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Triterpenic and phenolic acids production changed in *Salvia officinalis* via *in vitro* and *in vivo* polyploidization: A consequence of altered genes expression

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ABSTRACT

The induction of polyploidy is an efficient technique for creating a diversity of genetic, phenotypic, and phytochemical novelties in plant taxa. Sage (*Salvia officinalis* L.) is a well-known medicinal plant rich of valuable bioactive molecules such as triterpenic and phenolic acids. In the present study, the effect of *in vitro* and *in vivo* polyploidization on morphological characteristics, anatomical structures, phytochemical traits, and expression level of the genes involved in the biosynthesis of major triterpenic acids (ursolic, betulinic, and oleanolic acids) of the plant was studied. The sterile seeds treated with different concentrations (0, 0.05, 0.1, and 0.2%) of colchicine for 24 and 48 h were considered for polyploidy induction. Flow cytometry and chromosome counting were used to confirm the ploidy level of diploid ($2n = 2x = 14$, 2C DNA = 1.10 pg) and tetraploid ($2n = 4x = 28$, 2C DNA = 2.12 pg) plants after seven months. The highest polyploidy induction was obtained by applying 0.1% (w/v) colchicine for 48 h with an efficiency of 19.05% *in vitro* tetraploidy. Polyploids showed differences in leaf shape and color, leaf and stem thickness, trichrome density, root length, plant height, and number of leaves compared to diploid plants. There was also a significant decrease in rosmarinic acid content in polyploid (plants) as compared to diploid plants. Although a significant decrease in ursolic acid content was observed in polyploids, betulinic acid content associated with the expression levels of genes encoding enzymes being active in triterpene biosynthesis such as squalene epoxidase (*SQE*) and lupeol synthase (*LUS*). The expression of *SQE* and *LUS* was significantly increased in *in vitro* tertaploids (2.9-fold) and *in vivo* mixoploids (2.4-fold). The results confirm the idea that induced polyploidy can randomly alter breeding traits of plants as well as the content of bioactive compounds.

1. Introduction

Artificial polyploidy is a plant breeding process that produces unpredictable outcomes. In fact, this genetic manipulation method can produce new or various functions in different plant species (Lynch, 2007). Polyploid plants, compared with diploids, often exhibited some desirable agronomic traits including intense growth, resistance to biotic and abiotic stresses, greater adaptability, increased yields, and high content of specialised metabolites (Pan-pan et al., 2018; Chen et al., 2018; Mo et al., 2020). Doubling the number of chromosomes may have an impact on the genetic, phenotypic, growth and phytochemical characteristics of plants (Ma et al., 2015; Hoang et al., 2020). In addition, in polyploid plants, the expression level of genes may increase as compared to diploid plants, which could lead to a dosage-regulated expression. (Dhooghe et al., 2011; Osborn et al., 2003). So far, polyploidy has been used in many ornamental and medicinal plants species

Abbreviations: TAs, triterpenic acids; UA, ursolic acid; BA, betulinic acid; OA, oleanolic acid; RA, rosmarinic acid; SAA, salvianolic acid A; CA, caffeic acid; ChA, chlorogenic acid; *FDS*, farnesyl pyrophosphate synthase; *SQS*, squalene synthase; *SQE*, squalene epoxidase; *LUS*, lupeol synthase; *MFAS*, mixed-function amyrin synthase; *BAS*, β-amyrin synthase; PGRs, plant growth regulators; MS, Murashige and Skoog; HPLC, high performance liquid chromatography; FCM, flow cytometer.

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for different modification purposes (Mo et al., 2020; Wang et al., 2020; Kondo et al., 2020; Hannweg et al., 2016; Aqafarini et al., 2019; Sabzehzari et al., 2019; Chen et al., 2018). In recent decades, polyploidy in medicinal plants has been used with the aim of increasing the quantity and quality of specialised metabolites (Wei et al., 2018; Zhou et al., 2020a; Sadat Noori et al., 2017; Salma et al., 2018; Tavan et al., 2015). There are many protocols for polyploidy induction; however, the development of a suitable protocol depends on the antimitotic compound and its concentration, on the exposure period, the method of treatment chosen, the type of explant and plant species (Dhooghe et al., 2011).

Specialised metabolites, including terpenoids, phenols and alkaloids are accumulated by the plants under specific conditions (genotype, climate, and edaphic factors and so forth). These specific compounds play a critical role in the adaptation, environment interaction and resistance to biotic and abiotic stress of plants. They are often investigated due to their effects as antifungal, antimicrobial, insecticidal, and antioxidant (Osbourn et al., 2011; Moses et al., 2013; Biswas and Dwivedi, 2019). Polyphenols include flavonoids, phenolic acids, and tannic acids. These compounds have significant anti-oxidation, anti-inflammation and antibacterial properties, act as well as strong inhibitors of enzymes and of glycation, and exert frequently miRNA effects (Fraga et al., 2010; Xie and Chen, 2013; Xie et al., 2014; Tavan et al., 2020). Terpenoids including mono-, di-, sesqui-, tri-, and sesterterpenoids are also the most abundant and diverse natural compounds produced in many plants . Triterpenoids (C30H48) are widely distributed in plants and possess a broad range of biological activities (Topçu, 2006).

Betulinic acid (BA), ursolic acid (UA), and oleanolic acid (OA) are well-known triterpenic acids (TAs), which have been associated with diverse biological activities, based on antimicrobial, anti-inflammatory, anti-hyperlipidemic, anti-tumor, anti-HIV, and antidiabetic properties (Pisha et al., 1995; Misra et al., 1997; Jäger et al., 2009; Moghaddam et al., 2012).

The biosynthesis of triterpenoids begins with the production of specific precursors like squalene and oxidosqualene as derivatives of the cytoplasmic mevalonic acid (MVA) pathway. In other words, the cyclization of 2,3-oxidosqualene by specific oxidosqualene cyclases (OSCs) including α-amyrin synthase (AAS), β-amyrin synthase (BAS), and lupeol synthase (LUS) lead to the generation of α-amyrin, β-amyrin, and lupeol. Finally, cytochromes P450 (CYP450s) are involved in the formation of valuable triterpene acids such as UA, OA, and BA (Biswas and Dwivedi, 2019; Haralampidis et al., 2002; An et al., 2020). So far, some key genes in the biosynthesis pathway leading to TAs have been characterized from several important Laminaceae species, including *Ocimum basilicum* (Misra et al., 2014), *Salvia officinalis* L. (Ali et al., 2017), *Salvia guaranitica* (Ali et al., 2018), *S. officinalis*, *Rosmarinus officinalis*, and *Thymus persicus* (Aminfar et al., 2019).

