IMAM KHOMEINI INTERNATIONAL UNIVERSITY
IJGPB
Iranian Journal of Genetics and Plant Breeding
Print ISSN : 2251-9610
Online ISSN : 2676-346X

Analysis efficiency of Iranian Ajowan ecotypes on hairy root production mediated by different *Agrobacterium rhizogenesis* strains

Narges Moradi[1], Seyed Ahmad Sadat Noori[1]*, Ali Fadavi[2], Seyed Mohamad Mahdi Mortazavian[1], Ali Pakdin Parizi[3]

[1]Department of Agronomy and Plant Breeding Sciences, University of Tehran, Pakdasht, Tehran, Iran.
[2]Department of Food Science Technology, University of Tehran, Pakdasht, Tehran, Iran.
[3]Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.
**Corresponding author, 0000-0002-0860-5982. Email: noori@ut.ac.ir. Tel: +98-21-36040615.*

ABSTRACT INFO

Research Paper

Received: 02 Sep 2022

Accepted: 04 Dec 2022

ABSTRACT

Ajowan (*Trachyspermum Ammi* L.) is one of the most important medicinal plants native to the Middle East and Iran. The antibiotic properties of its essential oils are related to the relatively high content of thymol. In this study, the possibility of hairy root production was investigated in a pre-experimental study considering many factors affecting transgenic efficiency, including explant type, immersion time, and the type of culture medium. Optimal conditions for the pre-experiment were set up based on the completely randomized design with seven *Agrobacterium rhizogenes* strains (A3, A6, A7, 4404, AATCC15834, R1000, A4) and six selected Ajowan ecotypes (Ardebil, Shiraz, Arak, Sarbishe, Qom, and Rafsanjan). The root-related morphological characteristics were measured to study the effect of main factors on the hairy root production and the bacterial strains in terms of hairy root induction. Based on the results, the highest percentage of hairy roots in Ardebil ecotype was induced by ATCC15834 strain (50%) and the lowest percentage was related to Rafsanjan ecotype induced by A6 (10%). The highest positive and significant correlation was observed in dry and wet weights (r=0.95), root frequency percentage and root length (r=0.80). The highest amount of phenolic compounds in hairy roots (240 mg/g dry weight matter) was associated with the Ardebil ecotype induced by strain ATCC15834. In this study, Shiraz and Ardebil ecotypes were identified as the best ecotypes, A4 and ATCC15834 strains as the most suitable strains, and suggested for future studies.

Key words: *Agrobacterium rhizogenes,* Correlation, Phenol, *Trachyspermum Ammi* L.

How to cite this article:
Moradi N., Sadat Noori S. A., Fadavi A., Mortazavian S. M. M., and Pakdin Parizi A. (2021). Analysis efficiency of Iranian Ajowan ecotypes on hairy root production mediated by different *Agrobacterium rhizogenesis* strains. *Iranian Journal of Genetics and Plant Breeding,* 10(1): 117-127.
DOI: 10.30479/IJGPB.2022.17488.1325

INTRODUCTION

Medicinal plants are one of the major sources of antibiotics and chemicals (Alviano *et al.,* 2009). Indiscriminate use of medicinal plants causes a serious threat to these valuable resources in the future, so it is important to pay attention to germplasm conservation programs to preserve these valuable plants. Ajowan (*Trachyspermum Ammi* L.) is one of the most important medicinal plants belonging to the Middle East and Iran. In ancient times, the roots and seeds of Ajowan were used in traditional medicine. The effective ingredients of Ajowan essential oil are thymol and carvacrol (Zarshenas *et al*., 2013; Boskabady *et al*., 2014). The antibiotic, antispasmodic, and antifungal properties of Ajowan essential oil are due to 50% content of thymol (Zarshenas *et al*., 2013). The second active ingredient of Ajowan essential oil, carvacrol, is used as a cold medicine (Boskabady *et al*., 2014). This plant is also used to treat digestive problems (Krishnamoorthy and Madalageri, 1999). Plant tissue culture is one of the most important branches of biotechnology that can play an important role in the protection of medicinal plants (Sharma and Dubey, 2011). Optimization of tissue culture can play an important role in propagation and utilization (Boskabady *et al*., 2014). Hairy Root Culture Technology (HR) is a new biotechnological approach that aims to improve the genetics and biochemical properties of several important medicinal plants (Gantait *et al*., 2020). Hairy roots can produce stable secondary metabolites that can compete with the secondary metabolites produced by aerial parts. Hairy roots are produced by infecting wounded plants with *Agrobacterium rhizogenes* in laboratory environments. *Agrobacterium rhizogenes* is a Gram-negative bacterium that causes a disease that appears as hairy roots on plants. In this method, T-DNA from the root-inducing plasmid (Ri) is transferred to the plant genome and expressed in the plant, which is indicated by morphological changes and growth rates (Pavlova *et al*., 2014; Biswas *et al*., 2017). There are many secondary metabolites including weak anticancer compounds in plants (Carqueijeiro *et al*., 2020). In addition, many of these metabolites are synthesized only in specific tissues (Sun *et al*., 2019). Various methods such as cell and yeast cultures (Chandran *et al.,* 2020) have been used to increase the secondary metabolites. Hairy root culture is another method for this purpose. Extraction of secondary metabolites from hairy root cultures has been successfully reported (Gantait and Mukherjee, 2021). The secondary metabolite production in plant tissue cultures differs from that in plants because of changes in the enzymes active in the biosynthetic pathway of secondary metabolite production. However, there is no such limitation in the production of secondary metabolites by hairy roots, and on the other hand, rapid growth and easy maintenance increase the production efficiency (Giri *et al.,* 2000). Transgenic explants production and the induction of hairy roots with *Agrobacterium rhizogenes* are influenced by factors such as species, age, and type of explant (Georgiev *et al.,* 2010), bacterial strain, bacterial suspension concentration, and the presence of phenolic substances in plant tissues (Bulgakov *et al*., 2012). In the first report, callus production was performed on 4 explants (root, shoot, leaf, and cotyledon) in Ajowan. The stem explant was the most effective explant in the Ajowan tissue culture. The highest callus induction (100%) was obtained in the stem explants in the medium containing BAP (0.25 mg/L), and the highest callus production was obtained in 2 mg/L density of 2.4-D (Fazeli-Nasab, 2018). Ajowan is a traditional potential herb cultivated in Eastern countries and widely used in other countries (Bairwa *et al.,* 2012). Since there are many problems for harvesting plant materials from natural resources such as environmental stresses, time consuming, and weather conditions (Zhou *et al.,* 2006) more studies are requred to be carried out on secondary metabolite production in the hairy roots of Ajowan plants mediated by *Agrobacterium* (Vamenani *et al.,* 2020). The present study was conducted to investigate the effects of ecotypes and Agrobacterium strains on hairy root production. The establishment of a hairy root system for commercial production is very important and controlled by many factors (Zheng *et al.,* 2021), However, little information is available particularly when the hairy root production of Ajowan is desirable. There is a lack of information concerning the effects of different conditions such as ecotype, culture medium, type and age of explants, concentration, and the type of bacterial strains on hairy root induction in Adjowan plants. Accordingly, in the present study, these parameters were investigated in details. The most suitable ecotypes of Ajowan and the bacterial strains were introduced.

