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Production of phenolic acids in hairy root cultures of medicinal plant *Mentha spicata* L. in response to elicitors

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ABSTRACT

In this study, hairy root induction in leaf and stem explants of *Mentha spicata* using various *Agrobacterium rhizogenes* strains was established for the first time. Although inoculation of explants by immersion method resulted in tissue necrosis, direct injection of explants by all examined strains (A13,R318,A4,GMI 9534 and ATCC15834) was effective. All different parts of the stem were susceptible to *A. rhizogenes* infection. However, the middle and lower internodes showed a higher rate of transformation. Among the different strains, the strain A13 exhibited the highest infection efficiency (almost 75% of the explants). A13 and R318-infected hairy roots showed the highest biomass production (close to 60 mg/flask), while infection with GMI 9534 produced the highest content of phenolic acids. Finally, the effect of phytohormone elicitation on hairy root growth and phenolic acid biosynthesis was investigated. A substantial increase in root growth and phenolic acids accumulation was obtained followed by 0.3 mg L^{-1} IBA and 100 µM MeJA treatment, respectively.

Keywords: *Mentha spicata*; Phenolic acids; *Agrobacterium rhizogenes*; Hairy root; IBA; MeJA

INTRODUCTION

Phenolic acids with a wide variety of pharmacological activities constitute an important group of secondary metabolites [1]. These compounds are produced as a defense response to protect plants from adverse environmental conditions [2]. The main phenolic acids such as rosmarinic acid (RA), chlorogenic acid (CGA), caffeic acid (CA), lithospermic acid B (LAB), and cinnamic acid (CIA) are mainly produced through the phenylpropanoid pathway [3]. The deamination of phenylalanine by phenylalanine ammonia lyase (*PAL*) generates CIA [3-6] which is converted by cinnamic acid 4-hydroxylase (*C4H*) and *p*-coumarate 3-hydroxylase (*C3H*) to CA as an important intermediate of the phenylpropanoid pathway [7]. It has been suggested that two aromatic amino acids, phenylalanine and tyrosine contribute in the biosynthesis of RA via two parallel and conjunct pathways: The phenylpropanoid and tyrosine-derived pathways. Chemically, RA and CGA are the esters of CA and LAB is considered as a dimer of RA. Nevertheless, the details of the biosynthetic pathway of LAB from RA have not yet been characterized [3-5]. Phenolic acids are present in plants in various concentrations and

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the plant species belonging to the Lamiaceae family are considered as a rich source of phenolic acids [1].

Mentha spicata, commonly called spearmint belongs to the Lamiaceae (Labiateae) family and is a perennial herbaceous plant. This plant is widespread in temperate and semi-temperate regions of the world, including Asia, Europe, North America, and Australia [8-10]. Spearmint is found in the wild in most parts of Iran. Members of the genus Mentha are used in the pharmaceutical, cosmetic and food industries due to their volatile oil compositions [8, 10]. This medicinal plant possesses antioxidant, antiviral, antimicrobial, and anti-fever properties due to the presence of high amounts of secondary metabolites such as: RA, CA, LAB, CGA and CIA [8-10]. Iranian spearmint has been extensively used in traditional medicine from ancient times for therapeutic purposes [11].

Since the secondary metabolites are often produced in limited quantities in differentiated tissues of medicinal plants, the extraction, isolation, and purification of theses metabolites is generally a major problem. It is desirable to use an efficient biotechnological method to produce these metabolites in less time and higher yield [12, 13].

Hairy root cultures provide many advantages as a persistent source for the production of valuable secondary metabolites due to their rapid growth rate, ease of preservation, biochemical and genetic stability, and capability to synthesize higher quantities of secondary metabolites relative to natural plant roots. Hairy root disease which is induced by *Agrobacterium rhizogenes*, a gram-negative bacterium, has gained attention as an effective biotechnological method to produce active plant ingredients [4, 14, 15]. So far, different *A. rhizogenes* strains have been used for plant transformation. These strains are divergent in terms of virulence, growth rate and morphology. This is partially related to different root-inducing (Ri) plasmids being harbored by each Agrobacterium strain [16].

In the past few years, several strategies such as elicitation have been applied in hairy root cultures to increase the biomass and desirable secondary metabolite production [13]. The effect of phytohormones as abiotic elicitors on root growth and productivity has been widely studied [17-19]. It has been reported that elevated levels of auxins promote the growth rate of hairy roots [20] and among the different types of auxins, indole-3-butyric acid (IBA) is the most effective [21]. Some hormonal elicitors such as methyl jasmonate (MeJA) play an important role in the plant defense response and induce the production of secondary metabolites such as phenolic acids in hairy root cultures [4, 22, 23].