In traditional medicine, the genus *Salvia* L. has been recognized as an important medicinal plant taxa with remarkable healing properties (Dweck, 2000). *Salvia* species are an important source of biologically valuable compounds, such as terpenoids (Ulubelen, 2000), phenolic acids, flavonoids, and tannins (Dweck, 2000). There are fifty-eight *Salvia* species occurring in Iran, some of which have already been studied for their bioactive compounds and biological activity (Firuzi et al., 2013; Aghaei Jeshvaghani et al., 2015). Recently, the species of sage (*S. officinalis*) has been reported as a rich source of triterpenic acids (TAs) amongst several *Salvia* species studied (Abdollahi-Ghehi et al., 2019). So far, polyploidy induction has been performed in several *Salvia* species, including *S. miltiorrhiza* Bunge (Chen et al., 2018), *S. hians* Royle (Grouh et al., 2011), and *S. coccinea* Buchoz (Kobayashi et al., 2008), with the aim of improving growth and increase the content of specialised metabolites.

Salvia officinalis L. (Lamiaceae) is native of Middle East and Mediterranean areas and throughout the world, particularly in Europe and North America (Miura et al., 2001). In this study here, polyploidy was induced to improve the quantity and quality of TAs and phenolic acid contents in this plant as an important species of the *Salvia* genus. Furthermore, the expression changes of some key genes involved in the biosynthesis of TAs as well as the morphological, anatomical characteristics and the chromosome counts were evaluated in diploid and polyploid plants of this species.

2. Results and discussion

This study investigated ploidy level, chromosome count, growth traits, and changes in metabolites levels of UA, BA, OA, RA, SAA, ChA, CA, and quercetin as well as the expression changes of some key genes involved in the biosynthesis of TAs for *in vitro* and *in vivo* cultures of polyploid and diploid plants of *S. officinalis*.

2.1. In vitro and in vivo polyploidy induction and survival rate

The mortality rate of treated explants increased with the concentrations and duration of colchicine exposure, and a higher mortality rate was demonstrated at a concentration of 0.2%, after one month. The non-growing seedlings after root emergence were considered as dead. After *in vitro* plants proliferation, they were sub-cultured monthly on MS medium without PGRs. Also, *in vivo* plants in the true four-leaf stage were transferred to plastic pots containing a mixed medium of vermicompost: peat moss: perlite: sand (2:2:1:1, v/v). Flow cytometry (FCM) analysis is an accurate method to confirm the ploidy level of plants and it is the only technique that identifies mixoploid plants (Salma et al., 2017). Here, FCM analysis was used to confirm ploidy level of diploid, tetraploid, and mixoploid plants after seven-month (Table 1).

Out of 105 plants obtained from the subculture of the *in vitro* plants, 19.05% and 28.57% were tetraploid and mixoploid, respectively. On the other hand, out of 83 *in vivo* treated plants, 37.3% mixoploid plants were obtained. The most effective treatment for polyploidy induction of *in vitro* plants was at a concentration of 0.1% colchicine and for 48 h of treatment. In contrast, *in vivo* treatments did result only in mixoploid plants after treatments with concentrations of 0.1 and 0.2% colchicine (Table 2). There are several papers that reported that the concentration of antimitotic agent and exposure time are highly interdependent in affecting polyploidization (Salma et al., 2017; Widoretno, 2016). Indeed, an increase in the concentration of the antimitotic compound and longer treatment time enhance the mortality rate of treated explants (Mo et al., 2020; Zhou et al., 2020b; Wang et al., 2020).

For FCM analysis, the peak of diploid plants (2*x*) *S. officinalis* was adjusted in channel 34–37, tetraploid plants (4*x*) in channel 65–71, and mixoploid plants (2*x* + 4*x*) was set on both channels (Fig. 1A–C). The standard plant peak was set in the 80.25 channel. Mean 2C DNA content was obtained for *S. officinalis* diploid and tetraploid plants (1.10 ± 0.04 and 2.12 ± 0.07 pg, respectively). Different amounts of 2C DNA in the range of 0.95–3.23 pg have been reported for various species of the *Salvia* genus (Zonneveld, 2019; Siljak-Yakovlev et al., 2010).

Also, flow cytometry results of the diploid and tetraploid plants were

Table 1
The degenerate primers related to TAs biosynthesis genes designed by Aminfar et al. (2019) except for the *SQE* gene.

Gene name	Primer	Primer sequence (5' → 3')	Amplicon size
FDS	FDSFwd	CATCGCCGCATTGT**W**CAG	187
	FDSRev	CAATCTTTTC**R**GGCTCACC	
SQS	SQSFwd	GCTTGACAC**W**GTTGAGGA	111
	SQSRev	GTACC**R**CATGAAAAATGCCA	
SQE	SQEFwd	CTCTTGTTTCGTCGGTCTGAT	113
	SQERev	CTGTGCTGCTGATGGGATAA	
LUS	LUSFwd	ATCAGAA**Y**GAAGATGGAGG	200
	LUSRev	CCA**R**AACTTTCCCCACGA	
BAS	BASFwd	GGAATGAAGATGCAGAG**Y**TT	227
	BASRev	TGCCATCCATGATCTTG**R**TC	
MFAS	MFASFwd	TC**N**TGGGGAAAGAC**N**TAT	131
	MFASRev	CGGCA**R**TAACACCACAT	

Table 2
The number of regenerated diploid and polyploid plants of *S. officinalis* for both *in vitro* and *in vivo* cultures.

Colchicine concentration (%)	Exposure time (h)	No. of treated seeds	Germination (%)		No. of the ploidy levels of treated plants				
			In vitro	*In vivo*	*In vitro*			*In vivo*	
					2*x*	4*x*	2*x*+4*x*	2*x*	2*x*+4*x*
0	24	30	70.0	86.7	40	0	0	26	0
	48	30	70.0	93.3	35	0	0	28	0
0.05	24	30	46.7	66.7	20	0	0	20	0
	48	30	33.3	60.0	17	0	0	18	0
0.1	24	30	33.3	50.0	10	0	15	5	10
	48	30	26.7	40.0	8	2	15	4	8
0.2	24	30	0.0	33.3	0	0	0	3	7
	48	30	0.0	26.7	0	0	0	2	6

