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Enhanced phenolic acids production in regenerated shoot cultures of *Salvia virgata* Jacq. after elicitation with Ag^+ ions, methyl jasmonate and yeast extract

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ABSTRACT

Phenolic acids, among the major secondary metabolites of *Salvia* species, are important bioactive phytochemicals, namely for their application in pharmaceutical industries. Biosynthesis of secondary metabolites, including phenolic acids in plant *in vitro* cultures is affected by biotic and abiotic elicitors, leading mostly to higher levels than in non-elicited ones. The present study was initially focused on achievement to a suitable procedure for direct multiple shoot regeneration on multinodal explants from 50 days old seedlings of *Salvia virgata* Jacq. on Murashige and Skoog (MS) solid media supplemented with different concentrations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). Then, the effects of different concentrations of Ag^+ ions, yeast extract (YE) and methyl jasmonate (MeJA) on total phenolic and flavonoid contents, as well as some phenolic acids were studied in the regenerated shoots after 1,3 and 5 days of elicitation in liquid free hormone MS medium. Based on the results, the maximum number of regenerated shoots (4.67) per responsive explant was obtained on MS medium containing 2 ppm BAP and 0.05 ppm IAA. As an effective abiotic elicitor, Ag^+ ions could improve production of phenolic acids in the shoot cultures, while the highest content (26 mg/g DW) of rosmarinic acid (RA) was reported on day 5 after exposure of regenerated shoots to 2.5 ppm of Ag^+ ions. Also, the highest contents of salvianolic acid A (Sal-A) (3.72 mg/g DW) and caffeic acid (CA) (35.5 mg/g DW) were found after elicitation of regenerated shoots with MeJA (11.2 ppm) on day 3 and Ag^+ ions (2.5 ppm) on day 5, respectively. The results suggested that MeJA and Ag^+ ions had the considerable ability to stimulate the production of valuable phenolic acids such as RA in the regenerated shoot cultures of *S. virgata*.

1. Introduction

Salvia virgata Jacq. (wand sage), belongs to Lamiaceae family and is a perennial medicinal plant native to Asia (northeast of Iran) and southeastern Europe. There are few scientific reports about *S. virgata*. Some traditional uses of the preparations of this little known medicinal plant against skin diseases, wounds and blood cancer (leukemia) have been reported (Baytop, 1999). Recently, modern medical studies have conceived antioxidant ability (Kosar et al., 2008; Tepe, 2008), anti-inflammatory and anti-nociceptive activities of the extracts of this species (Akkol et al., 2008).

It has been identified that the most abundant and important bioactive compounds in the shoots of this species are phenolic acids, especially rosmarinic acid (RA) (Fig. 1) (Kosar et al., 2008).

Some studies have confirmed that RA, as the main bioactive compound in *Salvia* species, especially *S. miltiorrhiza* and *S. officinalis* had some therapeutic effects such as antioxidant (Tepe, 2008), anti-ischemic (Ozturk et al., 2014) anticancer (Sharmila and Manoharan, 2012), anti-inflammatory (Chu et al., 2012), antibacterial (Abedini et al., 2013) and antiviral properties (Dubois et al., 2008). It can also prevent Alzheimer's disease (Airoldi et al., 2013), and acts as a putative inhibitor of HIV-1 integrase (Hooker et al., 2001) and reverse transcriptase (Mazumder et al., 1997).

Abbreviations: Ag^+ ions, silver ions; BAP, 6-benzylaminopurine; CA, caffeic acid; IAA, indole-3-acetic acid; LAB, lithospermic acid B; MeJA, methyl jasmonate; MS, Murashige and Skoog; RA, rosmarinic acid; Sal-A, salvianolic acid A; Sal-B, salvianolic acid B; YE, yeast extract.

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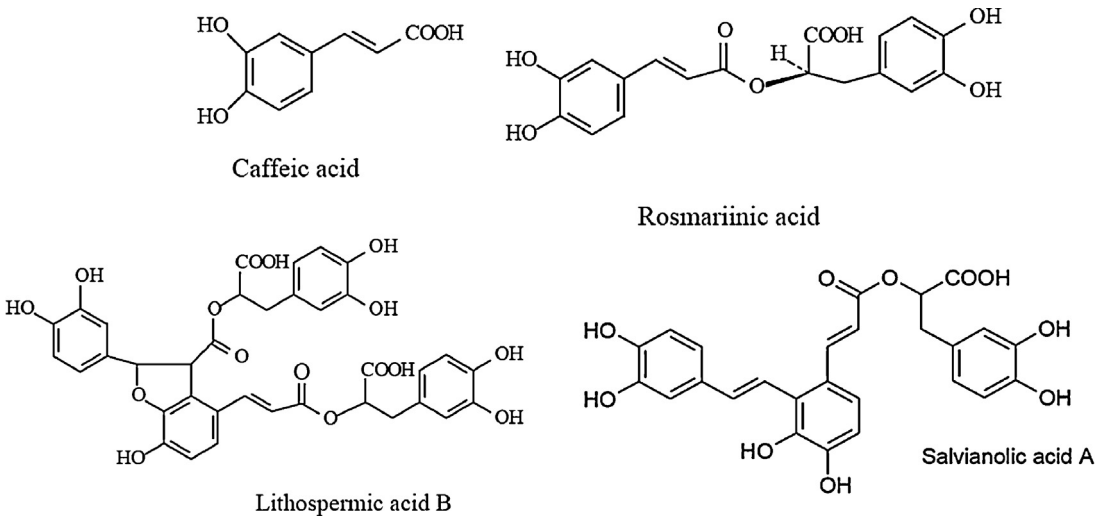

Fig. 1. Molecular structures of four phenolic acids in *Salvia* L. (Xing et al., 2015).

