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Research Article

Variation in phytochemical characteristics and some relevant gene expression in chromosome doubled *Salvia officinalis* L.

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Abstract

Polyploidy, as one of the main factors in the speciation and adaptation of plants, can increase the pharmaceutical constituents of medicinal plants. *Salvia officinalis* L. is a perennial plant from the Lamiaceae family with various medicinal properties. This study aimed to analyze and compare some phytochemical characteristics, such as essential oil and phenolic acid contents and examine the expression of some genes involved in the biosynthesis pathway of phenolic acids in diploid and induced tetraploid plants of *S. officinalis* by GC-MS and real-time PCR, respectively. The results revealed that *S. officinalis* essential oil components such as α-pinene, camphene, camphor, borneol, and fenchyl acetate were significantly increased ($P < 0.05$) in tetraploid plants compared with diploids. Furthermore, tetraploidy increased the level of phenolic acids of gallic, caffeic, rutin, coumaric, rosmarinic, quercetin, cinnamic, apigenin, and chlorogenic acids in *S. officinalis*. Also, based on RT-PCR results, a higher expression of *C4H* (Cinnamate 4-Hydroxylase), *PAL1* (Phenylalanine ammonia-lyase), *4CL* (4-Hydroxycinnamate coenzyme A ligase), *TAT* (Tyrosine aminotransferase), *HPPR* (Hydroxyphenylpyruvate reductase), and *CYP98A14* (cytochrome P450-dependent monooxygenase) genes involved in their biosynthesis pathway was observed tetraploids compared with diploids. The results confirmed that polyploidy breeding in medicinal plants could be applied to enhance secondary metabolite production.

Keywords: Essential oil, Gene expression, Polyploidy induction, *Salvia officinalis*, Secondary metabolites

Introduction

Autopolyploidy is a widespread phenomenon in plant species, in which plant populations have more than two complete sets of chromosomes or genomes in their cells due to the doubling of the whole genome, which, for example, can be caused by an abnormality in the cell cycle (Van de Peer *et al.*, 2017). Polyploidy, as an essential tool in the adaptation of plants to stress conditions (Chao *et al.*, 2013), can express new phenotypes that have not been seen in the diploid parents or exceed the range of their ancestral species in certain traits such as size, biomass, apomixes, etc. (Dar *et al.*, 2017; Horandl, 2022). These factors can give polyploids a great chance to survive in new environments and improve their agricultural production and quality.

S. officinalis L. is a perennial plant from the Lamiaceae family with $2n = 2x = 14$ chromosomes, which has been of particular interest since ancient times and is one of the most valuable medicinal plants, with fundamental therapeutic properties (Baricevic and Bartol, 2000). The active compounds of *S. officinalis* are divided into two groups: Water-soluble phenolic acids, such as rosmarinic acid, salvianolic acid, and lithospermic acid and diterpenoid tanshinones, which show various medical properties such as antioxidant, antitumor, and anti-inflammatory characteristics (Jiang *et al.*, 2023; Li *et al.*, 2023). The phenolic acids present in *Salvia* are synthesized through tyrosine-derived and phenylpropanoid pathways, and the tanshinones biosynthesis occurs through the MEP (2-C-methyl-D-erythritol 4-phosphate) and MVA (mevalonate) pathways in the cytosol or peroxisomes (Shi *et al.*, 2019).

Polyploidy can affect physiological and biochemical processes in plants, such as gene expression and the

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activity of isozymes. With increasing ploidy levels, the respiration rate is reduced, but photosynthesis, gene expression, and enzyme activity increase which, in turn, affects the quality and quantity of plant secondary metabolites (Madani *et al.*, 2019). Morphological, genome and gene expression variations were reported in induced autopolyploids of the *Chrysanthemum lavandulifolium* Fisch. ex Trautv. (Gao *et al.*, 2016). Successful induction of polyploidy using colchicine has been reported in various *Salvia* species such as *S. bowleyana* (Duan *et al.*, 2006), *S. miltiorrhiza* Bge (Gao *et al.*, 1996), *S. coccinea* L. (Kobayashi *et al.*, 2008), *S. hains* Royle ex Bentham (Grouh *et al.*, 2011), and *S. officinalis* (Hassanzadeh *et al.*, 2020; Tavan *et al.*, 2021).

Considering the importance of polyploidy breeding in medicinal plants and limited studies on its effect on gene expression and production of secondary metabolites in sage (*S. officinalis*), this study aimed to analyze and compare the essential oil components of diploid and induced tetraploid sage plants using the GC/MS technique and examine the expression of some genes involved in the biosynthesis of phenolic acids in diploid and tetraploid plants by real-time PCR.