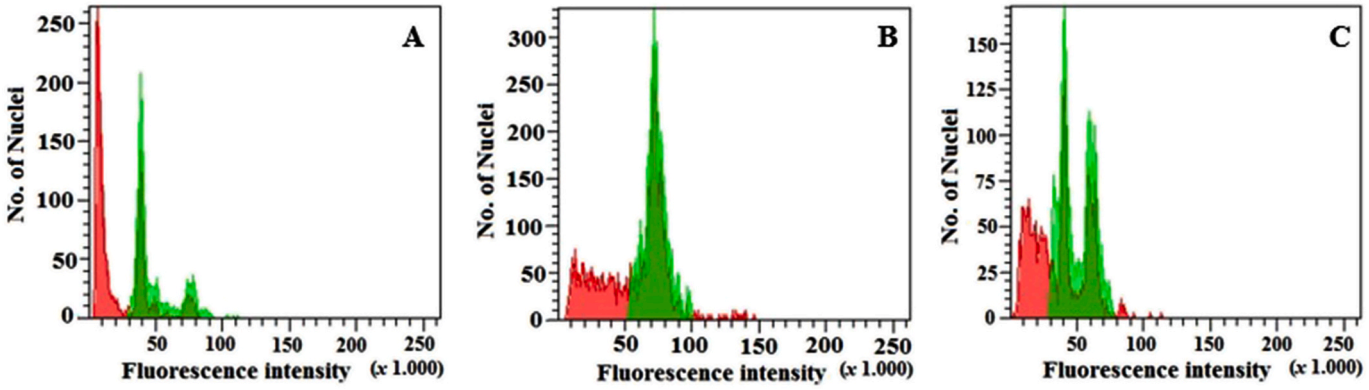

Fig. 1. Histograms of flow cytometric analysis of diploid (A), tetraploid (B) and mixoploid (C) plants of *S. officinalis*.

confirmed by chromosome counting. The results showed that the chromosome number of the *in vitro* natural diploids ($2n = 2x = 14$) (Fig. 2A) was duplicated after colchicine treatment ($2n = 4x = 28$) (Fig. 2B). Chromosome numbers in the *Salvia* genus are reported between $2n = 12$ and 64 (Haque, 1981). In addition, chromosome base numbers $x = 6$, 7, 8, 9, 10, 11, 13, 15, and 16 are determined in diverse cytological studies on the genus *Salvia*, and the ancestral chromosome base number seems to be $x = 7$ (Ranjbar et al., 2015).

2.2. Stomata and morphological characterization of diploid and polyploid plants

Generally, morphological changes are reliable indicators to confirm polyploidy in plants. Morphological changes include changes in leaf shape and color, leaf and stem thickness, trichomes density, root length, plant height, and number of shoots (Mo et al., 2020). In terms of appearance, *in vitro* tetraploid plants of *S. officinalis* had smaller, wider, and thicker leaves of a darker green color, and higher trichome density (Fig. 3). Also, *in vivo* mixoploid plants of *S. officinalis* had leaves with a lighter green color, less thickness, and lower trichome density (Fig. 4). Colchicine treatment increased internode length and resulted in the height enhancement of *in vivo* mixoploid plants. Conversely, *in vitro* polyploid plants had lower height due also to reduced root growth compared to diploids. However, the number of leaves in *in vitro* polyploids was higher than diploids but *in vivo* mixoploids had fewer leaves number (Table 3; Figs. 3 and 4). Our results are in agreement with other reports on *Lepidium sativum* (Aqafarini et al., 2019), *Antirrhinum majus* (Kondo et al., 2020), and are in disagreement with prior reports on *Plantago psyllium* (Sabzehzari et al., 2019) and *Bletilla striata* (Pan-pan et al., 2018). Unlike *in vivo* mixoploid plants, *in vitro* polyploid plants

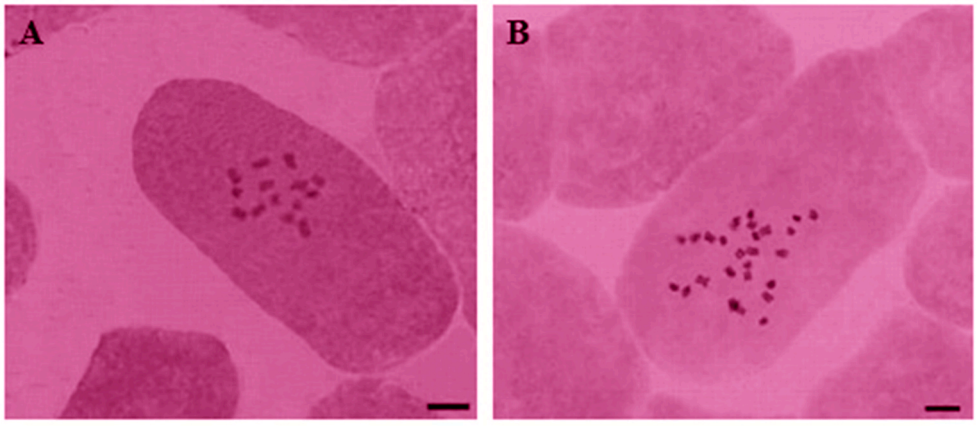

Fig. 2. Chromosome numbers of root tip cells from diploid plants $2n = 2x = 14$ (A); and tetraploid plants $2n = 4x = 28$ (B) of *S. officinalis*.