MATERIALS & METHODS

Preparation of explants

The seeds of different ecotypes of Ajowan (Ardebil, Shiraz, Arak, Sarbishe, Qom, and Rafsanjan) were prepared from the Gene Bank of Aburayhan college in Pakdasht (Latitude: 35.4817, Longitude: 51.6803). To sterilize seedlings, different steps were performed in order, 1) seeds were disinfected in sodium hypochlorite

for 12 minutes, 2) the washing was performed 3 times with sterile distilled water. 3) They were immersed in 70% alcohol for two minutes, and then washed three times with sterile distilled water. The disinfected seeds were completely dried and then placed on a solid medium (½ MS medium containing 3% sucrose and 0.8% agar with pH equal to 5.8). The cultured seeds were stored at 25 °C±1 in the culture chamber with 16 h light and 8 h darkness to produce sterile seedlings. The explants were obtained from the sterile seedlings after 21 days. These explants included stem, roots, cotyledon leaves, and main leaves with the size of 1 cm. They were used for inoculation with *Agrobacterium rhizogenes* and hairy roots induction.

Preparation of bacteria strains

Bacterial strains were prepared from Tarbiat Modarres University, National Genetic Research Institute, and Sari Research Institute. Various strains including A3, A6, A7, 4404, AATCC15834, R1000, and A4 *Agrobacterium rhizogenes* were used to induce hairy roots. Bacterial strains were cultured in the LB medium containing 50 mg/L rifampicin and kept at 28 °C and were shaken at 150 rpm. The suspension absorbance was measured at 700 nm and then was centrifuged at 4 °C and 3500 rpm. The supernatant was removed and the bacterial precipitate was suspended in the ½ MS medium containing 100 µM acetosyringone and this suspension was used to inoculate the explants.

Hairy roots induction

The explants were wounded with sterile scalpels and then immersed in bacterial suspension for 10-30 minutes. The inoculated explants were dried with sterile filter paper and then transferred to the MS and ½ MS media without any hormones. These media were placed at 25 °C for 48 h and in dark for better growth of bacteria. The formation of an aura around the explants indicates the occurrence of bacterial contamination. The *Agrobacterium rhizogenes* must be eliminated, for this purpose, explants were transferred to the same co-culture medium with 500 mg/L cefotaxime. The antibiotic concentration in the medium must be gradually decreased to eliminate the contamination, completely. The emergence of hairy roots in the inoculated explants was observed. The hairy roots number was counted and recorded daily. The roots that appeared in sizes 2-3 cm were transferred into a liquid culture medium of ½ MS without any hormones with 50 mg/L cefotaxime to prevent any possible contamination.

Pre-test details

To select the suitable medium and the type of explant, a pre-test was designed. This included a factorial, completely randomized design with two ecotypes (Shiraz and Arak), two strains of bacteria (ATCC15834 and A3), three types of explants (cotyledon leaves, stems, main leaves), and two culture media (MS and ½ MS). According to the results of this pretest, ½ MS, and stem explants were chosen (pretest result). In the second experiment, the effects of six ecotypes of Ajowan and seven strains of *Agrobacterium rhizogenes* were investigated on hairy root induction.

Confirmation of the transgenic hairy roots

To confirm the transgenicity of hairy roots, a fragment of the *rol* B gene was amplified and to ensure that there is no bacterial contamination, a fragment of the *vir* D gene was used for the polymerase chain reaction (PCR). Specific primers 5 ´-GCTCTTGCAGGCTAGTAGATTT-3 ´ and 5´-GAAGGTGCAAGCTCTCTC-3´ (Zhu *et al.,* 2001) were used for the proliferation of *rol*B gene and the primer pair used for *Vir* D was as follows 5 ´-GGAGTCTCTCAGCATGGAGCAA-3 ´ gene 5´-ATGTCCAAGGCAGTAAGCCCA-3´. (Ayadi and Tre´mouillaux-Guiller, 2003). The conditions of the PCR reaction included 94 °C for 5 min for the initial denaturation, and 35 cycles of 94 °C for 1 min, 55 °C for 1 min for annealing, 72 °C for 1 min for extension, and finally 72 °C for 7 min for the final extension. PCR products were observed on a 1% agarose gel after staining with ethidium bromide.

Measuring the wet and dry weights of the hairy roots

Based on the growth rate of the hairy roots, three samples were selected from each ecotype and the weight of induced hairy root was measured. For this purpose, a hairy root with an approximate weight of 10 mg was transferred to 100 mL flask Erlen containing ½ MS liquid medium (30 mL). After 45 days, the roots were washed in distilled water and placed on a filter paper to remove the free water attached to the roots, then their fresh weight was measured. To measure the dry weight, hairy root samples were kept at room temperature, then the dry weight of the samples was measured after 24 h.

Preparation of extracts

One gram of dried hairy roots was completely powdered with a mortar and then mixed with 10 ml methanol (95%) to provide a solution (1:10 g/ml), the solution was kept for 48 h, and after centrifugation at 13,000 rpm, the filtrate was used to measure the compounds.