Production of phenolic acids by hairy root cultures of some species from the Lamiaceae family have been reported [22-28]. With regard to the economic and medicinal importance of *M. spicata*, in this research, we investigated the induction of hairy roots and phenolic acid production through transformation of this plant via different strains of *A. rhizogenes*. The percentage of hairy root induction in five strains of *A. rhizogenes* (ATCC15834, GMI 9534, A13, A4 and R318), dry weight and the levels of RA, CA, LAB, CGA and CIA accumulation were studied. Moreover, the effects of IBA and MeJA on phenolic acid biosynthesis were evaluated.

MATERIALS AND METHODS

In this study, first, the susceptibility of two different types of explant (leaf and stem) was assessed using two inoculation methods (immersion and injection). In the next step, different parts of stems (lower, middle and upper internodes) were evaluated to choose the best part for hairy root induction. Moreover, five different strains of *A. rhizogenes* were screened for their hairy root induction capacity and the accumulation level of phenolic acids. Finally, the *A.rhizogenes* strain with the best induction capacity was selected for investigation of the phytohormone effect.

Plant material and sterilization: The cuttings of *M.spicata* were collected from Kerman, Iran. The leaves and shoots were both tested to select the more suitable explant for hairy root induction. Young leaves and stems (20 ± 6 cm in length) were taken from the plants grown *in vitro* at the phytotron of the National Institute of Genetic engineering and Biotechnology (NIGEB, Tehran, Iran). Leaf sections and internodes from different parts of the shoot (approximately 1.5 ± 0.5 cm segments) after a brief soaking in a soap solution were surface sterilized with ethanol 70% (3 min) and 1% sodium hypochlorite containing one drop of Tween 20% (3-4 min). Finally, the explants were rinsed three times with sterile double distilled water.

***Agrobacterium rhizogenes* growth and hairy root induction by two inoculation methods:** Five different strains of *A.rhizogenes* ATCC15834, GMI 9534, A13, A4 and R318 were provided by the bank of microbes at NIGEB (Tehran, Iran). The strain ATCC15834 was used for optimizing the type of explants and inoculation method. Hairy root induction was performed by two methods of inoculation (immersion and injection). In the first method, a single colony of bacteria was cultured overnight in 10 ml of liquid Luria-Bertani (LB) medium on a rotary shaker (180 rpm) at 28 ^{0}C. The bacterial pellet was resuspended in 25 ml of liquid MS medium containing 0.5% glucose and 100 µM acetosyringone. The culture was incubated at 28 ^{0}C on a rotary shaker (180 rpm) for approximately 2 hours. The suspension culture with an optical density of OD_{600}= 0.5 was used to infect explants. The leaf and stem sections were immersed in the suspension (3-5 min) followed by drying on sterile filter paper and culturing on full-strength MS solid medium containing 30 g L^{-1} sucrose and 8 g L^{-1} agar (pH= 5.7). In the injection method, the bacterial strain initiated from glycerol stocks were cultured in 5 ml of liquid Luria-Bertani (LB) medium on a rotary shaker (180 rpm) at 28 ^{0}C for 48 h. The bacterial strain was streaked on solidified LB medium and incubated at 28 ^{0}C for 48 h. Hairy root induction was performed by direct puncturing of the root side of internode segments and the midrib at the back part of the leaves using a needle infected by bacterial paste. The explants were cultured on full-strength MS solid medium by placing the infected side up at 25^{0}C in the dark. To eliminate bacteria, the infected explants were washed with 250 mg L^{-1} cefotaxime (Exir pharmaceutical co. Iran) solution and moved to full-strength MS solid medium containing 250 mg L^{-1} cefotaxime (pH= 5.7) after drying on sterile filter paper. Hairy roots appeared at the injured sites within about 14 ± 3 days. The emerging hairy roots were subcultured on full-strength MS solid medium followed by transferring the isolated roots to 50 ml of half-strength MS liquid medium (pH= 5.7) containing 30 g L^{-1} sucrose and 250 mg L^{-1} cefotaxime. All cultures were incubated at 25^{0}C on a gyratory shaker at 110 rpm in the dark. Hairy roots were transferred to fresh medium every two weeks and the concentration of antibiotic was gradually reduced. Finally, the hairy roots free of bacteria were harvested from the culture medium after approximately 3 months. Fresh weight (FW) was determined after washing the hairy roots by distilled water and blotted dry by filter paper. Dry weight (DW) and phenolic acids (RA, CA, LAB, CGA and CIA) content of hairy roots were measured after lyophilization. Three distinct flasks were used for each culture.

