



Elicitor-induced phenolic acids accumulation in *Salvia virgata* Jacq. hairy root cultures

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Received: 30 January 2021 / Accepted: 9 September 2021 / Published online: 27 September 2021
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Abstract

Phenolic acids, as the predominant secondary metabolites of *Salvia* plants, are largely used in pharmaceutical industries. The main aim of the study was to establish hairy root cultures of *Salvia virgata* Jacq. Also, the effects of methyl jasmonate (22.4 and 11.2 ppm), Ag⁺ ions (5 and 2.5 ppm) and yeast extract (100 and 50 ppm) were assessed on total phenol, total flavonoid, rosmarinic acid and salvianolic acid A contents in the hairy roots after 1, 3 and 5 days of exposure. Results showed that *Agrobacterium rhizogenes* strains (A4, ATCC15834, R1000, GM1534 and C58C1) had different potential to induce hairy roots on leaf explants. The transformed roots were molecularly confirmed using *rolC* gene, and the highest transformation frequency (56%) was obtained by ATCC15834 strain. Among the established hairy root lines, the highest amount of rosmarinic acid (0.45 ± 0.01 mg/g DW) and dry root biomass (2.29 ± 0.04 g) was obtained in AT3, the line which was induced by ATCC15834 strain. The maximum accumulation of total phenol (123.6 ± 0.93 mg GAE/g DW), total flavonoid (5.09 ± 0.07 mg QUE/g DW), rosmarinic acid (18.45 ± 0.8 mg/g DW) and salvianolic acid A (2.11 ± 0.04 mg/g DW) was observed in the hairy roots elicited with 22.4 ppm methyl jasmonate on day three after treatment. The results support that elicitation could be an effective procedure for the improvement of caffeic acid derivatives production in *S. virgata* hairy root cultures.

Key message

The results of this study approved that the application of elicitors is an effective procedure for the stimulation of phenolic acids production in the hairy root cultures of *Salvia virgata* Jacq.

Keywords *Agrobacterium rhizogenes* · Hairy root · Rosmarinic acid · *Salvia virgata* Jacq. · Salvianolic acid A

Abbreviations

CA	Caffeic acid
RA	Rosmarinic acid
Sal-A	Salvianolic acid A
Sal-B	Salvianolic acid B
HR	Hairy root
TP	Total phenol
TF	Total flavonoid

YE	Yeast extract
Ag ⁺ ions	Silver ions
MeJA	Methyl jasmonate

Introduction

Salvia virgata Jacq., an annual plant belonging to the Lamiaceae family, is native to Asia (northeast of Iran) and south-eastern Europe. The plant has traditionally been used to treat skin diseases, injuries and blood cancer (Baytop 1999). Some of the important biological properties of this species, including antioxidant (Karatoprak et al. 2016; Dehghani Latani et al. 2019), antimicrobial (Alizadeh 2013), antifungal (Bayar and Yilar 2019), anti-inflammatory and antinociceptive activities (Akkol et al. 2008) have been reported in the literature.

Based on in vitro and in vivo studies, the presence of some phenolic acids, such as caffeic acid (CA), rosmarinic

Communicated by Patricia Marconi.

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acid (RA), salvianolic acid A (Sal-A) and salvianolic acid B (Sal-B) has been reported in the whole plant and shoot cultures of *S. virgata* (Akkol et al. 2008; Ejtahed et al. 2015; Attaran Dowom et al. 2017; Fotovvat et al. 2018). Recent studies have described numerous biological/pharmacological properties for these phenolic acids, including antioxidant, antibacterial, antifungal (Grzegorzczuk-Karolak et al. 2018; Katanić Stanković et al. 2020), antiviral (Kim et al. 2020), anti-inflammatory (Liu et al. 2018), hypoglycemic (Huang et al. 2016) and anticancer (Swamy et al. 2018; Zhang et al. 2018; Katary et al. 2019) properties. Furthermore, it has been shown that RA and Sal-B could improve memory and cognitive impairment related to Alzheimer's disease (Gok et al. 2018).

Chemically, CA is the basic structural unit of phenolic acids in *Salvia* species. Rosmarinic acid is an ester of CA and β -3,4-dihydroxy phenyl-lactic acid (Danshensu). Salvianolic acid A is formed by two molecules of CA and one molecule Danshensu, and Sal-B is identified as a dimer of RA (Xu et al. 2014; Wang et al. 2019).

Phenolic acids are mainly synthesized via the phenylpropanoid and tyrosine-derived pathways (Di et al. 2013). Many of the genes encoding critical enzymes in the phenolic acids biosynthetic pathways, including phenylalanine ammonia-lyase (*PAL*), tyrosine aminotransferase (*TAT*), rosmarinic acid synthase (*RAS*), hydroxycinnamate: coenzyme A ligase (*4CL*), cinnamic acid 4-hydroxylase (*C4H*), 4-hydroxyphenylpyruvate reductase (*HPPR*), and a cytochrome P450-dependent monooxygenase (*CYP98A14*), were identified in *Salvia miltiorrhiza* Bunge (Di et al. 2013; Zhang et al. 2014; Wang et al. 2015; Xing et al. 2018).

Since the phenolic acids are generally produced in small quantities in differentiated tissues of medicinal plants, it is preferable to use an effective and profitable biotechnological method to produce these valuable compounds in less time with more efficiency (Grzegorzczuk-Karolak et al. 2018; Yousefian et al. 2020). Hairy root (HR) cultures can be considered as stable sources for the biosynthesis of secondary metabolites due to their genetic and biochemical stability, large biomass output and high biosynthetic capacity (Ono and Tian 2011).

