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ORIGINAL ARTICLE

Enhancement of phenolic acids accumulation in *Salvia abrotanoides* (Kar.) Sytsma shoot cultures under elicitation with nitric oxide

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Abstract

Salvia abrotanoides (Kar.) Sytsma is a medicinal plant that contains phenolic acids, especially rosmarinic acid (RA), with a wide variety of health benefits. In this research, the stimulatory effects of sodium nitroprusside (SNP), a donor of nitric oxide (NO), were examined on phenolic acids accumulation in the shoot cultures of the species after different times of exposure. The developed shoots on single-nodal explants in Murashige and Skoog solid medium supplemented with 0.5 mg L^{-1} kinetin and 0.05 mg L^{-1} indole-3-acetic acid were treated with SNP at concentrations of 25, 50 and 100 µM. The highest content of RA (10.45 ± 0.40 mg g^{-1} DW) was measured 144 h after elicitation of the shoots with 100 µM SNP. The maximum values of salvianolic acid A (Sal-A) (0.065 ± 0.00 mg g^{-1} DW) and salvianolic acid B (Sal-B) (0.42 ± 0.01 mg g^{-1} DW) were obtained in the shoots after 96 and 144 h exposure to 50 and 25 µM SNP, respectively. Also, elicitation with SNP at different concentrations significantly upregulated the crucial genes (*PAL*, *TAT*, *RAS* and *CYP98A14*) involved in phenolic acids biosynthesis in the shoots with distinct patterns, although no strong correlations were observed between transcription levels of the genes and phenolic acids accumulation. The findings of this study provide beneficial information about the impact of NO as an effective elicitor, which could be valuable for the in vitro improvement of phenolic acids production in *S. abrotanoides*.

Key Message

The results demonstrated that the elicitation with nitric oxide upregulated critical genes in the biosynthetic pathways and led to phenolic acids accumulation in the shoots of *Salvia abrotanoides* (Kar.) Sytsma.

Keywords *Salvia abrotanoides* · Nitric oxide · Phenolic acids · Gene expression · Shoot culture

Abbreviations

KIN	Kinetin
IAA	Indole-3-acetic acid
FSI	Frequency of shoot initiation
MSN	Mean shoot number
MSL	Mean shoot length
NO	Nitric oxide
SNP	Sodium nitroprusside
TPC	Total phenolic content
TFC	Total flavonoid content
RA	Rosmarinic acid
Sal-A	Salvianolic acid A
Sal-B	Salvianolic acid B
PAL	Phenylalanine ammonia-lyase
TAT	Tyrosine aminotransferase
RAS	Rosmarinic acid synthase
CYP98A14	Cytochrome P450-dependent monooxygenase

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Introduction

Medicinal plants are the primary sources of valuable bioactive compounds principally used in the pharmaceutical, cosmetic and food industries (Lubbe and Verpoorte 2011). Such biologically active compounds are abundant in many plants from the Lamiaceae family (Trivellini et al. 2016). *Salvia* L. is the largest and most widespread genus of the mint family that grows abundantly in the Mediterranean

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regions, South East Asia, and Central and South America (Georgiev and Pavlov 2017). Plants belonging to this genus produce important phenolic compounds, including phenolic acids, especially rosmarinic acid (RA) and salvianolic acids, with a broad spectrum of biological functions with medicinal and industrial applications (Wang et al. 2019).

Salvia abrotanoides (Kar.) Sytsma (synonym of *Perovskia abrotanoides* Karel) is an aromatic plant distributed in Central Asia, Iran, Afghanistan, Pakistan, India and Tibet (Jamzad 2012). The plant is being used in traditional medicine to treat various ailments, especially leishmaniasis, due to the presence of tanshinones in the roots (Beikmohammadi 2012). Also, some studies in the literature have demonstrated that the species contains biologically active constitutions, including essential oils with antioxidant and antimicrobial effects (Ashraf et al. 2014) and abietane-type diterpenes with anti-inflammatory activity (Alizadeh et al. 2019).

Rosmarinic acid, as the main polyphenolic compound in *Salvia* species and the precursor for salvianolic acids (Petersen 2013), has antioxidant, anti-inflammatory, anticancer, antibacterial, antiviral and antifibrotic activities. Also, this phenolic acid and its derivatives are potent agents for therapy of Alzheimer's, cardiovascular and cerebrovascular diseases (Shi et al. 2018; Habtemariam 2018). The constituent units for RA synthesis are provided through two parallel phenylalanine- and tyrosine-derived pathways, which phenylalanine ammonia-lyase (PAL) and tyrosine aminotransferase (TAT) are the two first regulatory enzymes in these pathways, respectively. 4-Coumaroyl-CoA and 4-hydroxyphenyllactic acid as the two intermediate products of these pathways are condensed by rosmarinic acid synthase (RAS) to provide 4-coumaroyl-4′-hydroxyphenyllactate. Then 4-coumaroyl-4′-hydroxyphenyllactate hydroxylates by a cytochrome P450-dependent monooxygenase (CYP98A14) to form RA (Wang et al. 2019). It is supposed that laccases isoforms are involved in catalyzing the conversion of RA to salvianolic acid B (Sal-B) in *Salvia miltiorrhiza* Bunge (Li et al. 2019). However, end routes to salvianolic acids in the biosynthetic pathway of phenolic acids have not been understood in detail.

Due to the pharmaceutical and industrial significance of the secondary metabolites, augmenting these compounds can raise their commercial value (Isah et al. 2018). Plant cell, tissue and organ cultures are effective techniques used to in vitro propagate medicinal plants for optimizing bioactive compounds production under controlled conditions (Marchev et al. 2014). In some plants, including *Salvia* species, the shoot culture technique is used to improve the accumulation of phenolic and terpenoid compounds (Grzegorczyk and Wysokińska 2009; Grzegorczyk et al. 2019).

The synthesis and subsequent accumulation of secondary metabolites in the plants mainly occur in response to environmental stresses (Verma and Shukla 2015), thus the application of elicitors mimicking stress-associated effects can be applied to achieve in vitro high yields of secondary metabolites (Ramirez-Estrada et al. 2016). Nitric oxide (NO) is an important signaling molecule involved in the responses of plants against biotic and abiotic stresses (Siddiqui et al. 2011; Bellin et al. 2013). It has also been reported that NO plays significant roles in the signal transduction pathways of some elicitors to induce secondary metabolites production in medicinal plants (Zhang et al. 2012). Some studies have shown that the application of exogenous NO as an elicitor promoted the production of indole alkaloids in *Catharanthus roseus* L. cell suspension cultures (Xu and Dong 2005), diterpenoid taxol in *Taxus chinensis* var. *mairei* cell cultures (Wang and Wu 2005), total tannins, saponins, phenols and total flavonoids in *Ginkgo biloba* L. cell suspension cultures (El-Beltagi et al. 2015), colchicine alkaloid in morphogenic cultures of *Gloriosa superba* L. (Jawahar et al. 2018) and essential oils and phenolic compounds in *Origanum majorana* L. under drought stress (Farouk and Al-Huqail 2020).