Salvianolic acid B (Sal-B) or lithospermic acid B (LAB), and salvianolic acid A (Sal-A) are dimeric derivatives of RA (Fig. 1), but major details of their biosynthesis pathway from RA have remained unknown (Xiao et al., 2010). Water-soluble phenolic acid, Sal-B, has been successfully isolated and purified from some Chinese *Salvia* plants such as *S. miltiorrhiza, S. bowleyana* Dunn, *S. cavaleriei* var. *simplicifolia* Stib. and *S. dabieshanensis* (Jiang et al., 2005; Min-Hui et al., 2008; Zhao et al., 2010) and Sal-A just has been derived from S.*miltiorrhiza* (Chen et al., 2006; Min-Hui et al., 2008). Among salvianolic acids, Sal-A and Sal-B are the most abundant components in *S. miltiorrhiza* and Sal-A has higher antioxidant activity than Sal-B at the same concentration (Wang et al., 2012).

There are some reports for *in vitro* enhancement of phenolic acids production using shoot cultures of plants (Abraham et al., 2011; Phatak and Heble, 2002; Skrzypczak-Pietraszek et al., 2014). Due to the presence of bioactive phenolic compounds, micropropagation of *S. virgata* through shoot *in vitro* culture could be an effective technique for the large-scale production of these valuable secondary metabolites (Akkol et al., 2008). In the case of RA production, shoot cultures are much better sources than cell suspension cultures (Roy and Mukhopadhyay, 2012). Grzegorczyk and Wysokinska (2009) have reported similar contents of RA for *in vivo* and *in vitro* grown shoots of *S. officinalis*.

Recent studies have shown that plant metabolism and consequently production of some secondary compounds can be affected by a wide range of elicitors, especially metabolites that would not usually be synthesized and accumulated in the parent plants in nature at higher amounts (Baskaran et al., 2012; Cui et al., 2012). Application of various elicitors such as silver ions (Ag^+ ions) and yeast extract (YE) in culture media was widely compatible to promote production and accumulation of secondary metabolites during the culture process (Wang et al., 2012).

Based on the reports of some researchers, YE and Ag^+ ions can stimulate the accumulation of RA and phenolic acids in the hairy root cultures of *S. miltiorrhiza* (Xiao et al., 2010; Xing et al., 2015; Yan et al., 2006). Moreover, it has been proved that production of phenolic acids can be promoted under methyl jasmonate (MeJA) in *S. officinalis* and some other plants (Bauer et al., 2009; Grzegorczyk and Wysokinska, 2009; Skrzypczak-Pietraszek et al., 2014).

Up to this time, there is only one available report (Ejtahed et al., 2015) about the effects of elicitors on phenolic acids production in the regenerated shoot cultures of *S. virgata*. For this reason, the present study was aimed to establish an effective *in vitro* direct shoot regeneration system for *S. virgata* and to investigate the effects of some elicitors on production of RA, CA and Sal-A in the regenerated shoots.

2. Materials and methods

2.1. Plant material

Mature seeds of *S. virgata* were collected from wild grown plants in the August 2013 at Reine village (Bojnoord, North Khorasan province, Iran) with the geographical specifications including latitude: 57° and 2 min North, longitude: 37° and 23 min East and altitude: 1765 m above sea level. The species was identified at the Ferdowsi University of Mashhad herbarium (FUMH), where voucher specimen (no. 38128) of the plant was deposited. Seeds were surface sterilized with 70% (v/v) ethanol for 1 min and 5% sodium hypochlorite (w/v) solution for 5 min. Then, they were rinsed three times in sterile distilled water. For germination, seeds were placed into glass jar containing 25 mL of MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and 0.7% agar. The pH of media was adjusted to 5.6–5.8 before adding agar, and the MS basal medium was autoclaved at 120 °C for 17 min. The glass jars were kept in the dark for 3 days at 25 ± 2 °C and after germination of the seeds, they were placed at 26 ± 2 °C and 16/8 h (light/dark) photoperiod (45 μM photons m^{-2} s^{-1} provided by cool white fluorescent lamps) in a culture room. These conditions were also applied for all the experiments described below.

2.2. Shoot induction and culture conditions

The multinodal explants (1 cm length with 2–3 nodes) were excised from 50-days-old sterile seedlings and were cultured on MS media supplemented with different concentrations of indole-3-acetic acid (IAA) (0, 0.05 and 0.1 ppm) and 6-benzylaminopurine (BAP) (0, 1, 1.5 and 2 ppm) to determine the best culture medium for direct shoot regeneration. Three replications were considered for each treatment and five multinodal explants were cultured in each jar. After 4 weeks, the best treatment for direct shoot regeneration was selected based on the mean shoot number per responsive explant (MSN), the mean shoot length (MSL) and the mean leaves number per regenerated shoot (MLN). Subsequently obtained shoots were moved to free hormone liquid MS media supplemented with 3% sucrose and elicitors (Ejtahed et al., 2015; Grzegorczyk and Wysokinska, 2009; Suarez et al., 2010).

