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

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Abstract

Phenolic acids, as the predominant secondary metabolites of *Salvia* species, 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, salvianolic acid A, and caffeic acid contents in the hairy roots after 1, 3 and 5 days of exposure. Results showed the used *Agrobacterium rhizogenes* strains (A4, ATCC15834, R1000, GM1534, and C58C1) differed in their ability 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 ATCC 15834 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 ATCC 15834 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), salvianolic acid A (2.11 ± 0.04 mg/g DW) and caffeic acid (2.61 ± 0.02 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.

1. Introduction

Salvia virgate Jacq., an annual plant belonging to the Lamiaceae family, is native to Asia (northeast of Iran) and southeastern Europe. The plant has traditionally been used for the treatment of skin diseases, wounds, and blood cancer (Baytop 1999; Poyraz and Koca 2006). Some of the important biological properties of this species, including antioxidant (Tepe 2008; karatoprak et al. 2016; Dehghani Latani et al. 2019), antimicrobial (Kursat et al. 2012; Alizadeh 2013; Golparvar et al. 2017), antifungal (Bayar and Yilar 2019), anti-inflammatory and antinociceptive activities (Akkol et al. 2008) have been reported in the literature.

Phenolic acids, including caffeic acid (CA), rosmarinic acid (RA), and salvianolic acid B (Sal-B) (also known as lithospermic acid B (LAB)), are principal polyphenolic compounds in *Salvia* species which exhibit multi-biological activities and health-benefit properties (Habtemariam 2018; Wang et al. 2019). Based on *in vitro* and *in vivo* studies, the presence of some phenolic acids such as CA, RA, Sal-A, and Sal-B, have been indicated in intact plant and shoot cultures of *S. virgata* (Tepe 2007; Kosar et al. 2008; Akkol et al. 2008; Ejtahed et al. 2015; Attaran Dowom et al. 2017; Fotovvat et al. 2018).

Recent studies have reported numerous biological/pharmacological properties for these phenolic acids, including antioxidant, antibacterial, antifungal (Weremczuk-Jeżyna et al. 2019; Grzegorczyk-Karolak et al. 2018; Katanić Stanković et al. 2020), antiviral (Ma et al. 2019; Kim et al. 2020), anti-inflammatory (Villalva et al. 2018; Liu et al. 2018), hypoglycemic (Huang et al. 2016; Jackson 2017; Salgueiro et al. 2018), and anticancer (Swamy et al. 2018; Zhang et al. 2018; Katary et al. 2019; Qin et al. 2019) activities.

Furthermore, it has shown that RA and Sal-B could improve memory and cognitive impairment related to Alzheimer's disease (Shen et al. 2017; 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 3, 4-dihydroxyphenyllactic acid, and Sal- B is identified as a dimmer of RA (Wang et al. 2019) (Fig. 1).

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

Due to the genetic stability, large biomass output, and high biosynthetic capacity, hairy root (HR) culture is considered as an alternative technique for secondary metabolite production, compared to native plant roots and cell/callus cultures (Hu and Du 2006; Ono and Tian 2011). Moreover, the application of HR cultures in many industrial and scientific fields, including designing of bioreactors, recombinant protein production, phytoremediation, and metabolic and genetic engineering, has been reported (Ashihara et al. 2011; Doran 2013; Kuma 2018).

It has demonstrated that the high amounts of phenolic acids, such as RA, are synthesized in the HR cultures of plants belonging to the Lamiaceae family including, *Salvia officinalis* L. (Grzegorczyk et al. 2006), *S. miltiorrhiza* Bunge (Yan et al. 2006; Zhao et al. 2011), *Ocimum basilicum* L. (Bais et al. 2002), *Coleus forskohlii* Briq. (Li et al. 2005), *Nepeta cataria* L. (Lee et al. 2010), as well as RA and LAB in *S. miltiorrhiza* (Xiao et al. 2010; Di et al. 2013).

Various elicitors including silver ions (Ag^+ ions), yeast extract (YE) and methyl jasmonate (MeJA) can widely use to stimulate production and accumulation of secondary metabolites in HR cultures (Ge and Wu 2005; Pirian and Piri 2013; Ahmadi Moghadam et al. 2014). According to some reports, production of different phenolic acids, such as CA, RA, and Sal-B have been improved in HR cultures of *C. blumei* (Bauer et al. 2009), *S. miltiorhiza* (Yan et al. 2006; Xiao et al. 2009; Xiao et al. 2010; Xing et al. 2015; Xing et al. 2018b), *S. officinalis* (Grzegorczyk and Wysokińska 2009), and *Mentha spicata* L. (Yousefian et al. 2020) after the elicitation with YE, Ag^+ ions and MeJA.

There is only one report (in Persian) available on the induction of HR cultures in *S. virgata* (Norouzi et al. 2017), and based on our knowledge, no report has been published 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 investigate the effects of biotic and abiotic elicitors on RA, CA, and Sal-A production in the transformed root cultures for the first time.

2. Materials And Methods

2.1. Plant material

Mature seeds of *S. virgata* were collected from wild-grown plants in August 2013 at Reine village (Bojnoord, North Khorasan province, Iran) located at latitude 57◦ 2 N, 37◦ 23 E longitude, and an elevation of 1765 m above sea level. The species was identified at the Ferdowsi University of Mashhad herbarium (FUMH), where a voucher specimen (No. 38128) of the plant was deposited. Seeds were surface sterilized with ethanol (70 %) for 1 min, followed by sodium hypochlorite (5 %) for 5 min, and rinsed three times with sterile distilled water. For germination, seeds were placed into glass jars containing 25 mL of Murashige and Skoog (MS) medium supplemented with sucrose (3 %) and agar (0.7 %). The pH of media was adjusted to 5.6–5.8 before adding agar, and the MS basal medium was autoclaved at 120 ºC for 17 min. The glass jars were kept in the dark for three days at 25 ± 2 ºC, and after germination of the seeds, they were placed at 26 ± 2 ºC and 16/8 h (light/dark) photoperiod (45 M photons m^{-2} s^{-1} provided by cool white fluorescent lamps) in a culture room. These conditions were also applied to all the experiments described below.