Despite the medicinal importance of *S. abrotanoides*, a few reports are available on the tissue culture of the species, including adventitious root culture (Zaker et al. 2015) and hairy root culture (Ebrahimi et al. 2017) for secondary metabolite production and direct organogenesis for micropropagation (Ghaderi et al. 2019).

So far, no report has been published regarding the stimulatory effect of NO on secondary metabolites production in shoot cultures of *S. abrotanoides*. In this research, we investigated the impact of SNP (sodium nitroprusside), as NO donor, on the accumulation of RA, Sal-A and Sal-B in the shoot cultures of *S. abrotanoides*, as well on the transcript levels of some critical genes in their biosynthesis pathways.

Materials and methods

Seed germination and shoot induction

Mature seeds of *S. abrotanoides* (Kar.) Sytsma were collected in September 2018 from a wild-grown population in the northeastern region of Iran (Kalat area, Razavi Khorasan province latitude 59°52′ N, longitude 36°38′ E and elevation 1756 m above sea level). The voucher specimen (FUMH90123) was deposited at the herbarium of Ferdowsi University of Mashhad. Surface sterilization of seeds was done with 70% (v/v) ethanol for 45 s and 5% sodium hypochlorite (w/v) solution for 3 min and then followed by washing them three times with sterile distilled water. The sterilized seeds were cultured in glass jars containing MS medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and 0.6% (w/v) agar. Subsequently, the jars were incubated at 23 ± 2 °C under 16 h light/8 h dark

photoperiod and a photosynthetic photon flux density of 400 µmol m^{-2} s^{-1} in a growth chamber for two months.

Single-node explants containing a pair of axillary buds were excised from sterile seedlings and cultured on MS media supplemented with different concentrations of kinetin (KIN) (0, 0.5, 1 and 2 mg L^{-1}) and indole-3-acetic acid (IAA) (0, 0.05 and 0.1 mg L^{-1}) to determine the best culture medium for shoot induction. All treatments were performed twice, and each treatment consisted of at least six replicates (jars) with three explants. The explants were cultured in jars containing 50 mL medium and incubated in the conditions described earlier for seeds culture. After four weeks, the best hormonal combination for stimulating the growth of axillary buds was assessed based on the frequency of shoot initiation (FSI), the mean shoot number per responsive explant (MSN) and the mean shoot length (MSL). Then, the well-developed shoots obtained in the optimum hormonal concentration for shoot development (0.5 mg L^{-1} KIN and 0.05 mg L^{-1} IAA) were subcultured on the solid hormone-free MS media for 30 days before treatment with the elicitor.

Elicitor treatment

The stock solution of the elicitor was prepared by dissolving SNP crystals (Merck, UK) in distilled water and then sterilizing by filtering through a 0.2 µm filter. The 2-month-old in vitro grown shoots were transferred to liquid MS media and subjected to various concentrations (0, 25, 50 and 100 µM) of SNP. Five replicates (glass jars) with six shoots per jar were used for each treatment. Shoots were cultured in 250-mL glass jars with 10 mL of the medium containing different concentrations of SNP under stationary culture. In each jar, shoots were placed in a vertical position, and only their cut ends were in contact with the surface of the culture media. Then cultures were incubated in the growth chamber under the conditions described earlier. The shoots in the control group were cultured in MS media without elicitor. Shoot samples were collected at 0, 8, 24, 48, 96 and 144 h after the treatment, instantly frozen in liquid nitrogen and kept at − 80 °C for molecular analyses. Alternatively, a constant weight of the dried shoots samples was used for phytochemical analyses.

Determination of total phenolic and flavonoid contents

Each dried shoot sample (0.1 g) was homogenized in a Falcon tube with 10 mL methanol (HPLC-grade) and then extracted in an ultrasonic bath (BANDELIN, Germany) at room temperature for 30 min. The extracts were centrifuged at 12,000 rpm for 10 min, and the collected supernatants were stored at − 20 °C until phytochemical analysis.

The total phenolic content (TPC) of the samples was determined using the Folin–Ciocalteu reagent (Singleton et al. 1999). The calibration curve was constructed by plotting the absorbance measurements of standard solutions of gallic acid (0.1, 0.2 and 0.3 mg mL^{-1}) at 750 nm against concentration. The total phenolic contents were measured using the regression equation of the standard curve ($y = 4.0104x$, $r^2 = 0.999$) and presented as mg gallic acid equivalent per g of dry weight (mg GAE g^{-1} DW).

The total flavonoid content (TFC) in the sample extracts was evaluated based on aluminum chloride colorimetric assay (Zhishen et al. 1999). The calibration curve was constructed by plotting the absorbance measurements of standard solutions of quercetin (0.1, 0.2 and 0.3 mg mL^{-1}) at 415 nm against concentration. The total flavonoid contents were measured using the regression equation of the standard curve ($y = 3.7511x$, $r^2 = 0.995$) and expressed as mg quercetin equivalent per g of dry weight (mg QE g^{-1} DW).

HPLC analysis of phenolic acids

Quantitative and qualitative determination of RA, Sal-A and Sal-B was carried out in a Smartline HPLC instrument (Knauer, Germany) consisting of a C18 MZ-Analysentechnik column (5 µm particle size, 250 mm length, 4.6 mm diameter). The solvent system used for separation was a mixture of acetonitrile (A) and water containing 0.1% orthophosphoric acid (B). The flow rate was 1 mL min^{-1}, and the injection volume was 20 µL. The detection of the peaks was performed using a UV detector at 280 nm. Before injection, the methanolic extracts were filtered through a 0.45 µm syringe filter (Millipore SAS, France). The linear gradient procedure was as follows: 10–25% A at 0–15 min, 25–80% A at 15 to 45 min, 80–100% A at 45–53 min, 100% A at 53–68 min, 100–10% A at 68–71 min and 10% A at 71–75 min. The corresponding peaks of RA, Sal-A and Sal-B were identified by comparing their retention times with those of the standard references in the chromatograms. The contents of phenolic acids in the shoots were calculated using calibration curves equations constructed by their standard solutions ($y_{RA} = 41606x$, $r^2 = 0.996$; $y_{Sal\text{-}B} = 2055x$, $r^2 = 0.995$; $y_{Sal\text{-}A} = 19497x$, $r^2 = 0.996$) and exhibited as mg g^{-1} of DW.