Materials and methods

Plant materials: The experimental materials included diploid and tetraploid plants of S. officinalis (C1 generation) induced by treatment of seeds with 0.25% (w/v) of colchicine for 48 h and confirmed through chromosome observations and flow cytometric analysis reported previously by Hassanzadeh *et al.* (2020).

Determination of phenolic acids by high-performance liquid chromatography (HPLC): Dried aerial parts of diploid and tetraploid plants (500 mg) were pulverized and extracted with 2 mL of methanol and 1% acid acetic (1:1, v:v), sonicated for 1 h and then kept at room temperature for 24 h. Phenolic acids, including gallic, caffeic, chlorogenic, rutin, coumaric, rosmarinic, quercetin, cinnamic, and apigenin acids, were detected and quantified through comparison with authentic standard curves. Standard solutions of these phenolic acids with dilutions of 0.1 mg mL^{-1} to 1.5 µg mL^{-1} were prepared (Sigma-Aldrich, Munich, Germany) for the calibration plots establishment. Isolation, identification, and quantification of phenolic acids were performed using high-performance liquid chromatography (HPLC) device, model 1100 series (Agilent, USA) on the octadecylsilane column, and Chemstation software was used to analyze the data. To better separation of the compounds, a washing program was used: first, the mobile phase began with a ratio of 10% acetonitrile and 90% solution of 1% acetic acid and a flow rate of 1 mL min^{-1}, at a ratio of 25% acetonitrile and 75% solution of 1% acetic acid with a flow rate of 1 mL min^{-1} within 5 min, then with 65% acetonitrile and 35% solution of 1% acetic acid with a flow rate of 1 mL min^{-1} was reached for 10 min. The total separation time was 15 min.

Essential oil extraction and identification of its chemical compounds: Essential oil of diploid and tetraploid *S. officinalis* plants were extracted from dried leaves and aerial parts (25 g) using the Clevenger apparatus. After about 3 h hydro-distillations, the essential oil was extracted from the water surface, dehumidified by anhydrous sodium sulfate, and stored in the refrigerator (4°C). The essential oil production percentage was calculated as essential oil weight per dry weight of plant material × 100 (Jaimand *et al.*, 2006). To determine essential oil components, a gas chromatography device connected to a mass spectrometer (Agilent 7890B, USA) was used. This device can inject liquid samples with the ability to dilute split/splitless inlet and is equipped with a spectrum detector. The mass detector (MSD) was used to identify the qualitative and quantitative compositions of the samples. The detector had an EI ionization system and a Quadrupole Single analyzer. The chromatographic conditions were as follows: The oven temperature increased from 60°C to 325 °C at a rate of 3 °C min^{-1}. The injector and detector temperatures were 240 °C and 250 °C, respectively. The compounds were identified by comparison of retention indices of the obtained mass spectra with the NIST and Wiley libraries.

RNA extraction: RNA extraction from leaf samples of diploid and tetraploid plants was performed using an RNX-PlusTM extraction kit (Sinaclon Company, Karaj, Iran), according to the manufacturer's instruction. Briefly, the frozen leaves at -70 °C were powdered in a mortar with liquid nitrogen, and 0.1 g of the powder was poured with 1 mL of RNX-Plus extraction buffer in a 1.5 mL nuclease-free microtube and vortexed for 10-15 s. Then, the microtubes were placed at 4 °C for 5 min, and 200 µL of cold chloroform was added and mixed well by shaking. After incubating on ice for 5 min, they were centrifuged at 13000 rpm at 4 °C for 15 min. Afterward, the aqueous phase was transferred to a new nuclease-free microtube, mixed with an equal volume of cold isopropanol, gently mixed, kept on ice for 15 min, and then centrifuged at 12,000 rpm at 4 °C for 15 min. Next, the supernatants were discarded, 1 mL of 75 % ethanol was added, and centrifuged again for 1 min at 4 °C at 7500 rpm. The supernatants were discarded, and the tubes dried at room temperature for a few minutes. Finally, the pellets were dissolved in 30 µL of nuclease-free water at 55-60 °C in a water bath for 7 min. Then centrifugation was performed at 10000 rpm at 4 °C for 2 min. The supernatant containing the extracted RNA was put in a new microtube.

After RNA extraction, all samples were treated with DNase using a specific DNA-free Kit (Thermo Yekta Tajhiz Azma, Tehran, Iran). The agarose gel electrophoresis was used to evaluate the quality of the extracted RNA. A 1% agarose gel was prepared using 1X TAE buffer dissolved in DEPC-treated water and supplemented with 0.5 µg mL^{-1} of ethidium bromide. RNA samples and ladder were mixed with an equal volume of RNA loading dye, incubated at 70 °C for 7

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Table 1. The sequences of the primers used in real-time PCR reactions.