2.3. Preparation of elicitors

Three elicitors were tested at 3 concentrations, including $AgNO_3$ (Ag^+ ions: 2.5, 5 and 10 ppm), yeast extract (YE: 50, 100 and 200 ppm) and MeJA (11.2, 22.4 and 44.8 ppm). Stock solutions of Ag^+ ions were prepared by dissolving certain amounts of $AgNO_3$ in deionized water (Zhao et al., 2010). Yeast extract carbohydrate fraction was prepared from commercial yeast extract (Cat. no.Y4250, Sigma) by ethanol precipitation as described by Hahn and Albersheim (1978). Briefly, 25 g yeast extract was dissolved in 125 mL distilled water and ethanol was added to a final concentration of 80% (v/v). The mixture was allowed to precipitate for 4 days at 4 °C in a refrigerator. The remaining gummy precipitate was redissolved in 250 mL distilled water and subjected to another round of ethanol precipitation. The final gummy precipitate was dissolved in distilled water, stored at 4 °C and used as stock solution. Methyl jasmonate was dissolved in 96% ethanol (Wang et al., 2015). All elicitor solutions were sterilized by filtering through 0.2 μm microfilters.

2.4. Elicitor treatment

For elicitation, various concentrations of elicitors were added at the selected doses to 30-days-old regenerated shoots in liquid free hormone MS medium. The regenerated shoots were harvested 1, 3 and 5 days after elicitor treatment. The same volume of water or ethanol was added to the control cultures and regenerated shoots were harvested at the same days as elicitor treated shoots. All cultures were maintained at the same mentioned conditions.

2.5. Extraction of phenolic compounds

The regenerated shoots were oven-dried at 40 °C until constant dry weight and grounded into fine powder. Plant materials were extracted with methanol (500 mg shoot material/10 mL solvent) by sonication for 30 min at room temperature. The extracts were then filtered through Whatman no.1 filter papers. After vacuum evaporation, the dried extracts were maintained at −20 °C.

2.6. Determination of total phenolic compounds

The total phenolic content of samples was determined by using Folin-Ciocalteu reagent and gallic acid as standard (Wojdylo et al., 2007). Briefly, 1 mg of dried extract was dissolved in 1 mL methanol, 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 extracts was measured at 765 nm against a reagent blank 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 construction of calibration curve. The concentration of total phenol in samples was measured using calibration equation ($y = 0.1427 + 0.0029x$, $r^2 = 0.999$) and expressed as mg of gallic acid equivalent (GAE) per g dry weight.

2.7. Determination of total flavonoid

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

2.8. Determination of rosmarinic acid content by spectrophotometric assay

Rosmarinic acid concentration of the samples was determined based on the complexation of RA with zirconium (Zr^{4+}) ions according to the procedure of Ozturk et al. (2010). The reaction mixture was formed by mixing 4.6 mL ethanol, 200 μL extract solution and 200 μL zirconium (IV) oxide chloride solution to make the final volume 5.0 mL. After 5 min, the absorbance of extracts was recorded against a reagent blank at 362 nm. The concentration of RA in the extracts was determined according to calibration curve equation ($y = -0.004 + 0.019x$, $r^2 = 0.999$) that was obtained from the standard RA graph at the concentration range of 0–14.4 ppm. Rosmarinic acid content in the extracts was expressed as mg per g dry weight.

2.9. HPLC analysis of phenolic acids

The content of phenolic acids was measured by 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 particle, 125 mm × 4 mm) coupled with a UV–vis detector (D-14163 model). Data were processed by Software ChromGate (V 3.1). Separation was performed using a mobile phase (concentration gradient).The 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. Initial condition was A-B (90:10, v/v), linearly changed to A-B (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. 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 base on the equation which was obtained from corresponding standards calibration curves. Stock solutions containing RA, Sal-A and CA (400 ppm) were prepared in ethanol and diluted to appropriate concentration range for construction of calibration curves. The concentration of RA, Sal-A and CA in samples were measured using calibration equations ($y_{RA} = 41606x$, $r^2 = 0.996$; $y_{Sal\text{-}A} = 19497x$, $r^2 = 0.996$; $y_{CA} = 7337.1x$, $r^2 = 0.995$). Evaluation of each sample was repeated three times.

2.10. Statistical analysis

Shoot regeneration study was designed in a factorial experiment based on a completely randomized design. 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 error (SE). A probability of $P \leq 0.05$ was considered to be significant.

Table 1
Shoot induction on multinodal explants of *S. virgata* after 4 weeks of culture on MS medium supplemented with different concentrations of BAP and IAA.