RNA extraction, cDNA synthesis and gene expression analysis

Frozen shoot samples were finely powdered in liquid nitrogen and were used to estimate the relative expression of responsible genes for phenolic acids biosynthesis consisting of *PAL*, *TAT*, *RAS* and *CYP98A14*. The *rbcL* gene (large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase) was chosen as the reference gene. Total RNA was extracted

from samples using the DENAzist total RNA isolation kit (Denazist Asia, Iran) according to the manufacturer's instructions. The integrity of RNA molecules was determined by 1% agarose gel electrophoresis, and their purity and concentrations were checked using a SPECTROstar Nano microplate reader (BMG LABTECH, Germany). Equal amounts of total RNA (1 µg) were used to synthesize cDNA using RevertAid reverse transcriptase (Thermo Fisher Scientific, USA) with a final concentration of 10 U μL^{-1}. The primers were designed based on conserved sequences from several related and close species in the Lamiaceae family. Conserved areas were detected through sequence alignment with Clustal Omega online software. The primers were designed using Gene Runner software, and then they were analyzed with BLAST software on NCBI (Table 1). The qRT-PCR assays were done using a Corbett Rotor-Gene 6000 Real-Time PCR system (Qiagen, USA) using the fluorescent dye SYBR®Green Master Mix 2×(Ampliqon, Denmark) following the manufacturer's protocol. Each amplification reaction (10 µL) contained 5 µL of 2×SYBR Green Mix, 0.5 µL of cDNA, 0.5 µM of the forward and reverse primers and 4 µL of nuclease-free PCR-grade water. The amplification program was initiated with the following temperature profile: 95 °C for 15 min, 40 cycles of 20 s at 95 °C, 20 s at 58–62 °C (annealing temperature) and 35 s at 72 °C, followed by gradient: 72–95 °C for 5 min. The relative transcript levels of the assessed genes were analyzed using the comparative cycle threshold (Ct) method, which uses the formula $2^{-\Delta\Delta CT}$. Furthermore, *PAL*, *RAS* and *CYP98A14* partial sequences were PCR-amplified and sequenced (Codon Genetic group, Iran). Partial nucleotide sequences of the genes have been deposited in National Center for Biotechnology Information (NCBI) database under accession numbers MZ274389.1, MZ274390.1, and MZ274391.1, respectively.

Table 1 Nucleotide sequences of primers used in this study

Primer name	Sequence (5′→3′)
PAL-RTF	ATGTGCAGAGCGCGGAG
PAL-RTR	AGTAGGTTGAAGACATGAGTTTAA
TAT-RTF	TCAGCACCTGAAAGAAGATTG
TAT-RTR	AACCAGGAACCAACCATCTC
RAS-RTF[a]	ATCGCCACGTGCGGCGA
RAS-RTR[a]	TCAAACGGCGCCGCCCA
CYP98A14-RTF	CTAAGGAGGTGCTGAAGGAG
CYP98A14-RTR	GTGGAGTCGTTGTAGATGGA
rbcL-RTF	TCACATTCACCGTGCAATGCAT
rbcL-RTR	AGTGAATATGATCTCCACCGGA
PAL-F[a]	CATGAGTAAGGGCACCGACAG
PAL-R[a]	GGGTTGTTCGTTGACGGAGTTG
CYP98A14-F[a]	CTAAGGAGGTGCTGAAGGAG
CYP98A14-R[a]	GATGATGGTGTCCTCGCTGA

PAL: phenylalanine ammonia-lyase, *TAT*: tyrosine aminotransferase, *RAS*: rosmarinic acid synthase, *CYP98A14*: cytochrome P450-dependent monooxygenase, *rbcL*: ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit

[a]Designed primers for PCR amplification and sequencing of the genes

Statistical analysis

The experiment was designed randomly with a factorial nature, and analyses were performed in three repetitions per treatment. Analysis of variance (ANOVA) using SPSS software (version 22) was used to interpret the data. Duncan's multiple range test was used at the $P \leq 0.05$ level to analyze significant differences between the means. Correlation analysis was performed to determine the association between the accumulation of phenolic acids and the expression of their biosynthetic genes.

Results

Shoot culture

In this assay, the sterilized seeds were germinated on basal MS medium after three days of culture. After two months of incubation, single nodal segments were cut from seedlings and used as explants. The explants were cultured on MS medium supplemented with 3% sucrose with various concentrations of KIN and IAA for shoot induction. The axillary buds growth was observed on nodal explants in all treatments, with the maximum number of two developed shoots on one explant. Among the tested hormonal treatments for shoot development, the best results for the calculated FSI, MSN and MSL indices were obtained in the medium containing 0.5 mg L^{-1} KIN and 0.05 mg L^{-1} IAA (Table 2; Fig. 1).

Effect of NO on total phenolic and flavonoid contents

The contents of phenolic compounds were measured in the shoots extracts of *S. abrotanoides* after exposure to different concentrations of SNP (Table 3). Significant time-dependent increases in TPC were observed in the shoots after exposure to all the applied SNP concentrations. However, a maximum value of 21.18 ± 0.15 mg GAE g^{-1} DW (1.33-fold of the non-elicited control group at the same exposure time) was achieved for TPC in the elicited shoots 144 h after treatment with 50 µM SNP.

Unlike TPC, exposure to SNP resulted in reduced levels of TFC in the shoots. The significant decreases were observed after 144 h of elicitation with 25 µM SNP, which was the minimum obtained TFC (1.7-fold lower than the

Table 2 Shoot formation on single-nodal explants from *S. abrotanoides* cultured on MS medium supplemented with different concentrations of KIN and IAA after four weeks

Treatment	KIN (mg L^{-1})	IAA (mg L^{-1})	FSI (%)	MSN	MSL (cm)
T0	0	0	60 ± 5.19^{b}	1.71 ± 0.11^{ab}	3.30 ± 0.75^{c}
T1	0.5	0	70 ± 8.66^{ab}	1.21 ± 0.05^{cd}	2.20 ± 0.57^{c}
T2	0.5	0.05	90 ± 5.77^{a}	2.00 ± 0.00^{a}	9.10 ± 1.09^{a}
T3	0.5	0.1	80 ± 4.61^{ab}	1.16 ± 0.09^{c}	3.12 ± 0.63^{c}
T4	1	0	80 ± 6.92^{ab}	1.88 ± 0.11^{a}	3.70 ± 0.36^{bc}
T5	1	0.05	70 ± 7.50^{ab}	1.21 ± 0.14^{cd}	2.82 ± 0.75^{c}
T6	1	0.1	60 ± 8.66^{b}	$1.44 \pm 0.05^{b-d}$	2.81 ± 0.57^{c}
T7	2	0	70 ± 2.88^{ab}	1.49 ± 0.16^{bc}	3.37 ± 0.64^{c}
T8	2	0.05	60 ± 6.35^{b}	1.16 ± 0.09^{c}	5.68 ± 0.70^{b}
T9	2	0.1	70 ± 11.54^{ab}	2.00 ± 0.00^{a}	5.57 ± 0.49^{b}