It has been demonstrated that the high amounts of phenolic acids, such as RA, are synthesized in the HR cultures of some members of Lamiaceae family, including *Salvia officinalis* L. (Grzegorzczuk et al. 2006), *S. miltiorrhiza* (Yan et al. 2006; Xiao et al. 2010; Zhao et al. 2011; Di et al. 2013), *Coleus forskohlii* Briq. (Li et al. 2005) as well as RA and Sal-B in *S. miltiorrhiza* (Xiao et al. 2010; Di et al. 2013). According to some reports, the accumulation of different phenolic acids, such as CA, RA, Sal-A and Sal-B, has been improved in HR cultures of *Coleus blumei* Benth. (Bauer et al. 2009), *S. miltiorrhiza* (Xiao et al. 2009, 2010; Hao et al. 2012; Zhang et al. 2015; Xing et al. 2015, 2018), *S.*

officinalis (Grzegorzczuk and Wysokińska 2009) and *Mentha spicata* L. (Yousefian et al. 2020) after elicitation with yeast extract (YE), silver ions (Ag^+), methyl jasmonate (MeJA) and plant growth regulators.

There is only one report (in Persian) available on the induction of HR cultures in *S. virgata* (Norouzi et al. 2017), and up to our knowledge, there is no report regarding the elicitation of phenolic acids biosynthesis in HR cultures of *S. virgata*. Therefore, this study was designed to develop an efficient HR culture system for *S. virgata*, using different *Agrobacterium rhizogenes* strains, and to examine the effects of biotic and abiotic elicitors on RA and salvianolic acids production in the transformed root cultures for the first time.

Materials and methods

Seed germination and growth conditions

In August 2013, the mature seeds of *S. virgata* were gathered from wild-grown plants in Reine region (Bojnord, Iran), which is situated at latitude 57°2' N, longitude 37°23' E and elevation 1765 m above sea level. The species' voucher specimen (No. 38128) was deposited at the Ferdowsi University of Mashhad Herbarium (FUMH). The mature seeds of *S. virgata* were surface sterilized in 70% (v/v) ethanol for 1 min followed by 5% (v/v) sodium hypochlorite solution for 5 min and then rinsed three times with sterile distilled water. Seeds were cultured in glass jars with 25 mL of solid Murashige and Skoog (MS) medium supplemented with 3% sucrose and 0.7% agar-agar for germination. The pH of the MS basal media was adjusted to 5.6–5.8 before the addition of agar, and then they were autoclaved at 120 °C for 17 min. The glass jars were kept in the dark for 3 days at 25 ± 2 °C and then transferred to a growth chamber at 26 ± 2 °C and 16/8 h (light/dark) photoperiod (45 $\mu\text{mol}/\text{m}^2/\text{s}$ given by cool white fluorescent lamps).

Growth of *A. rhizogenes*

Five *A. rhizogenes* strains (ATCC15834, R1000, A4, C58C1, and GM1534), which were obtained from the Biotechnology Research Center, Karaj, Iran, were incubated on Luria-Bertani (LB) agar medium (Vervliet et al. 1975) with 50 ppm rifampin at 28 °C for 48 h.

Induction of hairy root cultures

Leaf segments from aseptic in vitro grown 50-day-old seedlings were used as explants. The explants were pierced with a needle dipped in an *A. rhizogenes* culture. The bacterial culture was incubated on a fresh LB solid medium for 48 h under darkness before being used to transform the explants.

Uninfected control explants were inoculated with a sterile, bacteria-free needle. The explants from infected and control groups were cultured on hormone-free MS agar (0.7%) medium and incubated in the dark at 25 °C. After 2 days of inoculation, the explants were transferred to MS basal medium supplemented with sucrose (3%), cefotaxime (300 ppm), and Difco Bacto agar (0.7%). The cultures were incubated under a 16/8 h (light/dark) photoperiod (45 $\mu\text{mol}/\text{m}^2/\text{s}$ given by cool white fluorescent lamps) at 25 °C. The procedure was repeated three times for each bacterial strain with 25 explants.

The transformation frequencies (the percentages of explants forming roots after infection with *A. rhizogenes* strains) were determined 10–14 days after infection. After 30 days post-inoculation, fifteen fast-growing lines, induced by all bacterial strains, were selected. Excised HR tips (0.6 g) were transferred into the separate 250 mL Erlenmeyer flasks filled with 50 mL hormone-free 1/2 MS liquid medium containing 3% sucrose and 300 mg/L cefotaxime as described by Grzegorzczuk-Karolak et al. (2018), with some modifications. Three replicates were considered for each line. The cefotaxime concentration was gradually decreased to 200 and 100 mg/L during the second and third subcultures, respectively. The flasks were incubated at 25 °C in the dark on a rotary shaker (Noor Sanat Ferdows, Iran) at 100 rpm. After six subcultures during 2 months of culture, the dry weight and amount of RA were measured in HR lines induced by all strains. The content of RA in HR lines was measured by spectrophotometry according to Öztürk et al. (2010). Finally, one HR line with the highest biomass and RA content (line AT3 induced by ATCC15834 strain) was chosen and used for elicitor treatments.

Confirmation of transgenic hairy root lines

The genomic DNA (gDNA) from five putative transformed root lines of each strain and nontransformed roots was extracted from 100 mg of plant tissue following the procedures described by Sharp et al. (1988), with slight modifications. In this study, DNA from *A. rhizogenes* ATCC15834 strain served as the positive control, and DNA isolated from the roots of uninfected 50-day-old seedlings grown in MS medium was used as the negative control.

The Polymerase Chain Reaction (PCR) was carried out to amplify an internal *rolC* gene fragment (612 bp). The PCR was performed on the C1000 Touch TM 96-Well Thermal Cycler (Bio-Rad, USA). The PCR reaction was contained Taq PCR Master Mix Kit (Qiagen), gDNA (100 ng total DNA) and oligonucleotide primers (10 μM final concentration in the total volume of 15 μL). The primers used to detect the *rolC* gene were 5'-ATG GCT GAA GAC GAC CTG TGT T-3' and 5'-TTA GCC GAT TGC AAA CTT GCA C-3'. The PCR program was included a 4 min denaturing

step at 95 °C, 35 cycles of 60 s at 95 °C, 30 s at 55 °C and 60 s at 72 °C, followed by a final extension step of 7 min at 72 °C. Approximately 10 μL of the PCR products were electrophoresed on 1% (w/v) agarose gel, stained with Gel-Red, and visualized under UV on the Gel Imaging System (Bio-Doc, Germany).