2.2. Growth of *Agrobacterium 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 ºC for 48 h.

2.3. Establishment of hairy root cultures

Leaf explants were taken from *S. virgata* plants grown *in vitro* for 50 days. The explants were wounded with a needle dipped into *A. rhizogenes* cultures. Control explants were wounded with a sterile needle without bacteria. Infected and control explants were placed on hormone-free MS agar (0.7 %) medium and incubated in the dark at 25 ºC. After two days of inoculation, the explants were transferred to MS basal medium supplemented with sucrose (3 %), cefotaxime (300 ppm), and Difco Bacto agar (0.7 %) was used for solidifying medium. Root cultures were incubated under 16/8 h (light/dark) photoperiod (45 M photons m^{-2} s^{-1} provided by cool white fluorescent lamps) at 25°C.

The experiment was repeated three times; 25 explants were used for each treatment (n = 25). The transformation frequencies (the percentages of explants forming roots after infection with *A. rhizogenes* strains) were determined 10–14 days after infection. From each strain, three obtained roots, longer than 1 cm, were excised from explants and transferred individually into 250 mL Erlenmeyer flasks containing 50 mL 1/2 MS liquid medium without growth regulators and supplemented with 300 mg/L cefotaxime to prevent bacterial spread. The cefotaxime concentration was gradually decreased to 200 and 100 mg/L during the second and third subcultures, respectively. The cultures were maintained on an incubator shaker at 100 rpm at 25 ºC in the dark. After eight subcultures, when the antibiotic was eliminated from

the medium, one fast-growth root line with the most RA content was obtained (ATCC15834 line AT3) and used for elicitors treatments.

2.4. Confirmation of transgenic hairy root lines

The genomic DNA (gDNA) from five putative transformed root lines of each strain and untransformed roots (negative control) 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 seedling roots were used as the negative control.

The PCR was performed to amplify an internal *rolC* gene fragment (612 bp). The PCR analysis was on the C1000 Touch TM 96-Well Thermal Cycler (Bio-Rad, USA) and began with 15 µL reaction mixtures containing amplicon, gDNA (100 ng total DNA), and oligonucleotide primers in 10 µM final concentration. The primers for detecting 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 comprised of an initial denaturing step of 4 min at 95°C and 35 cycles, each consisting 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 GelRed, and visualized under UV on the Gel Imaging System (Bio-Doc, Germany). Predicted products of *rolC* were obtained apart from the control (non-induced root).

2.5. Elicitor treatment

Three elicitors, including Ag^+ ions, YE and MeJA, were tested at two concentrations. A stock solution of Ag^+ ions was prepared by dissolving AgCl in deionized water and added (2.5 and 5 ppm final concentrations) in liquid hormone-free 1/2 MS medium. Carbohydrate fraction of YE was made from commercial yeast extract (Cat. no.Y4250, Sigma) by ethanol precipitation as described by Hahn and Albersheim (1978) and added (50 and 100 ppm final concentration) in freshly prepared 1/2 MS liquid medium. A stock solution (0.1 M) of MeJA was prepared by dissolving it in 96 % ethanol and added (11.2 and 22.4 ppm final concentrations) in autoclaved 1/2 MS liquid medium (Wang et al. 2015). All the elicitor solutions were sterilized by filtering through 0.2 µM microfilters.

Hairy roots were cultured for 55 days (end of exponential growth phase), and then the old medium was substituted with 30 ml of fresh 1/2 MS liquid medium supplemented with Ag^+ ions, MeJA and YE. Half strength MS liquid medium with the same volume of water or ethanol (instead of elicitors) was added to control cultures. Hairy roots were harvested 1, 3, and 5 days after elicitor treatments. All cultures were maintained at the same mentioned conditions (part 2–3).

2.6. Extraction of phenolic compounds

Harvested hairy roots were oven-dried at 40 ºC to reach the constant dry weight and then grounded to a fine powder. Dried powders of plant materials were extracted with methanol (98 %) (500 mg root material/10 mL solvent) by sonication for 30 minutes at room temperature. The extracts were then

filtered using Whatman No.1 filter papers. After vacuum evaporation, the dried extracts were maintained at -20 ºC.

2.8. Determination of total phenolic compounds

The total phenolic (TP) content of the samples was determined by using Folin-Ciocalteu reagent and gallic acid as standard (Wojdylo et al. 2007). Briefly, 1 mg of the dried extract sample was dissolved in 1 mL methanol (1000 ppm), then 100 µL of the prepared solution was thoroughly mixed in a test tube with 2 mL water and 200 µL Folin-Ciocalteu reagent for 3 min, and the mixture was incubated with 1 mL of 20 % (w/v) sodium carbonate solution at room temperature for 1 h. The absorbance of the extracts was measured at 765 nm against a blank (a solution without the extract). A stock solution of gallic acid (800 ppm) was prepared in distilled water and diluted to appropriate concentrations (200–800 ppm) for the construction of a calibration curve. The concentration of TP in samples was measured using the calibration equation ($y = 0.1427 + 0.0029\ x$, $r^2 = 0.999$) and expressed as mg of gallic acid equivalent (GAE) per g dry weight.

2.9. Determination of total flavonoids

Flavonoid concentration in the samples was measured spectrophotometrically, according to the procedure of Chang et al. (2002). The reaction mixture was prepared by mixing 0.5 mL of methanolic solution of dried extract (1000 ppm) with 1.5 mL methanol, 0.1 mL 10 % (w/v) aluminum chloride, 0.1 mL 1 M potassium acetate and, 2.8 mL distilled water. After 30 min of incubation at room temperature, absorbance was measured at 415 nm against a blank without the extract. For the establishment of a calibration curve, different concentrations of quercetin (20–100 ppm) were prepared in distilled water using a stock solution (100 ppm). The total flavonoid (TF) content in samples was calculated by the standard curve equation ($y = -0.065 + 0.0088\ x$, $r^2 = 0.939$) and results were expressed as mg quercetin equivalent (QUE) per g dry weight.

