation of *A. sieberi* by *A. rhizogenes*. More studies are required to clarify how T-DNA transfer is controlled by mineral compounds. In early studies, the production of artemisinin in hairy root cultures of *A. dubia* and *A. annua* has also been investigated and they found a higher growth rate and biosynthesis of artemisinin content as compared to control untransformed roots (Liu et al. 1997; Ali et al. 2012). Arab et al (2006) reported artemisinin content in *A. sieberi* was 0.2% and 0.14% of dried weight (DW) of plant materials in summer and autumn (Arab, Rahbari et al. 2006).

Conclusion

The present study focused on developing an efficient protocol for *in vitro* organogenesis, regeneration, and *in vitro* artemisinin production in genetically transformed root culture of *Artemisia sieberi*. Direct shoot organogenesis is important because it can be used for breeding work of *Artemisia*, since effective shoot regeneration will improve the recovering shoots formed from transformed cells. The improved transformation protocol was achieved by modifying different factors (different strains of *A. rhizogenes* and types of *A. sieberi* explants, pre-culture, and co-culture conditions). To the best of our knowledge, this is the first report considering the effect of different factors on *A. rhizogenes* transformation of *A. sieberi*. These results can help to develop better strategies for the mass propagation of *A. sieberi* for gene transformation goals, production of large amounts of these valuable therapeutic compounds, and commercial cultivation.

Acknowledgements

The authors would like to thank Zanjan Pharmaceutical Biotechnology Research Center, Zanjan University of Medical Sciences, Zanjan, Iran, for funding this research (Project Code: A-12-848-4).

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

Aberham A, Pieri V, Croom Jr EM, Ellmerer E ,Stuppner H. 2011. Analysis of iridoids, secoiridoids and xanthones in *Centaurium*

erythraea, *Frasera caroliniensis* and *Gentiana lutea* using LC–MS and RP-HPLC. J. Pharm. Biomed. Anal., 54(3): 517-525.

Akkawi M, Aburemeleh Q, Jaber S, Qutob M, Lutgen P. 2014. The effect of *Artemisia sieberi* extracts on the Formation of β-Hematin. Br. J. Pharmacol., 5(1): 49-54.

Ali M, Kiani B, Mannan A, Ismail T ,Mirza B. 2012. Enhanced production of artemisinin by hairy root cultures of *Artemisia dubia*. J. Med. Plant Res., 6: 1619-1622.

Arab H, Rahbari S, Rassouli A, Moslemi M ,Khosravirad F. 2006. Determination of artemisinin in *Artemisia sieberi* and anticoccidial effects of the plant extract in broiler chickens. Trop. Anim. Health Prod., 38(6): 497-503.

Azadi P, Chin DP, Kuroda K, Khan RS ,Mii M. 2010. Macro elements in inoculation and co-cultivation medium strongly affect the efficiency of *Agrobacterium*-mediated transformation in *Lilium*. Plant Cell Tissue Organ. Cult., 101(2): 201-209.

Beigmohamadi M, Movafeghi A, Jafari S ,Sharafi A. 2019. Potential of the genetically transformed root cultures of *Plumbago europaea* for biomass and plumbagin production. Biotechnol. Prog.: e2905.

Beigmohammadi M, Sharafi A, Jafari S. 2019. An optimized protocol for *Agrobacterium rhizogenes*-mediated genetic transformation of *Citrullus colocynthis*. J. Appl. Biotechnol. Rep., 6(3): 113-117.

Buommino E, Baroni A, Canozo N, Petrazzuolo M, Nicoletti R, Vozza A, Tufano MA. 2009. Artemisinin reduces human melanoma cell migration by down-regulating αvβ3 integrin and reducing metalloproteinase 2 production. Invest New Drugs., 27(5): 412-418.

Chen D-H, Ye H-C ,Li G-F. 2000. Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci., 155(2): 179-185.

Chen L, Cai Y, Liu X, Guo C, Sun S, Wu C, Jiang B, Han T ,Hou W. 2018. Soybean hairy roots produced in vitro by *Agrobacterium rhizogenes*-mediated transformation. Crop J., 6(2): 162-171.

Danphitsanuparn P, Boonsnongcheep P, Boriboonkaset T, Chintapakorn Y ,Prathanturarug S. 2012. Effects of *Agrobacterium rhizogenes* strains and other parameters on production of isoflavonoids in hairy roots of *Pueraria candollei* Grah. ex Benth. var. candollei. Plant Cell, Tissue Organ. Cult., 111(3): 315-322.