Values are the means ± standard error (n = 3). Means within a column followed by the same superscript letter(s) are not significantly different at $P < 0.05$ based on Duncan's test

KIN: kinetin, IAA: indole-3-acetic acid, FSI: frequency of shoot initiation, MSN: mean shoot number, MSL: mean shoot length

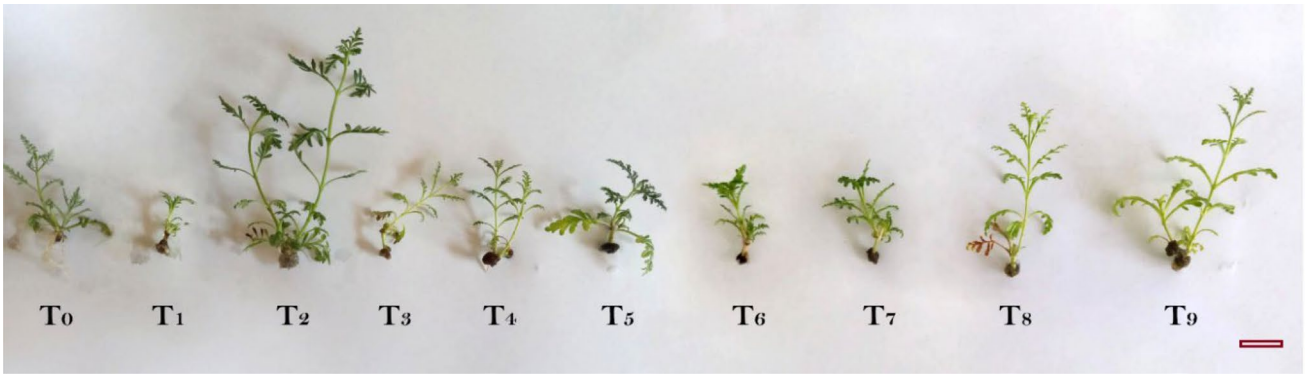

Fig. 1 In vitro shoot development on the single nodal segments of *S. abrotanoides* cultured on MS media supplemented with various concentrations of kinetin (KIN) and indole-3-acetic acid (IAA) for four weeks. Scale bar = 1 cm. T0-T9: Treatments with different hormonal combinations. T0: control, T1: 0.5 mg L^{-1} KIN, T2: 0.5 mg L^{-1} KIN and 0.05 mg L^{-1} IAA, T3: 0.5 mg L^{-1} KIN and 0.1 mg L^{-1} IAA, T4: 1 mg L^{-1} KIN, T5: 1 mg L^{-1} KIN and 0.05 mg L^{-1} IAA, T6: 1 mg L^{-1} KIN and 0.1 mg L^{-1} IAA, T7: 2 mg L^{-1} KIN, T8: 2 mg L^{-1} KIN and 0.05 mg L^{-1} IAA, T9: 2 mg L^{-1} KIN and 0.1 mg L^{-1} IAA

control), and after 48 h treatment with 50 and 100 µM elicitor (Table 3).

Effect of NO on phenolic acids content

Three phenolic acids (RA, Sal-A and Sal-B) were identified and determined by the HPLC method in the shoots of *S. abrotanoides* after elicitation with SNP. HPLC chromatograms of the methanolic extracts of untreated and SNP-treated *S. abrotanoides* shoots, compared with that of the standards of phenolic acids, are shown in Fig. 2. Analysis of data revealed that the accumulation of RA, Sal-A and Sal-B was enhanced by extending the duration of exposure to SNP in a concentration-dependent manner (Fig. 3).

As illustrated in Fig. 3a, a continuous increase in RA accumulation was observed when shoos were exposed to different concentrations of SNP in a time-progressive manner. The highest contents of this phenolic acid were recorded as 10.45 ± 0.40 mg g^{-1} DW (2.96-fold of the control) and 9.73 ± 0.35 mg g^{-1} DW (2.75-fold of the control) following 144 h elicitation with 100 and 50 µM, respectively.

As depicted in Fig. 3b, time-dependent increases and concentration-dependent decreases were found in the content of Sal-B in the shoots elicited with SNP; as a result, the highest amount of Sal-B (0.42 ± 0.01 mg g^{-1} DW), which was sixfold of the control, was obtained after a 144 h treatment with 25 µM SNP.

As shown in Fig. 3c, the significant increase in Sal-A content was started at 8 h after elicitation of the shoots with both 25 and 50 µM SNP, continued over the 144 h of the experiment for the 25 µM treatment group and under longer exposure times of 96 and 144 h for shoots elicited with 50 µM SNP. The content of this phenolic acid was significantly declined in the shoots at the most times of

Table 3 The effects of different concentrations of SNP on the total phenolic and flavonoid contents of *S. abrotanoides* shoots

SNP treatment (μM)	Time after treatment (h)	Total phenolic content (mg GAE g^{-1} DW)	Total flavonoid content (mg QUE g^{-1} DW)
Control	0	13.68 ± 0.75^{mn}	9.30 ± 0.27^{ab}
Control	8	$14.76 \pm 0.20^{i-m}$	$8.86 \pm 0.12^{a-c}$
Control	24	$14.59 \pm 0.19^{j-n}$	9.31 ± 0.15^{ab}
Control	48	$15.19 \pm 0.09^{h-l}$	$8.74 \pm 0.31^{a-c}$
Control	96	$16.12 \pm 0.26^{f-i}$	$8.88 \pm 0.57^{a-c}$
Control	144	$15.87 \pm 0.07^{g-j}$	$8.91 \pm 0.24^{a-c}$
25	0	$14.18 \pm 0.86^{k-n}$	9.03 ± 0.39^{ab}
25	8	$16.20 \pm 0.51^{f-i}$	8.47 ± 0.39^{bc}
25	24	$15.57 \pm 0.06^{g-k}$	7.97 ± 0.15^{cd}
25	48	$15.24 \pm 0.11^{h-l}$	$8.74 \pm 0.07^{a-c}$
25	96	$16.54 \pm 0.11^{e-h}$	$8.79 \pm 0.15^{a-c}$
25	144	$17.45 \pm 0.15^{d-f}$	5.23 ± 0.21^{h}
50	0	13.34 ± 0.96^{n}	9.27 ± 0.25^{ab}
50	8	$16.86 \pm 0.26^{e-g}$	9.56 ± 0.25^{a}
50	24	$16.07 \pm 0.17^{f-i}$	9.49 ± 0.38^{ab}
50	48	$15.65 \pm 0.41^{g-j}$	7.43 ± 0.24^{de}
50	96	19.12 ± 0.24^{bc}	5.73 ± 0.27^{gh}
50	144	21.18 ± 0.15^{a}	$5.99 \pm 0.17^{f-h}$
100	0	$13.98 \pm 0.76^{l-n}$	9.12 ± 0.25^{ab}
100	8	$15.53 \pm 0.31^{g-k}$	8.99 ± 0.30^{ab}
100	24	$16.54 \pm 0.47^{e-h}$	$8.73 \pm 0.19^{a-c}$
100	48	$17.85 \pm 0.46^{c-e}$	6.73 ± 0.41^{ef}
100	96	20.32 ± 0.36^{ab}	6.80 ± 0.19^{ef}
100	144	18.41 ± 0.46^{cd}	6.19 ± 0.30^{fg}

Values are the means ± standard error (n = 3). Means within a column followed by the same superscript letter(s) are not significantly different at $P < 0.05$ based on Duncan's test. SNP: sodium nitroprusside

exposure to 100 μM of SNP. The highest content of Sal-A (0.065 ± 0.00 mg g^{-1} DW), which was threefold of the control, was attained when the cultures were elicited with 50 μM SNP for 96 h.