Assay of phenolic compounds

Total phenolic compounds were measured based on the Folin-Ciocalteu staining method (Folin and Ciocalteu, 1927). The basis of this method is the creation of a blue complex due to Folin reagent reaction with phenolic compounds in alkaline media. This complex shows the maximum absorbance at 760 nm. About 20 µl of hairy root extract was mixed in a tube with 1160 µl distilled water and 100 µl Folin-Ciocalteu reagents. The samples were kept at room temperature for 5 min and then 300 µl sodium carbonate solution (20% w/w) was added. After shaking, the test tubes were placed at room temperature and in dark conditions and after 30 min, the absorbance was read at 760 nm using a spectrophotometer. The results were reported as equivalent to milligram of gallic acid per gram of dry weight. To plot the standard curve for gallic acid, its basic solutions were prepared as 0-10-20-30-40-50-60-70-80-90-100 µg/ml. After drawing the calibration curve of gallic acid, the total content of phenol in the extract was calculated by placing the amount of extract bsorbance in the linear equation related to the standard curve. The Y axis is the bsorbance and X axis is the amount of phenolic compounds (Figure 1). Finally, the data were expressed based on the miligram equivalent of gallic acid per gram of the extract.

Data analysis

The pre-test was a factorial experiment based on completely randomized design with five factors concluding (ecotype, bacteria, culture medium, immersion time, and explant type). In the pre-test, the effect of two ecotypes (Shiraz and Arak) and Agrobacterium strains (A3, 15834) in two culture media (MS, ½ MS) with three immersion times (10 minutes, 20 minutes and 30 minutes) and three explants (stem, leaf, cotildon) was investigated to determine the percentage of the produced hairy roots. The main experiment was factorial based on completely randomized design with six bacterial strains and seven ecotypes with three replicates (three Petri dishes each containing 10 explants). The percentage frequency of transgenic plants was calculated based on the ratio between the number of hairy roots and the number of infected explants. The LSD test was used to investigate the significant difference between treatments and R and SAS software were used to analyze and compare the means**.** Heatmap was performed by R software (R3.4 software model, Agricola Ggplot2 package).

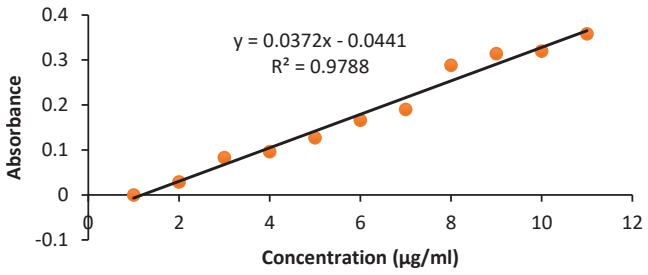

Figure 1. The standard curve of gallic acid to determine of total phenolic compounds.

Table 1. The analysis of variance for simple effects and their interactions on the number of roots in Ajowan.

Source of variation	df	Mean of square
Ecotype (A)	1	26.04**
Bacteria (B)	1	14.004**
Culture medium (C)	1	0.226^{ns}
Immersion time (D)	2	59.370**
Explant type(E)	2	117.509**
A×B×C	1	10.226*

^{ns}: not significant difference.
Significances are indicated *: P<0.05, **: P<0.01.

RESULTS & DISCUSSION

Pretest results

According to the results of the pretested analysis of variance, simple effects including ecotype and bacteria, immersion time, and type of explants on hairy root induction (%) were significant at 0.01 level, but the effect of culture medium on hairy root production was not significant (Table 1). The explant type had a significant effect on the number of hairy roots, in general, the percentage of transgenicity was lower in the explants of the main leaves and cotyledons than in the stem explants, and the lowest number of hairy roots was observed in the explants of the cotyledons (Figure 2). The best explants for hairy root production were stem explants. Success in inducing hairy roots by *Arobacterium* depends on several factors including species, tissue, and explants (Md Setamam *et al.,* 2014). Shiraz ecotype induced by strain ATCC15834 had the highest average number of hairy roots with stem explants (5 root - 50%) and the lowest average number of hairy roots was exhibited by the cotyledon and leaf explants of Shiraz ecotype induced by A3 strain (1.2 root- 12%). As a result, Farsi *et al.* (2018) reported that stem explants are the best explants for hairy root induction in *Datura stramonium*. According to the type of plant, Murthy reported that hairy roots

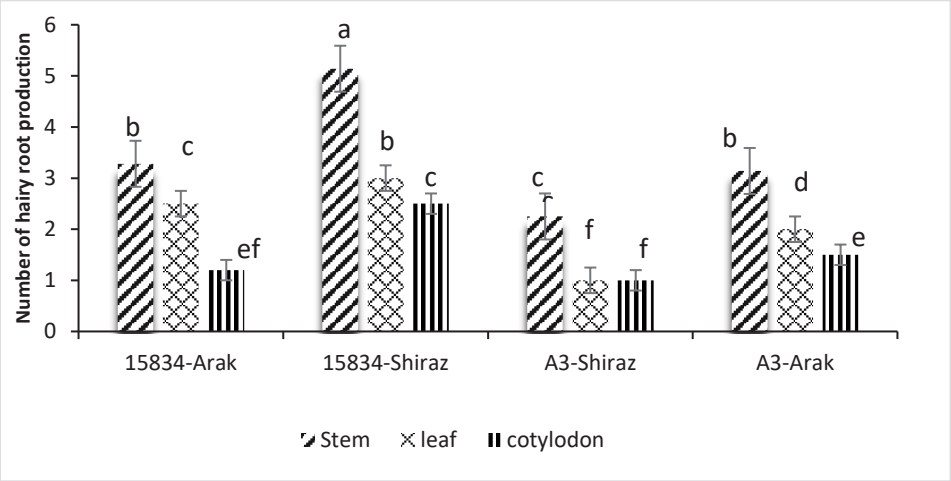

Figure 2. The effect of explants, ecotypes, and bacterial strains on the mean production of hairy roots in Ajowan.

Table 2. Analysis of variance effects of simple and interaction effects of bacteria strains and studied ecotypes on the production of hairy roots in Ajowan.