Faisal M, Anis M. 2006. Thidiazuron induced high frequency axillary shoot multiplication in *Psoralea corylifolia*. Biol. Plant., 50(3): 437-440.

Ferreira JF, Luthria DL, Sasaki T ,Heyerick A. 2010. Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. Molecules., 15(5): 3135-3170.

Georgiev MI, Agostini E, Ludwig-Müller J, Xu J. 2012. Genetically transformed roots: from plant disease to biotechnological resource. Trends Biotechnol., 30(10): 528-537.

Georgiev MI, Pavlov AI, Bley T. 2007. Hairy root type plant in vitro systems as sources of bioactive substances. Appl. Microbiol. Biotechnol., 74(6): 1175.

Han J-L, Wang H, Ye H-C, Liu Y, Li Z-Q, Zhang Y, Zhang Y-S, Yan F ,Li G-F. 2005. High efficiency of genetic transformation and regeneration of *Artemisia annua* L. via *Agrobacterium tumefaciens*-mediated procedure. Plant Sci., 168(1): 73-80.

Hosseini B, Alizadeh M, Hassani A, Jafari M, Rahimi A. 2016. High-Frequency in Vitro Direct Shoot Regeneration from Nodal Explants of *Hyssop* Plant (*Hyssopus officinalis* L.). J. Med. plants By-prod., 5(2): 187-193.

Ivanescu B, Miron A, Corciova A. 2015. Sesquiterpene lactones from *Artemisia* genus: biological activities and methods of analysis. J. Anal. Methods Chem., 2015(12): 1-21.

Jaber S, Abu-Lafi S, Lutgen P, Abu-Remeleh Q, Akkawi M. 2015. Bicarbonate In-Vitro Effect on Beta-Hematin Inhibition by *Artemisia sieberi* Aqueous Infusion. J. Pharm. Pharmacol., 3(2): 63-72.

Jana S, Sivanesan I, Jeong BR. 2013. Effect of cytokinins on in vitro multiplication of *Sophora tonkinensis*. Asian Pac. J. Trop. Biomed., 3(7): 549-553.

Khawar K, Sarhin E, Sevimay C, Cocu S, Parmaksiz I, Uranbey S, Ipek A, Kaya M, Sancak C, Ozcan S. 2005. Adventitious shoot regeneration and micropropagation of *Plantago lanceolata* L. Period. Biol., 107(1): 113-116.

Kim Y, Wyslouzil BE ,Weathers PJ. 2002. Secondary metabolism of hairy root cultures in bioreactors. In Vitro Cell. Dev. Biol. Plant., 38(1): 1-10.

Liu B, Wang H, Du Z, Li G ,Ye H. 2011. Metabolic engineering of artemisinin biosynthesis in *Artemisia annua* L. Plant Cell Rep., 30(5): 689-694.

Liu C, Murch S, El-Demerdash M ,Saxena P. 2003. Regeneration of the Egyptian medicinal plant *Artemisia judaica* L. Plant Cell Rep., 21(6): 525-530.

Liu C, Wang Y, Ouyang F, Ye H, Li G. 1997. Production of artemisinin by hairy root cultures of *Artemisia annua* L. Biotechnol. Lett., 19(9): 927-929.

Magyar-Tábori K, Dobránszki J, Da Silva JaT, Bulley SM, Hudák I. 2010. The role of cytokinins in shoot organogenesis in apple. Plant Cell Tissue Organ. Cult., 101(3): 251-267.

Manuhara YSW, Kristanti AN, Utami ESW ,Yachya A. 2015. Effect of sucrose and potassium nitrate on biomass and saponin content of *Talinum paniculatum* Gaertn. hairy root in balloon-type bubble bioreactor. Asian Pac. J. Trop. Biomed., 5(12): 1027-1032.

Mirzaee H, Sharafi A, Sohi HH. 2016. In vitro regeneration and transient expression of recombinant sesquiterpene cyclase (SQC) in *Artemisia annua L*. S. Afr. J. Bot., 104(1): 225-231.

Nahrevanian H, Sheykhkanlooye Milan B, Kazemi M, Hajhosseini R, Soleymani Mashhadi S, Nahrevanian S. 2012. Antimalarial effects of Iranian flora *Artemisia sieberi* on *Plasmodium berghei* in vivo in mice and phytochemistry analysis of its herbal extracts. Malar. Res. Treat., 2012.