Treatment Name	BAP (ppm)	IAA (ppm)	Mean shoot number/responsive explant ± SE	Mean shoot length ± SE (cm)	Mean number of leaves/responsive explant ± SE
T0	0	0	1 ± 0.00^{d}	1 ± 0.16^{d}	8 ± 0.82^{d}
T1	1	0	2.67 ± 0.47^{bc}	2.17 ± 0.24^{bc}	25.33 ± 1.25^{bc}
T2	1.5	0	3.67 ± 0.47^{b}	1.83 ± 0.24^{bc}	26.67 ± 2.36^{bc}
T3	2	0	3.00 ± 0.00^{bc}	2.27 ± 0.21^{bc}	27.33 ± 2.05^{bc}
T4	1	0.05	2.33 ± 0.47^{bc}	2.30 ± 0.16^{b}	24.00 ± 2.94^{bc}
T5	1.5	0.05	3.00 ± 0.00^{bc}	2.33 ± 0.24^{b}	27.67 ± 2.05^{b}
T6	2	0.05	4.67 ± 0.47^{a}	3.50 ± 0.16^{a}	36.67 ± 2.36^{a}
T7	1	0.1	2.67 ± 0.47^{bc}	2.00 ± 0.00^{bc}	22.67 ± 2.05^{c}
T8	1.5	0.1	3.33 ± 0.47^{bc}	1.50 ± 0.41^{c}	25.33 ± 2.05^{bc}
T9	2	0.1	3.33 ± 0.47^{c}	2.40 ± 0.08^{b}	27.67 ± 2.05^{b}

Each value represents mean ± SE. Within a column, means followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's Multiple Range Test. BAP: 6-benzylaminopurine, IAA: indole-3-acetic acid.

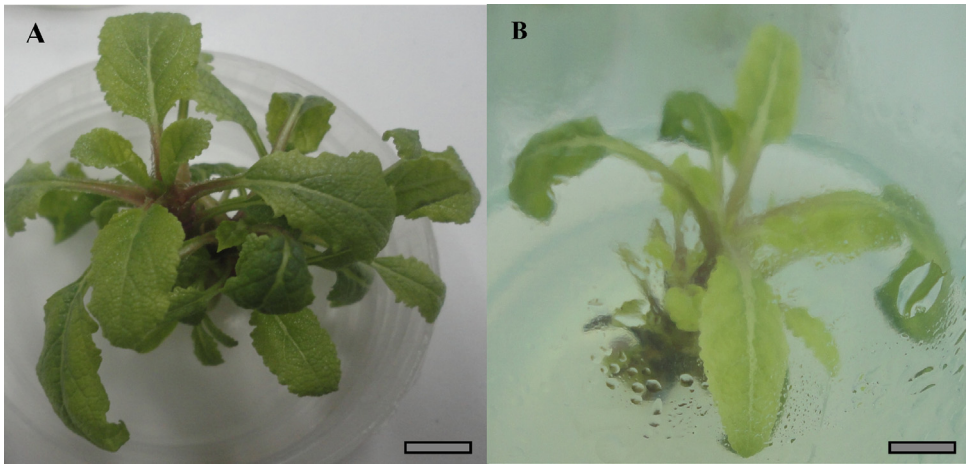

Fig. 2. Shoot regeneration on multinodal explants of *S. virgata* on MS basal medium supplemented with IAA (0.05 ppm) and BAP (2 ppm) (A) and on hormone free MS basal medium (B) 4 weeks after culture. Scale bar = 1 cm.

3. Results

3.1. Shoot regeneration

Seeds germinated on free hormone MS medium after 3 days in dark. Then, cultures were transferred to light to complete seedling development. After 50 days of seed culture on hormone free MS basal medium, nodal explants were cultured in the media supplemented with various concentrations of BAP and IAA. After 4 weeks of incubation, all multinodal explants produced regenerated shoots and MSN, MLN and MSL were evaluated for them. According to the results and under the similar conditions, MSN, MLN and MSL indices varied between 1 ± 0.00–4.67 ± 0.47, 1 ± 0.16–3.5 ± 0.16 and 8 ± 0.82–36.67 ± 2.36, respectively in the different treatments. Based on the MSN, MLN and MSL indices, the medium contained 2 ppm BAP and 0.05 ppm IAA was selected as the best medium for shoot regeneration (Table 1, Fig. 2).

3.2. Effects of elicitors on total phenolic and flavonoid contents

Significant differences ($P \leq 0.05$) were found between total phenolic compounds of the regenerated shoots in the different treatments. The highest contents of phenolic compounds were obtained in the treated shoots with 11.2 ppm of MeJA and 50 ppm of YE on 3th day and 2.5 ppm of Ag^{+} ions on 5th day after elicitation, with the values of 93.6 ± 0.81, 93.6 ± 1.36 and 123.1 ± 0.44 mg GAE/g DW respectively, which were 2.07–2.88 times higher than the obtained value for the control (Table 2).

Five days after elicitation with 2.5 ppm of Ag^{+} ions, a 1.71 fold increase was observed in the flavonoids content (9.66 ± 0.07 mg QUE/g DW) of regenerated shoots (Table 2), which was the maximum amount of flavonoids obtained from elicitation treatments. While, the highest amounts of total flavonoids in the regenerated shoots treated with 50 ppm of YE (7.47 ± 0.09 mg QUE/g DW) and 11.2 ppm MeJA (7.47 ± 0.09 mg QUE/g DW) were reported 3 days after treatment, which were 1.32 and 1.46 times higher than the values of the corresponding controls, respectively (Table 2).

3.3. Effects of elicitors on phenolic acids content

Based on the spectrophotometric results, the best treatment time for the effect of each elicitor was selected for quantitative analysis of RA content in the samples by HPLC. Among the different exposure periods, best results were observed for Ag^{+} ions 5 days after treatment and for MeJA and YE, 3 days after elicitation (Table 2). HPLC chromatograms of the extracts from the regenerated shoots after elicitation with Ag^{+} ions (2.5 ppm), untreated shoots and the standards solution are shown in Fig. 3.