Source of variation	df	Mean of square			
		Hairy root frequency	Hairy root length	Number of days to observe hairy roots	Hairy root dry weight
Ecotype (A)	5	7.40**	8.10**	43.62**	2.42**
Bacteria (B)	6	1.05^{ns}	2.77**	6.64^{ns}	0.83^{ns}
(A×B)	30	2.86*	3.06**	10.35**	1.77**
Error	84	1.60	1.07	4.38	0.51

^{ns}: not significant difference.
Significances are indicated *: P<0.05, **:P<0.01.

were only induced from cotyledons and leaves in *Withania sominifera* (Murthy *et al.,* 2008). The highest frequency of hairy root induction (75%) was reported in *Teucrium chamaedrys* on leaves with A13 strain (Bernousi *et al.,* 2016) The age of explants also rought about different results, for example, the explant of stems of 21-day-old seedlings produced more hairy roots than older explants, therefore, stem explants collected from the 21-day-old seedlings were selected as the best explants for the main experiment. The age of the tissue, hormonal balance, and type of explants are important factors in the induction of hairy roots (Moehninsi and Navarre, 2018). The effect of the culture medium on hairy root induction (%) was not significant, and ½ MS was preferred to MS for the main experiment due to its lower cost. The results of the pretest showed that the type of bacterial strain had a significant effect on the number of hairy roots induction, consequently more strains were selected and their effects were studied in the main experiment. The strain ATCC15834 was able to produce the higher number of hairy roots relative to strain A3 (Figure 2). Similar results were reported in another experiment on the induction of hairy roots of *Althea officinalis* on different explants including leaf, petiole, and shoot, the maximum transformation was observed on shoot explants with ATCC15834 strain (Tavassoli and Afshar, 2018).

Main test results

Different types of strains and ecotypes caused different responses in terms of number and length of hairy roots. There was a significant difference in the number and length of hairy roots in different ecotypes and strains. The type of bacterial strain had a significant effect on the production of transgenic hairy roots (Table 2). Ardebil ecotype had the highest potential for hairy root production, and strain ATCC15834 had the highest potential to induce hairy roots. The highest percentage of hairy roots was obtained with strains ATCC15834 on ecotype Ardebil (50%), and the lowest one resulted by Rafsanjan ecotype induced by A4 strain (10%) (Figure 3A). Different bacterial strains had different abilities to induce hairy roots in this plant. A similar result was reported that strain ATCC 15834 was the most efficient

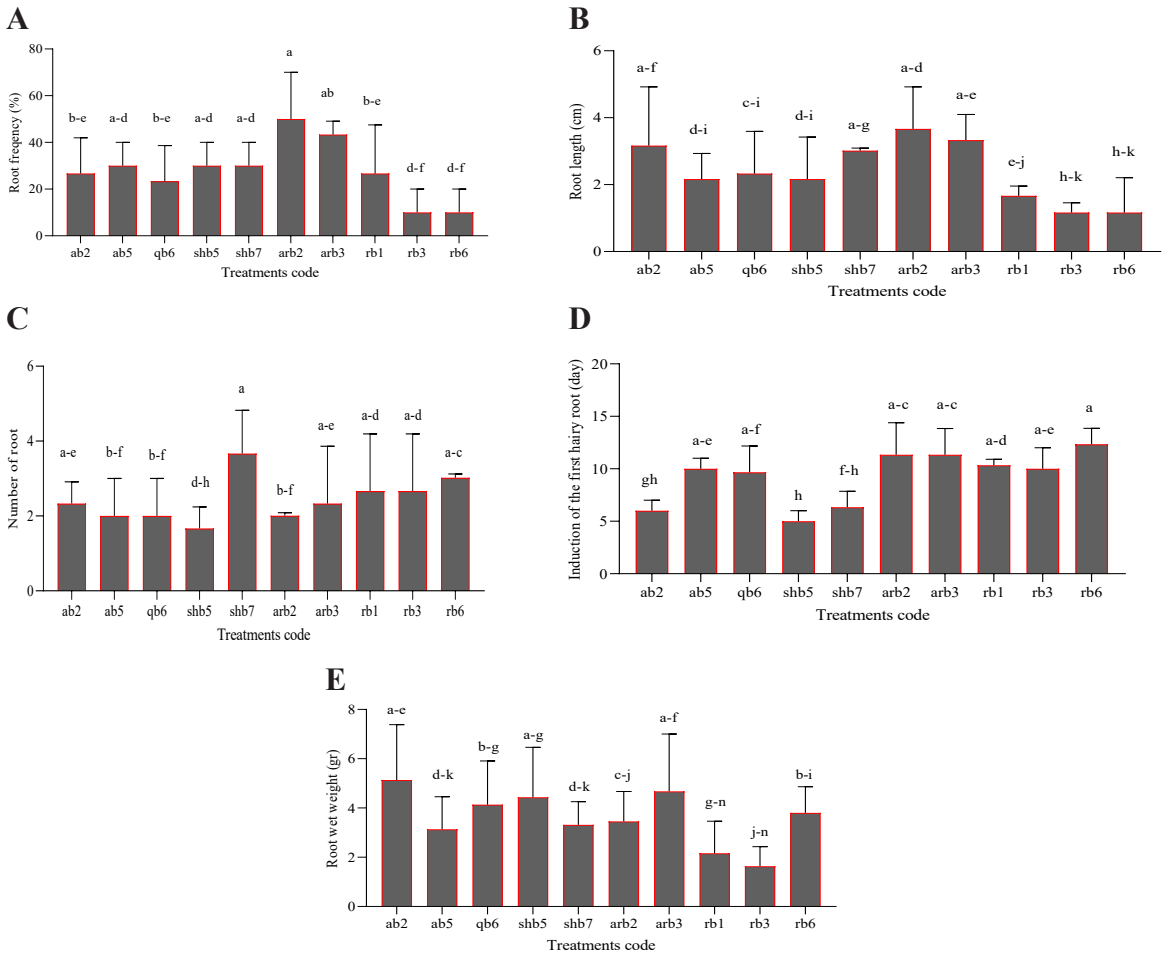

Figure 3. A: Frequency diagram of hairy roots, **B:** hairy root length, **C:** number of roots, **D:** number of days to the first root, **E:** fresh weighted diagram of hairy roots after 2 months.
The codes of ecotypes: a: Arak, q: Qom, sh: Shiraz, ar: Ardebil, r: Rafsanjan.
The codes of bacterial strains: b1: R1000, b2: ATCC15834, b3: A4, b5: A3, b6: A6, b7: A7.