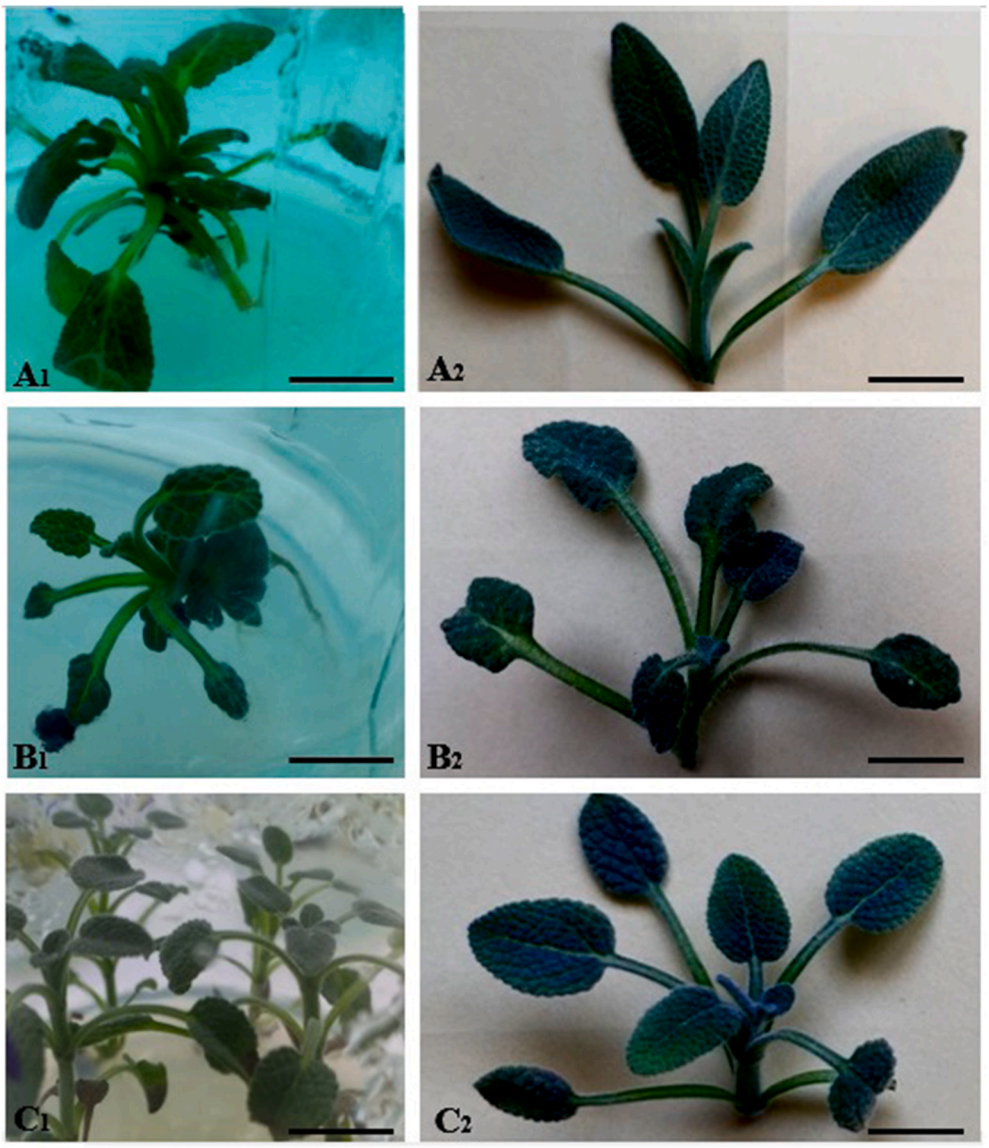

Fig. 3. Comparison of morphological characteristics of *in vitro* plants of diploid (A1,2), tetraploid (B1,2), and mixoploid (C1,2) of *S. officinalis*. Bars = 1 cm.

had a higher dry and fresh weight than diploid plants (Table 3).

Another method to confirm the increased ploidy level is the analyses of anatomical changes including changes in length and width of the stomata guard cell and stomatal density. A lower stomata density along with greater length and width of the stomata guard cells were observed in polyploid plants compared with diploid plants in both *in vitro* and *in vivo* plants (Table 3; Figs. 5 and 6). These findings were similar to previous reports on other plants species (Mo et al., 2020; Pan-pan et al., 2018; Zhou et al., 2020b; Li et al., 2020).

2.3. Effect of polyploidy on content of TAs and phenolic acids

Several factors are responsible for the metabolic content of medicinal plants such as genotype, climate, and edaphic factors. Although the effects of induced polyploidy are unpredictable and each species should be examined individually, this breeding method through doubling chromosome numbers and altering the activity of biosynthesis enzymes plays an important role in increasing metabolites content (Salma et al., 2017; Tavan et al., 2015).

Salvia species contain valuable specialised metabolites including TAs (UA, BA, and OA) and phenolic acids (RA, SAA, ChA, CA, and quercetin) in the aerial parts (Topçu, 2006). In the present study, after confirming the ploidy level of plants, the effect of polyploidy on the content of important specialised compounds was determined by HPLC analysis (Table 4).

There was a significant difference in the content of TAs between diploid and polyploid plants of *S. officinalis* in both *in vitro* and *in vivo* conditions separately and in comparison with each other ($P \leq 0.01$) (Table 4). The content of UA in polyploid plants decreased compared with the corresponding diploids, whereas a higher content of BA was observed in polyploid plants in both *in vitro* and *in vivo* conditions compared with those of the diploid counterparts. Also, *in vivo* mixoploid plants showed an increase in the content of OA compared to diploid plants (Table 4).

Although there was no significant difference in the contents of SAA, CA, ChA, and quercetin between diploid and polyploid plants of *S. officinalis* in both *in vitro* and *in vivo* condition, the content of RA decreased in polyploid plants (Table 4).

Zhou et al. (2020) reported that the content of total protein, of the enzymes catalase and of proline and malondialdehyde significantly increased in tetraploid plants of *Zingiber officinale*, in the contrary the contents of the superoxide dismutase and peroxidase decreased in tetraploid plants. Chen et al. (2018) showed that there was no significant difference in the content of salvianolic acid B between tetraploid and

Fig. 4. The morphological characteristics of *in vivo* plants of diploid (A), and mixoploid (B) of *S. officinalis*.

Table 3
Effect of ploidy level on the morphological and stomata characteristics of *in vitro* and *in vivo* plants of *S. officinalis*.

Characteristics	*In vitro*			*In vivo*	
	2*x*	4*x*	2*x*+4*x*	2*x*	2*x*+4*x*
Length of guard cells (μm)	21.6 ± 0.8^{c}	30.1 ± 0.9^{a}	25.1 ± 0.7^{b}	22.1 ± 0.7^{c}	25.2 ± 0.8^{b}
Width of guard cells (μm)	19.2 ± 0.8^{d}	26.2 ± 0.9^{a}	22.9 ± 0.7^{b}	20.2 ± 0.8^{c}	23.2 ± 0.6^{b}
Stomatal density (μm)	25.5 ± 1.1^{a}	16.3 ± 0.7^{d}	22.4 ± 1.8^{b}	21.4 ± 1.2^{b}	18.6 ± 1.3^{c}
Plant height (cm)	5 ± 0.4^{c}	3.65 ± 0.4^{d}	4.05 ± 0.3^{d}	25.78 ± 1.1^{b}	40.2 ± 1.1^{a}
Leaf (No.)	17 ± 1.1^{d}	27.4 ± 0.9^{a}	19.2 ± 0.8^{c}	22 ± 1.3^{b}	14.1 ± 1.3^{e}
Root length (cm)	6.15 ± 0.7^{c}	4.25 ± 0.5^{d}	4.8 ± 0.5^{d}	24.25 ± 1.2^{a}	16.7 ± 0.9^{b}
Plant dry weight (mg)	276.5 ± 44.0^{d}	382.5 ± 49.3^{c}	320 ± 45.3^{d}	1420 ± 76.6^{a}	1268 ± 85.7^{b}
Plant fresh weight (mg)	1160 ± 250.3^{d}	1550 ± 254.9^{c}	1280 ± 181.3^{cd}	7295 ± 516.6^{a}	6890 ± 440.8^{b}

The data (mean ± standard deviation) were evaluated from 10 plants each of polyploids and diploids. Means were compared using Duncan's multiple range tests at $p < 0.05$.

diploid plants of *Salvia miltiorrhiza*, but the concentration of dihydrotanshinone and total tanshinones in tetraploid plants was higher than in diploid plants. In tetraploid plants of *Trachyspermum ammi*, the yield of essential oil obtained was two-and-a half times higher than in diploid plants. Also, the essential oil of diploids plants contained α-Terpieol, while this compound was absent in tetraploid plants (Noori et al., 2017). Wei et al. (2018) identified a higher content of matrine and oxymatrine in tetraploid lines of *Sophora tonkinensis* compared with diploid plants.