In most cases, Ag^{+} ions were much more effective than YE and MeJA in stimulating the accumulation of RA in the regenerated shoots. The stimulating effect of all three elicitors was stronger at the lowest doses (2.5 ppm of Ag^{+} ions, 50 ppm of YE and 11.2 ppm MeJA, respectively) (Fig. 4). Rosmarinic acid content in the regenerated shoots was attained 26 ± 1.09 mg/g DW (on day 5), 20 ± 0.56 mg/g DW (on day 3) and 25 ± 2.52 mg/g DW (on day 3) after exposure to 2.5 ppm Ag^{+} ions, 50 ppm YE and 11.2 ppm MeJA,

Table 2
The effect of different concentrations of various elicitors on RA, total phenol and total flavonoid contents in the regenerated shoots of *S. virgata*.

Elicitors (ppm)	Days after treatment	RA content (mg/g DW)	Phenols content (mg GAE/g DW)	Flavonoids content (mg QUE/g DW)
Ag^+ 10	1	0.77 ± 0.02^{kl}	48.46 ± 1.30^{o}	4.98 ± 0.07^{p}
Ag^+ 10	3	0.91 ± 0.01^{i}	72.56 ± 0.65^{hi}	6.58 ± 0.30^{h}
Ag^+ 10	5	1.06 ± 0.01^{f}	90.69 ± 0.93^{cd}	7.21 ± 0.08^{ef}
Ag+ 5	1	0.66 ± 0.01^{op}	51.82 ± 0.42^{n}	5.61 ± 0.08^{lm}
Ag^+ 5	3	0.88 ± 0.02^{ij}	78.28 ± 0.65^{fg}	6.88 ± 0.14^{g}
Ag^+ 5	5	1.17 ± 0.02^{d}	106.57 ± 0.71^{b}	7.39 ± 0.26^{de}
Ag^+ 2.5	1	0.73 ± 0.01^{lm}	60.45 ± 0.53^{m}	6.82 ± 0.07^{l}
Ag^+ 2.5	3	1.18 ± 0.02^{d}	90.58 ± 0.23^{cd}	7.96 ± 0.07^{f}
Ag^+ 2.5	5	1.44 ± 0.03^{a}	123.13 ± 0.44^{a}	9.66 ± 0.07^{a}
YE 200	1	0.80 ± 0.02^{k}	61.71 ± 0.52^{m}	5.59 ± 0.07^{m}
YE 200	3	1.00 ± 0.03^{gh}	73.40 ± 1.36^{hi}	6.70 ± 0.15^{h}
YE 200	5	0.88 ± 0.01^{ij}	64.03 ± 0.46^{lm}	5.39 ± 0.10^{n}
YE 100	1	0.70 ± 0.04^{mn}	62.56 ± 0.50^{m}	5.59 ± 0.11^{m}
YE 100	3	$1.04 \pm 0.04f^{g}$	85.55 ± 0.39^{e}	6.64 ± 0.06^{ef}
YE 100	5	0.86 ± 0.04^{j}	71.26 ± 0.44^{ij}	6.25 ± 0.10^{ij}
YE 50	1	0.87 ± 0.02^{ij}	80.05 ± 0.49^{f}	6.06 ± 0.13^{k}
YE 50	3	1.12 ± 0.01^{e}	93.66 ± 1.36^{c}	7.47 ± 0.09^{cd}
YE 50	5	0.98 ± 0.06^{h}	86.49 ± 0.60^{e}	6.34 ± 0.06^{i}
MeJA 44.8	1	1.06 ± 0.03^{f}	61.71 ± 0.53^{hi}	5.69 ± 0.07^{lm}
MeJA 44.8	3	1.14 ± 0.03^{de}	73.40 ± 0.49^{gh}	6.70 ± 0.15^{jk}
MeJA 44.8	5	0.86 ± 0.03^{c}	64.03 ± 0.36^{m}	5.39 ± 0.10^{n}
MeJA 22.4	1	1.26 ± 0.04^{c}	67.15 ± 0.50^{kl}	5.60 ± 0.11^{h}
MeJA 22.4	3	1.29 ± 0.04^{h}	87.65 ± 0.57^{de}	7.25 ± 0.06^{c}
MeJA 22.4	5	0.96 ± 0.02^{h}	73.92 ± 0.21^{hi}	6.25 ± 0.11^{i}
MeJA 11.2	1	0.96 ± 0.02^{b}	66.13 ± 0.49^{kl}	6.06 ± 0.06^{i}
MeJA 11.2	3	1.36 ± 0.02^{j}	93.66 ± 0.81^{c}	7.47 ± 0.09^{b}
MeJA 11.2	5	0.86 ± 0.02^{no}	68.03 ± 0.24^{jk}	5.21 ± 0.06^{o}
Control 1	1	0.68 ± 0.68^{no}	45.95 ± 0.80^{op}	4.73 ± 0.10^{q}
Control 1	3	0.66 ± 0.66^{no}	45.06 ± 0.41^{op}	5.63 ± 0.03^{lm}
Control 1	5	0.62 ± 0.62^{p}	42.71 ± 0.39^{p}	5.64 ± 0.05^{lm}
Control 2	1	0.42 ± 0.01^{q}	27.66 ± 0.12^{r}	3.94 ± 0.03^{r}
Control 2	3	0.66 ± 0.03^{op}	37.65 ± 0.27^{q}	5.09 ± 0.04^{op}
Control 2	5	0.73 ± 0.01^{lm}	37.81 ± 0.44^{q}	4.70 ± 0.04^{q}

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. Regenerated shoots 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 shoots, Control 2: ethanol-treated shoots.