Preparation and treatment of phytohormones: IBA (Duchefa Biochemie, Netherland) and MeJA (Sigma-Aldrich, USA) were dissolved in NaOH and distilled water, respectively. IBA was filter-sterilized (Orange scientific 0.2 µm) and added in 50 ml of autoclaved half-strength MS liquid medium (pH= 5.7) with a final concentration of 0.3 mg L^{-1}. In order to investigate the effect of IBA on growth rate and phenolic acids accumulation, a single hairy root line with an initial biomass of 0.3 g was cultured in half-strength MS medium supplemented with IBA for approximately 87 days. Half-strength MS medium without IBA was considered as control culture. MeJA treatment was performed on day 87 post-inoculation (the end of exponential growth phase) and the medium was substituted with 50 ml of half-strength MS liquid medium (pH= 5.7) supplemented with filter-sterilized MeJA with a final concentration of 100 µM. Control roots were grown in half-strength MS medium without MeJA. Phenolic acids

production was measured after 4 days (96 hr) of culture. Three independent biological replicates were considered for experiments.

Genomic DNA extraction and PCR: Genomic DNA of *M.spicata* transformed hairy roots and non-transformed roots (as negative control) were extracted by the CTAB method [29]. The presence of the *rol*B gene in *M.spicata*'s hairy roots was approved by PCR. Moreover, PCR analysis with the *vir*G gene was performed to confirm any bacterial contamination. The sequences of primers which were designed to amplify *rol*B and *vir*G are listed below.

Primers for *rol*B gene

F:5'- GCTCTTGCAGTGCTAGATTT-3' and R: 5'- GAAGGTGCAAGCTACCTCTC-3'

Primers for *vir*G gene

F:5'- GGTCGCTATGCGGCATC-3' and R: 5'- CCTGAGATTAAGTGTCCAGTCAG-3'

Extraction and HPLC analysis of RA, CA, LAB, CGA and CIA: About 50 mg of freeze-dried hairy root samples were finely powdered and blended in 10ml of 60% ethanol. Thereafter, the ultrasonic (Elma Ultrasonics, Germany) extraction method at 40 ^{0}C for 30 min was used as an appropriate extraction method for phenolic components followed by centrifugation at 10000 rpm for 30 min. The supernatant was separated, and the residue was macerated in 60% ethanol and the rest of steps were done as mentioned previously. The supernatant from both fractions were pooled, filtered and the solvent was evaporated via a rotary evaporator (Buchi rotavapor, Switzerland) at 40 ^{0}C. HPLC analysis of extracts was carried out by a Knauer HPLC system (Berlin, Germany) equipped with a K-1001 HPLC pump, a Rheodyne injector including 20 µL sample loops model 7725i and a K-2800 photodiode array detector (PDA). Chromatography assessments were performed with a stainless steel Reversed-Phase C_{18} column (150 mm × 4.6 mm, 5 µm particle size, 100 A, Beckman, USA) at room temperature. EZChrom Elite software was used to monitor and acquire chromatographic data. The injection volume of the sample was 20 µl and the desired phenolic acid compounds were detected at 257, 275, 300 and 320 nm, while the flow rate was 0.7 ml min^{-1}. The mobile phase was consisted of solvent (A): HPLC-grade water with 0.02% trifluoroacetic acid (TFA) and solvent (B): 0.02% TFA in methanol. The separation of five major phenolic acids was achieved using the following gradient elution: 0-5 min, 5% solvent B which reaches to 15% within 15 min; 15-25 min, 35% solvent B; 25-45 min, 55% solvent B, and at 45-55 min solvent B reaches to 100%.

Standard curves: A multi-level calibration method was performed. Standard solutions of RA, CA, LAB, CGA and CIA were prepared at five concentrations in the range of 50, 25, 12.5, 6.25 and 1.5 mg L^{-1} to construct standard curves. The linearity of the HPLC method was checked by plotting 5-point calibration curves. Each point of linearity curve was obtained as the average of three consecutive injections. The regression equations and correlation coefficients (R^2) were as follows: Rosmarinic acid [y = 47042x – 13498] (R^2 = 0.9968); Caffeic acid [y = 133303x – 141312] (R^2 = 0.9976); Lithospermic acid B [y = 25776x - 66519] (R^2 = = 0.9729); Chlorogenic acid [y = 67993x – 43319] (R^2 = 0.9995); Cinnamic acid [y = 180282x – 381069] (R^2 = 0.9894). It should be noted that the standards were purchased from Sigma-Aldrich (USA).