Elicitor treatment

Three elicitors, including Ag^+ ions, YE, and MeJA, were tested at two concentrations. A stock solution of Ag^+ ions was made by dissolving AgCl in deionized water and adding a liquid hormone-free 1/2 MS medium to the desired concentrations (2.5 and 5 ppm final concentrations). The carbohydrate fraction of YE was obtained by ethanol precipitation from yeast extract (Cat. No. Y4250, Sigma) as defined by Hahn and Albersheim (1978) and then added to a freshly prepared 1/2 MS liquid medium (50 and 100 ppm final concentrations). A stock solution (0.1 M) of MeJA was prepared by dissolving it in 96% ethanol and then adding to an autoclaved 1/2 MS liquid medium (11.2 and 22.4 ppm final concentrations) (Wang et al. 2015). The elicitor solutions were passed through 0.2 μm pore-size filters.

To determine the optimal time for elicitor treatments, a growth curve was established for HR line AT3. For this purpose, root tips (0.6 g) were excised from 1-month-old HRs and cultured into 250 mL flasks containing 50 mL 1/2 MS liquid medium with 3% sucrose. The cultures were kept at 25 °C under continuous darkness, with shaking at 100 rpm. The flasks were harvested at weekly intervals during the 70 days culture period to measure HR fresh weight and dry weight. A growth curve was plotted using HR dry weights (data were not shown).

The elicitation was carried out in 250-mL Erlenmeyer flasks containing 50 mL of 1/2 MS liquid medium with 3% sucrose and inoculated with 0.6 g HRs line AT3. The flasks were incubated on a rotary shaker at 100 rpm and maintained at 25 °C in the dark. The elicitor treatments were performed at the end of the exponential phase of HR growth curve on day 56. Prior to elicitation, the spent medium was discarded and replaced with 50 mL of fresh one, supplemented with different concentrations of elicitors as described before. For the control groups, the same amounts of water (solvent of Ag^+ and YE) and ethanol (solvent of MeJA) were applied to the 50 mL of 1/2 MS liquid medium. Hairy roots were collected on the 1st, 3rd, and 5th days following elicitor treatments.

Phenolic compounds extraction

Harvested HRs were dried in an oven at 40 °C to achieve a steady dry weight before being ground to a fine powder. The dried HRs (0.5 g) were extracted with 10 mL methanol

(98%) by 30 min of sonication at room temperature and filtered through Whatman No. 1 papers. The extracts were dried using vacuum evaporation and stored at -20°C .

Total phenol and flavonoid determination

Total phenol (TP) content in the samples was assessed with the Folin–Ciocalteu method (Wojdyło et al. 2007), and total flavonoid (TF) concentration was determined using the aluminum chloride colorimetric method (Chang et al. 2002), with slight modification (Attaran Dowom et al. 2017). The standard curve equations ($y = 0.1427 + 0.0029x$, $r^2 = 0.999$) and ($y = -0.065 + 0.0088x$, $r^2 = 0.939$) were used to measure the TP and TF contents in samples and the data were expressed as mg of gallic acid equivalent (GAE) and quercetin equivalent (QUE) per g of dry weight, respectively.

Phenolic acids analysis by HPLC

The content of phenolic acids in the samples was determined using the HPLC method. The HPLC system was a Smartline model (Knauer, Germany) with a quaternary pump and a reversed-phase column C18 Eurospher-100 (5 μm particles, 125 mm \times 4 mm), as well as a UV–Vis detector (D-14163 model). Software ChromGate (V 3.1) was used to process the results. Deionized water with 0.2% glacial acetic acid (solvent A) and acetonitrile (solvent B) was used as the separation's mobile phase. The flow rate remained constant at 1 mL/min. At the 0 and 15 min, the conditions were 90/10 (v/v) A/B and 75/25 (v/v), respectively. The level of mobile-phase A had dropped to 20% at 40 min, then to 0% at 45 min. This ratio remained constant until 50 min, after which the percentage of mobile-phase A rose linearly to 90% in the next 5 min. The injection volume was 20 μL , and the peaks were measured at 280 nm. Before injection, the samples were filtered using a 0.45 μm pore size hydrophilic PTFE membrane filter. Standards of phenolic acid were obtained from Sigma-Aldrich company and used to identify and quantify RA, Sal-A and Sal-B. Peaks in the HPLC chromatograms of samples were identified according to the retention times of the corresponding standards. The content of each phenolic acid was determined based on the equation derived from the corresponding standard calibration curve. For the creation of calibration curves, standards of RA, Sal-A and Sal-B were dissolved in ethanol (400 ppm) and then were diluted to reach the required concentration range. Calibration equations ($y_{\text{RA}} = 41,606x$, $r^2 = 0.99$; $y_{\text{Sal-A}} = 19,497x$, $r^2 = 0.99$; $y_{\text{Sal-B}} = 537.58x$, $r^2 = 0.99$) were used to determine the concentrations of RA, Sal-A and Sal-B in samples. Analyses were repeated three times in each treatment.

Statistical analysis

In a completely randomized design, each experiment was replicated at least three times. Using SPSS program version 16.0, the data were put through one-way ANOVA. Duncan's Multiple Range Test was applied for comparisons between mean values, recorded as means \pm standard deviation (SD). The significant differences between data were determined at $P \leq 0.05$.

Results

Establishment of *S. virgata* hairy root cultures and confirmation of transgenic status

In this study, detached leaf explants from the in vitro 50-day-old plants were inoculated with five strains of *A. rhizogenes*. Hairy roots initials appeared on the incision sites of the explants within 10 days. After 4 weeks of inoculation, all of the bacterial strains used in this study successfully induced hairy roots at the wounded sites of leaf explants. No root formation was observed in the control explants (Fig. 1). As shown in Fig. 2, all of the hairy roots, induced by five different strains, were confirmed to have the *rolC* gene in their genomes.

According to the results, selected strains of *A. rhizogenes* showed a significant difference in their ability to induce HRs. The infection frequency varied from 20.4 ± 0.77 to $56 \pm 2.67\%$ depending on the bacterial strain. The highest infection frequency (56%) was found in the leaf explants infected with ATCC15834 strain, while the lowest infection frequency (20.4%) was obtained in the explants inoculated with C58C1 strain (Fig. 3).