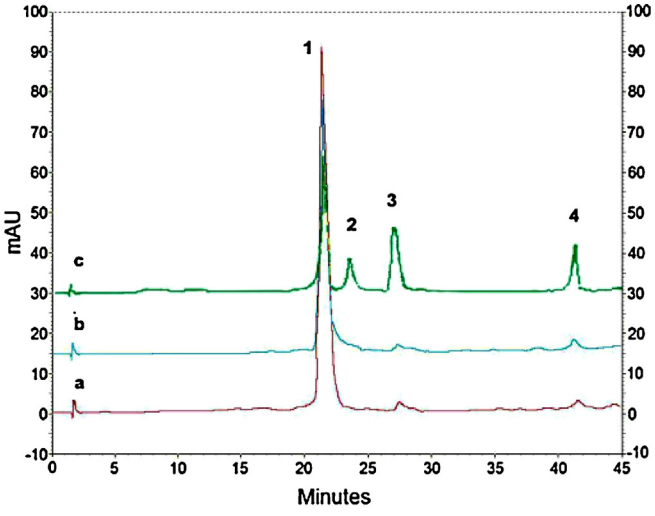

Fig. 3. HPLC chromatograms of methanolic extract of *S. virgata* regenerated shoots exposed to 2.5 ppm Ag^+ ions (a), untreated shoots (control) (b) and standards solution (c). Peak 1: rosmarinic acid, peak 2: salvianolic acid B, peak 3: salvianolic acid A, peak 4: caffeic acid.

respectively. The contents of RA at these treatments were 1.73, 1.17 and 1.66 times higher than those in the control, respectively (Fig. 4).

At the concentration of 2.5 ppm Ag^+ ions, Sal-A production increased up to 1.61 ± 0.05 mg/g DW which was 2.5 fold greater than that obtained for control. The highest concentration of Sal-A (3.73 ± 0.06 mg/g DW) was measured in the shoots treated with 11.2 ppm MeJA, which was about 2.5 times higher than the amount (1.46 ± 0.22 mg/g DW) evaluated in the control samples. The lowest concentration of YE (50 ppm) increased Sal-A accumulation in the regenerated shoots up to 1.17 times, as compared with the control. In particular, exposure to Ag^+ ions and MeJA significantly enhanced the Sal-A content in the regenerated shoots (Fig. 4). The results indicated that Sal-A content in the regenerated shoots after exposure to 44.8 ppm MeJA and 100 and 200 ppm YE was considerably decreased. The content of Sal-B was very low in the regenerated shoots of *S. virgata* and was not detected in this experiment.

In this project, the results strongly indicated that elicitation with Ag^+ ions, YE and MeJA induced accumulation of CA in the regenerated shoots. Maximum CA accumulation (35.5 ± 3.83 mg/g DW) was achieved with the addition of 2.5 ppm Ag^+ ions in the culture medium. As compared with the control, a 3.4 fold increase was found in the content of CA in this treatment.

The application of Ag^+ ions led to a significant increase in CA accumulation at the 2.5 and 5 ppm concentrations, but at 10 ppm there were no statistically significant differences in the CA content of the treated shoots, as compared with the control (Fig. 4). The shoots treated with 50 ppm YE had the highest CA content (24.1 ± 0.46 mg/g DW), which was significantly different from the CA content in the control (6.9 ± 0.29 mg/g DW). Among the different MeJA concentrations tested, a significant enhancement was found in the accumulation of CA in the shoots treated with 11.2 and 22.4 ppm of MeJA, when compared with the control. Treatment with Ag^+ ions at 10 ppm, YE at 200 ppm and MeJA at 44.8 ppm failed to increase the CA content in the *in vitro* shoot cultures. Consequently, Ag^+ ions (2.5 ppm), YE (50 ppm) and MeJA (11.2 ppm) at their lowest concentrations were more effective for stimulation of CA in the *S. virgata* shoot cultures.