Statistical analysis: This study was conducted based on a completely randomized design. The values represent the average ± SD of three independent biological replicates. The data were analyzed using IBM SPSS 23 statistical software. The data were compared by analysis of variance and the Duncan's multiple range tests were used to compare means (P≤0.05).

RESULTS

According to the results shown in figure 1, between the two different explants of leaves and shoots tested, shoots demonstrated a higher rate of transformation and were selected as the more

suitable explants for hairy root induction. The induction of hairy roots through the immersion method was not effective for either of the two types of explants. Necrosis occurred in all explants in a short time after *Agrobacterium* infection with the immersion method, due to the hypersensitive reaction of spearmint's delicate tissues. The next experimental procedures were carried out by injection, as an efficient method in internodal segments of the stem.

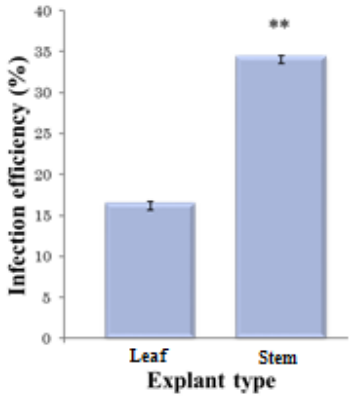

Figure 1: Comparative statistical positive responses of the leaf and stem explants of *M. spicata* to direct injection by *A.rhizogenes*.

All different parts of stem (lower, middle and upper internodes) were susceptible to *A.rhizogenes* infection. There were no statistically significant differences in terms of hairy root formation between the different parts. However, as shown in figure 2, the middle and lower internodes exhibited a higher rate of transformation: 18.82% and 25.6% respectively. The percentage of transformation was calculated as the number of explants inducing hairy root over the total number of infected explants.

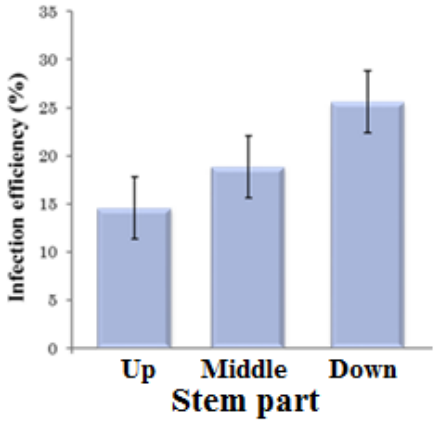

Figure 2: The percentage of hairy root induction in different parts of stem.

Five different strains of *A.rhizogenes* were used to induce hairy roots in stem explants. Hairy root induction through the mentioned method was efficient in all the strains; however, the hairy root formation rate, dry weight, root elongation, and the morphology of hairy roots were different in each strain. Among the different strains of *A.rhizogenes*, A13 exhibited the highest infection efficiency followed by R318. The percentage of rooting in strains A13, R318, A4, GMI 9534 and ATCC 15834 were 75%, 56.25%, 24.32%, 13.33% and 10%, respectively (Fig. 3a). A13 and R318-infected hairy roots indicated the highest growth rates (60.56 and 59.53 mg/flask, respectively) (Fig. 3b). Furthermore, according to the Table 1, different root morphologies were observed between the five strains. Short and few lateral branches were

observed for the A4 and R318 strains, while the number of long lateral branches in hairy roots which were induced by GMI 9534, ATCC 15834, and A13 were more than the other strains.