Elicitors effects on the content of total phenols and flavonoids

Hairy root line AT3, induced by ATCC15843 strain, showed the highest biomass and RA content and was selected for elicitor treatments (Table 1). Contents of total phenols and flavonoids in the line AT3 were measured after 1 to 5 days of treatment with different concentrations of MeJA, YE and Ag^+ ions. According to Table 2, MeJA-elicited HRs accumulated higher levels of TP and TF after 1, 3 and 5 days of elicitation, compared with control cultures. With the duration of MeJA exposure time, variations were detected in TP and TF contents of HRs, so significant increases were observed on the 3rd day, followed by significant decreases on day 5 of elicitation. Based on the MeJA dosage and exposure time, the concentrations of TP and TF changed

Fig. 1 Hairy root induction from leaf explants of *S. virgata* on MS-based medium, **a** 1 week and **b** 4 weeks after inoculation with *A. rhizogenes* strain ATCC15834. **c** Untransformed explants (control) after 4 weeks, **d** 2-month-old hairy root cultures in 1/2 MS liquid medium. Arrows indicate developing hairy roots on the leaf explants (scale bar = 1 cm)

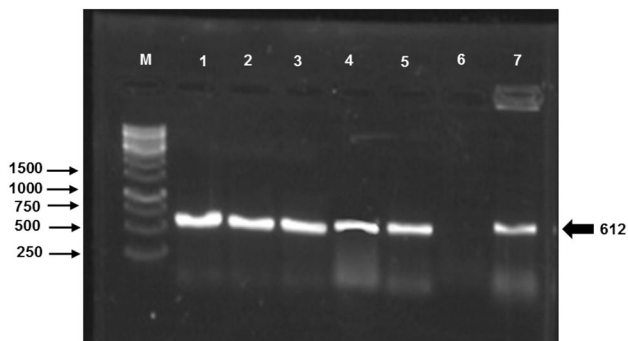
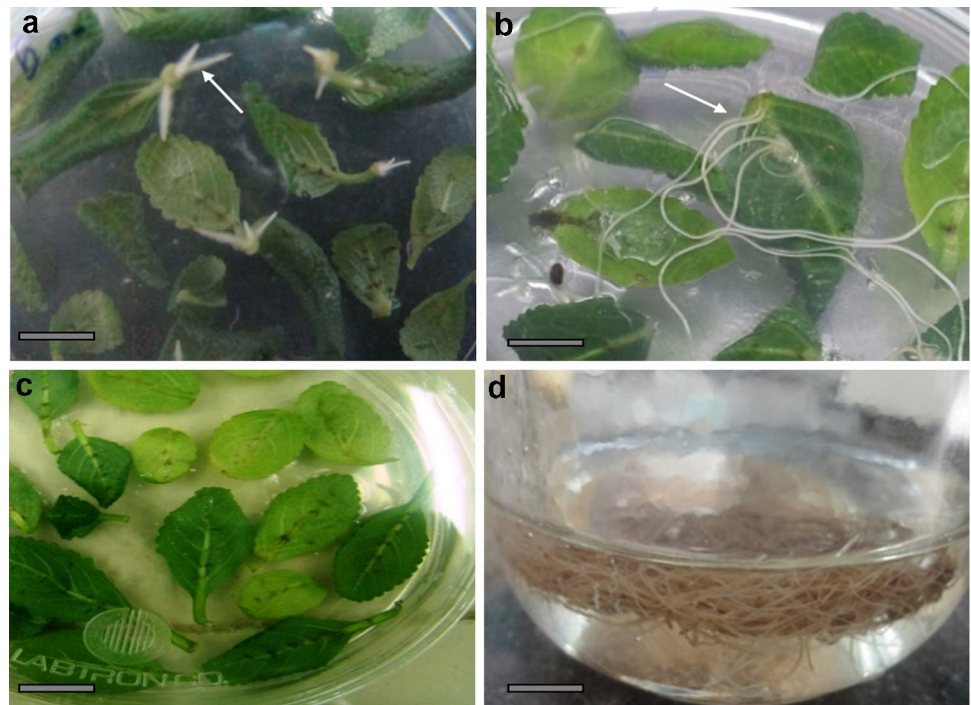


Fig. 2 PCR analysis of *S. virgata* hairy roots transformed by different strains of *A. rhizogenes*. ATCC15834 strain (lane 1), R1000 strain (lane 2), A4 strain (lane 3), C58C1 strain (lane 4), GM1534 strain (lane 5), non-transformed roots (lane 6). Lane 7 is the genomic DNA from *A. rhizogenes* ATCC15834 strain (positive control). Lane M is the molecular weight marker (1 kb DNA ladder). Leftward arrow shows the amplified fragment of *rolC* (612 bp) gene

between 76.14 ± 0.96 to 123.66 ± 0.93 mg GAE/g DW and 2.66 ± 0.11 to 5.09 ± 0.07 mg QUE/g DW, respectively. The highest accumulation of TP (2.06-fold of control) and TF (2.72-fold of control) was achieved with 22.4 ppm MeJA after 3 days of elicitation (Table 2).

As shown in Table 2, following 50 ppm YE treatment, the content of TP on day 3 (90.39 ± 0.77 mg GAE/g DW) and day 5 (88.63 ± 1.03 mg GAE/g DW) of elicitation was significantly enhanced (1.98- and 1.61-fold of control, respectively). Application of 100 ppm YE to HRs significantly elevated the amount of TP from 49.66 ± 0.69 mg GAE/g

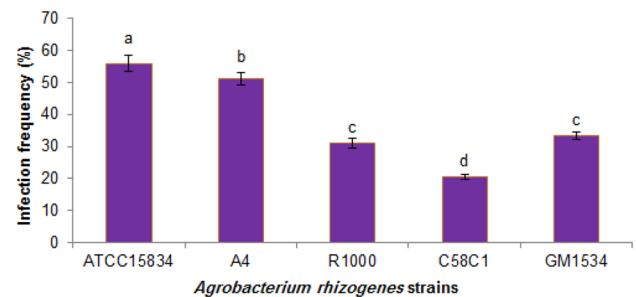


Fig. 3 Hairy root induction frequency from *S. virgata* leaf explants. Data were collected 4 weeks after infection with various strains of *A. rhizogenes*. Each value indicates the mean \pm SD of three replicates. Consistent with Duncan's Multiple Range Test, columns labeled with the same letter are not significantly different ($P \leq 0.05$)