Gene	Sequence (5′ → 3)	Tm (ºC)	Amplicon length (bp)
C4H	FW: CCAGGAGTCCAAATAACAGAGC RV: GCCACCAAGCGTTCACCAAGAT	61	183
PAL1	FW: TACCTCGTCGCCCTATGCCAAG RV: ATTGACGCCCATTGTGAGAGTT	61	117
HPPR	FW: TGACTCCAGAAACAACCCACATT RV: CCCAGACGACCCTCCACAAG	59.5	138
CYP98A14	FW: CAGGATTTGATTTGGGCGGACTA RV: AACCTTTTCCCAAATACCAGCCT	59	244
TAT	FW: CAACTGCTGGTCTTCCACAAC RV: GCGAGCCAAAACGGACA	58	142
4CL	FW: CCAGTTGCTTTTGTTGTGAGATCG RV: CCTGATGGAGACTTGGGAATTGC	61.5	140
18srRNA	FW: CCAGGTCCAGACATAGTAAG RV: GTACAAAGGGCAGGGACGTA	58	177

min, and cooled on ice. Afterward, samples were loaded onto the gel and run for 45 min at 70 V. The 28 S and 18 S rRNAs were visualized with a UV transilluminator (Gel Doc, Bio-Rad, Feldkirchen, Germany). Also, the quality of the extracted RNA was controlled using a nanodrop spectrophotometer (Shimadzu UV-2501PC) by determining the absorption at 260 and 280 nm wavelengths.

cDNA synthesis: For cDNA synthesis, the Revert Aid First-strand cDNA synthesis kit (Fermantaz Company, Germany) was used following the manufacturer's proposed protocol. For 20 µL PCR reaction, 5 µL of nuclease-free water, 6 µL of extracted RNA, and 1 µL of Oligo dT primers were placed at thermocycler at 65 °C for 5 min to connect Oligo-dT primers to RNA strands. Then 4 µL of buffer reaction (5x), 1 µL of Ribolook RNase Inhibitor enzyme (20 U μL^{-1}), 2 µL of dNTP mix (10 mM), and 1 µL of Revert Aid RT (200 U μL^{-1}) enzyme buffer were added to the tube and placed in a thermocycler at 42 °C for 60 min. Then, the synthesized cDNA was stored at -20°C for subsequent gene expression studies.

Real-time PCR analysis: To perform the qRT-PCR, the Rotor gene Q-pure Detection Qiagen model 6000 (QIAGEN, USA) was used. The *18S rRNA* gene was used as the reference gene for data normalization. RT-PCR reaction was performed in a volume of 12.5 µL, including 6.25 µL of the Maxima SYBR Green/Fluorescein qPCR master mix kit (5x), 4 µL of nuclease-free water, 0.5 µL of the primers, and 1.25 µL of cDNA. RT-PCR reaction was performed using forward and backward primers of *C4H*, *PAL1*, *4CL*, *TAT*, *HPPR*, and *CYP98A14* genes and *18srRNA* as a housekeeping gene in diploid and tetraploid plants of *S. officinalis* (Table 1). The primers for the *C4H*, *PAL1*, *TAT*, *HPPR*, and *CYP98A14* genes were designed according to Zhou *et al.* (2017); and for *4CL* by Oligo 7 (https://www.oligo.net/) and Primer blast (www.nlm.nih.gov/tools/primer-blast) software and synthesized by Cinaclon Company (Karaj, Iran).

The thermal program for gene amplification by RT-PCR was the initial denaturation step at 95 °C for 10 min for 1 cycle, followed by 40 cycles (95 °C for 40 s, the primer binding to cDNA at the specified temperatures for 30 s, and expansion at 72 °C for 1 min) for amplification. Reactions were done in triple biological and three technical replications. After the accomplishment of the RT-PCR, the amplification and melting curve was plotted for each gene by increasing the temperature from 45 to 95 °C, with a rate of 0.5 °C per second. The gene amplification was confirmed through the melting curve analysis, and gel electrophoresis was used to affirm proliferation accuracy. Serial dilutions of cDNA were taken through real-time PCR to be sure that the concentrations of the primers used to generate the cycle threshold (CT) values were consistent with the corresponding cDNA dilutions.

Statistical analysis: The significant differences between diploid and tetraploid plants for biochemical measurements was performed through *t*-test using SPSS19 software, and the data were shown as mean ± standard error (SE). To investigate the increase or decrease in gene expression, RT-PCR data were analyzed using REST software (Pfaffl *et al.*, 2002). The *18srRNA* gene was used as the reference gene to normalize real-time PCR data

Results and discussion

Phenolic acids and essential oil compounds: Isolation and identification of phenolic acids in diploid and tetraploid plants through HPLC analysis showed that the contents of phenolic acids, including gallic, caffeic, rutin, coumaric, rosmarinic, quercetin, cinnamic, apigenin, and chlorogenic acids are significantly greater (from 1.32 fold increase in chlorogenic acid to 2.33 fold in coumaric acid) in tetraploids than in diploid plants based on *t*-test (Table 2).