Table 1: Effect of different *A. rhizogenes* strains on hairy root morphology

Strain	Hairy root length (cm)	Number of lateral roots/root
A13	20.2 ± 0.36^{a}	40 ± 1^{e}
R318	10.33 ± 0.85^{d}	9.66 ± 0.57^{b}
A4	7.46 ± 0.7^{e}	5.33 ± 1.15^{a}
9534	13.05 ± 0.63^{c}	33.33 ± 1.52^{d}
15834	15.33 ± 0.92^{b}	26 ± 1^{c}

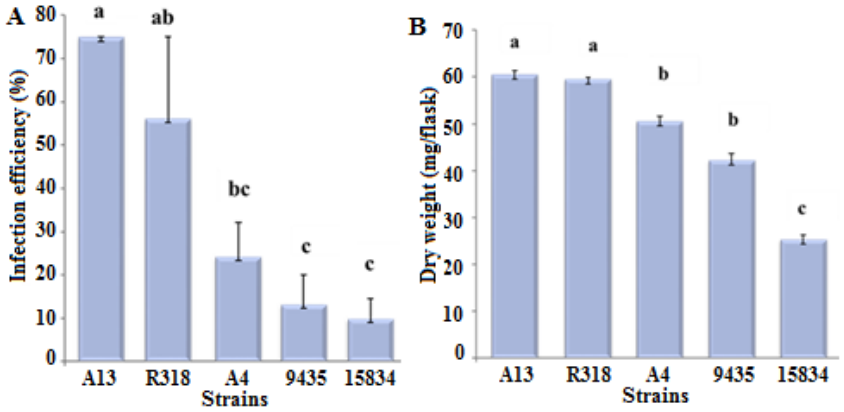

Figure 3: (A) The percentage of hairy root induction in different strains of *A. rhizogenes*. (B) Growth rate of different strains of *A. rhizogenes*. Different letters on columns show significant difference between the strains.

The integration of T-DNA into the plant hairy roots genome was confirmed by amplification of a 430bp fragment related to *rol*B gene in transformed roots. The *A.rhizogenes* plasmid was used as positive control, while the expected fragment was not amplified in the genomic DNA of non-transformed roots as negative control. PCR analysis of hairy roots with *vir*G primers exhibited no contamination with *A. rhizogenes*.

The individual levels of phenolic acids (RA, CA, LAB, CGA and CIA) in hairy roots of *Mentha spicata* were detected by HPLC (Fig. 4). Regardless of the different strains, all five phenolic acids were identified in the hairy root samples. The results indicated that CA, LAB and CIA comprise the dominant amount of phenolic acids in *Mentha spicata*'s hairy root infected with different strains of *A. rhizogenes*. Among the strains, infection with GMI 9534 produced the highest content of CA, LAB, CIA, RA, and CGA (106.76, 60.22, 44.02, 20.08, and 13.53 µg g^{-1} DW (dry weight of hairy roots), respectively). Following by GMI 9534 strain, the content levels of CGA (9.65 µg g^{-1} DW) and CIA (34.08 µg g^{-1} DW) were significantly higher in hairy roots infected with A13 compared to other strains. However, there were no significant variations among A13, A4, and ATCC15834 strains in terms of CA and RA content (p-value < 0.05). LAB content was significantly higher in the A13 (37.54 µg g^{-1} DW) and A4 (27.12 µg g^{-1} DW) strains compared to the ATCC15834 (22 µg g^{-1} DW) and R318 (22.69 µg g^{-1} DW) strains. Furthermore, in the case of R318 strain, the observed levels of RA, CA and CIA were the lowest.

The growth rate of control hairy roots was very slow, while a significant increase in the growth rate of *M. spicata* hairy roots was observed after exogenous auxin treatment (Table 2). The results indicated that supplementation of 0.3 mg L^{-1} IBA significantly (P<0.05) induced CA and LAB accumulation about 3-fold and 1.5-fold more than the control samples, respectively. There were no statistically significant differences in terms of RA and CGA content between the control and IBA-treated samples, whereas the content of CIA significantly

decreased about 1.5-fold lower than the control samples. This may be due to the role of CIA as the CA precursor.