2.10. HPLC analysis of phenolic acids

The content of phenolic acids was measured by the HPLC method. The HPLC apparatus was a Smartline model (Kenuer, Germany) with a quaternary pump and a reversed-phase column C18 Eurospher-100 (5 µm particles, 125 mm × 4 mm) coupled with a UV-VIS detector (D-14163 model). The data were processed by Software ChromGate (V 3.1). The separation was performed using a mobile phase consisted of water with 0.2 % glacial acetic acid (solvent A) and acetonitrile (solvent B). The flow rate was kept at 1 mL/min. The initial condition was 90/10 (v/v) A/B and 75/25 (v/v) at 15 min. The percentage of mobile-phase A decreased to 20 % at 40 min and reached 0 % at 45 min. This ratio remained stable until 50 min, and in the next 5 min, the percentage of mobile-phase A increased linearly to 90 %. The injection volume was 20 µL, and peaks were monitored at 280 nm. The samples were filtered through a hydrophilic PTFE membrane filter with a 0.45 µm pore size before injection. Peaks were identified by congruent retention times compared with those of standards. Rosmarinic acid, Sal-A, and CA (Fig. 1) were detected and quantified using authentic standards obtained from Sigma. The content of each phenolic acid was calculated based on the equation, which was obtained from the corresponding

standard calibration curve. The stock solutions of RA, Sal-A, and CA (400 ppm) were prepared in ethanol and diluted to appropriate concentration range for the construction of calibration curves. The concentrations of RA, Sal-A, and CA in samples were measured using calibration equations (y_{RA} = 41606 x, r^2 = 0.996; y_{Sal-A} = 19497 x, r^2 = 0.996; y_{CA} = 7337.1 x, r^2 = 0.995). Each treatment was performed in three replicates.

2.11. Statistical analysis

All the tissue culture experiments were repeated at least three times in a completely randomized design. The data were subjected to one-way ANOVA using SPSS software version 16.0. Mean values were compared by Duncan's Multiple Range Test and reported as means ± standard errors (SE). A probability of $P \leq 0.05$ was considered to be significant.

3. Results

3.1. 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 four weeks of the 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. 2). As shown in Fig. 3, all of the hairy roots, induced by five different strains, were confirmed to have *rolC* gene in their genomes.

According to the results, selected strains of *A. rhizogenes* showed a significant difference in their ability to induce HRs, and the infection frequency varied from 20.4 ± 0.77 to 56 ± 2.67 percent 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. 4).

In the next step of the present study, three fast-growing hairy root lines were selected from all five groups of HR lines (induced by 5 different strains of *A. rhizogenes*), transferred to 1/2 MS liquid medium, and then analyzed for their growth and RA content after 2 months. The highest dry weight and RA content were obtained in the HR line AT3, which was induced by ATCC15834 strain, so it was selected as the best line for elicitor treatments (Table 1).

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

Bacterial strain	Hairy root line	Rosmarinic acid (mg/g DW)	Dry Weight (g)
ATCC15834	AT1	$0.24 \pm 0.01^{e-g}$	1.23 ± 0.06^{b}
ATCC15834	AT2	0.22 ± 0.01^{fg}	0.95 ± 0.05^{d}
ATCC15834	AT3	0.45 ± 0.01^{a}	2.29 ± 0.04^{a}
A4	A1	$0.27 \pm 0.03^{c-e}$	0.66 ± 0.02^{g}
A4	A2	$0.27 \pm 0.01^{d-g}$	0.83 ± 0.00^{e}
A4	A3	0.32 ± 0.02^{b}	1.13 ± 0.06^{c}
R1000	R1	$0.32 \pm 0.01^{e-g}$	0.04 ± 0.02^{j}
R1000	R2	$0.24 \pm 0.04^{e-g}$	0.72 ± 0.02^{f}
R1000	R3	0.32 ± 0.01^{b}	0.08 ± 0.03^{e}
C58C1	C1	$0.24 \pm 0.00^{e-g}$	0.11 ± 0.00^{i}
C58C1	C2	$0.29 \pm 0.01^{b-d}$	0.20 ± 0.00^{k}
C58C1	C3	0.21 ± 0.01^{g}	$0.13 \pm 0.0l^{i}$
GM1534	GM1	$0.27 \pm 0.04^{c-e}$	$0.04 \pm 0.04_{i}$
GM1534	GM2	$0.26 \pm 0.02^{c-f}$	$0.70 \pm 0.00f^{g}$
GM1534	GM3	0.30 ± 0.03^{bc}	0.50 ± 0.00^{h}

Each value represents mean ± SE of three replicates. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test

3.2. Effects of elicitors on total phenolic and flavonoid contents

Total phenol and total flavonoid contents in the ATCC15834 strain-induced HRs (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 the 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 between 76.14 ± 0.96 to 123.6 6 ± 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 three 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 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 one and day five after elicitation, respectively. The increase in the exposure time of elicitation with 50 ppm YE from one day to three 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 TF accumulation in HRs was significantly stimulated by both concentrations of YE. The yeast extract-treated HRs contained higher levels of TF on day 3 and 5, compared to the 1st day after elicitation. The contents of TF varied from 2.13 ± 0.02 mg QUE/g DW (on day one after elicitation with 50 and 100 ppm YE) to 3.20 ± 0.3 mg QUE/g DW (on day three 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 one day to five days (Table 2). However, no significant differences were observed between the TP content of HRs on day three and day five 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 one where TP content showed 1.05-fold increase. The maximum contents of TP (98.88 ± 0.91) and TF (3.50 ± 0.36) 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).

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

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}

Elicitors (ppm)	Days after treatment	Total phenol content (mg GAE/g DW)	Total flavonoid content (mg QUE/g DW)
Control 2	3	59.92 ± 0.91^{o}	1.87 ± 0.05^{ij}
Control 2	5	45.65 ± 0.77^{m}	1.91 ± 0.11^{ij}

Each value represents mean ± SE of three replicates. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test. Hairy roots were sampled at 1, 3 and 5 days after the addition 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

3.3. Effects of elicitors on phenolic acids content

Hydromethanolic extracts of elicited and non-elicited HRs (line L3) were utilized for quantitative analysis of phenolic acids (RA, Sal-A and, CA) using the HPLC method. The HPLC chromatograms of phenolic acids from some treatment as compared to the control are shown in Fig. 5. The results obtained from HPLC analysis revealed that RA, CA and, Sal-A were present in three elicited and non-elicited HRs of *S. virgata*. Rosmarinic acid was the most abundant phenolic acid in the extracts, followed by CA and Sal-A. Salvianolic acid B was not detected in the HR samples (Fig. 5).