2.4. Expression analysis of TAs biosynthesis genes in different ploidy levels

Three enzymes of farnesyl pyrophosphate synthase (FDS), squalene synthase (SQS), and squalene epoxidase (SQE) at the beginning of the pathway and three enzymes of mixed-function amyrin synthase (MFAS), lupeol synthase (LUS), and β-amyrin synthase (BAS) at the end of the biosynthesis pathway play an important role in the formation of TAs (UA, BA, and OA). qRT-PCR was used to demonstrate the relationship of UA, BA, and OA content with the expression pattern of key genes in the biosynthesis pathway of these TAs and in different ploidy levels of both *in vitro* and *in vivo* conditions of *S. officinalis*.

In vitro mixoploid plants had higher expression of *FDS*, *SQS*, *SQE*, and *LUS* (2.75, 7.83, 1.51, and 1.84 fold, respectively) compared to diploid plants. Also, *in vitro* tetraploid plants had higher expression of *SQS*, *SQE*, and *LUS* (1.88, 5.15, and 4.34 fold, respectively) compared to diploid plants (Fig. 7). On the other hand, higher expression of *SQE*, *LUS*, and *BAS* (2.39, 13.03, and 2.34 fold, respectively) genes was observed in *in vivo* mixoploid plants compared to diploids (Fig. 7). In the case of three genes (*SQE*, *MFAS*, and *LUS*) the same expression pattern (increasing, reducing, and increasing of expression, respectively) was observed in *in vitro* and *in vivo* polyploids compared with those of diploid counterparts.

There are reports that overexpression of *HMGR*, *FDS*, and *SQS* genes in the biosynthesis pathway of the isoprenoid increases triterpenoids production (Seo et al., 2005; Muñoz-Bertomeu et al., 2007; Kim et al., 2010). Also, *SQE* overexpression, as a new strategy, has already been used to raise triterpenoid production in plants (Dong et al., 2018).

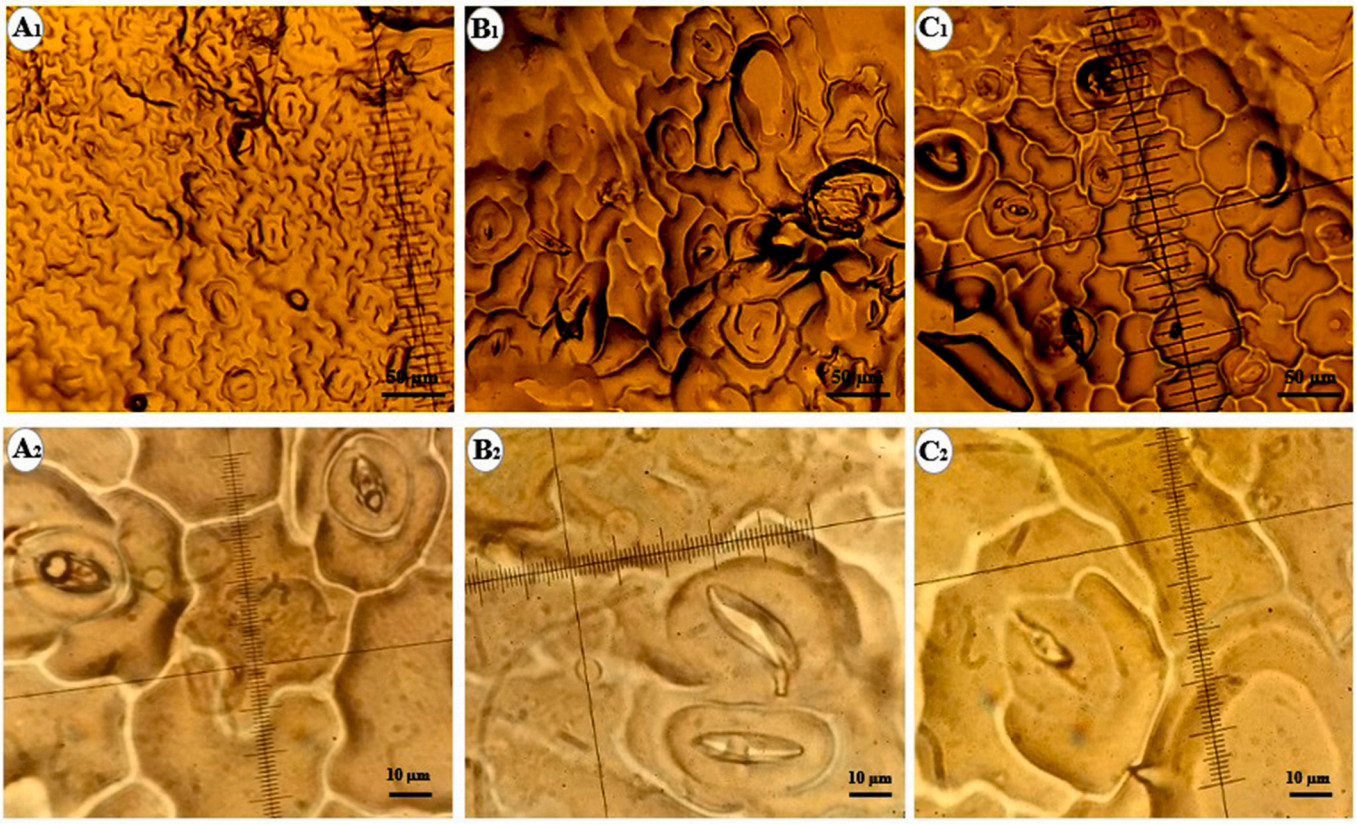

Fig. 5. The observation of stomata characteristics *in vitro* plants of diploid (A1,2), tetraploid (B1,2), and mixoploid (C1,2) of *S. officinalis*. Bars = 50 and 10 µm.