one (Vamenani *et al.,* 2020). Contact and interaction of *Agrobacterum* with plant cells is a basic requirement for *Agrobacterium*-mediated gene transfer (Samadi *et al.,* 2012). The ability of *Agrobacterium rhizogens* to infect and produce hairy roots depends on its strains (Sharafi *et al*., 2014). Results showed that the roots obtained from stem explants inoculated with strain ATCC15834 had the highest length (3.8 cm) and growth rate in the solid medium (Figure 3B). Hairy root production results showed that all bacterial strains used in this experiment were able to produce hairy roots. *Rol* B gene expressions cause hairy root appearance, their longitudinal growth and determine the phenotype (Altamura, 2004). The highest hairy root weight was obtained for ecotype Arak induced by ATCC15834 *Agrobacterium* (~5 gr) in liquid culture and the lowest hairy root weight was obtained for ecotype Rafsanjan induced by A4 *Agrobacterium* (1.5 g) (Figure 3E). Arafa *et al.* (2015) reported that A4 was the best *A. rhizogenes* strain for hairy root induction in *Nepeta cataria*. In another report, the young leaves of *Przewalskia tangutica* showed the highest sensitivity to A4 (100%) for the induction of hairy roots (Lan & Quan, 2010). Further, ATCC 15834 has also been reported as a most widely used *A. rhizogenes* strain for strong root induction ability (Kumar *et al.,* 2014). The hairy root of Ardebil ecotype induced by ATCC15834 had no significant difference in root length with ecotype Arak induced by ATCC15834 and the highest root length belonged to ecotype Ardebil induced by ATCC15834 (Figure 3B). This means that probably the bacterial strain has a greater effect than the plant ecotype. In a similar result reported on the hairy root induction of *Semecarpus anacardium* L. using *A. rhizogenes* strains LBA 9402, A4 and ATCC 15834, the rate of transformation was higher in ATCC15834 strain compared to those of A4 and LBA 9402 (Panda *et al.,* 2017). Also, in an experiment for the optimization of hairy root induction

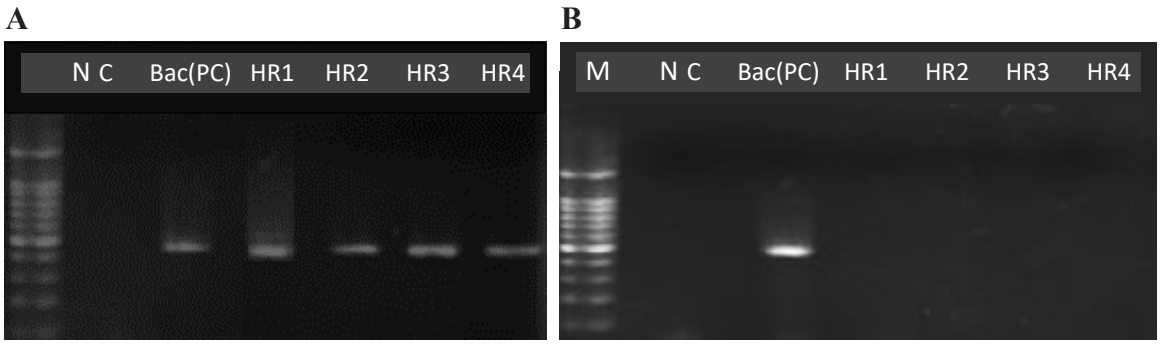

Figure 4. A: Confirmation of transgenic status of the *Trachyspermum Ammi* (L.) hairy roots by *rol*B gene specific primers, **B:** evaluation of hairy roots bacterial contamination by *Vir* D gene specific primers.
(M: DNA size Marker, NC: Negative control, DNA extracted from non-transgenic explant, Bac(PC): Positive control, DNA extracted from bacteria, HR: DNA extracted from different hairy roots).

in *Callerya speciose*, 4 strains, A4, LBA9402, R1601 and 4 explants, hypocotyls, cotyledons, leaves and excised stems were used. The maximum rate of hairy root induction was obtained in cotyledon explants by strain LBA9402 (Yao *et al.,* 2016). The first hairy roots appeared in Shiraz ecotype explants after 5 days of induction with A7 and A3 strains. After induction. The emersion of hairy root of Ardebil ecotype, induced with ATCC15834 and A4 bacteria, required the longest period (~ 12 days). The highest length of hairy roots and the longest period of Ardebil ecotype can be contributed to the more successful gene transfer to this ecotype. The economical production is important in any process, when the hairy root appearance is short, more time saving and more efficient production are feasible (Weber *et al.,* 2011).

Transgenic hairy roots confirmation

PCR method can be used simply for detecting T-DNA sequences in hairy roots (Samadi *et al.,* 2012). In this study, the presence of the *rol* B gene in the hairy root lines was confirmed by PCR analysis (Figure 4A). The *rolB* and *rolC* genes are absolutely essential for the induction of hairy roots (Georgiev *et al.,* 2007). Hairy roots are determined by the identification of the *rol*-B gene (Altamura, 2004). In this experiment, *Agrobacterium* were used as a positive control and the natural root of the non-transgenic seedling was used as a negative control. In the selected transgenic hairy root, a band was amplified, but no such amplicon was observed in the untransformed root sample (negative control). To ensure that the hairy roots were transgenic and the absence of bacterial contamination in the hairy roots, PCR analysis with *Vir* D was also performed. In the transgenic hairy roots containing the *rol* B gene, there was no amplification for the *Vir* D gene, indicating that the hairy roots were free of bacterial contamination. (Figure 4B). This result indicated that Ajowan is susceptible for transformation with *Agrobacterium rhizogenes* and the roots and stem explants respond quite efficiently to transformation by Agrobacterium.

Root morphology

From the appearance point of view, the strain ATCC15834 induced roots with more hairy branches, whereas roots induced by strain A4 were hairless and had few branches (Figures 5 and 6). There was a considerable difference in terms of root induction and growth at different bacterial strains. The hairy roots varied in terms of phenotypic and morphological characteristics (the number of branching and density of root) (Zhang *et al*., 2006). The reason for this apparent difference can be attributed to the amount of T-DNA inserted, the ability of the ecotypes to take up the bacterial gene, and the differential expression of T-DNA genes in the host plant (Smolka *et al.*, 2010). In addition, researchers have reported that the number of secondary metabolites vary depending on the type of entry site and the stimulation of T-DNA in the host plant. The differences in morphology and growth rate of hairy roots were attributed to the diversity of plasmids introduced by the different bacterial strains, which is related to the action of *rol* B genes. Expression of these genes alters the host plant's auxin and cytokinin hormones contents and also the sensitivity of plant cells to growth hormones (Tiwari *et al.,* 2007).