Comparison of essential oil production also showed significant differences between diploid and tetraploid plants. The yield of essential oil production in tetraploid plants (0.91%) was significantly ($P < 0.05$) higher than in diploids (0.80%) (Table 2). The chemical compositions of essential oils of diploid and tetraploid *S. officinalis* plants are shown in Table 3. Compounds such as α-Pinene, Camphene, Camphor, Borneol, and

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Table 2. Comparison of phenolic acids and essential oil contents between diploid and tetraploid plants of *S. officinalis* L.

Phenolic acids (mg kg^{-1})	Tetraploid (4*x*)	Diploid (2*x*)	Significance
Gallic acid	64.95 ± 5.50 [a]	40.87 ± 2.02 [b]	*
Caffeic acid	39.54 ± 2.88 [a]	27.28 ± 0.92 [b]	*
Chlorogenic acid	19.49 ± 0.53 [a]	14.74 ± 0.83 [b]	**
Rutin acid	10.11 ± 0.47 [a]	7.26 ± 0.79 [b]	*
Coumaric acid	88.57 ± 11.78 [a]	38.08 ± 5.57 [b]	**
Rosmarinic acid	791.82 ± 56.52 [a]	492.45 ± 30.85 [b]	**
Quercetin acid	12.19 ± 0.92 [a]	7.70 ± 0.49 [b]	*
Cinnamic acid	3.30 ± 0.36 [a]	1.45 ± 0.09 [b]	*
Apigenin	49.42 ± 4.71 [a]	26.88 ± 3.54 [b]	**
Essential oil (%)	0.91 ± 0.05[a]	0.80 ± 0.02 [b]	**

Data are shown as means ± SE (*n* = 6). *, **, show significantly differences according to two-sample Student's *t*-test at P < 0.05 and P < 0.01, respectively.

Table 3. Chemical composition (%) of leaf essential oil in diploid and tetraploid plants of *S. officinalis* L.

No.	Compound	CAS\#	Retention time (min)	Retention index (RI)	% of Total		Sig.
					Tetraploids	Diploids	
1	Tricyclene	508-32-7	5.65	919	0.12 ± 0.01	0.09 ± 0.00	ns
2	α-Pinene	80-56-8	5.97	936	2.75 ± 0.12	1.94 ± 0.08	*
3	Camphene	79-92-5	6.37	952	3.73 ± 0.26	1.02 ± 0.30	**
4	β-Pinene	127-91-3	7.17	964	1.27 ± 0.06	0.98 ± 0.12	ns
5	β-Myrcene	123-35-3	7.60	986	0.30 ± 0.01	0.22 ± 0.01	ns
6	α-Terpinene	99-86-5	8.44	1012	0.11 ± 0.00	0.10 ± 0.00	ns
7	o-Cymene	527-84-4	8.68	1017	0.11 ± 0.01	0.11 ± 0.01	ns
8	D-Limonene	138-86-3	8.82	1031	0.25 ± 0.02	0.29 ± 0.04	ns
9	1,8-Cineole	470-82-6	9.02	1033	6.65 ± 0.48	8.74 ± 0.55	**
10	γ-Terpinene	99-85-4	9.99	1055	0.17 ± 0.00	0.14 ± 0.00	ns
11	Thujone	471-15-8	12.16	1091	35.25 ± 1.22	36.04 ± 1.84	ns
12	Camphor	76-22-2	13.40	1120	12.41 ± 1.23	6.51 ± 0.01	**
13	Pinocamphone	15358-88-0	13.89	1141	0.05 ± 0.01	0.03 ± 0.00	ns
14	Borneol	507-70-0	14.15	1147	3.08 ± 0.52	1.25 ± 0.00	**
15	Terpinene-4-ol	562-74-3	14.60	1162	0.14 ± 0.01	0.14 ± 0.24	ns
16	α-Terpineol	98-55-5	15.20	1171	0.10 ± 0.02	0.06 ± 0.03	ns
17	Myrtenol	515-00-4	15.46	1194	0.11 ± 0.00	0.10 ± 0.00	ns
18	Fenchyl acetate	4057-31-2	19.28	1220	1.26 ± 0.20	0.38 ± 0.00	**
19	Caryophyllene	87-44-5	23.87	1418	1.44 ± 0.14	2.07 ± 0.22	*
20	α-Humulene	6753-98-6	24.95	1439	4.41 ± 0.33	6.57 ± 0.50	**
21	Aromadendrene	489-39-4	25.11	1440	0.11 ± 0.00	0.19 ± 0.01	ns
22	d-Cadinene	483-76-1	26.90	1524	0.09 ± 0.00	0.10 ± 0.00	ns
23	Caryophyllene oxide	1139-30-6	28.56	1572	0.15 ± 0.02	0.19 ± 0.02	ns
24	Ledol	577-27-5	28.90	1573	0.10 ± 0.00	0.09 ± 0.00	ns
25	Viridiflorol	552-02-3	28.93	1590	11.02 ± 0.50	14.61 ± 1.17	**
26	α-Humulene oxide	19888-33-6	29.29	2038	0.82 ± 0.05	1.07 ± 0.17	*
27	Manool	596-85-0	39.30	2055	12.81 ± 0.69	14.77 ± 3.20	**
28	β-Selinene	17066-67-0	39.81	2252	0.23 ± 0.01	0.25 ± 0.04	ns
Total identified components (%)					99.48	99.59	