Effect of NO on gene expression

The expression levels of four *PAL*, *TAT*, *RAS* and *CYP98A14* genes encoding the key enzymes involved in the phenolic acids biosynthesis were examined in the SNP-exposed shoots of *S. abrotanoides* (Fig. 4).The results of Real-time PCR analyses approved that expression of all studied genes were remarkably enhanced after exposure to the various concentrations of SNP. As illustrated in Fig. 4a, the expression of *PAL* was upregulated in response to NO and reached the maximum level (7.94-fold of the control) 24 h after treatment with 50 μM SNP. *PAL* expression in the elicited shoots with 50 μM SNP showed a biphasic pattern so that the transcript levels of the enzyme were significantly increased in 24 and 96 h and declined in 48 and 144 h after exposure. The relative expression level of *TAT* was stimulated and attained the highest level (8.08-fold compared with control) 24 h after exposure to 50 μM SNP (Fig. 4b). The expression of *RAS* and *CYP98A14* genes were stimulated in shoots in response to elicitation with SNP at different exposure times (Fig. 4c, d). The highest transcript level of *RAS* (61.68-fold of the control) was perceived at 8 h after treatment with 25 μM SNP, while the maximum level of *CYP98A14* expression (27.22-fold of the control) was achieved after 24 h of elicitation with 50 μM SNP. In this experiment, significant but not strong correlations were observed between the transcript levels of all examined genes and phenolic acids contents in the treated shoots.

Discussion

In vitro micropropagation via the activation of preexisting meristems of the nodal explants is the basic strategy for shoot multiplication of many plant species to assure of keeping genetic fidelity (Rani and Raina 2000). It has been proven that in the presence of cytokinins in the media alone or combination with an appropriate auxin, the capacity and frequency of shoot formation on explants could

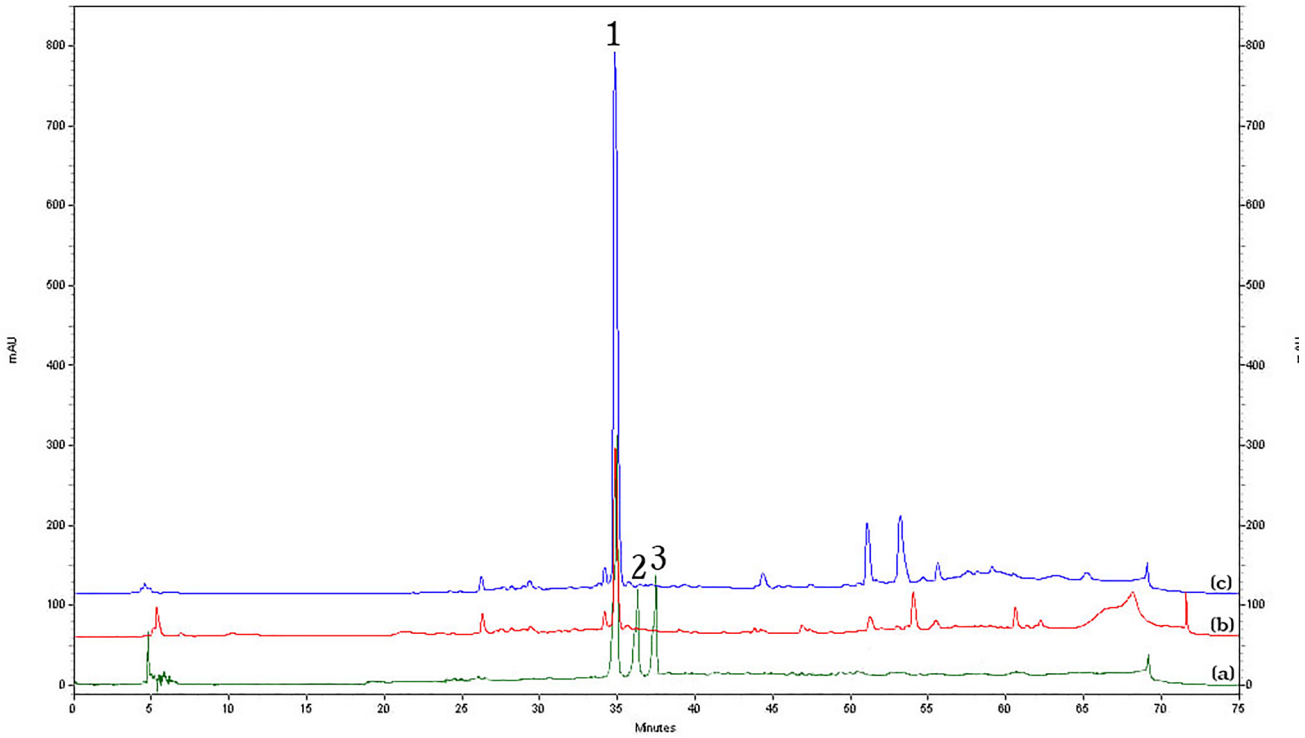

Fig. 2 HPLC chromatograms of the three phenolic acids in the standards solution (**a**), methanolic extract of control (**b**) and shoots of *S. abrotanoides* exposed to 100 µM sodium nitroprusside after 144 h (**c**). Peak 1: rosmarinic acid, Peak 2: salvianolic acid B, Peak 3: salvianolic acid A

be increased (Yildiz 2012). These tissue culture protocols could be used for the applicable propagation and commercial production of medicinal plants (Chaturvedi et al. 2007).