Correlation of traits

Based on the mean of the data, the correlation coefficient matrix between the studied traits was calculated for all six ecotypes. The value of positive and significant correlation between all attributes are: dry and wet weights (r=0.95*), root frequency and root length (r=0.80*), root length and dry weight

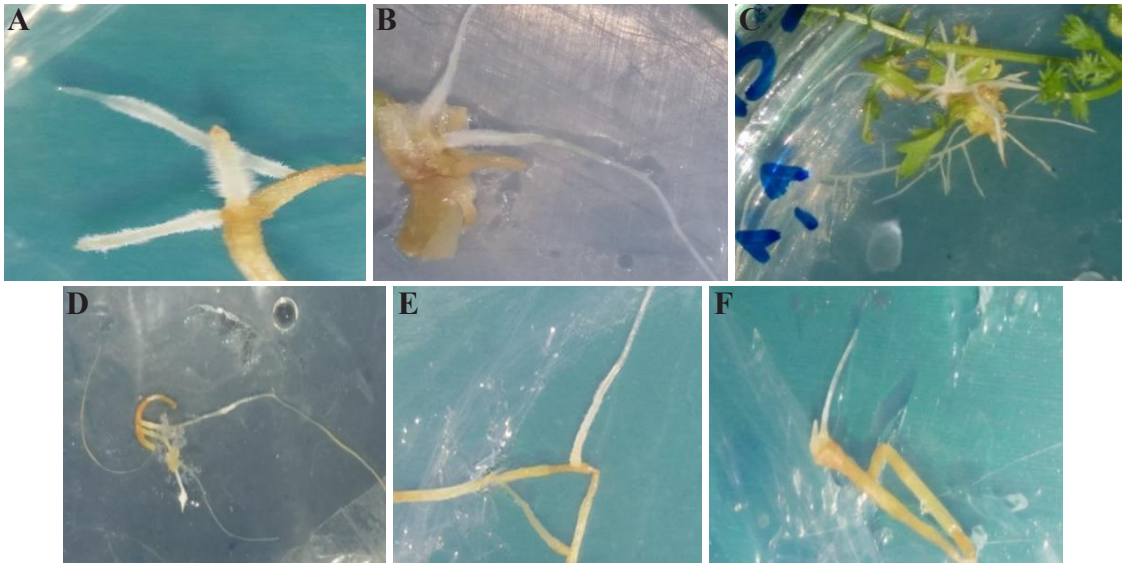

Figure 5. Emergence and growth of hairy roots of Ajowan medicinal plant inoculated with *Agrobacterium rhizogenes* in the solid medium 15 days after explant contamination of stems. **A, B, C:** hairy roots obtained after inoculation with *Agrobacterium rhizogenes* in Ardebil ecotype by strain ATCC15834, **D, E, F:** stem explants of hairy roots observed due to inoculation with *Agrobacterium rhizogenes* in Shiraz ecotype A4 stem explants.

Figure 6. A: Growth of Ardebil ecotype hairy roots in ½ MS liquid medium in the first week inoculated with *Agrobacterium rhizogenes* ATCC15834, **B:** growth of Ardebil ecotype hairy roots in ½ MS liquid, **C:** growth of Ardebil ecotype hairy roots induced by A4 bacteria in ½ MS liquid medium.

(r=0.75**), root length and wet weight (r=0.63**), dry weight and root number (r=0.39*) and wet weight and root number (r=0.36*). It should be noted that the number of days to observe primary hairy roots had no significant correlation with the other traits (Figure 7). On the other hand the observations showed that the lowest negative and non-significant correlation was observed between dry weight and number of days to observe primary hairy roots (r=0.16^{ns}).

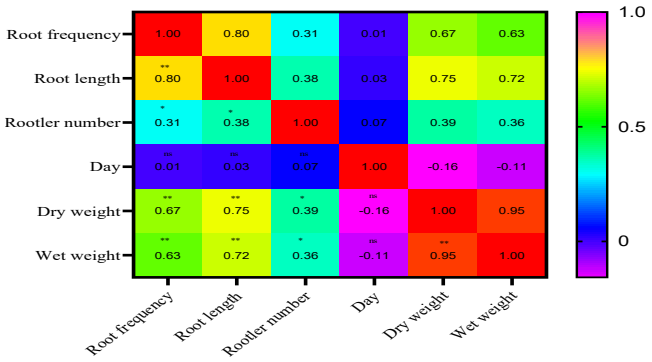

Figure 7. Correlation of traits using heatmap.

Total phenol

Hairy roots produce secondary plant compounds that equal or exceed those of plant organs (Kim *et al.*, 2002). The phenolic compounds are important factors in determining the antioxidant properties of the extract.

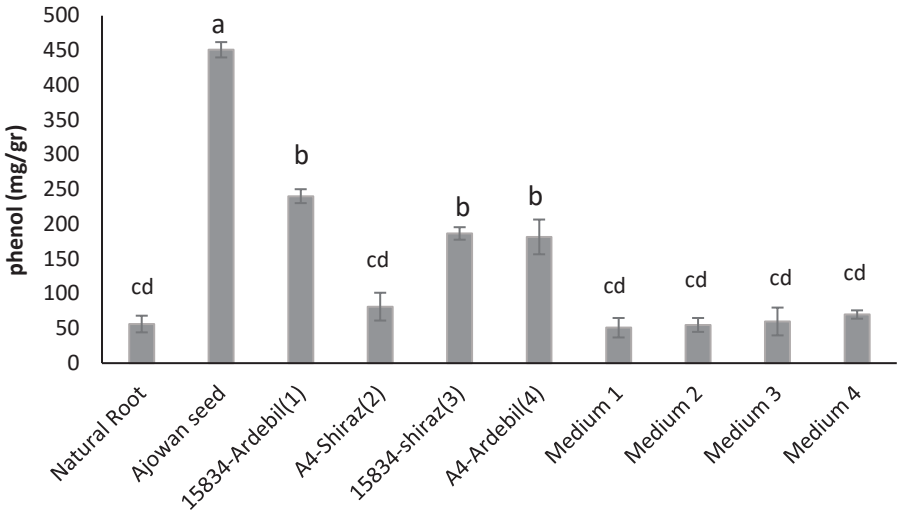

Figure 8. Comparison of phenolic compounds of natural roots, hairy roots, hairy root medium and seeds.