Data are shown as means ± SE (*n* = 6). *, **, show significant differences according to two-sample Student's *t*-test at P < 0.05 and P < 0.01, respectively, ns shows no significant differences

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Fenchyl acetate were significantly ($P < 0.01$) higher in tetraploid plants than in diploids. Likewise, diploid plants had significantly higher amounts of 1,8-Cineole, α-Humulene, Veridiflorol, α-Humulene oxide, and Caryophyllene than that tetraploids. The most abundant components of sage essential oil in tetraploid plants were as: Thujone> Manool> Camphor> Viridiflorol> 1,8-Cineole> α-Humulene, and in diploids was as: Thujone> Manool> Viridiflorol> 1,8-Cineole> α-Humulene> Camphor. Other compounds such as β-Myrcene, α-Terpinene, γ-Terpinene, Pinocamphone, Terpinene-4-ol, α-Terpineol, Myrtenol, Aromadendrene, d-Cadinene, Ledol, and some others were also detected in the studied samples, which their contents were smaller (< 0.5%) without any significant differences between tetra- and diploid plants (Table 3). A GC-MS chromatogram of *S. officinalis* essential oil for identification of the compounds in di- and tetraploid plants of this medicinal species based on the retention time and index is given in Figure 1 a, b.

Real-time PCR analysis: Qualitative and quantitative evaluation of RNA extracted from the leaves of the diploid and tetraploid *S. officinalis* plants using 1% agarose gel electrophoresis and nanodrop showed that the extracted RNAs had a good quality without fragmentation or contamination. According to the nanodrop results, the light absorption at 230 and 280 nm was the minimum, and at 260 nm was the maximum. The A_{260}/A_{280} ratio of the extracted RNA was 1.8 to 2, which indicates the absence of protein or phenolic contamination in RNA samples and the adequate purity of the extracted RNAs.

According to the amplification diagrams obtained from the real-time PCR reaction using forward and backward primers (Table 1) of *C4H, PAL1, 4CL, TAT, HPPR*, and *CYP98A14* target and *18srRNA* as internal control genes in diploid and tetraploid plants of *S. officinalis*, it was revealed that the primers accurately attached to the target sites at the specified temperature and caused them to amplify. Melting curve analysis showed the specific amplification of each gene. Due to the presence of only one peak in all PCR products of the studied genes and *18S rRNA*, it can be said that the amplification of all fragments was done specifically and without nonspecific products such as primer dimers. 1.5% agarose gel electrophoresis of real-time PCR products of diploid and tetraploid samples (Figure 2) showed the expected sizes for all products. Comparing the expression rate of *C4H*, *PAL1*, *4CL*, *TAT*, *HPPR*, and *CYP98A14* genes between diploid and tetraploid plants showed a significant ($P < 0.05$) increase (from 2.30 fold change in *PAL* gene to 13.16 fold change in *HPPR* gene) in tetraploid plants, which shows that the expressions of these genes were significantly higher in tetraploid plants (Figure 3).

Polyploid plants often display advantageous features, including greater adaptability and resistance, and also they have giant flowers, fruits, and other prominent features compared with their diploid relatives (Manzoor *et al.*, 2019). If these changes occur in the commercial parts of the plant, as in medicinal and vegetable plants, they may become a valuable feature for plant improvement. Tetraploid plants in our study had a higher leaf length, leaf width, and plant height (Hassanzadeh *et al.*, 2020). In addition, according to HPLC analysis, the polyploid plants had a higher content of phenolic acids, which shows polyploidy plays a crucial role in increasing the yield of this medicinal plant. It has been reported that the tanshinone contents in tetraploid plants of *S. miltiorrhiza* Bge were higher than the control diploid plants (Gao *et al.*, 1996). Also, Mishra *et al.* (2010) showed that the alkaloid profile in induced autotetraploid plants of *Papaver somniferum* L. showed a significant enhancement in morphine content from 25% to 50%.