According to the results shown in Fig. 6, the levels of three studied phenolic acids in HRs treated with both concentrations (11.2 and 22.4 ppm) of MeJA were significantly elevated over three 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 three 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, 2.61 ± 0.02 mg/g DW CA, and 2.11 ± 0.04 mg/g DW Sal-A over the 3-day, nearly 1.81-, 4.35- and 3.76-fold of untreated HRs on the same day (Fig. 6).

The data collected from YE-treated HRs, presented in Table 3 or Fig. 7, revealed that the increase in the contents of RA and Sal-A, which was observed during the first three days of elicitation with 50 and 100 ppm YE, was followed by a decrease on day five after treatment. The application of 50 ppm YE for three days was the best treatment for the production of RA and Sal-A. 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. 6). Elicitation with YE at the concentration of 50 ppm was more effective than 100 ppm to accumulate CA in HRs. Caffeic acid accumulation in HRs treated with 50 ppm YE significantly increased on days 3 and 5 of the elicitation period, as compared to day 1. The highest accumulation of CA (1.03 ± 0.01 mg/g DW) was achieved on the 5th day of treatment (1.74-fold of control) (Fig. 6).

As shown in Fig. 6, during five 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 the production of phenolic acids in HRs cultures, as compared to 5 ppm Ag^{+}. A progressive

time-dependent enhancement in the contents of RA, Sal-A, and CA was found throughout elicitation with 2.5 ppm Ag^+. The highest production of RA (16.01 ± 0.09 mg/g DW), CA (1.99 ± 0.01 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-, 2.45- and 3.37-fold of control, respectively) (Fig. 6).

4. 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 %) frequencies of infection were obtained with ATCC15834 and C58C1 strains, respectively. Different *A. rhizogenes* strains have diverged in their abilities to induce HRs in plant species (Lee et al. 2010; Pirian et al. 2012; Shirazi et al. 2013; Setamam et al. 2014). 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 LBA 9402. Higher virulence of ATCC15834 strain (46 %) over other strains, A4 and SA79, (42 % and 21 %, respectively) of *A. rhizogenes* was also found in 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 many different medicinal plants, including *Althaea officinalis* L. (Tavassoli and Afshar 2018), *Perovskia abrotanoides* Karel. (Ebrahimi et al. 2017), *Helicteres isora* L. (Kumar et al. 2014), *Solenostemon scutellarioides* L. (Saleh and Thuc 2009*), Ipomoea batatas* L. (Chandran and Potty 2008) and *S. officinallis* (Grzegorczyk et al. 2006). Different strains of *A. rhizogenes* display different transforming potentials, which can be attributed to their different plasmids (Giri and Narasu 2000; Pirian et al. 2012; Shirazi et al. 2013; Setamam et al. 2014; Tavassoli and Afshar 2018). 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. rhizogenesis.* 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 in comparison with mannopine-type strains (Hong et al. 2006; 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 (Saleh and Thuc 2009; Pal et al. 2013; Singh et al. 2018). Variation in phenotype, growth pattern and secondary metabolites production in HRs have been attributed to the diversity of Ri plasmids, T-DNA binding sites, and different numbers of T-DNA copies in different strains of *A. rhizogenesis* (Gupta et al. 2015; Thwe et al. 2016; Hassan and Belbasi 2017; Figlan and Makunga 2017). Moreover, the efficiency of different *Agrobacterium* strains for developing HRs is strongly dependent on plant species and must be determined empirically (Park et al. 2017; Thwe et al. 2016).

In the present survey, among 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 (Grzegorczyk et al. 2006; Grzegorczyk-Karolak et al. 2018). The growth variations in HR clones might be originated from a different expression of T-DNA genes in individual root lines, which could adjust the biosynthesis of endogenous hormones or the susceptibility of plant cells to growth regulators (Ono and Tian 2011; Grzegorczyk-Karolak et al. 2018). Formation and growth of HRs are the consequences of excessive biosynthesis of endogenous auxins and cytokinins and enhanced sensitivity of transformed plant cells to these hormones, and *rol* genes appear to play a critical role in these processes (Hashem 2009). Gene *rolA* has a promoter region similar to those of some auxin-regulated genes, and its product is probably a DNA binding protein and stimulator of growth (Matveeva et al. 2015). Primary studies proposed that *rolB* protein is a β-glucosidase releasing auxin (IAA) from its inactive conjugated forms, thus increasing auxin sensitivity (Esruch et al. 1991). Moreover, the *rolB* gene-encoded product was shown to exhibit tyrosine phosphatase activity (Filippini 1996; Dilshad et al. 2015) and to interact with 14-3-3 proteins (Moriuchi et al. 2004; Matveeva et al. 2015), thus taking part in auxin signaling, enhancing the sensitivity of transformed cells toward auxin. It has been suggested that the product of *rolC* gene is a glucosidase liberating cytokinins from their bound forms (Estruch et al. 1991; Rangslang et al. 2018). Besides, the individual or combined *rol* genes are recognized to be an efficient activator of secondary metabolites biosynthesis in plants (Sarkar et al. 2018). Products of these genes can activate the biosynthesis of different types of secondary metabolites in transgenic HRs, including alkaloids, anthraquinones, isoflavonoids, and ginsenosides (Bulgakov et al. 2013; Matveeva et al. 2015). 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). It has been reported that transformed root cultures can accumulate high levels of secondary metabolites typical of the mother plant or even new metabolites after elicitation with various biotic andabiotic elicitors (Naik et al. 2016a). Various factors, such as elicitor type and concentration, duration of elicitation, and age or stage of the culture at the time of elicitation, need to be optimized for the best production of target secondary metabolites (Naik et al. 2016b; Halder et al. 2019).