In this experiment, the phenolic compounds of all hairy roots were determined by the gallic acid method. The phenolic content of hairy roots is influenced by several biological and abiotic factors (Bruni *et al.,* 2009). The results showed that Ajowan seed had the highest amount of phenolic compounds, Ardebil ecotype hairy roots induced by strain ATCC15834 had the highest content of phenolic compounds and the natural root contained very low phenolic compounds of about 50 mg/g of dry matter. Similar to this result, El-Esawi (2017) reported that transgenic hairy roots exhibited a 54.8–96.7% increase in the total phenolic content, when compared with the non-transgenic roots. In overall, the total amount of phenolics measured in hairy roots was higher than normal roots, which is consistent with Joseph *et al.* (2020) findings. Plant roots release a wide range of compounds that are not directly involved in plant growth and development, but are very important for plants in stress conditions. Among these materials are phenolic compounds and flavonoids (pang *et al*., 2007). The hairy roots produced in this study released the phenolic compound into the liquid media. The liquid ½ MS in which hairy roots were grown also had phenolic compounds equal to or higher than the normal roots of the Ajowan. In other words, total phenolic compounds of hairy roots and culture medium are comparable in the amount of phenolic compounds obtained from seeds (Figure 8).

ACKNOWLEDGMENTS

The authors acknowledge the support of vice-presidency for science and technology (Iran national science foundation).

REFERENCES

Altamura M. M. (2004). *Agrobacterium rhizogenes rol*B and *rol*D genes: regulation and involvement in plant development. *Journal of Plant Cell Tissue Organ Culture,* 77: 89-101.

Alviano D. (2009). Search for new alternatives to treat microbial diseases. *Journal of Current Pharmaceutical Biotechnology*, 10(1): 106-121.

Arafa N. M., Gabr A. M. M., Ibrahim M. M., Shevchenko Y., and Smetanska I. (2015). Study the effect of hairy root transformation on rapid growth (growth morphology) of *Nepeta cataria in vitro* cultures. *Journal of Innovations in Pharmaceuticals and Biological Sciences,* 2(4): 439-450.

Ayadi R., and Tremouillaux-Guiller J. (2003). Root formation from transgenic calli of Ginkgo biloba. *Journal of Tree Physiol*ogy, 23(10): 713-718.

Bairwa R., Sodha R. S., and Rajawat B. S. (2012). *Trachyspermum ammi*. *Pharmacognosy Reviews,* 6(11): 56-60. DOI: https://doi.org/10.4103/0973-7847.95871.

Biswas T., Mathur A. K., and Mathur A. (2017). A literature update elucidating production of Panax ginsenosides with a special focus on strategies enriching the anti-neoplastic minor ginsenosides in ginseng preparations. *Applied Microbiology and Biotechnology,* 101: 4009-4032. DOI: https:// doi.org/10.1007/s00253-017-8279-4.

Boskabady M. H., Alitaneh S., and Alavinezhad A. (2014). *Carum copticum* L.: a herbal medicine with various pharmacological effects. *BioMed Research International,* 2014: 569087. DOI: https://doi.org/10.1155/2014/569087.

Bruni R., and Sacchetti G. (2009). Factors affecting polyphenol biosynthesis in wild and field grown St. John'sWort (*Hypericum perforatum* L. Hypericaceae/ Guttiferae). *Molecules*, 14(2): 682-725. DOI: 10.3390/molecules14020682.

Bulgakov V., Gorpenchenko T., Veremeichik G., Shkryl Y.,

Tchernoded G., Bulgakov D., Aminin D., and Zhuravlev N. (2012). The *rol*B gene suppresses reactive oxygen species in transformed plant cells through the sustained activation of antioxidant defense. *Plant Physiology,* 158(3): 1371-1381. DOI: 10.1104/pp.111.191494.

Carqueijeiro I., Langley Ch., Grzech D., Koudounas K., Papon N., EO'Connor S., and Courdavault V. (2020). Beyond the semi-synthetic artemisinin: metabolic engineering of plant-derived anticancer drugs. *Current Opinion in Biotechnology,* 65: 17-24. DOI: https://doi.org/10.1016/j.copbio.2019.11.017.

Chandran H., Meena M., Barupal T., and Sharma K. (2020). Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. *Biotechnology Reports*, 26: e00450.

El-Esawi M. A., Elkelish A., Elansary H. O., Ali H. M., Elshikh M., Witczak J., Ahmad M. (2017). Genetic Transformation and Hairy Root Induction Enhance the Antioxidant Potential of *Lactuca serriola* L. *Oxidative Medicine and Cellular Longevity,* 2017: 5604746. DOI: 10.1155/2017/5604746.

Farsi M., Moshtaghi N., Shahriari F. A., and Raeisi M. (2005). Investigation on growth stability and alkaloid content of transformed hairy roots in *Datura stramonium. Agricultural Sciences and Technology,* 19(2): 47-56.

Gantait S., Mitra M., and Chen J. T. (2020). Biotechnological interventions for ginsenosides production. *Journal of Biomolecules,* 10: 538.

Gantait S., and Mukherjee E. (2021). Hairy root culture technology: applications, constraints and prospect. *Applied Microbiology and Biotechnology,* 105(1): 35-53. DOI: https://doi.org/10.1007/s00253-020-11017-9.

Georgiev M., Pavlov A., and Bley T. (2007). Hairy root type plant in vitro systems as sources of bioactive substances. *Applied Microbiology and Biotechnology*. 74: 1175-1185. DOI: 10.1007/s00253-007-0856-5.

Georgiev M. I., Jutta L. M., and Bley T. (2010). Hairy root culture: copying nature in new bioprocesses. *Medicinal Plant Biotechnology*. DOI: https://doi.org/10.1079/9781845936785.0156.

Giri A., and Narasu M. L. (2000). Transgenic hairy roots: recent trends and applications. *Biotechnology Advances,* 18: 1-22.

Kim Y., Wyslouzil B. E., and Weathers P. J. (2002). Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cellular & Developmental Biology-Plant,* 38: 1-10. DOI: 10.1079/IVP2001243.

Krishnamoorthy V., and Madalageri M. B. (1999). Bishop weed (*Trachyspermum ammi*): an essential crop for north Karnatka. *Journal of Medicinal and Aromatic Plant Sciences,* 21(4): 996-998.

Kumar V., Desai D., and Shriram V. (2014). Hairy root induction in *Helictere sisora* L. and production of Diosgenin in hairy roots. *Journal of Natural Products and Bioprospecting,* 4: 107-112.