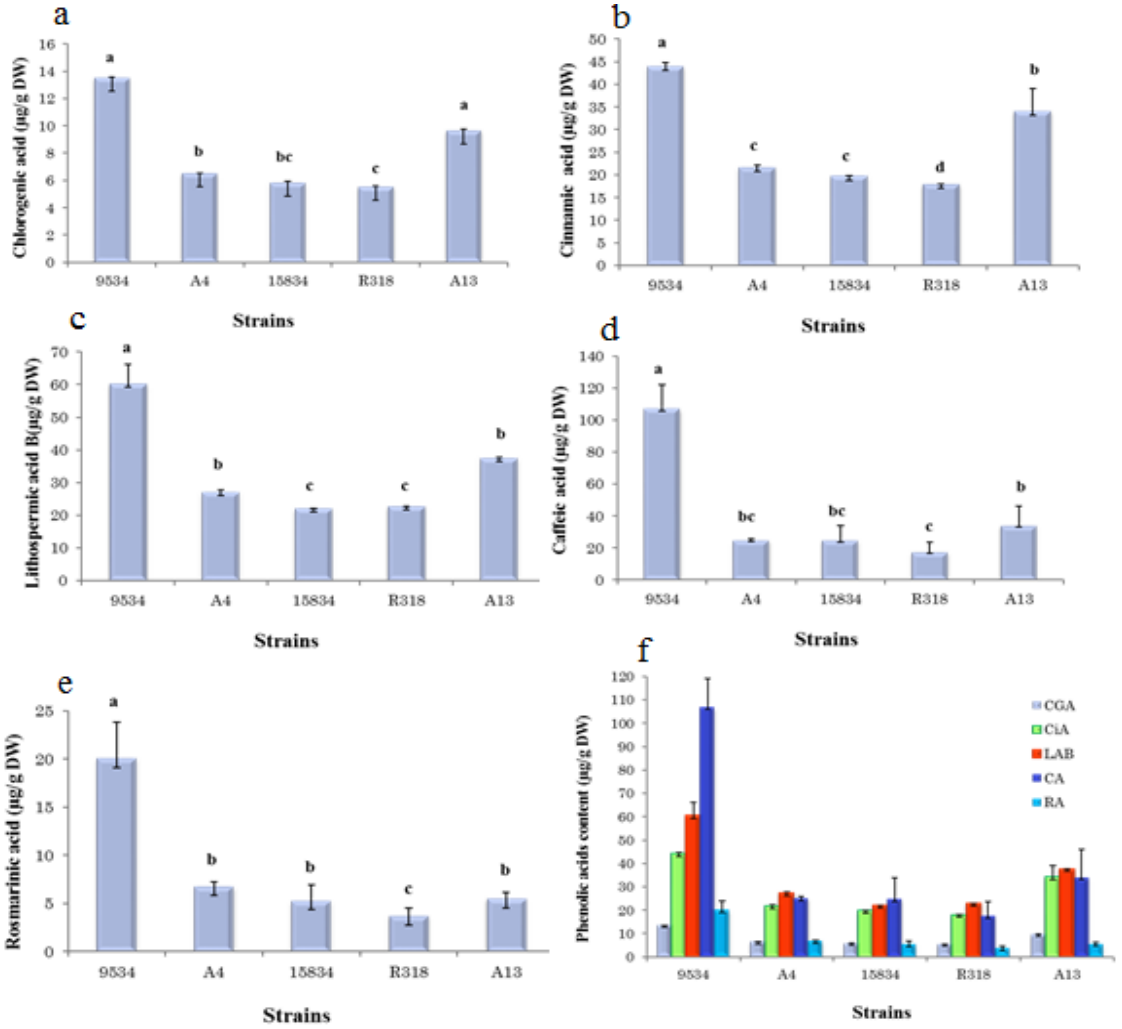

Figure 4: The content level of CGA (a), CIA (b), LAB (c), CA (d) and RA (e) in response to different *A. rhizogenes* strains. The comparison of five phenolic acids content among different strains (f). Different letters on columns show significant difference between the strains.

Table 2: Effect of IBA on growth rate and phenolic acids accumulation after 87 days of culture

Elicitation	Fresh mass (g/50 ml)	CGA (µg g^{-1} DW)	CA (µg g^{-1} DW)	RA (µg g^{-1} DW)	CIA (µg g^{-1} DW)	LAB (µg g^{-1} DW)
IBA (0.3 mg L^{-1})	8.13 ± 1.05**	9.15 ± 1.51	107.63±19.23**	4.67 ± 0.69	21.74 ± 0.21	57.69± 10.11*
Control	1.1 ± 0.2	9.65 ± 0.1	33.99 ± 12.09	5.49 ± 0.66	34.08 ± 4.97*	37.54 ± 0.12

The asterisks indicate statistically significant differences at $P< 0.05$ ($0.01<P< 0.05$*, $P<0.01$**) between the IBA treated cultures and that in the corresponding controls.

Phenolic acids accumulation was determined in hairy roots elicited with MeJA and control samples (non-elicited with MeJA) after 96 hours (Table 3). MeJA application significantly induced CGA, RA, and CIA content about 1.4-fold, 4.4-fold, and 1.9-fold more than the control samples, respectively. There was no statistically significant difference in terms of LAB content between the control and MeJA-treated samples, whereas the content of CA significantly decreased about 1.5-fold lower than the control samples.