The current study revealed that the production of TP, TF, RA, CA, 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 enhanced phenolic acids contents, especially RA, in HR cultures of *S. miltiorrhiza* (Yan et al. 2006; Zhang et al. 2014; Xing et al. 2015; Xing et al. 2018b), *M. spicata* (Yousefian et al. 2020) and *C. forskohlii* (Li et al. 2005). Differential effects of elicitors on the phenolic acid accumulation in HRs were observed in the present study. Although YE and Ag^+ ions had a stimulatory effect on the production 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 been also 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, CA, and Sal-A in MeJA-elicited (at 22.4 ppm for three days) HRs of *S. virgata* were 2.06, 2.72, 1.81, 4.35 and, 3.76-fold of the control culture, respectively. Similarly, Li et al. (2005) indicated that compared with YE, MeJA (at 22.4 ppm for seven days) was the most effective elicitor for the production of RA in *C. forskohlii* HR cultures, stimulating RA contents about 3.4 times greater than the control group. According to the report of Xiao et al. (2009), the application of MeJA, at 22.4 ppm for six days, enhanced not only RA but also LAB accumulation in *S. miltiorrhiza* HR cultures by approximately 2- and 6.5-fold, respectively. Furthermore, the addition of 22.4 ppm MeJA improved the production of RA (on day 5) and Sal-B (on day 2) about 1.5-fold and 1.7-fold of the control in HR cultures of *S. miltiorrhiza*, respectively (Zhang et al. 2014). Xing et al. (2018b) also reported that RA and Sal-B concentrations reached their highest levels in *S. miltiorrhiza* HRs on days 3 and 6 after treatment with 22.4 ppm MeJA, respectively.

Based on the results of the current study, compared with the control cultures, accumulation of CA (4.35-fold-increase) and 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 major phenolic acid, accumulated in the roots compared with CA and Sal-A.

It is believed that MeJA, as a signaling molecule, stimulates the key enzymes taking part in the biosynthesis of secondary metabolites. During the MeJA-triggered signal transduction, several processes such as reactive oxygen species (ROS) production, calcium fluxes, protein phosphorylation, biosynthesis and activation of transcription factors, and expression of secondary metabolite biosynthetic genes have been reported by several researchers (Baenas et al. 2014; Giri et al. 2016; Ho et al. 2020). Recently, it has been revealed that methyl jasmonate-responsive transcription factors regulate RA and salvianolic acid biosynthesis (Yang et al. 2017). For instance, overexpression of SmMYC2, a basic helix-loop-helix transcription factor, and an activator of the jasmonate signaling pathway can induce the expression of target genes including *PAL*, *TAT*, and *CYP98A14*, as well as an increase in Sal-B accumulation in *S. miltiorrhiza* (Du et al. 2018). A jasmonate-responsive transcription factor (a novel AP2/ERF) has been reported to activate the expression of *RAS* gene and positively regulates salvianolic acid biosynthesis in *S. miltiorrhiza* HRs (Sun et al. 2019). The results of a study by Deng et al. (2020) indicated that SmMYB2 activates the expression of cytochrome P450, thereby promoting MeJA-mediated biosynthesis of RA and Sal-B in *S. miltiorrhiza* HRs.

Yeast extract is the effective biotic elicitor widely used in plant cell and tissue cultures to increase secondary metabolism (Shi et al. 2007; Sørensen and Sondergaard 2014; Pastore et al. 2020). The stimulatory effect of YE on the production of secondary metabolites may be associated with the presence of various peptide and polysaccharide components in it such as chitin, N-acetyl glucosamine oligomers, beta-glucans, glycopeptides, and ergosterol (Zhao et al. 2011; Eskandari et al. 2012; Baenas et al. 2014). Besides, the metal ions (Ca^{2+}, Co, Zn) contained in YE can probably act as abiotic elicitors, as proposed by Srivastava and Srivastava (2014), and Kochan et al. (2017).

In the present study, significant enhancements in TP, RA, and Sal-A levels (1.98-, 1.44- and 2.42-fold of the control level, respectively) were found when YE (50 ppm) was applied to the *S. virgata* HR cultures for three days, and 1.74-fold-increase in the CA content was observed after five days. Similar to these results, a previous study reported that YE increased RA in HR cultures of *S. miltiorrhiza* (Zhao et al. 2011). According to the report of Chen et al. (2001), the accumulation of RA and LAB in *S. miltiorrhiza* HR cultures was about 3.2- and 2.8-fold of the control on the 7th day after elicitation with 5000 ppm YE, respectively. 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 10000 ppm YE, seven 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 4- and 8-days treatment with 200 ppm YE, respectively. According to Bauer et al. (2009), RA contents in two YE-induced (5000 ppm YE for one day) HR clones of *C. blumei* were 20 % and 44 % higher than that obtained in the control group.

Yeast-derived elicitors can stimulate some of the key enzymes from the phenylpropanoid and tyrosine-derived pathways (Yan et al. 2006; Zhai et al. 2016). Some reports showed YE improves RA biosynthetic pathway via the enhancement of PAL, TAT, and RAS activities (Sumaryono et al. 1991; Mizukami et al. 1992; Yan et al. 2006; Sahu et al. 2013). Based on a review by Zhai et al. (2016), it can be verified that various signal components such as ROS, G proteins, Ca^{2+} influx, jasmonate, and protein kinases are involved in the YE-induced production of secondary metabolites. Gene expression studies by Park et al. (2016) revealed a close relationship between the increased expression levels of phenylpropanoid biosynthetic pathway genes (*PAL*, *C4H*, and *4CL*) and RA accumulation in the YE-elicited cell cultures of *Agastache rugosa.* Although different families of transcription factors (WRKY, bHLHs, AP2-ERF, and MYBs) have been proposed as YE-responsive transcription factors which regulate the biosynthesis of some plant secondary metabolites (Yang et al. 2013; Gao et al. 2014; Zhou et al. 2017), it has not elucidated whether they are responsible for the YE-induced accumulation of phenolic acids.

Silver ions, as one of the most potent abiotic elicitor, is believed to stimulate the production of plants secondary metabolites (Naik and Al-Khayri 2016b). In the current study, after the addition of 2.5 ppm Ag^{+} (for five days), the accumulation of TP, TF, RA, CA, and Sal-A was estimated to be 1.8-, 1.88-, 1.54-, 2.45- and 3.37-fold of the control, respectively. Similarly, as reported by Xing et al. (2015), the maximum concentrations of RA (1.3-fold of the control) and CA (about 1.4-fold of the control) in HRs of *S. miltiorrhiza* were determined after elicitation with 2.5 ppm Ag^{+} ions (for six days). In another study involving *S. miltiorrhiza* HR cultures, TP and RA reached to 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), while Sal-B (approx. 3.5 times higher than the control) dramatically responded to this elicitor.