In this assay, the combination of the low concentrations of KIN (0.5 mg L^{-1}) and IAA (0.05 mg L^{-1}) in the culture media was more effective in promoting elongation of the formed shoots and increasing their FSI and MSN indices. The findings of this work are congruent with the results of similar studies about the stimulatory effects of KIN along with an auxin on the growth of axillary buds and shoot elongation in *Salvia przewalskii* Maxim. (0.5–2 mg L^{-1} KIN and 0.1 mg L^{-1} IAA) (Skała et al. 2007), *Salvia santolinifolia* Boiss. (2 mg L^{-1} KIN and 1 mg L^{-1} NAA) (Jan et al. 2020) and *Salvia sclarea* L. (1–2 mg L^{-1} KIN and 1 mg L^{-1} NAA) (Erişen et al. 2020). In the present work, compared to similar studies on *Salvia* species, the combination of the lower concentrations of KIN (0.5 mg L^{-1}) and IAA (0.05 mg L^{-1}) in the culture media was more effective on the elongation of the developed shoots. However, in other *Salvia* species such as *Salvia officinalis* L. (Petrova et al. 2015), *Salvia brachyodon* Vandas (Misic et al. 2006), *Salvia nemorosa* L. (Skała and Wysokińska 2004), *Salvia virgata* Jacq. (Dowom et al. 2017), *Salvia dolomitica* Codd (Bassolino et al. 2015) and *S. sclarea* (Ghanbar et al. 2016), the combinations of other cytokinins, especially 6-benzyl amino purine (BAP), with an auxin in the culture medium were more effective for improving the shoot multiplication.

Due to the various biological activities and therapeutic effects of phenolic compounds, improvement of their production seems to be necessary (Kumar and Goel 2019). Numerous studies have indicated elicitation with stress-related compounds (methyl jasmonate, salicylic acid and NO) on the improvement of bioactive phenolic compounds production in the plant cell, tissue and organ cultures (Gorelick and Bernstein 2014). It has been shown that when the plant cells are exposed to pathogens, abiotic stress and exogenous NO, the levels of phenolic compounds, including flavonoids and anthocyanins, are elevated by activating the phenylpropanoid biosynthesis pathway (Li et al. 2017a; Sharma et al. 2019).

Treatment with exogenous NO caused the enhancement of TPC and TFC in some plant culture systems, such as *Scutellaria baicalensis* Georgi suspension cultures (Zhang et al. 2014a), *Melissa officinalis* L. shoot cultures (Rezaei 2018), *Ginkgo biloba* callus cultures (Hao et al. 2009) and *Raphanus sativus* L. sprouts (Wu et al. 2016). Our findings indicated that treatment with different concentrations of SNP exerted stimulatory effects on TPC in the elicited shoots. The highest TPC (1.33-fold of the control) was found in the exposed shoots to 50 µM SNP after 144 h. However, a significant decrease in TFC was detected after 48 h treatment

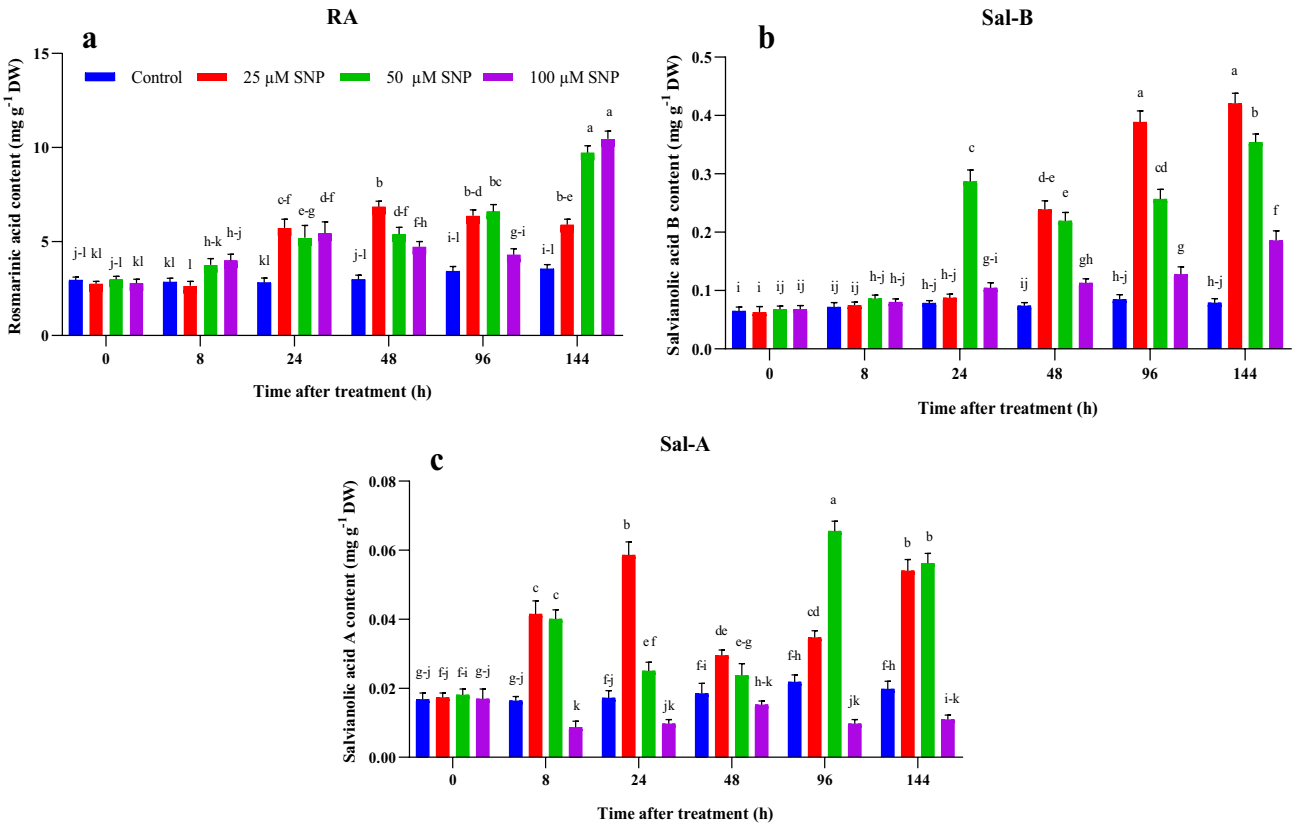

Fig. 3 Changes in the contents of rosmarinic acid (**a**), salvianolic acid B (**b**), and salvianolic acid A (**c**) in *S. abrotanoides* shoots during elicitation with different concentrations of sodium nitroprusside (SNP). Values are the means ± standard error (n = 3). Different superscript letter(s) denote significant differences at $P < 0.05$ based on Duncan's test

with 50 and 100 µM SNP and at 144 h exposure to 25 µM SNP when compared with the control. Since the biosynthesis of different groups of phenolic compounds depends on the common precursors, it can be suggested that treatment of the *S. abrotanoides* shoots with NO could direct the substrate flow to biosynthesis pathways of other phenolic compounds, such as phenolic acids.