Nakano Y. 2017. Effect of Acetosyringone on *Agrobacterium*-mediated Transformation of *Eustoma grandiflorum* Leaf Disks. Jpn. Agric. Res. Q., 51(4): 351-355.

Oksman-Caldentey K-M, Inzé D. 2004. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends Plant Sci., 9(9): 433-440.

Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, Mcphee D, Leavell M, Tai A, Main A ,Eng D. 2013. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature., 496(7446): 528-532.

Paniego N, Giulietti A. 1994. *Artemisia annua* L.: dedifferentiated and differentiated cultures. Plant Cell Tissue Organ. Cult., 36(2): 163-168.

Ram M, Jain D, Mishra H, Mandal S, Abdin M. 2014. in: Springer, pp. 173-204.

Sakipova Z, Wong NSH, Bekezhanova T, Shukirbekova A ,Boylan F. 2017. Quantification of santonin in eight species of *Artemisia* from Kazakhstan by means of HPLC-UV: Method development and validation. PloS one., 12 (3): e0173714.

Sharafi A, Hashemi SH, Sharafi A, Azadi P ,Mousavi A. 2014a. Tissue culture and regeneration of an antimalarial plant, *Artemisia sieberi* Besser. Res. J. Pharmacogn., 1(3): 15-20.

Sharafi A, Sohi HH, Azadi P, Sharafi AA. 2014b. Hairy root induction and plant regeneration of medicinal plant *Dracocephalum kotschyi*. Physiol. Mol. Biol. Plants., 20(2): 257-262.

Sharafi A, Sohi HH, Mirzaee H, Azadi P. 2014c. In vitro regeneration and *Agrobacterium* mediated genetic

transformation of *Artemisia aucheri* Boiss. Physiol. Mol. Biol. Plants., 20(4): 487-494.

Sharafi A, Sohi HH, Mousavi A, Azadi P, Khalifani BH, Razavi K. 2013a. Metabolic engineering of morphinan alkaloids by over-expression of codeinone reductase in transgenic hairy roots of *Papaver bracteatum*, the Iranian poppy. Biotechnol. Lett., 35(3): 445-453.

Sharafi A, Sohi HH, Mousavi A, Azadi P, Razavi K, Ntui VO. 2013b. A reliable and efficient protocol for inducing hairy roots in *Papaver bracteatum*. Plant Cell Tissue Organ. Cult., 113(1): 165-168.

Shi JQ, Zhang CC, Sun XL, Cheng XX, Wang JB, Zhang YD, Xu J, Zou HQ. 2013. Antimalarial Drug Artemisinin Extenuates Amyloidogenesis and Neuroinflammation in APP swe/PS 1dE9 Transgenic Mice via Inhibition of Nuclear Factor- κ B and NLRP 3 Inflammasome Activation. CNS Neurosci Ther., 19(4): 262-268.

Srinivasan M, Kumar K, Karunanandham M, Ponnusamy M. 2017. Influence of acetosyringone concentration on induction of carrot hairy root by *Agrobacterium rhizogenes*. Afr. J. Microbiol., 8: 2486-2496.

Sudha C, Sherina T, Anand VA, Reji J, Padmesh P, Soniya E. 2013. *Agrobacterium rhizogenes* mediated transformation of the medicinal plant *Decalepis arayalpathra* and production of 2-hydroxy-4-methoxy benzaldehyde. Plant Cell Tissue Organ. Cult., 112(2): 217-226.

Tang K, Shen Q, Yan T, Fu X. 2014. Transgenic approach to increase artemisinin content in *Artemisia annua* L. Plant Cell Rep., 33(4): 605-615.

Thiruvengadam M, Rekha K, Chung I-M. 2016. Induction of hairy roots by *Agrobacterium rhizogenes*-mediated transformation of *spine gourd* (*Momordica dioica Roxb*. ex. willd) for the assessment of phenolic compounds and biological activities. Sci. Hortic., 198(1): 132-141.

Valimehr S, Sanjarian F, Sharafi A, Sabouni F. 2014. A reliable and efficient protocol for inducing genetically transformed roots in medicinal plant *Nepeta pogonosperma*. Physiol. Mol. Biol. Plants., 20(3): 351-356.

Willoughby JA, Sundar SN, Cheung M, Tin AS, Modiano J, Firestone GL. 2009. Artemisinin blocks prostate cancer growth and cell cycle progression by disrupting Sp1 interactions with the cyclin-dependent kinase-4 (CDK4) promoter and inhibiting CDK4 gene expression. J. Biol. Chem., 284(4): 2203-2213.