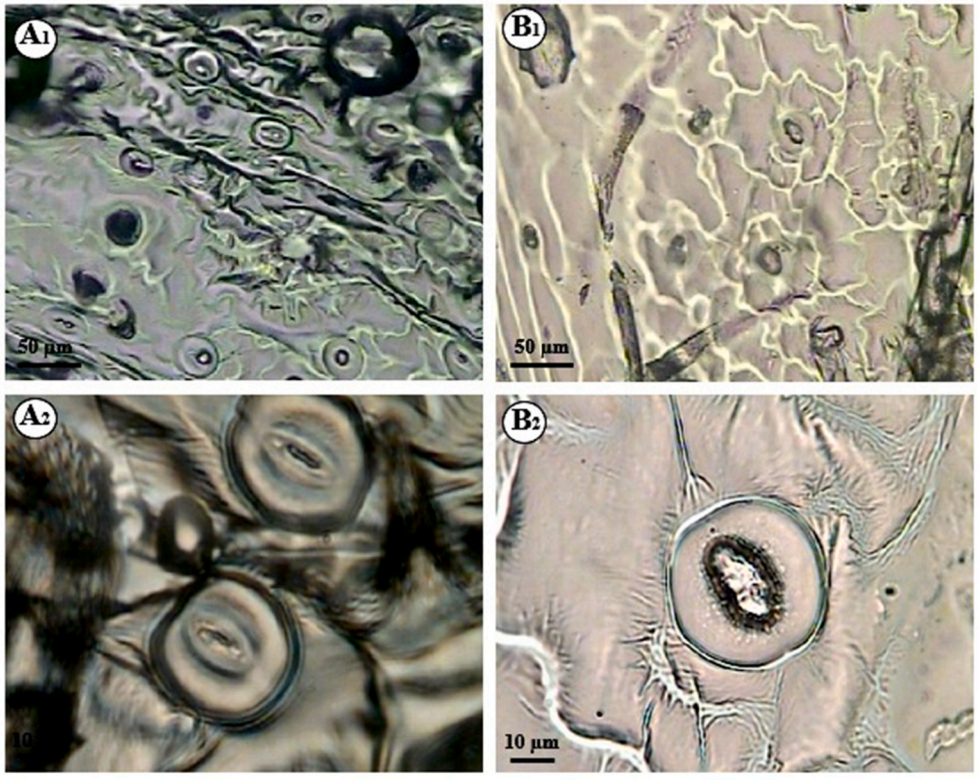

Fig. 6. The observation of stomata characteristics *in vivo* plants of diploid (A1,2) and mixoploid (B1,2) of *S. officinalis*. Bars = 50 and 10 µm.

Table 4
Comparison of TAs and phenolics contents of *in vitro* and *in vivo* diploid and polyploid plants of *S. officinalis*.

Composition (mg/g DW)	*In vitro*			*In vivo*	
	2*x*	4*x*	2*x*+4*x*	2*x*	2*x*+4*x*
Ursolic acid	6.12 ± 0.60^{a}	2.66 ± 0.29^{c}	3.08 ± 0.27^{c}	5.92 ± 0.22^{a}	4.34 ± 0.18^{b}
Betulinic acid	3.16 ± 0.40^{c}	9.25 ± 0.44^{a}	4.15 ± 0.32^{b}	0.88 ± 0.09^{e}	2.15 ± 0.18^{d}
Oleanolic acid	2.47 ± 0.20^{a}	1.05 ± 0.10^{b}	1.27 ± 0.10^{b}	0.74 ± 0.08^{c}	1.23 ± 0.12^{b}
Rosmarinic acid	9.71 ± 1.86^{a}	4.53 ± 2.18^{bc}	7.59 ± 2.27^{ab}	5.45 ± 0.07^{bc}	3.66 ± 0.08^{c}
Salvianolic acid A	0.74 ± 0.03^{a}	0.65 ± 0.02^{b}	0.69 ± 0.01^{b}	0.61 ± 0.01^{c}	0.69 ± 0.01^{b}
Chlorogenic acid	0.65 ± 0.02^{c}	0.71 ± 0.03^{ab}	0.74 ± 0.04^{a}	0.67 ± 0.01^{bc}	0.63 ± 0.01^{c}
Caffeic acid	0.51 ± 0.02^{b}	0.68 ± 0.01^{a}	0.46 ± 0.09^{b}	0.47 ± 0.01^{b}	0.44 ± 0.01^{b}
Quercetin	0.08 ± 0.02^{a}	0.02 ± 0.02^{b}	ND	ND	ND

The data (mean ± standard deviation) were obtained from 3 replications of each of polyploids and diploids. Means were compared using Duncan's multiple range tests at $p < 0.05$. ND, not determined.

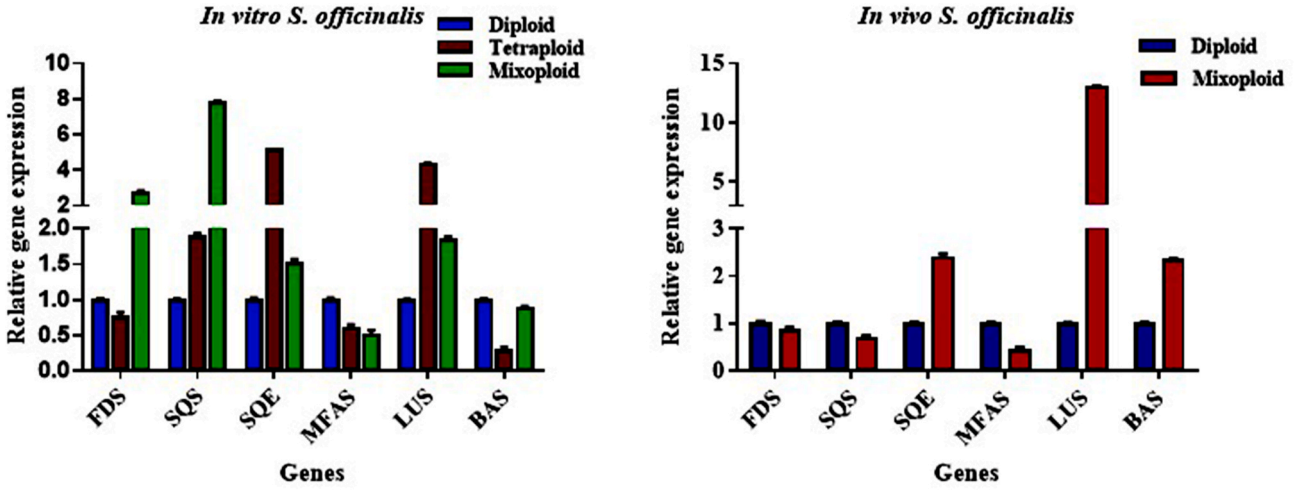

Fig. 7. Relative expression of six genes related to the biosynthesis of TAs, including farnesyl diphosphate synthase (*FDS*), squalene synthase (*SQS*), squalene epoxidases (*SQE*), lupeol synthase (*LUS*), β-amyrin synthase (*BAS*), and mixed function amyrin synthase (*MFAS*) genes in the different ploidy levels of *in vitro* and *in vivo* conditions of *S. officinalis*. Error bars are shown as standard deviation (n = 3).