DW and 54.82 ± 0.32 mg GAE/g DW in control roots to 75.61 ± 0.37 mg GAE/g DW and 76.22 ± 0.63 mg GAE/g DW in the elicited HRs on day 1 and day 5 after elicitation, respectively. The increase in the exposure time of elicitation with 50 ppm YE from 1 day to 3 days resulted in a significant increase in TP content, followed by a significant decrease on day 5. However, in the presence of 100 ppm YE, no significant change was found in TP content during the whole period of elicitation. The highest content of TP (90.39 ± 0.77 mg GAE/g DW) was observed in the cultures containing 50 ppm YE three days after elicitation (Table 2). The results represented that both concentrations of YE significantly stimulated TF accumulation in HRs. The yeast extract-treated HRs contained higher levels of TF on

Table 1 Effects of different *A. rhizogenes* strains on dry weight and rosmarinic acid content in hairy root cultures of *S. virgata* after 2 months

Bacterial strain	Hairy root line	Rosmarinic acid (mg/g DW)	Dry weight (g)
ATCC15834	AT1	2.94 ± 0.16 ^{d-g}	1.23 ± 0.06 ^b
ATCC15834	AT2	2.77 ± 0.10 ^{fg}	0.95 ± 0.05 ^d
ATCC15834	AT3	5.47 ± 0.16 ^a	2.29 ± 0.04 ^a
A4	A1	3.38 ± 0.34 ^{c-e}	0.66 ± 0.02 ^g
A4	A2	3.09 ± 0.14 ^{d-g}	0.83 ± 0.00 ^e
A4	A3	4.02 ± 0.17 ^b	1.13 ± 0.06 ^c
R1000	R1	2.85 ± 0.42 ^{e-g}	0.37 ± 0.02 ^j
R1000	R2	2.96 ± 0.42 ^{d-g}	0.72 ± 0.02 ^f
R1000	R3	3.96 ± 0.15 ^b	0.83 ± 0.03 ^c
C58C1	C1	3.03 ± 0.03 ^{d-g}	0.11 ± 0.00 ⁱ
C58C1	C2	3.63 ± 0.11 ^{bc}	0.20 ± 0.00 ^k
C58C1	C3	2.63 ± 0.08 ^g	0.13 ± 0.01 ⁱ
GM1534	GM1	3.40 ± 0.43 ^{cd}	0.45 ± 0.04 ⁱ
GM1534	GM2	3.30 ± 0.24 ^{c-f}	0.70 ± 0.00 ^{fg}
GM1534	GM3	3.71 ± 0.34 ^{bc}	0.50 ± 0.00 ^h

Each value indicates the mean ± SD of three replicates. Consistent with Duncan's Multiple Range Test, means labeled with the same letter inside a column are not significantly different ($P \leq 0.05$)

day 3 and day 5 compared with the 1st day after elicitation. The contents of TF varied from 2.13 ± 0.02 mg QUE/g DW (on day 1 after elicitation with 50 and 100 ppm YE) to 3.20 ± 0.3 mg QUE/g DW (on day 3 after elicitation with 100 ppm YE) (Table 2). Based on the results obtained from the treatment of HRs with Ag^+ (2.5 and 5 ppm), TP and TF contents were significantly enhanced with increasing exposure time from 1 day to 5 days (Table 2). However, no significant variations were detected between the TP content of HRs on day 3 and day 5 after treatment with 5 ppm of Ag^+ ions. Generally, following the rise of the Ag^+ ion concentration in the culture medium from 2.5 ppm to 5 ppm, no significant increase was observed in either TP or TF contents of HRs, except for day 1, where TP content showed a 1.05-fold increase. The maximum contents of TP (98.88 ± 0.91 mg GAE/g DW) and TF (3.50 ± 0.36 mg QUE/g DW) were obtained in HRs treated with 2.5 ppm Ag^+ on the 5th day after elicitation (1.80- and 1.88-fold of control, respectively) (Table 2).

Elicitors effects on the content of phenolic acids

Hydromethanolic extracts of elicited and non-elicited HRs (line AT3) were utilized to quantitative analysis of phenolic acids (RA, Sal-A and Sal-B) using the HPLC method. The HPLC chromatograms of phenolic acids from some treatments compared with the control are shown in Fig. 4. The

Table 2 Effect of various concentrations of the applied elicitors on total phenol and total flavonoid contents in *S. virgata* hairy roots (line AT3)

Elicitors (ppm)	Days after treatment	Total phenol content (mg GAE/g DW)	Total flavonoid content (mg QUE/g DW)
Ag^+ 5	1	77.31 ± 0.12 ⁱ	2.12 ± 0.06 ^{hi}
Ag^+ 5	3	88.39 ± 0.58 ^{ef}	2.72 ± 0.05 ^{fg}
Ag^+ 5	5	89.80 ± 0.9 ^{de}	3.15 ± 0.04 ^{de}
Ag^+ 2.5	1	73.41 ± 0.79 ^k	2.15 ± 0.06 ^h
Ag^+ 2.5	3	86.89 ± 0.91 ^f	2.94 ± 0.04 ^{ef}
Ag^+ 2.5	5	98.88 ± 0.91 ^c	3.50 ± 0.36 ^c
YE 100	1	75.61 ± 0.37 ^j	2.13 ± 0.02 ^{hi}
YE 100	3	77.14 ± 2.42 ^{ij}	3.20 ± 0.30 ^d
YE 100	5	76.22 ± 0.63 ^{ij}	2.24 ± 0.05 ^h
YE 50	1	41.55 ± 0.70 ^m	2.13 ± 0.08 ^{hi}
YE 50	3	90.39 ± 0.77 ^d	2.77 ± 0.09 ^{fg}
YE 50	5	88.63 ± 1.03 ^e	2.66 ± 0.08 ^g
MeJA 22.4	1	76.14 ± 0.96 ^{ij}	2.77 ± 0.07 ^{fg}
MeJA 22.4	3	123.66 ± 0.93 ^a	5.09 ± 0.07 ^a
MeJA 22.4	5	98.14 ± 1.14 ^c	4.28 ± 0.08 ^b
MeJA 11.2	1	80.02 ± 0.27 ^h	3.10 ± 0.08 ^{de}
MeJA 11.2	3	103.05 ± 1.05 ^b	3.26 ± 0.22 ^d
MeJA 11.2	5	82.00 ± 0.78 ^g	2.66 ± 0.11 ^g
Control 1	1	49.66 ± 0.69 ^o	1.52 ± 0.02 ^k
Control 1	3	45.58 ± 1.20 ⁱ	1.75 ± 0.10 ^j
Control 1	5	54.82 ± 0.32 ^o	1.86 ± 0.12 ^j
Control 2	1	46.25 ± 0.68 ⁿ	1.85 ± 0.07 ^j
Control 2	3	59.92 ± 0.91 ^o	1.87 ± 0.05 ^j
Control 2	5	45.65 ± 0.77 ^m	1.91 ± 0.11 ^{ij}