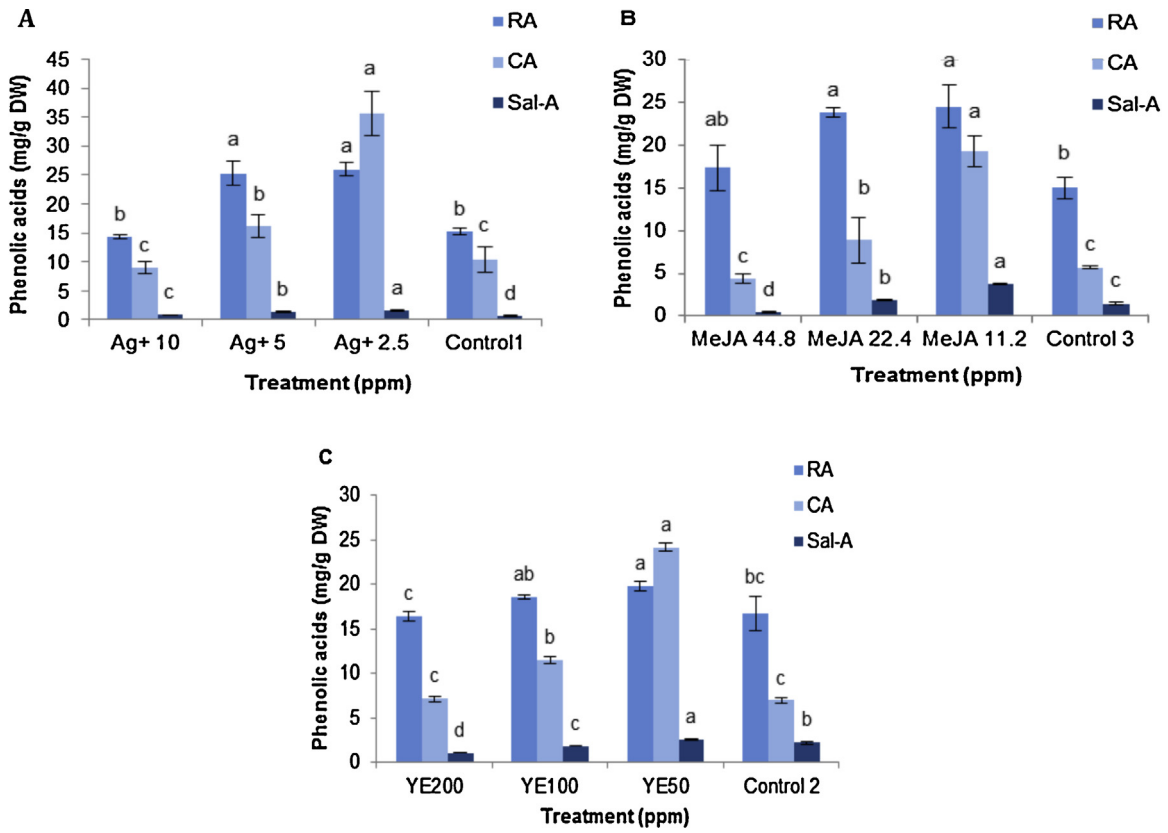

Fig. 4. Effects of different concentrations of elicitors on the RA (rosmarinic acid), CA (caffeic acid) and Sal-A (salvianolic acid A) contents in the regenerated shoots of *S. virgata* 5 days after treatment with Ag^+ ions (A), 3 days after treatment with MeJA (B) and YE (C). Error bars for standard errors (n = 3). Ag^+: silver ions, YE: yeast extract, MeJA: methyl jasmonate, Control 1: untreated shoots, Control 2: untreated shoots, Control 3: ethanol-treated shoots.

4. Discussion

Due to the presence of active meristems, nodal segments are generally suitable explants for micropropagation of many plant species; especially through direct multiple shoot regeneration. Clonal fidelity can be maintained by such shoots (Frabetti et al., 2009; Patil et al., 2013). For stimulating a high frequency of direct shoot proliferation on nodal segments, among various cytokinins tested in many plant species, outstanding results have been obtained with BAP (Faisal et al., 2007; Stefaan et al., 1994). According to the previous reports, low concentrations of an auxin in combination with a cytokinin could positively change the frequency of shoot induction and elongation (Cocu et al., 2004; Xu et al., 2008). In this work, direct shoot regeneration of *S. virgata* from multinodal explants was successfully established. In agreement with the results reported for *S. nemorosa* shoot cultures (Skala and Wysokinska, 2004), all the tested concentrations of BAP in present study, completely inhibited the rooting of *S. virgata* shoots.

In this investigation, the maximum number of shoots (4.67) obtained per responsive explants was greater than those of other *Salvia* species, such as *S. blancoana* (1.5) (Cuenca and Amo-Marco, 2000) and *S. officinalis* (3.2) (Santos-Gomes et al., 2002) and was comparable with the number of regenerated shoots (5.7) reported for *S. nemorosa* (Skala and Wysokinska, 2004). The promotive effect of BAP in shoot production was also reported earlier for several other species of the Lamiaceae family. The optimum concentration of BAP for shoot regeneration in *S. virgata* was 2 ppm (8.9 μM), which was higher than those reported for *S. canariensis* (0.1 μM) (Mederos et al., 1997), *S. sclarea* (2.2 μM) (Liu et al., 2000), *S. przewalskii* (2.2 μM) (Skala et al., 2007) and *S. officinalis* (6.7 μM) (Santos-Gomes et al., 2002); while was as the same concentration that has been used for efficient shoot induction in *S. nemorosa* (8.9 μM) (Skala and Wysokinska, 2004). These results indicated that *S. virgata* and *S. nemorosa* have the same potential for shoot regeneration, as a growth response, in *in vitro* cultures.

In this study, it was found that biotic and abiotic elicitors can stimulate total phenol and flavonoids content in the shoot cultures of *S. virgata*. Similar investigations showed YE, MeJA and Ag^+ ions significantly enhanced the total phenol and flavonoid content in cell and tissue cultures of some other plants (Abraham et al., 2011; Wang et al., 2015; Yan et al., 2006).

The results of present study showed that YE as a biotic elicitor and Ag^+ ions and MeJA as two abiotic elicitors can stimulate an increased accumulation of phenolic compounds, especially phenolic acids such as RA, CA and Sal-A in the shoot cultures of *S. virgata*. According to these findings, the content of RA, total phenols and flavonoids reached the highest levels in the regenerated shoots 3 days after MeJA and YE elicitation and 5 days after Ag^+ ions treatment. All of the used elicitors had a stimulatory effect on the production of examined phenolic acids in a negative dose dependent manner on the production of examined phenolic acids. Type and dosage of the elicitors, and the period of elicitation were the factors that affected RA accumulation in the shoot cultures (Wang and Wu, 2013).