Therefore, it can be concluded that overexpression of genes at the entry of a biosynthesis pathway leading to triterpenoids can differently affect the activity of *OSC* genes, such as *MFAS*, *LUS*, and *BAS*. Indeed, OSCs use 2,3-oxidosqualene as a common biosynthesis intermediate for converting it into different pentacyclic triterpenoids including BA, OA, and UA. In other words, OSCs can be either single functional such as BAS, AAS, and LUS that catalyze oleanane, ursane, and lupane skeletons, respectively, or they can be multifunctional such as MFAS cyclizing 2,3-oxidosqualene to α-amyrin and β-amyrin in a 5:3 ratio (Misra et al., 2014; Aminfar et al., 2019). Polyploidization could affect gene expression and gene regulation and these effects have been elaborated in a number of polyploid plants such as *Isatis indigotica* (Lu et al., 2006), *Helianthus decapetalus* (Church and Spaulding, 2009), *Cymbopogon* spp (Lavania et al., 2012), and *Dendrocalamus latiflorus* Munro (Qiao et al., 2017). So far, there has been no report about the effect of ploidy level on the genes expression involved in the biosynthesis pathway of terpenoids. Nevertheless, Javadian et al. (2017) indicated that the expression level and enzyme activity of genes related to podophyllotoxin biosynthesis pathway increased in two leaf and stem organs of tetraploids compared to those of the diploids of *Linum album*. In addition, the expression of some known genes related to artemisinin, morphinan, and thebaine biosynthesis in synthesis tetraploids of *Artemisia annua* L., *Papaver somniferum* L., and *Papaver bracteatum* L. increased compared to diploids (Mishra et al., 2010; Lin et al., 2011; Tarkesh Esfahani et al., 2021). Therefore, the up-regulation of some genes of the biosynthesis pathway for functional specialised metabolites in the artificial autotetraploids further demonstrate that this new type of medicinal plants with boosted targeted metabolites and biomass is promising (Zhou et al., 2015; Tarkesh Esfahani et al., 2021).

The calculation of the correlation coefficient showed a strong and positive relationship between gene expression of *MFAS* and *LUS* with the production of UA and BA, respectively, in different ploidy levels and in both *in vitro* and *in vivo* conditions (Fig. 8). Also, there was a weak and negative correlation between gene expression of *FDS*, *SQS*, and *SQE* with TAs content; however it is important to mention that this correlation was investigated in both cross-ploidy level and cross-growth condition dimensions. In general, TAs accumulation may be related to the expression of each of the six genes of the biosynthesis pathway of these compounds.

In this study, stability of *in vitro* tetraploid plants (100%) was confirmed after three times of subculture. Therefore, consecutive vegetative propagation keep the different levels of ploidy while there is more tendency for the preservation of stability in terms of DNA content and viable seed production for progenies of polyploidized plants in second generation (Pereira et al., 2017).

3. Conclusion

The present study is the successful report of *in vitro* and *in vivo* induced polyploidy of *S. officinalis*. The highest tetraploid induction efficiency (19.05%) for *in vitro* conditions was achieved after 48 h of exposure to 0.1% colchicine concentration, whereas only mixoploid plants were observed for *in vivo* conditions using the maximum colchicine concentration (0.2%). Polyploidy induction caused a significant increase in gene expression of *SQE* that plays a key role in *OSCs* expression and TAs production. On the other hand, the phenolic content decreased. These results showed that increased ploidy levels may not

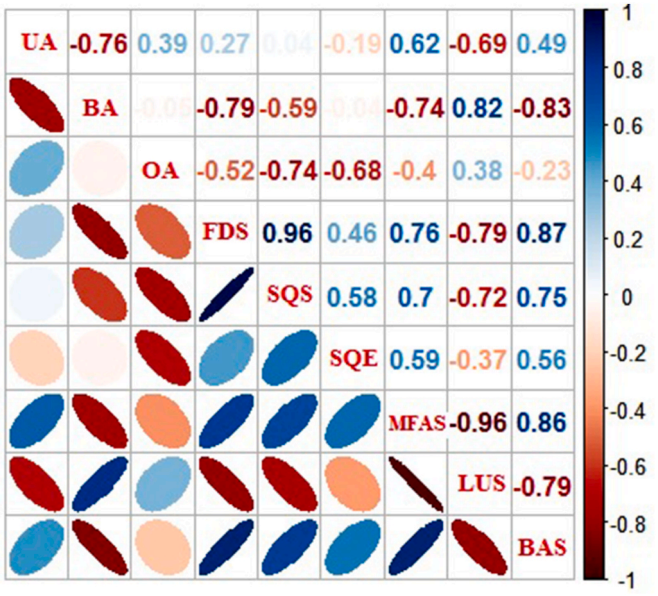

Fig. 8. Pearson correlation and the relationship between TAs contents of betulinic acid (BA), ursolic acid (UA), and oleanolic acid (OA) and gene expression related to triterpenic acids biosynthesis pathway including farnesyl diphosphate synthase (*FDS*), squalene synthase (*SQS*), squalene epoxidases (*SQE*), lupeol synthase (*LUS*), β-amyrin synthase (*BAS*), and mixed function amyrin synthase (*MFAS*) genes in different ploidy levels of *in vitro* and *in vivo* conditions of *S. officinalis*.

always be associated with the enhancement of specialised metabolites and should be carefully verified. The further researches will be necessary to evaluate the influence of increased ploidy level on the transcriptome and metabolome in different species.

4. Experimental

4.1. Chemicals and reagents

Murashige and Skoog (MS) medium including vitamins was purchased from Duchefa Biochemie (Haarlem, Netherland). Colchicine, agar, sucrose, plant growth regulators (PGRs), acetic acid glacial, hydrochloric acid, dimethylsulphoxide, phosphoric acid, methanol, acetonitrile, and water of HPLC grade were obtained from Merck (Darmstadt, Germany) as well as authentics of betulinic acid, oleanolic acid, ursolic acid, rosmarinic acid, salvianolic acid A, chlorogenic acid, caffeic acid, and quercetin, were provided from Sigma (Sigma-Aldrich Corporation, MO, USA).