Table 3: Effect of MeJA on phenolic acids accumulation

Elicitation	CGA (µg g^{-1} DW)	CA (µg g^{-1} DW)	RA (µg g^{-1} DW)	CIA (µg g^{-1} DW)	LAB (µg g^{-1} DW)
MeJA (100 µM)	13.62 ± 0.28*	71.51 ± 11.19	20.86 ± 0.2**	42.81 ± 0.17**	57.60 ± 0.39
Control	9.15 ± 1.51	107.63 ± 19.23*	4.67 ± 0.69	21.74 ± 0.21	57.69 ± 10.11

The asterisks indicate statistically significant differences at $P<0.05$ ($0.01<P<0.05$*, $P<0.01$**) between the MeJA treated cultures and that in the corresponding controls.

DISCUSSION

Over the past few decades, the medicinal and commertial importance of secondary metabolites have been resulted in a great interest in production of phytochemicals by means of plant tissue culture techniques. Several biotechnological strategies such as optimizing culture conditions, metabolic engineering and elicitation can be applied to improve the production of important secondary metabolites [30].

Elicitation is defined as the stimulation of plant stress response to enhance secondary metabolites production in *in vitro* plant cultures [13]. The plant tissue cultures can be elicited by biotic (pathogen-derived elicitors) or abiotic elicitors (signaling molecules such as phytohormones) [31, 32]. Phenolic acids as defense agents play a key role in plant responses to biotic and abiotic stresses to protect them against any threats [32, 33].

The pathogen invasion and wounding, such as infection by *A. rhizogenes* induce the biosynthesis of jasmonates, particularly MeJA, that activates a signal transduction process [32, 31]. Followed by infection, the flux through the phenylpropanoid pathway increases that results in accumulation of antimicrobial compounds, especially phenolic acids. However, the plant response to infection differs among species [34].

Several studies have reported that the species of the Lamiaceae family are valued as rich sources of natural antioxidants [35-37]. Within this family, the pharmacological properties of *Mentha spicata* such as the antibacterial and antimutagenic activities have been widely described due to its phenolic acid content [9, 35-37]. As previously mentioned, hairy root cultures are considered as an alternative approach for producing valuable secondary metabolites. Hairy root induction is highly related to tissue type, plant genotype, and the interaction of bacterial strains with the plant species [38]. Therefore, it is imperative to evaluate the suitable types of explants and bacterial strains to optimize the transformation event [17, 39]. In the present investigation, the susceptibility of two different types of spearmint explants (leaf and stem) to infection by *A. rhizogenes* using two inoculation methods (immersion and injection) was investigated. It has been presumed that phloem cells that are located deep in plant organs contain high levels of sucrose and auxin and could be suitable target sites for *A. rhizogenes* infection [38, 40]. Stems that were wounded deeply demonstrated a higher rate of transformation and were selected as a more suitable explant for hairy root induction. In our study, the immersion method was not successful for hairy root induction due to necrotic reactions in explants. Necrosis reaction and cell death, which is observed in many *Agrobacterium*-infected plants, may be the consequence of the plant's defense in response to bacteria, called the hypersensitive reaction [41].

It has been well documented that *A. rhizogenes* strains have different capabilities to stimulate the production of various secondary metabolites in hairy root cultures [16, 42, 43]. Here in this research, the phenolic acids production was compared among five different *A. rhizogenes* strains: ATCC15834, 9534, A13, A4, and R318. Our results revealed that *A. rhizogenes* strain 9534 was the best in terms of CA, LAB, CIA, RA, and CGA content (106.76, 60.22, 44.02, 20.08, and 13.53 µg g^{-1} DW, respectively). It can be concluded that the potential of the strain 9534 as a biotic elicitor to induce the production of phenolic acids is higher than the other strains. With respect to CA (33.99 µg g^{-1} DW), LAB (37.54 µg g^{-1} DW), CIA (34.08 µg g^{-1} DW), and CGA (9.65 µg g^{-1} DW) accumulation levels, the strain A13 was in the second place.