In the present survey, for the first time, the effects of SNP were tested on the accumulation of RA, Sal-A and Sal-B in the shoot culture of *S. abrotanoides*. According to the results, after elicitation of the shoots with SNP, the contents of RA, Sal-B and Sal-A improved 2.96-, 6- and 3-fold of the control groups, respectively. These results are in good agreement with those of some previous reports about the stimulatory effects of NO on phenolic acids accumulation in plant tissue culture systems. The stimulatory effect of SNP (40 µM) on RA production (twofold of control) in *Onosma paniculatum* Bur. et Franch. cell suspension cultures has been described by Wu et al. (2009). Guo et al. (2014) indicated that the synthesis of Sal-B in *S. miltiorrhiza* cell cultures was elicited by SNP (0.5 mM), and its amount increased 6.86 times when compared with control. Exposure to SNP (> 5 mM) led to an enhancement in RA production in *M. officinalis* shoot cultures and subsequently improved their antioxidant capacity (Rezaei 2018). Moreover, based on the findings of Wu et al. (2007), the levels of caffeic acid derivatives elevated 1.22-fold of control in the adventitious roots of *Echinacea purpurea* (L.) Moench in response to 100 µM SNP.

To achieve the maximum yields of the desired compounds in elicitation procedures, factors such as concentration of elicitor and exposure times are critical (Narayani and Srivastava 2017). For instance, Khezerluo et al. (2018) showed that the production of hyoscyamine and scopolamine reached the maximum levels after treatment of hairy root cultures of *Hyoscyamus reticulatus* L. with 50 µM SNP for 48 h and 100 µM SNP for 24 h, respectively, compared with control. Similarly, Mahendran et al. (2021) have reported the highest contents of gymnemagenin and deacylgymnemic acid production in cell suspension cultures of *Gymnema sylvestre* (Retz.) R. Br. ex Sm. after 96 h exposure to 10 and 20 µM SNP, respectively. According to the results of the current work, both the SNP concentration and the incubation time were significant contributing factors in the enhancement of phenolic acids in the shoots of *S. abrotanoides*. Overall, analyses showed that

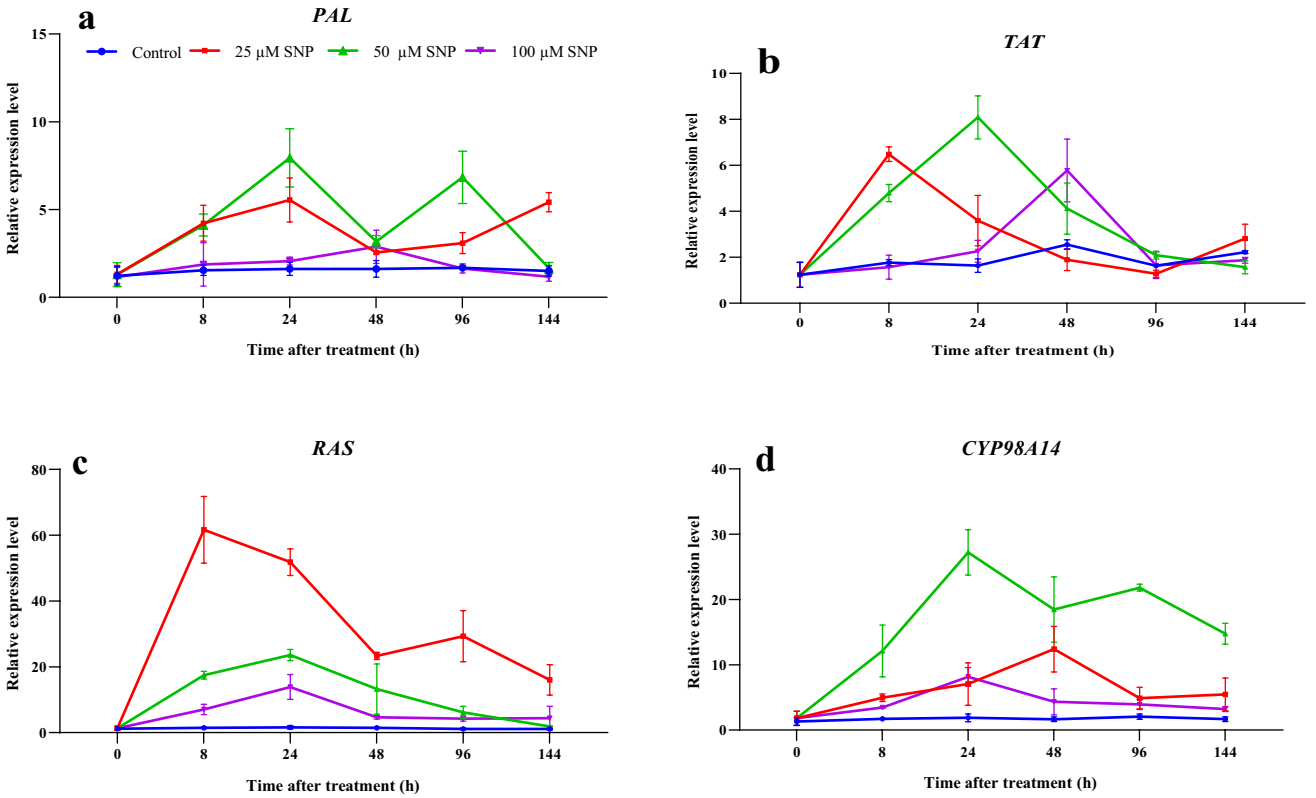

Fig. 4 Changes in the gene expression levels of phenolic acid biosynthetic genes in *S. abrotanoides* shoots during elicitation with different concentrations of sodium nitroprusside (SNP). Values are the means ± standard error (n = 3). *PAL*: phenylalanine ammonia-lyase (**a**), *TAT*: tyrosine aminotransferase (**b**), *RAS*: rosmarinic acid synthase (**c**), *CYP98A14*: cytochrome P450-dependent monooxygenase (**d**)

elicitation with SNP at 50 µM over a 144 h time course could effectively improve the accumulation of RA, as the main components of the shoots extracts, as well as other examined phenolic acids.

Some researchers have revealed the regulatory effects of NO on crucial enzymes in the biosynthesis of phenylalanine-derived phenolic compounds, including phenolic acids and flavonoids. Li et al. (2017b) illustrated that increase in NO levels after treatment with brassinosteroids and SNP (200 µM) enhanced the gene expression and activity of the PAL enzyme in *Camellia sinensis* L. leaves and consequently resulted in the enhanced accumulation of flavonoids. Jiao et al. (2016) have also demonstrated that the enzymes involved in the phenylpropanoid pathway, including PAL, were upregulated in the *Glycine max* L. sprouts after exposure to NO or UV-B radiation. Tossi et al. (2011) has proposed that NO establishes a link between stress and the phenylpropanoid pathway by activating transcription factors. To date, the stimulatory effect of NO on *TAT* expression has not been investigated, and the present study is the first report about the influence of this signaling molecule on the expression level of this gene. The obtained results indicated that elicitation with NO could stimulate the synthesis of phenolic acids in the shoot cultures of *S. abrotanoides* by upregulation of *PAL*, *TAT*, *RAS* and *CYP98A14* genes.