Salvia essential oil is used as an antimicrobial agent. Increasing thujone, camphor, and 1,8-Cineole percentage in essential oil, its antimicrobial activity increases (Senatore *et al.*, 2005). According to Bougatsos *et al.* (2004), a high percentage of camphor is associated with effective antimicrobial activity. Viridiflorol and *α*-humulene of essential oils have antifungal properties cell toxic activity against some tumor cells (Silva *et al.*, 2007), and an anti-acetylcholinesterase activity which makes them appropriate for the treatment of Alzheimer's disease (Miyazawa *et al.*, 1998). So, polyploid induction, as a valuable method, can be used in the breeding programs of important economic medicinal plants for increasing essential oils and their valuable components (Gao *et al.*, 1996; Mo *et al.*, 2020).

The expression rate of all studied genes showed a significant ($P < 0.05$) increase (from 2.30 fold change in the *PAL1* gene to 13.16 fold change in the *HPPR* gene) in tetraploid plants as compared with diploids ones (Figure 3). These genes are involved in phenolic acids biosynthesis (Luo, 2019; Ma *et al.*, 2015). The *PAL* encodes phenylalanine ammonia-lyase (PAL), the first enzyme which catalyzes the L-phenylalanine to produce trans-cinnamic acid, which plays a more significant role in the phenylpropanoid pathway. Cinnamate 4-hydroxylase (C4H) is involved in the catalysis of the hydroxylation of cinnamate to 4-coumarate for the synthesis of p-coumaric acid. Also, 4-Coumarate: CoA ligase (4CL) catalyzes 4-coumaroyl acid to form 4-coumaroyl-CoA. Tyrosine aminotransferase (TAT) and 4-hydroxyphenylpyruvate reductase (HPPR) are involved in making 3,4-dihydroxyphenyllactic acid (DHPL) in the tyrosine-derived pathway. Tyrosine aminotransferase (TAT) is the first enzyme in the tyrosine-derived pathway of phenolic acids biosynthesis. 4-Hydroxyphenylpyruvate reductase (HPPR), the second enzyme in the tyrosine-derived pathway, catalyzes 4-hydroxyphenylpyruvic acid to form 4-hydroxyphenyllactic acid. Cytochrome P450-dependent monooxygenase (CYP98A14) participates in the synthesis of rosmarinic acid by catalyzing 4C-DHPL (Ma *et al.*, 2015; Wang *et al.*, 2015).

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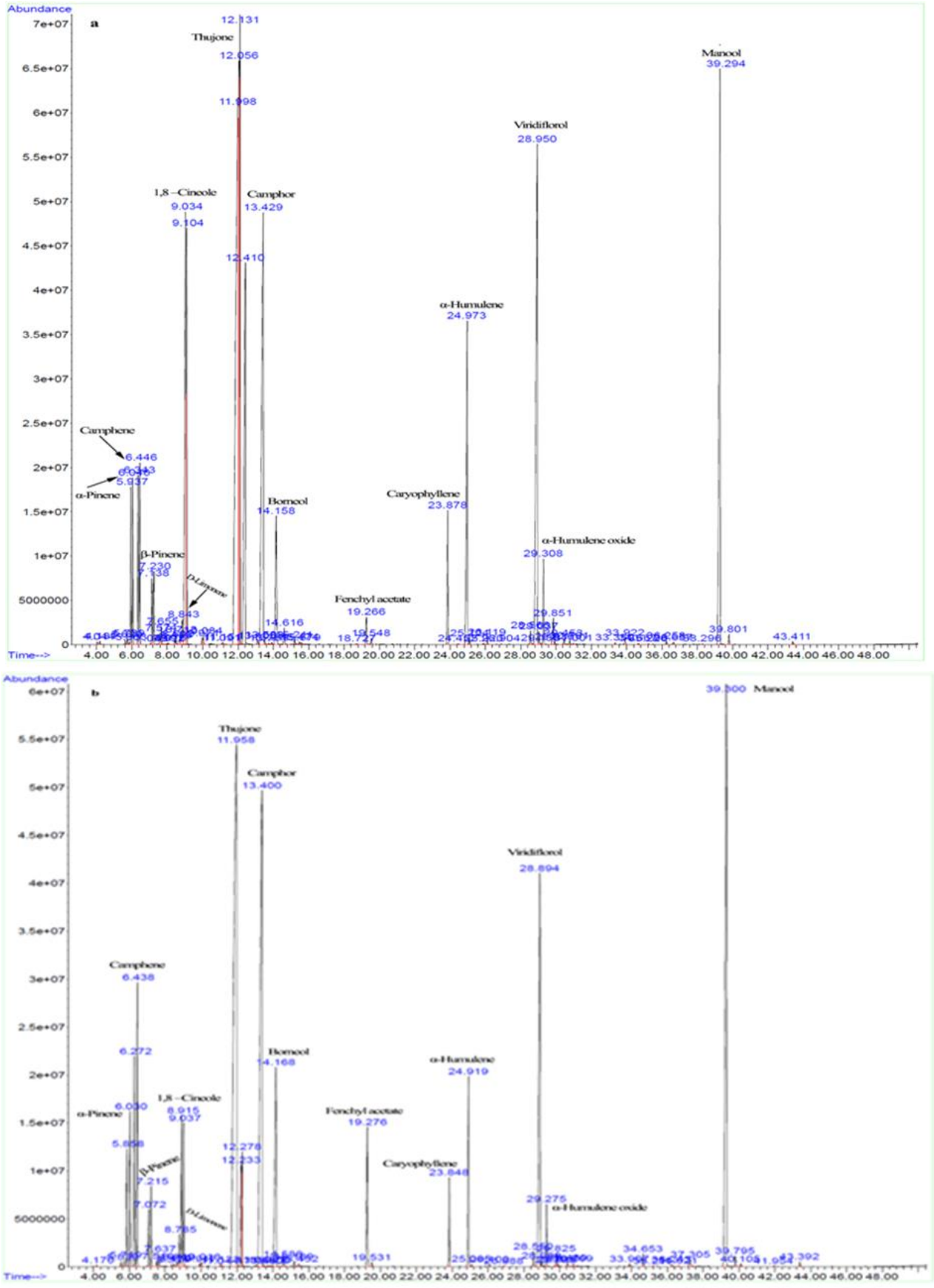