The stimulating effects of elicitors such as YE, MeJA and Ag^+ ions on RA accumulation have also been observed in hairy root cultures of *S. miltiorrhiza* (Yan et al., 2006), shoot culture of *S. officinalis* (Grzegorczyk and Wysokinska, 2009) and in cell cultures of other plant species including *Exacum affine* (Skrzypczak-Pietraszek et al., 2014), *Lithospermum erythrorhizon* (Ogata et al., 2004) and *Coleus blumei* (Szabo et al., 1999).

Yeast extract is one of the most widely used biotic elicitors to motivate production of different groups of secondary metabolites including cardiac glycosides (Patil et al., 2013), phenolics (Thaweesak et al., 2011), phenolic terpenes (Chen and Chen, 2000; Ge and Wu, 2005; Zaker et al., 2015) and alkaloids (Eskandari et al., 2012; Wungsintaweekul et al., 2012) in plant cells and tissue cultures.

In this study, YE showed only a moderate stimulating effect on phenolic acids accumulation. Among the three applied concentrations, only 50 ppm of YE had significant effect on production of RA in the proliferated shoots, as compared with the control. These results are in accordance with the results reported by Nasiri-Bezenjani et al. (2014) for *Melissa officinalis* seedlings. The positive effects of this elicitor on the biosynthesis of RA in *C. blumei* (Szabo et al., 1999), hairy root of *S. miltiorrhiza* (Chen et al., 2001; Yan et al., 2006), cell cultures of *L. erythrorhizon* (Ogata et al., 2004) have been previously reported. However, addition of 100 and 200 ppm YE to the culture media exhibited a negative effect on Sal-A production. Yeast extract is made of different components including minerals, amino acids and vitamins. It has been suggested that the concentrations of cations such as Ca^{2+}, Co^{2+} and Zn^{2+} in YE may be the reason for stimulating effect of this elicitor on secondary metabolites production (Eskandari et al., 2012). The accumulation of phenolic acids under YE elicitation is due to its effects on phenylpropanoid pathway and mainly through a tyrosine-derived pathway (Yan et al., 2006).

Based on the obtained results, it was indicated that addition of Ag^{+} ions greatly improved the accumulation of phenolic aids such as RA, CA and Sal-A in the regenerated shoots of *S. virgata*. This observation is in agreement with the results of Yan et al. (2006) and Xing et al. (2015) on *S. miltiorrhiza*. Nevertheless, it has been reported that Ag^{+} ions did not affect on RA accumulation, but efficiently enhanced LAB in *S. miltiorrhiza* hairy root cultures (Xiao et al., 2010). So whether Ag^{+} ions have meaningful effect on phenolic acids remains unclear (Xing et al., 2015). On the contrary, significant accumulation of tanshinones (diterpene quinones) has been reported in adventitious root cultures of *Perovskia abrotanoides* Karel. (Zaker et al., 2015) and in *S. miltiorrhiza* hairy root cultures (Xing et al., 2015) under Ag^{+} ions elicitation.

In the present survey, the results showed that MeJA at the lower concentrations (11.2 and 22.4 ppm) significantly increased the phenolic acids contents in the shoots. The positive effect of MeJA on phenolic acids production has also been reported in *E. affine* shoot cultures (Skrzypczak-Pietraszek et al., 2014). Also, similar results were obtained for RA accumulation in *S. officinalis* shoot cultures (Grzegorczyk and Wysokinska, 2009), *L. erythrorhizon* cell cultures (Ogata et al., 2004) and hairy root cultures of *C. blumei* (Bauer et al., 2009). Recently, several scientific reports have shown that variety of secondary products such as phenylpropanoids (Suresh and Ravishankar, 2005), flavonoids (Wang et al., 2015), alkaloids (Eskandari et al., 2012; Wungsintaweekul et al., 2012) and terpenoids (Grzegorczyk and Wysokinska, 2009; Zaker et al., 2015; Zhao et al., 2010), are induced with MeJA signaling pathway in plant cell and tissue cultures. According to some studies, different concentrations of MeJA, Ag^{+} ions and YE change plant gene expression of key enzymes in the phenylpropanoid and tyrosine derived pathways and the time required to reach the maximum level of gene expression is different for each biosynthetic pathway and even each type of chemical compounds (Yan et al., 2006; Xing et al., 2015).

5. Conclusion

In conclusion, it was successfully established a tissue culture procedure with high efficiency for *in vitro* shoot regeneration of *S. virgata* through multinodal explants cultures on MS medium contained certain concentration of BAP and IAA. Also, the present study displayed that different elicitors had various effects on phenolic acids production in the regenerated shoots of *S.virgata*. Silver ion, at low concentration, was the most effective elicitor for stimulating phenolic acids production, followed by MeJA and YE. Among the phenolic acids, the highest increase was observed in the content of CA after treatment with the tested elicitors. However, in the most cases, RA was the most dominant phenolic acid accumulated in the elicited shoots as compared with CA and Sal-A. Therefore, pharmacologically valuable metabolites in *S. virgata* can be produced by these efficient *in vitro* systems. However, further studies are needed to identify the genes for the key enzymes involved in the elicitor-induced biosynthetic pathways.

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