Ag^{+}-induced accumulation of different phenolic acids, including CA, RA, and LAB, in HRs of *S. miltiorrhiza*, were 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 mitogen-activated protein kinases, thereby resulting in phosphorylation and activation of downstream transcription factors (Paeizi et al. 2018). Recent studies showed that certain members of WRKY transcription factors in *Arabidopsis thaliana* L. (Kohan-Baghkheirati and Geisler-Lee 2015) and bZIP transcription factors in *S. miltiorrhiza* (Zhang et al. 2018) are responsive to Ag^+ ions and regulate plant secondary metabolism in response to the abiotic stress. Based on some reports, Ag^+-induced accumulation of secondary products can be related to the cross-talk with jasmonate signaling (Paeizi et al. 2018) and to the inhibition of ethylene signal transduction (Li et al. 2016; Khalili et al. 2010; Zhang and Wu 2003).

5. Conclusion

This study illustrated, for the first time, an efficient method for the production of phenolic acids 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 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, CA, and Sal-A in HR cultures, and the highest accumulation of these phenolic components was achieved at 22.4 ppm MeJA after three days. Subsequent investigations relevant to elicitation and mechanism of phenolic acids biosynthesis need to be conducted to further utilize the potency of *S. virgata* HR cultures to produce more bioactive compounds.

Abbreviations

- HR: hairy root
- RA: rosmarinic acid
- CA: caffeic acid
- Sal-A: salvianolic acid A
- TP: total phenol
- TF: total flavonoid
- MeJA: methyl jasmonate
- YE: yeast extract
- Ag^+ ions: silver ions
- PCR: polymerase chain reaction

Declarations

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124. **Elicitor-induced phenolic acids accumulation in *Salvia virgata* Jacq. hairy root cultures**
125. **Plant Cell, Tissue and Organ Culture**
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Figures