Each value indicates the mean ± SD of three replicates. Consistent with Duncan's Multiple Range Test, means labeled with the same letter inside a column are not significantly different ($P \leq 0.05$). Hairy roots were collected 1, 3, and 5 days after the application of selected elicitors

GAE gallic acid equivalent, QUE quercetin equivalent, Ag^+ ions: silver ions, YE yeast extract, MeJA methyl jasmonate, Control 1 untreated roots, Control 2 ethanol-treated roots

results obtained from HPLC analysis revealed that RA and Sal-A were present in three elicited and non-elicited HRs of *S. virgata*. Rosmarinic acid was found in higher concentrations in the extracts than Sal-A. Salvianolic acid B was not detected in the HR samples (Fig. 4).

According to the results shown in Fig. 5, the levels of studied phenolic acids in HRs treated with both concentrations (11.2 and 22.4 ppm) of MeJA were significantly elevated over a 3-day period and then decreased on the 5th day after elicitation. The best stimulating effect of MeJA was achieved at the dose of 22.4 ppm, and the analyzed phenolic acids tended to increase to a maximum level on day 3 post elicitation. Hairy roots elicited with 22.4 ppm MeJA accumulated up to 18.45 ± 0.8 mg/g DW RA and

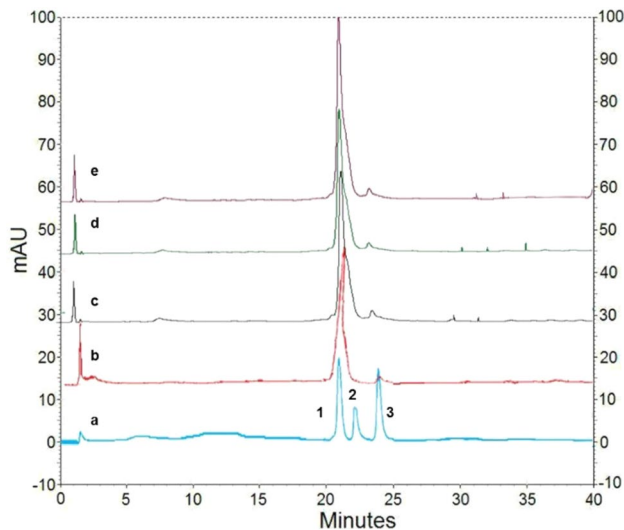


Fig. 4 HPLC chromatograms of hydromethanolic extracts from *S. virgata* hairy roots (line AT3). **a** standard solution, **b** non-elicited hairy roots (control), **c** hairy roots elicited with 50 ppm YE for 3 days, **d** hairy roots elicited with 2.5 ppm Ag^+ ions for 5 days, **e** hairy roots elicited with 22.4 ppm MeJA for 3 days. Peak 1: rosmarinic acid, Peak 2: salvianolic acid B, Peak 3: salvianolic acid A

2.11 ± 0.04 mg/g DW Sal-A over the course of 3 days, nearly 1.81- and 3.76-fold of untreated HRs on the same time (Fig. 5).

The data collected from YE-treated HRs, presented in Fig. 5, revealed the increase in RA and Sal-A contents, which was observed during the first 3 days of elicitation with 50 and 100 ppm YE, was followed by a decrease on day

5 after treatment. The application of 50 ppm YE for 3 days was the best treatment for RA and Sal-A production. Maximum amounts of RA (15.58 ± 0.01 mg/g DW) and Sal-A (1.65 ± 0.01 mg/g DW) were 1.44- and 2.42-fold of control, respectively (Fig. 5).

As shown in Fig. 5, during 5 days, elicitation with Ag^+ ions (2.5 and 5 ppm), in most cases, resulted in a higher accumulation of phenolic acids in HRs than the control. Elicitation with 2.5 ppm Ag^+ was more effective on producing phenolic acids in HRs cultures than 5 ppm Ag^+ . A progressive time-dependent enhancement in the contents of RA and Sal-A was found throughout elicitation with 2.5 ppm Ag^+ . The highest production of RA (16.01 ± 0.09 mg/g DW) and Sal-A (1.52 ± 0.06 mg/g DW) were attained after a 5-day exposure of HRs to 2.5 ppm Ag^+ (1.54- and 3.37-fold of control, respectively) (Fig. 5).