Figure 1. GC-MS chromatogram of *S. officinalis* essential oil based on retention times and indices, (a) diploid, and (b) tetraploid plants. (Some remarkable metabolites were indicated on the peaks).

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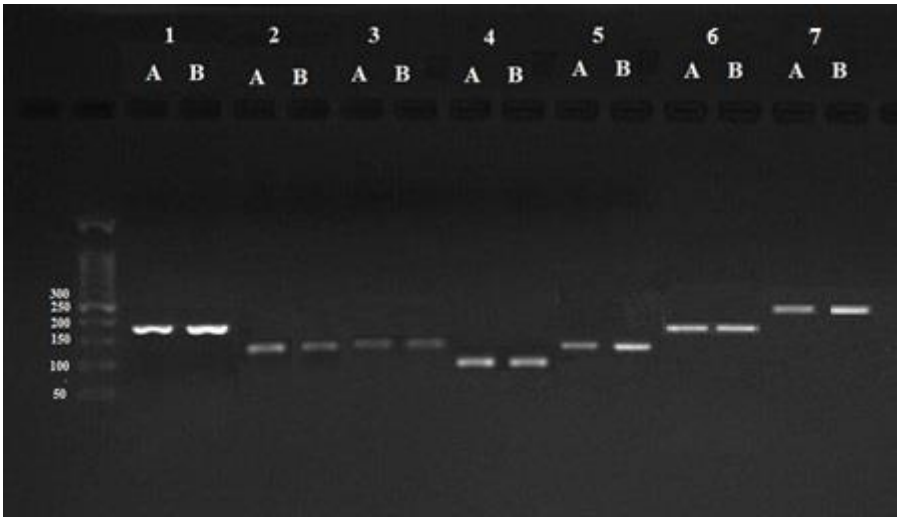


Figure 2. Agarose gel electrophoresis (1.5%) of PCR products in diploid (a) and tetraploid (b) samples of *S. officinalis* L.: 1 *18srRNA*, 2 *C4H*, 3 *HPPR*, 4 *PAL1*, 5 *CYP98A14*, 6 *TAT*, and 7 *4CL*. The size of the leader is 50 bp.