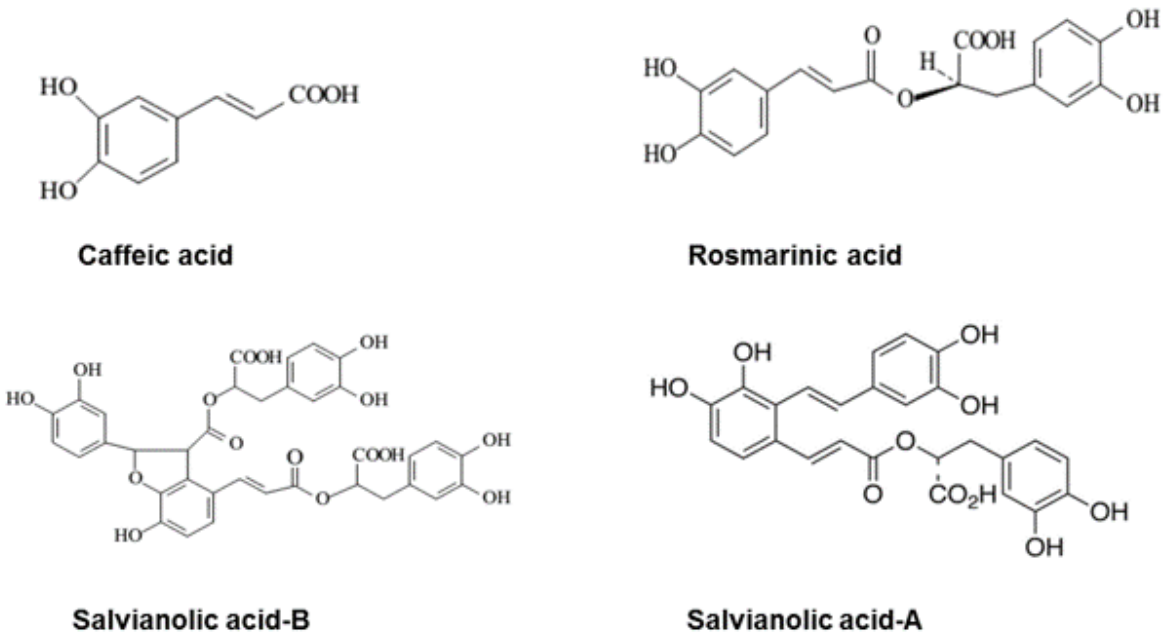

Figure 1

Chemical structures of caffeic acid and some of its derivatives in Salvia genus

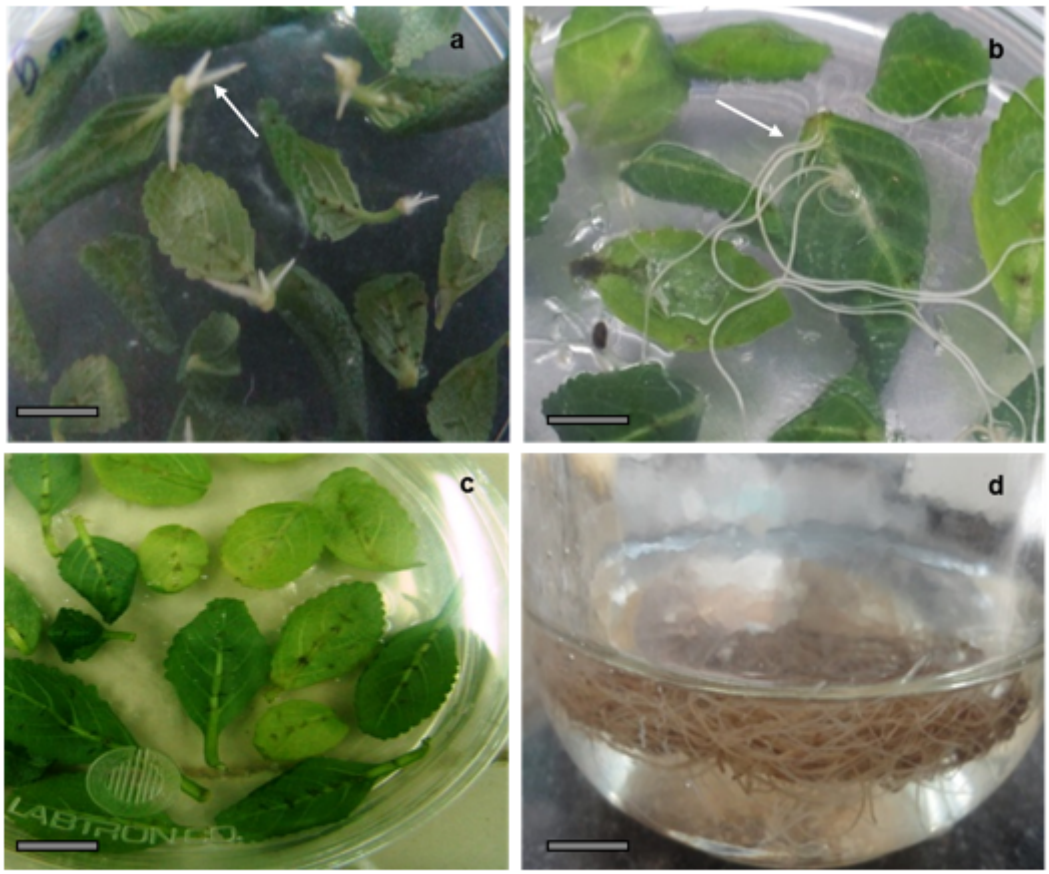

Figure 2

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

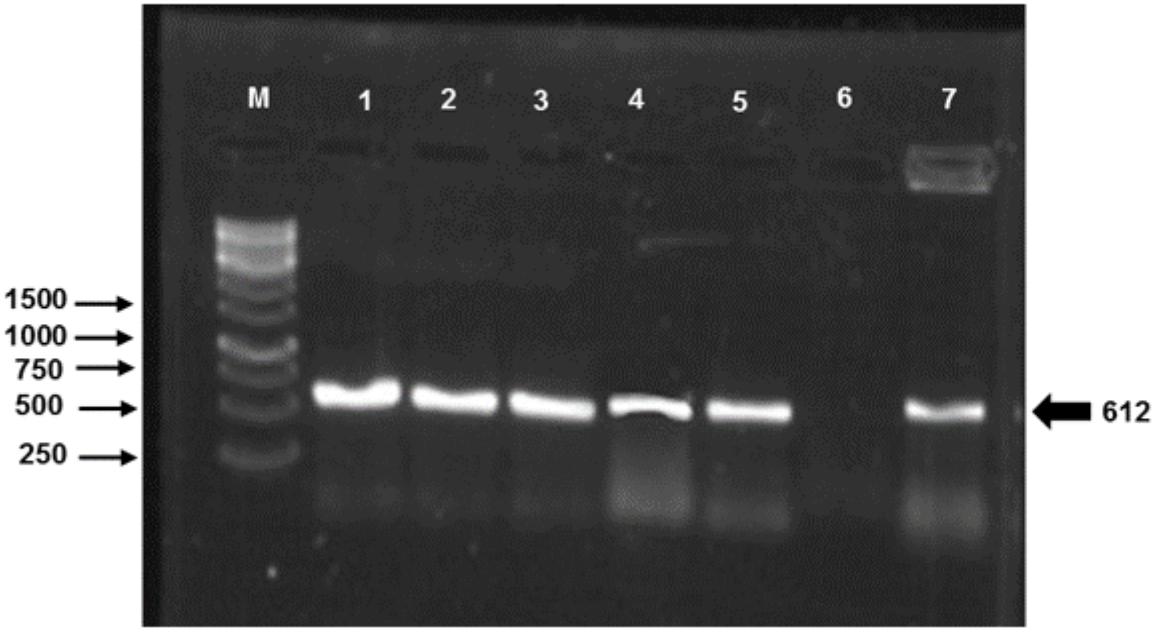

Figure 3

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). Arrowhead shows amplified fragment of rolC (612 bp) gene

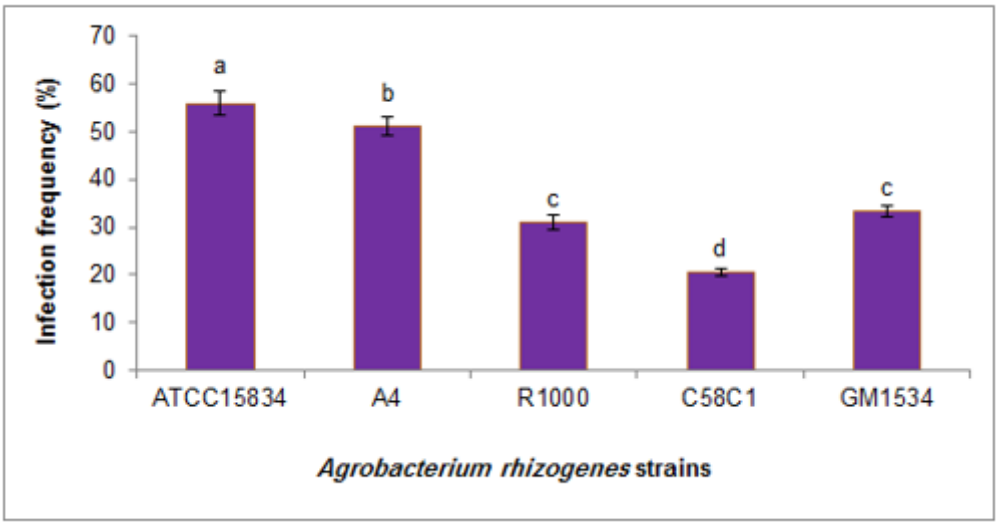

Figure 4

Hairy root induction frequency from S. virgata leaf explants. Data were collected 4 weeks after infection with different strains of A. rhizogenes. Each value represents mean ± SE of three replicates. Means with the same letter are not significantly different (P≤ 0.05) according to Duncan's Multiple Range Test