Discussion

This study aimed to establish HR cultures of *S. virgata* and, for the first time, to increase the production of phenolic acids based on the biotic and abiotic elicitor application. The findings revealed that all the tested strains of *A. rhizogenes* (ATCC15834, A4, R1000, C58C1 and GM1534) could generate HRs on the leaf segments. However, the infection frequency was significantly different among the five strains. The highest (56%) and the lowest (20.4%) values of infection frequencies were obtained with ATCC15834 and C58C1 strains, respectively. According to Norouzi et al. (2017), among 4 bacteria strains evaluated for induction of HRs on

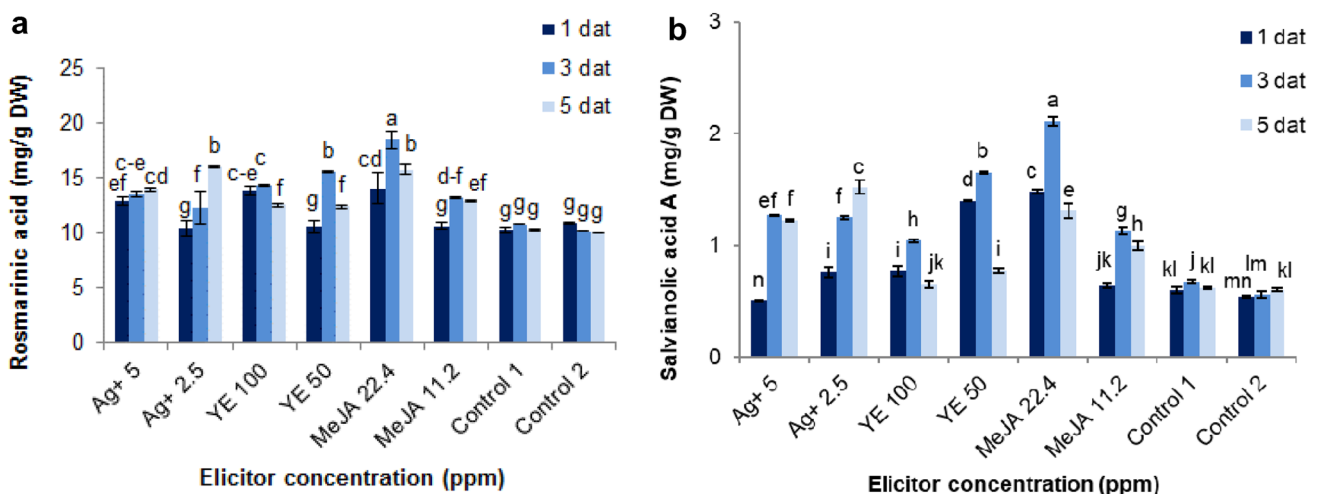


Fig. 5 Impact of various elicitor concentrations on phenolic acids production in *S. virgata* hairy root (line AT3) cultures during periods of treatments. The data represent the mean \pm SD of three replicates. Consistent with Duncan's Multiple Range Test, the bars which

are assigned with a common letter are not significantly differed at $P \leq 0.05$ value. **a** rosmarinic acid, **b** salvianolic acid-A. Ag^+ silver ions, YE yeast extract, MeJA methyl jasmonate, Controls 1 untreated roots, Control 2 ethanol-treated roots, dat day after treatment

leaf explants of *S. virgata*, both A4 and 2659 strains showed the higher HR induction frequency (76%) compared with ATCC15834 and 1724 strains (72%).

Different *A. rhizogenes* strains have diverged in their abilities to induce HRs in plant species (Srivastava et al. 2018). Panda et al. (2017) reported that the optimum transformation efficiency (61%) was attained in leaf explants of *Semecarpus anacardium* L. with ATCC15834 strain, in comparison with strains A4 and LBA9402. Higher virulence of ATCC15834 strain (46%) over other strains, A4 and SA79 (42 and 21%, respectively), of *A. rhizogenes* was also found in the case of *Boerhaavia diffusa* L. leaf explants (Gupta et al. 2015). Similarly, a higher frequency of HR induction in explants infected with ATCC15834 strain than those infected with other strains has been reported in different medicinal plants, including *Perovskia abrotanoides* Karel. (Ebrahimi et al. 2017), and *S. officinallis* (Grzegorzczak et al. 2006). Different strains of *A. rhizogenes* display different transforming potentials, which can be attributed to their different plasmids (Gupta et al. 2015; Thwe et al. 2016; Figlan and Makunga 2017). Findings from the present study showed a more successful HR formation in *S. virgata* with the agropine-type (ATCC15834, R1000 and A4) than the mannopine-type (C58C1) strains of *A. rhizogenes*. It has been proved that agropine-type strains, which have two T-DNA regions on their Ri plasmid (T_L and T_R), have more infection ability than mannopine-type strains (Verma et al. 2012). The genes encoding auxin have been localized on the T_R -DNA of the agropine-type Ri plasmid (Rawat et al. 2019). Therefore, agropine-types of *A. rhizogenes* strains are less dependent on endogenous auxin in explants, and this additional auxin source supports the HR formation (Pal et al. 2013). Moreover, the efficiency of different *Agrobacterium* strains for developing HRs is strongly dependent on plant species and must be evaluated experimentally (Park et al. 2017; Thwe et al. 2016).

In the present survey, among the three distinct HR lines induced by ATCC15834 strain, line AT3 was the best-grown line with maximum biomass production and highest RA accumulation. It has been revealed that different transformed root clones have various capacities for biomass accumulation and secondary metabolites biosynthesis (Grzegorzczak et al. 2006; Grzegorzczak-Karolak et al. 2018). The growth rate and biosynthetic characteristics of HRs are controlled by the products of individual or combined *rol* genes (Dilshad et al. 2015; Matveeva et al. 2015; Sarkar et al. 2018).

Elicitation is a successful and probably the most widely applied approach for the induction of secondary metabolites biosynthesis in plant HR cultures (Halder et al. 2019). Various factors, such as elicitor type and concentration, period of elicitation and culture age at the time of elicitation, may all be optimized for the best development of target secondary metabolites (Naik and Al-Khayri 2016; Halder et al. 2019).