In this experiment, a biphasic time-course expression pattern of *PAL* was observed in the elicited shoots with 50 µM SNP. This pattern could be due to the distinctive and time course-dependent gene expression of different PAL isoenzymes in response to NO elicitation. Phenylalanine ammonia-lyase is encoded by a small multigene family in different plant species (Kong 2015). In *S. miltiorrhiza*, three *PAL* genes (*SmPAL1-3*) have been identified that are expressed in an organ-specific manner and usually participate in the biosynthesis of distinct compounds in the phenylpropanoid pathway. Also, it has been shown that treatment with methyl jasmonate and polyethylene glycol could induce all *PAL* genes in the roots in different degrees and in a time-dependent mode (Hou et al. 2013). Three *TAT* genes have also been identified in *S. miltiorrhiza,* whose expression patterns were tissue-specific and differently affected by methyl jasmonate and had distinctive roles in the biosynthesis of secondary compounds (Wang et al. 2015).

Some investigators have revealed the significance of *PAL* and *TAT* genes in phenolic acids production in the other members of the Lamiaceae family (Nourozi et al. 2019; Fatemi et al. 2019). Although, the contribution of phenylalanine- and tyrosine-derived pathways in the biosynthesis of phenolic acids can be different depending on the plant species, culture system and the used elicitation techniques. For example, in *S. miltiorrhiza,* silencing the *PAL* gene with RNAi technology reduced the accumulation of RA and Sal-B contents, indicating that *PAL* has a critical role in the biosynthesis of phenolic compounds in this species (Song and Wang 2011). However, based on the results of Ejtahed et al. (2015), no positive correlation was detected between *PAL* transcription level and RA accumulation in *S. virgata* and *S. officinalis*. Findings of an investigation on two genotypes of *S. miltiorrhiza* with different phenolic acids contents indicated that the synthesis of RA and Sal-B are regulated at the distinct molecular levels. Rosmarinic acid accumulation in the genotype with higher contents of the phenolic acids correlated with the *PAL* expression level, and the content of Sal-B was mainly dependent on the transcript level of *TAT*. However, the opposite results were attained for the other genotype (Song and Li 2015). Also, among the two different system cultures of *S. miltiorrhiza*, the transcriptional level of *TAT* and activity of the enzyme were more correlated with the production of phenolic acids in hairy root cultures (Zhang et al. 2014b), compared to the cell culture system (Dong et al. 2010). Moreover, Rahmani et al. (2020) have demonstrated that the synthesis of caffeic acid and RA in *Salvia verticillata* L. leaves under multi-walled carbon nanotubes treatment has a positive correlation with the expression level of *TAT*.

In the Current investigation, the transcript levels of *PAL* and *TAT* significantly increased after treatment with different concentrations of SNP. Given that there were no strong correlations between the gene expression levels of *PAL* or *TAT* with the contents of RA, Sal-A and Sal-B, it could be proposed that both phenylalanine- and tyrosine-derived pathways participated in the final accumulation of these phenolic acids in the NO-exposed *S. abrotanoides* shoot cultures. The expression of *RAS* and *CYP98A14*, encoding the two final enzymes in the biosynthesis of RA, intensely enhanced after elicitation with NO. Nevertheless, following the induction of *RAS* and *CYP98A14* (8 h after treatment with NO), no increase was observed in the content of RA; however, the accumulation of Sal-A was enhanced. The findings of Wu et al. (2009) implied that exposure to 40 µM of SNP could stimulate the expression of *RAS* and *CYP98A6*, leading to the biosynthesis of RA and Sal-B, in the cell suspension cultures of *O. paniculatum*. There is no further report in the literature about the effect of NO on the regulation of gene expression of crucial enzymes in RA biosynthesis and subsequent steps leading to the production of salvianolic acids.

Some recent studies have been confirmed the importance of *RAS* and *CYP98A14* in the biosynthetic pathway of RA and its derivatives in other *Salvia* species. Di et al. (2013) found that inhibition of *CYP98A14* expression in *S. miltiorrhiza* transgenic hairy root lines led to significant decreases in the contents of RA and Sal-B. Moreover, Fu et al. (2020) have shown that *CYP98A14*-overexpressing hairy roots lines of *S. miltiorrhiza* produced higher RA and Sal-B contents than *RAS*-overexpressing ones. Their results illustrated the probably more potent effect of CYP98A14 on the biosynthetic pathway of phenolic acids. Besides, the results of Pesaraklu et al. (2021) showed that the relative expression levels of *PAL*, *TAT*, *RAS* and *CYP98A14* in *S. officinalis* and *S. verticillata* leaves were increased after treatment with methyl jasmonate, and the production of phenolic acids was strongly correlated with *TAT* and *CYP98A14* expression levels.

In the present work, same as *PAL* and *TAT*, no strong correlations were statistically observed between the expression levels of *RAS* and *CYP98A14* and the contents of RA, Sal-A and Sal-B in SNP-treated shoots. Recently Stafiniak et al. (2021) revealed no significant correlation between the expression levels of *PAL*, *TAT*, *RAS* and *CYP98A14* and the RA content in the leaves of field-grown *S. abrotanoides* and *Salvia yangii* B.T. Drew under seasonal variations. Based on the available knowledge and according to the results of this study, it can be proposed that different isoforms of the examined genes may be involved in the biosynthesis of phenolic acids in the shoot cultures of *S. abrotanoides*, whose expression patterns were affected by the SNP concentration and exposure time.

Conclusion

In this work, the stimulation of axillary bud growth for shoot formation successfully occurred on single nodal explants of *S. abrotanoides* through the application of KIN and IAA hormones in culture media. For the first time, the findings presented here revealed that SNP (as NO donor) could act as an elicitor to induce *PAL*, *TAT*, *RAS* and *CYP98A14* in the biosynthesis pathway of phenolic acids, which in turn led to enhance the accumulation of RA, Sal-A and Sal-B. In most cases, the accumulation of each phenolic acid was dependent on the applied concentration and exposure time of the shoots to SNP. Further experiments are needed to identify the rate-limiting steps and regulatory factors in the phenolic acids biosynthesis pathway in *S. abrotanoides* shoots and the precise molecular mechanism through which the NO affects the accumulation of phenolic acids for application purposes.

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Author contributions Contributor 1 (FR) and Contributor 2 (TR) conceived this research and designed experiments; Contributor 1 (FR) performed experiments and analyses; Contributor 1 and Contributor 2 wrote the paper and Contributor 3 (PA) participated in the revisions of it. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors. Any ethical rights have been observed in writing this article, and the scientific contribution of all individuals in this research has been fully clarified and agreed upon by all.

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