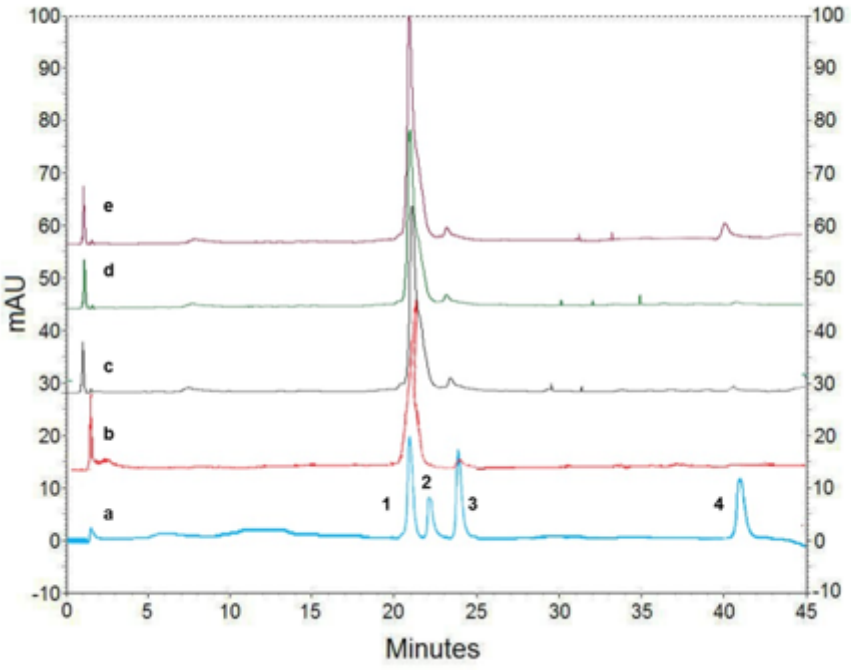

Figure 5

HPLC chromatograms of hydromethanolic extracts from S. virgata hairy roots. 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; Peak 4: caffeic acid

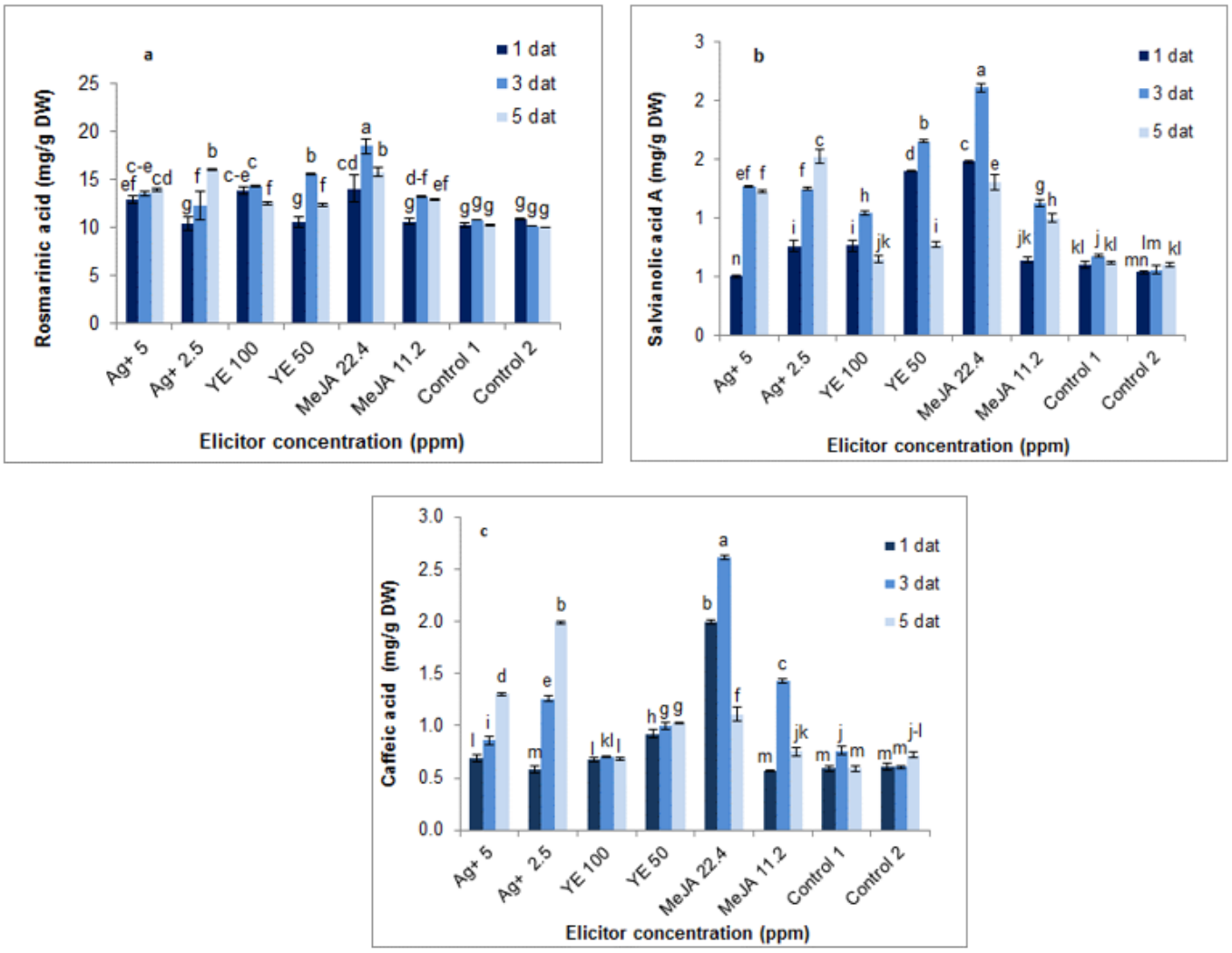

Figure 6

Effects of different concentrations of the applied elicitors on phenolic acids production in the hairy root cultures of S. virgata during periods of treatments. The data represent mean ± SE of three replicates. The different letters denote a statistically significant difference (P≤ 0.05) according to Duncan's Multiple Range Test. a Rosmarinic acid, b salvianolic acid-A, c caffeic acid. Ag+, silver ions; YE, yeast extract; MeJA, methyl jasmonate; Controls 1, untreated roots; Control 2, ethanol-treated roots; dat, day after treatment

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