It is well reported that the difference in the ability of hairy root induction among the *A. rhizogenes* strains is specifically related to differential expression and integration of T-DNA genes into the plant genome [16, 39, 43, 45]. It has been confirmed that hairy root stimulation and phenolic compound accumulation are strain-specific and the selection of an effective strain is dependent on plant species [16]. In our experiments, five different *A. rhizogenes* strains were capable of hairy root formation through the applied method with various transformation efficiencies and hairy root morphologies. Our findings showed that among the employed strains, A13 and R318 were more effective for hairy root induction in spearmint. Diverse root morphologies have been ascribed to the different types of root-inducing (Ri) plasmids being harbored by each Agrobacterium strain [16, 42]. Generally, our results indicated that the *A. rhizogenes* strain 9534, despite the higher levels of phenolic acids production, was not efficient in hairy root induction. Overall, A13 has been suggested as a potent candidate for the induction of hairy root in stem explants of spearmint due to high infection efficiency, growth rate and appropriate accumulation level of important phenolic acids. Subsequently, induced hairy roots with the strain A13 were further investigated for the phytohormones effect.

We constructed a heatmap to visualize the changes in individual amounts of phenolic acids in response to different *A. rhizogenes* strains (as biotic elicitors) and elicitation with phytohormones (as abiotic elicitors) (Fig. 5). It has been proven that MeJA is a signal molecule that induces the production of various plant secondary metabolites through the activation of related biosynthetic genes [22]. As can be seen in figure 5, MeJA efficiently enhanced the content of CGA, RA, and CIA in hairy root cultures of *M. spicata*, induced by the strain A13 of *A. rhizogenes*. As stated earlier, CGA is a CA ester. An induction in CGA content in MeJA-treated hairy roots was observed in parallel with the reduction of CA accumulation. It has been documented that application of plant growth regulators in hairy root cultures affects the biomass and secondary metabolites accumulation [18, 21]. Based on several published literature among the plant growth regulators of auxin, IBA is greatly responsible for secondary root formation in hairy root cultures [44]. In our experiments, despite the dramatic effect of IBA on the growth rate of spearmint hairy roots, its effect on phenolic acids accumulation was only remarkable on CA and LAB production. The content of RA was not affected by IBA treatment. This was reasonable due to the role of RA as the LAB precursor. Increased production of LAB resulted in more precursor transformation.

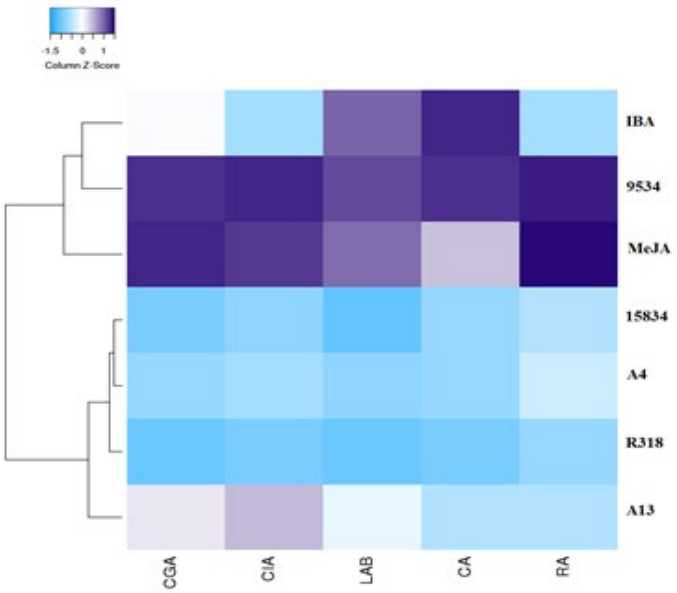

Figure 5: Heatmap represents the changes in phenolic acids content in response to different *A. rhizogenes* strains and elicitation. The relative levels of each metabolite are indicated by the intensity of the blue color in which light blue and dark blue represent low and high concentrations of phenolic acids, respectively.

In conclusion, our findings in this study represent an efficient method for hairy root induction and phenolic acid production in Iranian spearmint using five different strains of *A. rhizogenes*. Transformation efficiency, growth rate, hairy roots morphology, and phenolic acid production were affected by bacterial strains. According to the obtained results, the strain GMI 9534 was an efficient strain for phenolic acid production, whereas the strain A13 was recommended as a suitable strain for mass production of hairy roots with an appropriate accumulation level of important phenolic acids. IBA treatment along with MeJA elicitation is suggested as an effective approach for fast growth and stimulation of phenolic acids production in *M. spicata* hairy root cultures.

New surveys are in progress in our laboratory to increase the accumulation levels of valuable compounds like rosmarinic acid through investigating the molecular mechanism of key genes involved in phenolic acids biosynthetic pathways.

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Conflict of Interest: The authors declare that this article has no conflicts of interest.

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