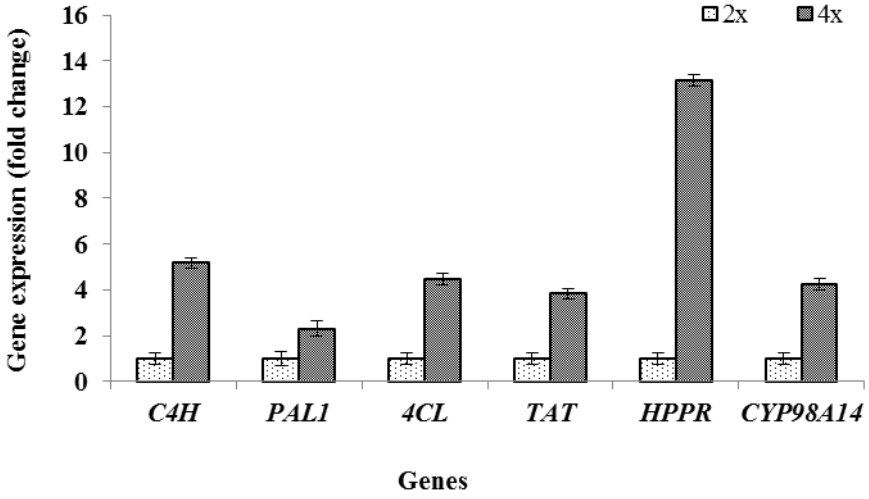


Figure 3. Comparing of expression of *C4H, PAL1, 4CL, TAT, HPPR*, and *CYP98A14* genes in diploid and tetraploid plants of *S. officinalis* L. Bars represent means ± SE (*n* = 3). * shows significant differences according to the *p*-value of the alternate hypothesis that the difference between sample (tetraploid) and control (diploid) groups is due only to chance using REST software.

According to the gene expression analysis, chromosome doubling affected gene expression, and significant up-regulation was observed in the transcription of these genes in *S. officinalis* tetraploid plants as compared with diploids. The changes in gene expression between diploid and tetraploid plants have been reported in various studies. It has been reported that the *tdc*, *g10h*, *sls*, *str*, *dat*, and *prx1* genes had a higher expression level in the tetraploid plants of *Catharanthus roseus* L. than that in diploids (Xing *et al.*, 2011). Studies by Mishra *et al.* (2010) on the expression level of genes involved in morphine biosynthesis in *Papaver somniferum* also showed an increase in the expression of these genes in tetraploid plants. Tavan *et al.* (2021) also reported that the expression level of *SQE* and *LUS* genes was significantly increased in tetra- and mixoploids of *S. officinalis* in comparison with diploid ones.

Polyploids having larger C-values than their diploid counterparts were expected to show increased gene expression (Leitch and Bennett, 2004; Madani *et al.*, 2019). Allen *et al.* (2008) showed that overexpression of some genes involved in morphine synthesis increases the production of these alkaloids. In agreement with our finding, Adams and Wendel (2005) declared that polyploidy has considerable effects on gene expression, including silencing and up/down-regulation of gene expression. In an analysis of gene expression in colchicine-treated sorghum plants, Murali *et al.* (2013) showed up-regulated expression of most of the genes encoding sucrose synthases, and only one gene exhibited a lower expression level. Yang *et al.* (2011) indicated that polyploids show gene expression bias at the genome level. Also, It has been found that the differential gene expression between the control and colchicine-treated plants may be partially due to the

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changes in gene dosage (Murali *et al.*, 2013) and the high copy number of genes (Sattler *et al.*, 2016). Variations in gene expression caused by manipulation of ploidy levels can be instigated via pseudogenization, subfunctionalization, neofunctionalization, and epigenetic factors such as DNA methylation, changes in histones and chromatin structure, microRNAs (miRNAs), and small interfering RNAs (siRNAs) which in turn, affects gene expression (Osborn *et al.*, 2003; Salmon and Ainouche, 2010; Song and Chen, 2015).

As shown in Table 2, the production of phenolic acids was increased in tetraploid plants, and it can be a result of the increment of the expression of genes involved in the phenolic acids production pathway. The effect of polyploidization on gene expression, either using microarray technology at the transcriptome level or two-dimensional electrophoresis at the proteome level, has been studied (Albertin *et al.*, 2006; Riddle *et al.*, 2010). All the studies have shown that polyploidy may induce variation in some gene expressions. This phenomenon may be because chromatin is more in contact with the nuclear membrane after polyploidy, thus increasing gene activity (Levin, 2002). Cells with higher ploidy are larger and expected to have greater transcription levels than lower ploidy cells. Similar findings have been found in *Atropa belladonna*, where all hyoscyamine has been converted to scopolamine due to increased expression of the gene encoding hyoscyamine-6β-hydroxylase (Yun *et al.*, 1992).

Conclusion

In all, it was concluded that some *S. officinalis* essential oil components differed between tetraploid and diploid plants, and tetraploidy increased the level of phenolic acids in *S. officinalis* and caused higher expression of genes involved in their biosynthetic pathway, especially *C4H*, *PAL1*, *4CL*, *TAT*, *HPPR*, and *CYP98A14* genes. So, polyploidy induction in medicinal plants can be helpful to increase the expression of genes involved in the biosynthesis of phenolic acids and increase the production of secondary metabolites of medicinal plants.

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