The current study revealed that the production of TP, TF, RA and Sal-A in the HR cultures of *S. virgata* is stimulated by biotic (YE) and abiotic (Ag^+ ions and MeJA) elicitors. Similar studies have shown that YE, MeJA and Ag^+ ions significantly improved phenolic acids contents, especially RA, in HR cultures of *S. miltiorrhiza* (Yan et al. 2006; Zhang et al. 2014; Xing et al. 2015, 2018), *M. spicata* (Yousefian et al. 2020) and *C. forskohlii* (Li et al. 2005). Our findings showed that the used elicitors had different effects on the phenolic acid accumulation in HRs. Although YE and Ag^+ ions had a stimulatory influence on the synthesis of phenolic acids, the concentrations of these compounds were inversely proportional to the elicitor concentration. In contrast, MeJA had a positive dose-dependent impact on phenolic acids accumulation. Phenolic acid accumulation in HR cultures of *C. blumei* (Bauer et al. 2009) and *S. miltiorrhiza* (Chen et al. 2010) have also been related to the exogenous application of MeJA. The level of MeJA-induced phenolic acid accumulation in the present study was higher than those observed in the YE- and Ag^+ -elicited HRs. The optimal contents of TP, TF, RA and Sal-A in MeJA-elicited HRs (at 22.4 ppm for 3 days) of *S. virgata* were 2.06-, 2.72-, 1.81- and 3.76-fold of the control culture, respectively. Similarly, Li et al. (2005) discovered that, when compared with YE, MeJA (at 22.4 ppm for 7 days) was the most powerful elicitor for the production of RA in *C. forskohlii* HR cultures, raised RA levels approximately 3.4 times higher than the control group. According to several reports on the HR cultures of *S. miltiorrhiza*, after the application of MeJA at 22.4 ppm, the accumulation of RA increased by 2-fold (on day 6) (Xiao et al. 2009), 1.5-fold (on day 5) (Zhang et al. 2014) and 1.5-fold (on day 3) (Xing et al. 2018) of the control cultures depending on the elicitation time course.

According to the results of current study, compared with the control cultures, accumulation of Sal-A (3.76-fold-increase) in the transgenic roots treated with 22.4 ppm MeJA was more affected than RA (1.81-fold-increase). However, RA was the most predominant phenolic acid in the transgenic roots. It is proposed that MeJA, as a signaling molecule, stimulates the biosynthesis of RA and salvianolic acids through the activation of MeJA-responsive transcription factors (Sun et al. 2019; Deng et al. 2020). The molecular events (reactive oxygen species (ROS) production, changes in calcium fluxes and protein phosphorylation) involving in MeJA signaling have been stated by some scholars (Baenas et al. 2014; Ho et al. 2020). The stimulatory effect of YE on the secondary metabolites production may be related to its organic (peptide, polysaccharide) (Baenas et al. 2014) and metal ion (Ca^{2+} , Co and Zn) components (Srivastava and Srivastava 2014; Kochan et al. 2017).

In the present study, significant increases were found in TP, RA and Sal-A levels (1.98-, 1.44- and 2.42-fold of the control level, respectively) when the *S. virgata* HR cultures

were exposed to YE (50 ppm) for 3 days. Similar to these results, previous studies reported that YE increased RA in HR cultures of *S. miltiorrhiza* (Yan et al. 2006; Zhao et al. 2011). According to the report of Chen et al. (2001), the accumulation of RA in *S. miltiorrhiza* HR cultures was about 3.2-fold of the control on the 7th day after elicitation with 5000 ppm YE. The highest accumulation of RA (1.9 times higher than the control) in HR cultures *C. forskohlii* was achieved at both concentrations of 100 and 10,000 ppm YE, 7 days after elicitation (Li et al. 2005). In the study of Yan et al. (2006) on HR cultures of *S. miltiorrhiza*, TP and RA contents increased by 1.4- and 1.6-fold at the end of day 4 and day 8 after treatment with 200 ppm YE, respectively. According to Bauer et al. (2009), RA contents in two YE-induced (5000 ppm YE for 1 day) HR clones of *C. blumei* were 20 and 44% higher than that obtained in the control group. Yeast-derived elicitors can stimulate gene expression of the key enzymes from both phenylpropanoid (*PAL* and *C4H*) and tyrosine-derived (*TAT* and *HPPR*) pathways (Park et al. 2016).

In the current study, after the adding of 2.5 ppm Ag⁺ (for 5 days), the accumulation of TP, TF, RA, and Sal-A was estimated to be 1.8-, 1.88-, 1.54- and 3.37-fold of the control, respectively. Similarly, as reported by Xing et al. (2015), the maximum concentration of RA (1.3-fold of the control) in HRs of *S. miltiorrhiza* was determined after elicitation with 2.5 ppm Ag⁺ ions (for 6 days). In another study involving *S. miltiorrhiza* HR cultures, TP and RA reached the maximal levels (about 1.2- and 1.3-fold of the control, respectively) when HRs were treated with 2.5 ppm Ag⁺ ions for 4 and 8 days, respectively (Yan et al. 2006). Nevertheless, Xiao et al. (2010) reported that RA accumulation in HRs of *S. miltiorrhiza* was not affected by Ag⁺ ions (2.5 ppm). Ag⁺-induced accumulation of different phenolic acids in HRs of *S. miltiorrhiza* was found to be coincident with the up-regulation of several genes (especially *TAT*, *HPPR* and *C4H*) in tyrosine-derived and phenylpropanoid pathways (Xiao et al. 2010; Xing et al. 2015). It has been proposed that the ROS reaction cascade triggered by Ag⁺ can activate the phosphorylation and activation of downstream transcription factors (Kohan-Baghkheirati and Geisler-Lee 2015; Paeizi et al. 2018).

Conclusions

In this study, for the first time, we presented an efficient protocol for the phenolic acids production in the well-established HR cultures of *S. virgata* using the elicitation technique. Among the five different strains of *A. rhizogenes* (ATCC15834, R1000, A4, C58C1 and GM1534), the ATCC15834 strain showed the highest capability to induce HR formation and RA production. The elicitation technique utilized here revealed the inducible nature of phenolic acids

production in HRs of *S. virgata*. All tested elicitors (MeJA, Ag⁺ and YE) positively influenced the contents of TP, TF, RA, and Sal-A in HR cultures, and the highest accumulation of these phenolic components was achieved at 22.4 ppm MeJA after 3 days. Subsequent investigations relevant to elicitation and mechanism of phenolic acids biosynthesis need to be conducted further to utilize the potency of *S. virgata* HR cultures to produce more bioactive compounds.

Acknowledgements A research grant (No. 3/25607) for PhD thesis provided by Ferdowsi University of Mashhad supported this work. The authors also are grateful to Biotechnology Laboratory of Tehran University for HR cultures and Shahed University's Plant Physiology Laboratory for HPLC measurements.

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