4.2. Plant material

The plant seeds of *Salvia officinalis* L. (Lamiaceae) were provided from the garden of medicinal plants of Hamedan, Iran. A voucher specimen (Number: A-Hort-97-SO) has been deposited in the herbarium of Bu-Ali-Sina University, Hamedan, Iran.

4.3. In vitro induction of polyploidy

The plant seeds (n = 30) for each treatment were disinfected *in vitro* according to Ejtahed et al. (2015). They were then immersed in filter-sterilized colchicine solution at 0.05, 0.1, and 0.2% (w/v) concentrations for 24 and 48 h on an orbital shaker as described previously Tavan et al. (2015). The seeds immersed in sterilized distilled water were considered as control. Treated seeds were washed three times in sterile conditions and were then cultured on MS medium free of PGRs.

The seedlings of *S. officinalis* were cultured on MS medium with 0.5 mg/l BAP and 0.1 mg/l IAA for shoot proliferation. After one month, for root proliferation, the explants were transferred to MS medium with 1 mg/l IBA PGR. Treated regenerants along with control plants were sub-cultured monthly on MS medium without PGRs and were kept under 16/8 h (light/dark) conditions.

4.4. In vivo induction of polyploidy

The plant seeds (n = 30) for each treatment were disinfected and then treated with colchicine solution as mentioned in the prior section. Treated seeds were washed three times by sterile distilled water and were then placed on filter paper in Petri dishes. After 4 days, all germinated seeds from each certain Petri dish were planted into a mixed medium of vermicompost: peat moss: perlite (2:2:1, v/v) and plants were further protected under greenhouse conditions. After seven months, the plants (both *in vitro* and *in vivo* conditions) were used for flow cytometric analysis, the chromosome count, morphological and anatomical traits as well as phytochemical analysis and gene expression.

4.5. Flow cytometric analysis

About 25 mg leaf tissue of *in vitro* and *in vivo* plants of *S. officinalis* species along with the same mass of *Glycine max* L. (as an internal standard with 2C = 2.50 pg of DNA) were used for flow cytometric (FCM) analysis. FCM analysis was performed according to previously reported protocols by Loureiro et al. (2007) and its modification by Tavan et al. (2015). Also, genomic 2C DNA content was calculated based on previously reported equation (Doležel and Bartoš, 2005; Dolezel et al., 2003).

4.6. Chromosome counting

The root tips were cut from *in vitro* plantlets approved as diploid and tetraploid by FCM. The steps of chromosome observation was performed according to previously reported protocols by ; Abedi et al. (2015).

4.7. Stomata and morphological characterization of diploid and polyploid plants

After analysis of FCM and the chromosome counts, the plants that were confirmed as polyploids were grown for several months and investigated for anatomical and morphological traits. The morphological traits including leaf shape and color, leaf and stem thickness, trichomes density, root length, plant height, leaf number, fresh and dry weight of plant were compared between control and polyploid plants. For analysis of the stomata guard cell, epidermal cells of leaves were assayed from the lower area by the nail varnish method (Hamill et al., 1992).

4.8. Extraction, HPLC and HPLC-MS analysis

The aerial parts of the control and polyploid plants of *S. officinalis* were taken from *in vitro* and *in vivo* cultures, weighed, and air dried at room temperature. Extraction and high performance liquid chromatography (HPLC) analysis for phenolic compounds of rosmarinic acid (RA), salvianolic acid A (SAA), chlorogenic acid (ChA), caffeic acid (CA), and quercetin were investigated as described by Skendi et al. (2017) and for TAs compounds of UA, BA, and OA were assayed as described by Bakhtiar et al. (2014). The content of components was determined using their standards calibration curve. In addition, for justifying the HPLC results; a mass spectrometer (MS) connected to the HPLC system (HPLC-MS) was used as described by Bendif et al. (2020). Briefly, a Varian 212 binary pump equipped with a Varian Prostar 430 autosampler and coupled to a Varian 500 Ion Trap mass detector (MS) was employed. The mass spectrometer was equipped with an Electrospray Ionisation (ESI) ion source, operating in negative ion mode. Separation

of phytocompounds was achieved using an Agilent Eclipse plus C18 column (2.1 × 150 mm, 3.5 μm) as stationary phase and a gradient mixture of acetonitrile (A) and 0.1% formic acid in water (B) as mobile phase. Flow rate was 0.4 ml/min. In total, 14 compositions were detected and 8 were identified by comparing their retention time, UV–vis, and mass spectra with authentic standards (Table S1; Fig. S1).

4.9. Total RNA extraction and gene expression analyses

Total RNA was extracted from *in vitro* and *in vivo* explants of *S. officinalis* using the RNX-Plus Kit (CINNAGEN, Tehran, Iran) according to the manufacturer's instructions. Moreover, the extracted RNA was treated with Sinaclon RNase-free DNase (Sinaclon, MO5401, Tehran, Iran) according to the company's recommendations to eliminate any genomic DNA contamination. The quality and quantity of RNA were assessed using agarose gel electrophoresis and Nanodrop (Thermo Scientific, Germany) spectrophotometer analyses, respectively. The first-strand cDNA was synthesized from 1 μg of total RNA using reverse transcriptase with Oligo (dT)-18 primers according to the instructions of the Yekta Tajhiz Azma First-Strand cDNA synthesis kit (Yekta Tajhiz Azma, YT4500, Tehran, Iran). Gene-specific primers used for qRT-PCR were from the partial cDNA sequences (Aminfar et al., 2019) except the SQE gene that was designed using the OligoArchitect online software (Table 1).

qRT-PCR was performed to determine the expression of six genes encoding TAs biosynthesis enzymes in different ploidy levels of *S. officinalis* as described by D'Agostino et al. (2019). The Actin gene was selected as a housekeeping gene for data normalization. All reactions were performed with three biological replicates and two technical replicates. The relative expression of each gene (Fold changes) was calculated using the $2^{-\Delta\Delta Ct}$ method.

4.10. Statistical analysis

In the present study, calculations of statistical analysis were performed with SAS Ver. 9.1 (Cary, NC, USA) software. Means were compared using Duncan's multiple range tests at $p < 0.05$. The Pearson correlation coefficient was calculated to determine the relationship between the expression of six genes and three TAs content in both *Salvia* species and in different ploidy levels that was represented in a correlogram by RStudio statistical software.

Declaration of competing 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2021.112803.

Author contributions

AA designed the research; MT performed the experiments, analyzed the data, and wrote the manuscript; MHM and HS assisted in the phytochemical experiments; MMR assisted in anatomical experiments and qPCR experiments; HS, MHM, and MMR edited the manuscript.

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