Lan X. Z., and Quan H. (2010). Hairy root culture of *Przewalskia tangutica* for enhanced production of pharmaceutical tropane alkaloids. *Journal of Medicinal Plants Research,* 4: 1477-1481.

Md Setamam N., Jaafar Sidik N., Abdul Rahman Z., and Che Mohd Zain C. R. (2014). Induction of hairy roots by various strains of *Agrobacterium rhizogenes* in different types of *Capsicum* species explants. *Journal of BMC Research Notes*, 7: 414.

Moehninsi A., and Navarre D. A. (2018). Optimization of hairy root induction in *Solanum tuberosum*. *American Journal of Potato Research,* 95(6): 650-658.

Mulabagal V., and Tsay H-S. (2004). Plant cell cultures - an alternative and efficient source for the production of biologically important secondary metabolites. *International Journal of Applied Science and Engineering*, 2(1): 29-48.

Murthy H. N., Dijkstra C., Anthony P., White D. A., Davey M. R., Power J. B., Hahn E. J., and Paek K. Y.(2008). Establishment of *Withania somnifera* hairy root cultures for the production of withanolide A. *Journal of Integrative Plant Biology,* 50: 975-981.

Niazian M., Sadat-noori S. A., Tohidfar M., and Galuszka P. (2019). Agrobacterium-mediated genetic transformation of ajowan (*Trachyspermum ammi* (L.) Sprague): an important industrial medicinal plant. *Industrial Crops & Products,* 132(February): 29-40. DOI: https://doi.org/10.1016/j.indcrop.2019.02.005.

Panda B. M., Mehta U. J., and Hazra S. (2017). Optimizing culture conditions for establishment of hairy root culture of *Semecarpus anacardium* L. 3 *Biotech,*7: 21. DOI: 10.1007/s13205-017-0608-x.

Pang J., Cuin T., Shabala L., Zhou M., Mendham N., and Shabala S. (2007). Effect of secondary metabolites associated with anaerobic soil conditions on ion fluxes and electrophysiology in barley roots. *Plant Physiology,* 145: 266-276.

Pavlova O. A., Matveyeva T. V., and Lutova L. A. (2014). *rol*-Genes of *Agrobacterium rhizogenes*. *Russian Journal of Genetics: Applied Research,* 4: 137-145. DOI: https://doi. org/10.1134/S2079059714020063.

Rocha J., Eduardo-Figueira M., Barateiro A., Fernandes B. D., and Rosario B. (2015). Anti-inflammatory effect of rosmarinic acid and an extract of Rosmarinus officinalis in rat models of local and systemic inflammation. *Basic & Clinical Pharmacology & Toxicology,* 116(5): 398-413. DOI: 10.1111/bcpt.12335.

Sahayarayan J. J., Udayakumar R., Arun M., Ganapathi A., Alwahibi M., Aldosari N., Abubaker M., and Morgan A. (2020). Effect of different *Agrobacterium rhizogenes* strains for in-vitro hairy root induction, total phenolic, flavonoids contents, antibacterial and antioxidant activity of (*Cucumis anguria* L.). *Saudi Journal of Biological Sciences,* 27(11): 2972-2979. DOI: https://doi.org/10.1016/j.sjbs.2020.08.050.

Samadi A., Carapetian J., Heidary R., Jafari M., and Hssanzadeh A. (2012). Hairy root induction *in Linum mucronatum* ssp. an anti-tumor lignans production plant. *Nothlae Botanicae Hortiagrobatanici Cluj-Napaca*, 40(1): 125-131.

Sharafi A., Sohi H. H., Azadi P., and Sharafi A. A. (2014).

Hairy root induction and plant regeneration of medicinal plant *Dracocephalum kotschyi*. *Physiology and Molecular Biology of Plants,* 20: 257-262.

Sharma K., and Dubey S. (2011). Biotechnology and conservation of medicinal plants. *Journal of Experimental Sciences,* 2(10): 60-61.

Sun M., Shi M., Wang Y., Huang Q., Yuan T., Wang Q., Wang C., Zhou W., and Kai G. (2019). The biosynthesis of phenolic acids is positively regulated by the JA-responsive transcription ERF115 in *Salvia miltiorrhiza*. *Journal of Experimental Botany,* 70(1): 243-254. DOI: 10.1093/jxb/ery349.

Tiwari R. K., Trivedi M., Guang Z. C., Guo G. Q., and Zheng G. C. (2007). Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. *Journal of Plant Cell Reports,* 26: 199-210.

Vamenani R., Pakdin-Parizi A., Mortazavi M., and Gholami Z. (2020). Establishment of hairy root cultures by *Agrobacterium rhizogenes* mediated transformation of *Trachyspermum ammi* L. for the efficient production of thymol. *Biotechnology and Applied Biochemistry*, 67(3): 389-395. DOI: https://doi.org/10.1002/bab.1880.

Weber R. L. M., and Bodanese-Zanettini M. H. (2011). Induction of transgenic hairy roots in soybean genotypes by *Agrobacterium rhizogenes*-mediated transformation. *Journal of Pesquisa Agropecuária Brasileira,* 46: 1070-1075.

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 Organ Culture*, 126: 177-186.

Zarshenas M. M., Moein M. R., Samani S. M., and Petramfar P. (2013). An overview on ajwain (*Trachyspermum ammi*) pharmacological effect: modern and traditional. *Journal of Natural Remedies*, 14(1): 98-105.

Zhang Y., Mian M. R., and Bouton J. H. (2006). Recent molecular and genomic studies on stress tolerance of forage and turf grasses. *Crop Science,* 46(2): 497-511.

Zheng Q., Xu Z., Sun M., Liang H., WangY., Liu W., Huang P., and Zeng J. (2021). Hairy root induction and benzylisoquinoline alkaloid production in *Macleaya microcarpa*. *Plant Cell, Tissue and Organ Culture (PCTOC*), 147: 189-196. DOI: https://doi.org/10.1007/s11240-021-02109-z.

Zhu L. H., Holefors A., Ahlman A., Xue Z. T., and Welander M. (2001). Transformation of the apple rootstock M.9/29 with the *rol*B gene and its influence on rooting and growth. *Plant Science,* 160(3): 433-439. DOI: 10.1016/S0168-9452(00)00401-5.

Zhou L. G., and Wu J. Y. (2006). Development and application of medicinal plant tissue cultures for production of drugs and herbal medicinals in China. *Natural Product Reports*, 23(5): 789-810.